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(71) Applicant: **DANA-FARBER CANCER INSTITUTE, INC.** [US/US]; 450 Brookline Avenue, Boston, MA 02215-5450 (US).

(72) Inventors; and
(71) Applicants: **HAINING, William, N.** [GB/US]; 100 Homer Street, Newton, MA 02459 (US). **MANGUSO, Robert** [US/US]; 210 Calumet Street, Apt 2, Boston, MA 02120 (US).

(74) Agent: **SMITH, DeAnn, F.** et al.; Foley Hoag LLP, 155 Seaport Boulevard, Boston, MA 02210-2600 (US).

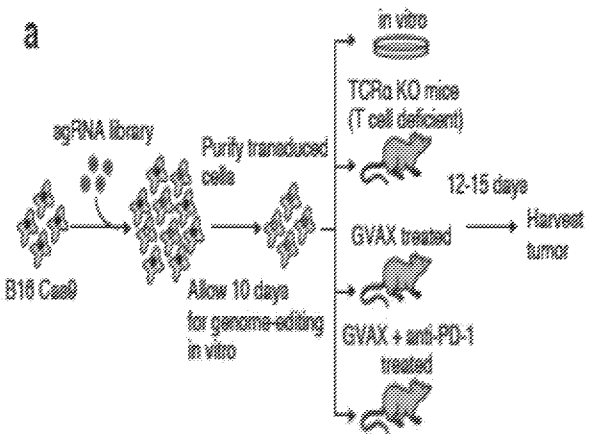
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(54) **Title:** MODULATING BIOMARKERS TO INCREASE TUMOR IMMUNITY AND IMPROVE THE EFFICACY OF CANCER IMMUNOTHERAPY

Figure 1



(57) **Abstract:** The present invention relates, in part, to methods of treating a cancer in a subject comprising administering to the subject a therapeutically effective amount of an agent that inhibits one or more biomarkers listed in Table 1, such as regulators of TNF signaling/NF-κB activation (*e.g.*, RIPK1, BIRC2, TBK1, TRAF3, RNF31, RBCK1, OTULIN, TRAF6, TAB2, and TNIP1), in combination with an immunotherapy.

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MODULATING BIOMARKERS TO INCREASE TUMOR IMMUNITY AND IMPROVE THE EFFICACY OF CANCER IMMUNOTHERAPY

Cross-Reference to Related Applications

This application claims the benefit of U.S. Provisional Application No. 62/532,593, filed on 14 July 2017; the entire contents of said application are incorporated herein in their entirety by this reference.

Background of the Invention

The striking clinical success of cancer immunotherapy with checkpoint blockade suggests it is likely to form the foundation of curative therapy for many malignancies (Reck *et al.* (2016) *N. Engl. J. Med.* 375:1823-1833; Hodi *et al.* (2010) *N. Engl. J. Med.* 363:711-723; Postow *et al.* (2015) *N. Engl. J. Med.* 372:2006-2017; Wolchok *et al.* (2013) *N. Engl. J. Med.* 369:122-133; Ferris *et al.* (2016) *N. Engl. J. Med.* 375:1856-1867; Brahmer *et al.* (2012) *N. Engl. J. Med.* 366:2455-2465; Nghiem *et al.* (2016) *N. Engl. J. Med.* 374:2542-2552; Topalian *et al.* (2012) *N. Engl. J. Med.* 366:2443-2454); Motzer *et al.* (2015) *N. Engl. J. Med.* 373:1803-1813). However, despite these successes, checkpoint blockade does not achieve sustained clinical response in most patients (Tumeh *et al.* (2014) *Nature* 515:568-571; Kelderman *et al.* (2014) *Mol. Oncol.* 8:1132-1139; Zaretsky *et al.* (2016) *N. Engl. J. Med.* 375:819-829). Additional therapeutic strategies are therefore needed to increase the clinical efficacy of immunotherapy. Moreover, the optimal strategy for combining emerging cancer immunotherapies with checkpoint blockade remains uncertain.

A relatively small number of genes, such as PD-L1, that enable tumors to evade the immune system have been discovered and most of these are already the focus of intense efforts to develop new immunotherapies (Freeman *et al.* (2000) *J. Exp. Med.* 192:1027-1034; Hirano *et al.* (2005) *Cancer Res.* 65:1089-1096; Dong *et al.* (2002) *Nat. Med.* 8:793-800; Balachandran *et al.* (2011) *Nat. Med.*17:1094-1100; Spranger *et al.* (2013) *Sci Transl Med.* 5:200ra116; Holmgaard *et al.* (2013) *J. Exp. Med.* 210:1389-1402; Sockolosky *et al.* (2016) *Proc. Natl. Acad. Sci. U.S.A.* 113:E2646-654; Liu *et al.* (2015) *Nat. Med.* 21:1209-1215; Weiskopf *et al.* (2016) *J. Clin. Invest.* 126:2610-2620; Tseng *et al.* (2013) *Proc. Natl. Acad. Sci. U.S.A.* 110: 11103-11108; Sica *et al.* (2003) *Immunity* 18:849-861; Zang *et al.* (2007) *Proc. Natl. Acad. Sci. U.S.A.* 104, 19458-19463). Although cancer cells could, in

theory, express many more genes that regulate their response or resistance to tumor immunity, strategies to systematically discover such genes are lacking.

Loss-of-function genetic screens have been increasingly used to study the functional consequences of gene deletion on tumor cells (Howard *et al.* (2016) Functional Genomic Characterization of Cancer Genomes. *Cold Spring Harb. Symp. Quant. Biol.* (2016); Ebert *et al.* (2008) *Nature* 451:335-339; Cowley *et al.* (2014) *Scientific Data* 1:article number 140035). These approaches include pooled genetic screens using CRISPR-Cas9-mediated genome editing that simultaneously test the role of a large number of genes on tumor cell growth, viability or drug resistance (Wang *et al.* (2014) *Science* 343:80-84; Shalem *et al.* (2014) *Science* 343:84-87). However, these screens have generally been conducted *in vitro*, where the contribution of the immune system is absent, or have studied phenotypes such as metastasis that do not directly evaluate the role of tumor immunity (Hart *et al.* (2015) *Cell* 163:1515-1526; Yu *et al.* (2016) *Nat. Biotechnol.* 34:419-423; Chen *et al.* (2015) *Cell* 160:1246-1260).

Despite the dramatic clinical success of cancer immunotherapy with PD-1 checkpoint blockade, most patients do not experience sustained clinical benefit from treatment. Accordingly a great need in the art exists for additional therapeutic strategies.

Summary of the Invention

The present invention is based, at least in part, on the discovery that inhibiting or blocking one or more biomarkers listed in Table 1, such as one or more regulators of TNF signaling and/or NF-κB activation (*e.g.*, receptor-interacting serine/threonine-protein kinase 1 (RIPK1), BIRC2, TBK1, TRAF3, RNF31, RBCK1, OTULIN, TRAF6, TAB2, and TNIP1), in combination with an immunotherapy, results in a synergistic therapeutic benefit for treating cancers that is unexpected given the lack of such benefit observed for the immunotherapy alone.

In one aspect, a method of treating a subject afflicted with a cancer comprising administering to the subject a therapeutically effective amount of an agent that inhibits one or more biomarkers listed in Table 1 or a fragment thereof, in combination with an immunotherapy, is provided.

Numerous embodiments are further provided that can be applied to any aspect of the present invention and/or combined with any other embodiment described herein. For example, in one embodiment, the agent described herein decreases the copy number, the

expression level, and/or the activity of one or more biomarkers in Table 1 (*e.g.*, one or more regulators of TNF signaling and/or NF-κB activation (*e.g.*, RIPK1, BIRC2, TBK1, TRAF3, RNF31, RBCK1, OTULIN, TRAF6, TAB2, and TNIP1). In another embodiment, the agent selectively decreases the activity of one or more biomarkers in Table 1, such as decreasing the serine/threonine-protein kinase activity and/or the receptor-binding activity of one or more regulators of TNF signaling and/or NF-κB activation (*e.g.*, receptor-interacting serine/threonine-protein kinase 1 (RIPK1), BIRC2, TBK1, TRAF3, RNF31, RBCK1, OTULIN, TRAF6, TAB2, and TNIP1). In still another embodiment, the agent described herein is a small molecule inhibitor, CRISPR single-guide RNA (sgRNA), RNA interfering agent, antisense oligonucleotide, peptide or peptidomimetic inhibitor, aptamer, or intrabody. In another embodiment, the RNA interfering agent is a small interfering RNA (siRNA), CRISPR RNA (crRNA), a small hairpin RNA (shRNA), a microRNA (miRNA), or a piwi-interacting RNA (piRNA). In still another embodiment, the RNA interfering agent is a CRISPR single-guide RNA (sgRNA). In yet another embodiment, the sgRNA comprises a nucleic acid sequence selected from the group consisting of nucleic acid sequence listed in Table 2. In one embodiment, the agent described herein comprises an intrabody, or an antigen binding fragment thereof, which specifically binds to the one or more biomarkers in Table 1 and/or a substrate of the one or more biomarkers in Table 1. In another embodiment, the intrabody, or antigen binding fragment thereof, is murine, chimeric, humanized, composite, or human. In still another embodiment, the intrabody, or antigen binding fragment thereof, is detectably labeled, comprises an effector domain, comprises an Fc domain, and/or is selected from the group consisting of Fv, Fav, F(ab')2, Fab', dsFv, scFv, sc(Fv)2, and diabodies fragments. In yet another embodiment, the intrabody, or antigen binding fragment thereof, is conjugated to a cytotoxic agent. In one embodiment, the cytotoxic agent is selected from the group consisting of a chemotherapeutic agent, a biologic agent, a toxin, and a radioactive isotope. In still another embodiment, the agent described herein increases the sensitivity of the cancer cells to an immunotherapy. In another embodiment, the immunotherapy and/or a cancer therapy is administered before, after, or concurrently with the agent. In still another embodiment, the immunotherapy comprises an anti-cancer vaccine and/or virus. In yet embodiment, the immunotherapy is cell-based. In one embodiment, immunotherapy inhibits an immune checkpoint. In another embodiment, the immune checkpoint is selected from the group consisting of CTLA-4, PD-1, VISTA, B7-H2, B7-H3, PD-L1, B7-H4, B7-H6, ICOS,

HVEM, PD-L2, CD160, gp49B, PIR-B, KIR family receptors, TIM-1, TIM-3, TIM-4, LAG-3, GITR, 4-IBB, OX-40, BTLA, SIRP, CD47, CD48, 2B4 (CD244), B7.1, B7.2, ILT-2, ILT-4, TIGIT, HHLA2, butyrophilins, IDO, CD39, CD73 and A2aR. In still another embodiment, the immune checkpoint is selected from the group consisting of PD-1, PD-L1, and PD-L2. In yet another embodiment, the immune checkpoint is PD-1. In another embodiment, the one or more biomarker described herein comprises a nucleic acid sequence having at least 95% identity to a nucleic acid sequence listed in Table 1 and/or encodes an amino acid sequence having at least 95% identity to an amino acid sequence listed in Table 1. In still another embodiment, the one or more biomarker is human, mouse, chimeric, or a fusion. In yet another embodiment, the agent reduces the number of proliferating cells in the cancer and/or reduces the volume or size of a tumor comprising the cancer cells. In another embodiment, the agent increases the sensitivity of the cancer to the immunotherapy. In still another embodiment, the one or more biomarkers comprise an amino acid sequence listed in Table 1, optionally wherein the amino acid sequence is selected from the group consisting of SEQ ID Nos: 2, 4, 6, 9, 11, 14, 16, 18, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 42, 44, 47, 49, 51, 54, 57, 60, 62, 64, 66, 68, 73, 76, 78, 80, 83, 86, and 88. In yet another embodiment, the one or more biomarkers are encoded by a nucleic acid sequence listed in Table 1, optionally wherein the nucleic acid sequence is selected from the group consisting of SEQ ID Nos: 1, 3, 5, 7, 8, 10, 12, 13, 15, 17, 19, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 41, 43, 45, 46, 48, 50, 52, 53, 55, 56, 58, 59, 61, 63, 65, 67, 69-72, 74, 75, 77, 79, 81, 82, 84, 85, and 87. In one embodiment, the cancer is melanoma. In another embodiment, the subject is an animal model of the cancer, preferably a mouse model, or a human. In still another embodiment, the method described herein further comprises administering to the subject at least one additional cancer therapy or regimen. In another embodiment, the at least one additional cancer therapy or regimen is administered before, after, or concurrently with the agent and/or the immunotherapy. In yet another embodiment, the agent described herein is administered in a pharmaceutically acceptable formulation.

In another aspect, a method of killing cancer cells comprising contacting the cancer cells with an agent that inhibits the copy number, the expression level, and/or the activity of one or more biomarkers listed in Table 1 or a fragment thereof, in combination with an immunotherapy, is provided.

As described above, numerous embodiments are further provided that can be applied to any aspect of the present invention and/or combined with any other embodiment described herein. For example, in one embodiment, the agent described herein decreases the copy number, the expression level, and/or the activity of one or more regulators of TNF signaling and/or NF-κB activation (*e.g.*, RIPK1, BIRC2, TBK1, TRAF3, RNF31, RBCK1, OTULIN, TRAF6, TAB2, and TNIP1). In another embodiment, the agent selectively decreases activity of one or more biomarkers in Table 1, such as decreasing the phosphatase activity and/or the substrate binding activity of one or more kinase signaling inhibitors. In still another embodiment, the agent is a small molecule inhibitor, CRISPR single-guide RNA (sgRNA), RNA interfering agent, antisense oligonucleotide, peptide or peptidomimetic inhibitor, aptamer, or intrabody. In one embodiment, the RNA interfering agent described herein is a small interfering RNA (siRNA), CRISPR RNA (crRNA), a small hairpin RNA (shRNA), a microRNA (miRNA), or a piwi-interacting RNA (piRNA). In another embodiment, the RNA interfering agent is a CRISPR single-guide RNA (sgRNA). In still another embodiment, the sgRNA comprises a nucleic acid sequence selected from the group consisting of nucleic acid sequence listed in Table 2. In one embodiment, the agent described herein comprises an intrabody, or an antigen binding fragment thereof, which specifically binds to the one or more biomarkers in Table 1 and/or a substrate of the one or more biomarkers in Table 1. In another embodiment, the intrabody, or antigen binding fragment thereof, is murine, chimeric, humanized, composite, or human. In still another embodiment, the intrabody, or antigen binding fragment thereof, is detectably labeled, comprises an effector domain, comprises an Fc domain, and/or is selected from the group consisting of Fv, Fav, F(ab')2, Fab', dsFv, scFv, sc(Fv)2, and diabodies fragments. In yet another embodiment, the intrabody, or antigen binding fragment thereof, is conjugated to a cytotoxic agent. In one embodiment, the cytotoxic agent is selected from the group consisting of a chemotherapeutic agent, a biologic agent, a toxin, and a radioactive isotope. In one embodiment, the agent described herein increases the sensitivity of the cancer cells to an immunotherapy. In another embodiment, the cancer cells are contacted with an immunotherapy and/or a cancer therapy before, after, or concurrently with the agent. In still another embodiment, the immunotherapy comprises an anti-cancer vaccine and/or virus. In one embodiment, the immunotherapy is cell-based. In another embodiment, the immunotherapy inhibits an immune checkpoint. In still another embodiment, the immune checkpoint is selected from the group consisting of CTLA-4, PD-

1, VISTA, B7-H2, B7-H3, PD-L1, B7-H4, B7-H6, ICOS, HVEM, PD-L2, CD160, gp49B, PIR-B, KIR family receptors, TIM-1, TIM-3, TIM-4, LAG-3, GITR, 4-IBB, OX-40, BTLA, SIRP, CD47, CD48, 2B4 (CD244), B7.1, B7.2, ILT-2, ILT-4, TIGIT, HHLA2, butyrophilins, IDO, CD39, CD73 and A2aR. In yet another embodiment, the immune checkpoint is selected from the group consisting of PD-1, PD-L1, and PD-L2. In one embodiment, the immune checkpoint is PD-1. In another embodiment, the biomarker described herein comprises a nucleic acid sequence having at least 95% identity to a nucleic acid sequence listed in Table 1 and/or encodes an amino acid sequence having at least 95% identity to an amino acid sequence listed in Table 1. In still another embodiment, the one or more biomarker is human, mouse, chimeric, or a fusion. In one embodiment, the agent described herein reduces the number of proliferating cells in the cancer and/or reduces the volume or size of a tumor comprising the cancer cells. In another embodiment, the agent increases the sensitivity of the cancer to the immunotherapy. In still another embodiment, the one or more biomarkers comprise an amino acid sequence listed in Table 1, optionally wherein the amino acid sequence is selected from the group consisting of SEQ ID Nos: 2, 4, 6, 9, 11, 14, 16, 18, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 42, 44, 47, 49, 51, 54, 57, 60, 62, 64, 66, 68, 73, 76, 78, 80, 83, 86, and 88. In yet another embodiment, the one or more biomarkers are encoded by a nucleic acid sequence listed in Table 1, optionally wherein the nucleic acid sequence is selected from the group consisting of SEQ ID Nos: 1, 3, 5, 7, 8, 10, 12, 13, 15, 17, 19, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 41, 43, 45, 46, 48, 50, 52, 53, 55, 56, 58, 59, 61, 63, 65, 67, 69-72, 74, 75, 77, 79, 81, 82, 84, 85, and 87. In one embodiment, the cancer is melanoma. In another embodiment, the subject is an animal model of the cancer, preferably a mouse model, or a human. In still another embodiment, the method described herein further comprises administering to the subject at least one additional cancer therapy or regimen. In yet another embodiment, the at least one additional cancer therapy or regimen is administered before, after, or concurrently with the agent and/or the immunotherapy. In another embodiment, the agent described herein is administered in a pharmaceutically acceptable formulation.

In still another aspect, a method of determining whether a subject afflicted with a cancer or at risk for developing a cancer would benefit from inhibiting the copy number, amount, and/or activity of at least one biomarker listed in Table 1 is provided, the method comprising a) obtaining a biological sample from the subject; b) determining the copy number, amount, and/or activity of at least one biomarker listed in Table 1; c) determining

the copy number, amount, and/or activity of the at least one biomarker in a control; and d) comparing the copy number, amount, and/or activity of the at least one biomarker detected in steps b) and c); wherein the presence of, or a significant increase in, the copy number, amount, and/or activity of, the at least one biomarker listed in Table 1 in the subject sample relative to the control copy number, amount, and/or activity of the at least one biomarker indicates that the subject afflicted with the cancer or at risk for developing the cancer would benefit from inhibiting the copy number, amount, and/or activity of the at least one biomarker listed in Table 1. In one embodiment, the method described herein further comprises recommending, prescribing, or administering an agent that inhibits the at least one biomarker listed in Table 1 if the cancer is determined to benefit from the agent. In another embodiment, the method described herein further comprises administering at least one additional cancer therapy that is administered before, after, or concurrently with the agent. In still another embodiment, the method described herein further comprises recommending, prescribing, or administering cancer therapy other than an agent that inhibits the at least one biomarker listed in Table 1 if the cancer is determined to not benefit from the agent. In yet another embodiment, the cancer therapy is selected from the group consisting of immunotherapy, targeted therapy, chemotherapy, radiation therapy, hormonal therapy, an anti-cancer vaccine, an anti-cancer virus, and a checkpoint inhibitor. In one embodiment, the control sample is determined from a cancerous or non-cancerous sample from either the patient or a member of the same species to which the patient belongs. In another embodiment, the control sample comprises cells.

In yet another aspect, a method for predicting the clinical outcome of a subject afflicted with a cancer expressing one or more biomarkers listed in Table 1 or a fragment thereof to treatment with an immunotherapy is provided, the method comprising a) determining the copy number, amount, and/or activity of at least one biomarker listed in Table 1 in a subject sample; b) determining the copy number, amount, and/or activity of the at least one biomarker in a control having a good clinical outcome; and c) comparing the copy number, amount, and/or activity of the at least one biomarker in the subject sample and in the control; wherein the presence of, or a significant increase in, the copy number, amount, and/or activity of, the at least one biomarker listed in Table 1 in the subject sample as compared to the copy number, amount and/or activity in the control, is an indication that the subject has a poor clinical outcome.

In another aspect, a method for monitoring the progression of a cancer in a subject, wherein the subject is administered a therapeutically effective amount of an agent that inhibits the copy number, amount, and/or activity of at least one biomarker listed in Table 1 and an immunotherapy is provided, the method comprising a) detecting in a subject sample at a first point in time the copy number, amount, and/or activity of at least one biomarker listed in Table 1; b) repeating step a) at a subsequent point in time; and c) comparing the amount or activity of at least one biomarker listed in Table 1 detected in steps a) and b) to monitor the progression of the cancer in the subject.

In still another aspect, a method of assessing the efficacy of an agent that inhibits the copy number, amount, and/or activity of at least one biomarker listed in Table 1 and an immunotherapy for treating a cancer in a subject is provided, comprising a) detecting in a subject sample at a first point in time the copy number, amount, and/or or activity of at least one biomarker listed in Table 1; b) repeating step a) during at least one subsequent point in time after administration of the agent and the immunotherapy; and c) comparing the copy number, amount, and/or activity detected in steps a) and b), wherein the absence of, or a significant decrease in, the copy number, amount, and/or activity of, the at least one biomarker listed in Table 1, in the subsequent sample as compared to the copy number, amount, and/or activity in the sample at the first point in time, indicates that the agent and immunotherapy treats the cancer in the subject.

As described above, numerous embodiments can be applied to any aspect of the present invention. For example, in one embodiment, between the first point in time and the subsequent point in time, the subject has undergone treatment, completed treatment, and/or is in remission for the cancer. In another embodiment, the cancer treatment is selected from the group consisting of immunotherapy, targeted therapy, chemotherapy, radiation therapy, hormonal therapy, an anti-cancer vaccine, an anti-cancer virus, and a checkpoint inhibitor. In still another embodiment, the first and/or at least one subsequent sample is selected from the group consisting of *ex vivo* and *in vivo* samples. In yet another embodiment, the first and/or at least one subsequent sample is a portion of a single sample or pooled samples obtained from the subject. In another embodiment, the sample described herein comprises cells, serum, peritumoral tissue, and/or intratumoral tissue obtained from the subject. In another embodiment, the one or more biomarkers listed in Table 1 comprise RIPK1, BIRC2, TBK1, TRAF3, RNF31, RBCK1, OTULIN, TRAF6, TAB2, and TNIP1. In still another embodiment, the cancer is melanoma. In yet another embodiment, the cancer is in

a subject and the subject is a mammal. In one embodiment, the mammal is a mouse or a human. In another embodiment, the mammal is a human.

In still another aspect, an agent that inhibits one or more biomarkers listed in Table 1, such as one or more regulators of TNF signaling and/or NF-κB activation (*e.g.*, RIPK1, BIRC2, TBK1, TRAF3, RNF31, RBCK1, OTULIN, TRAF6, TAB2, and TNIP1), is provided for treating a cancer in a subject, in combination with an immunotherapy. Such agent may comprise a small molecule inhibitor, an RNA interfering agent, an antisense oligonucleotide, a peptide or peptidomimetic inhibitor, an aptamer, and/or an intrabody, as described herein.

In yet another aspect, a vector comprising an agent that inhibits one or more biomarkers listed in Table 1, such as one or more regulators of TNF signaling and/or NF-κB activation (*e.g.*, RIPK1, BIRC2, TBK1, TRAF3, RNF31, RBCK1, OTULIN, TRAF6, TAB2, and TNIP1), for treating a cancer in a subject, in combination with an immunotherapy, is provided.

In another aspect, a host cell which comprises an agent that inhibits one or more biomarkers listed in Table 1, such as one or more regulators of TNF signaling and/or NF-κB activation (*e.g.*, RIPK1, BIRC2, TBK1, TRAF3, RNF31, RBCK1, OTULIN, TRAF6, TAB2, and TNIP1), for treating a cancer in a subject, in combination with an immunotherapy, is provided.

In still another aspect, a host cell which comprises a vector comprising an agent that inhibits one or more biomarkers listed in Table 1, such as one or more regulators of TNF signaling and/or NF-κB activation (*e.g.*, RIPK1, BIRC2, TBK1, TRAF3, RNF31, RBCK1, OTULIN, TRAF6, TAB2, and TNIP1), for treating a cancer in a subject, in combination with an immunotherapy, is provided.

In yet another aspect, a device or kit comprising the agent that inhibits one or more biomarkers listed in Table 1, such as one or more regulators of TNF signaling and/or NF-κB activation (*e.g.*, RIPK1, BIRC2, TBK1, TRAF3, RNF31, RBCK1, OTULIN, TRAF6, TAB2, and TNIP1), for treating a cancer in a subject, in combination with an immunotherapy, is provided.

Brief Description of the Drawings

Figure 1 includes 6 panels, identified as panels A, B, C, D, E, and F, which show that *in vivo* pooled loss-of-function screening using CRISPR/Cas9 in tumor cells recovers

known mediators of immune evasion. Panel A shows a schematic diagram of the *in vivo* screening system using the B16 transplantable tumor model. Tumor volumes (in mm^3) were compared under each conditions, averaged for each group at each time point (Panel B, left) or for individual animals on the day of sacrifice (Panel B, right). Bars represent means, while whiskers represent standard deviation. Enrichment analysis was carried out using a hypergeometric test to show functional classes of genes, (from the Gene Ontology Consortium database (GO)) targeted by sgRNAs, that were enriched or depleted in tumors in animals, including animals treated with irradiated tumor cell vaccine (GVAX) and anti-PD-1 antibody and the $TCR\alpha^{-/-}$ animals (Panel C). Frequency histogram (Panel D, top) and collapsed histograms (Panel D, middle) of enrichment or depletion (normalized as Z scores) are shown for all 9,992 sgRNAs screened. Enrichment/depletion scores are averaged from 10 mice per condition. sgRNAs targeting PD-L1 are indicated by the red lines (Panel D, middle). PD-L1 expression is compared among Cas9-expressing B16 tumor cells transfected with one of the four sgRNAs targeting PD-L1 (red) or a control sgRNA (grey) (Panel D, bottom). Similar to Panel D, Panel E shows the depletion of CD47 by its specific sgRNAs (indicated in red (top and middle) and CD47 expression after CRISPR editing with sgRNAs targeting CD47 (bottom). Panel F compares tumor volumes over time between CD47 null (red) and control (grey) tumors growing in mice treated with GVAX and PD-1 blockade (average and standard error of the mean; n = 10 animals per group). ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Figure 2 includes 6 panels, identified as panels A, B, C, D, E, and F, which show the performance analysis of the screening in Figure 1. Panel A shows Western blot of B16 cell lysate for Cas9 and β-ACTIN with or without transduction with a lentiviral vector encoding Cas9. A pie chart shows the fraction of genes targeted in the screening in each of the GO term categories indicated (Panel B). Two-dimensional histograms show the pair-wise distribution of sgRNAs abundance (averaged for each condition) (Panel C). Saturation analysis of animal replicates from the three *in vivo* screening conditions is shown in Panel D. Pearson correlations are calculated for the library distribution in one animal vs. any other animal, then for two animals averaged versus any other two averaged, and so on. Saturation approaches r = 0.95. A matrix of the Pearson correlations of the library distribution from one animal compared to any other animal for B16 Pool 1 is shown (Panel E). Expression of CD47 by B16 cells transfected with either CD47-targeting (red) or control (grey) sgRNA is compared (Panel F).

Figure 3 includes 3 panels, identified as panels A, B, and C, which show that loss of TNF signaling and/or NF-κB activation regulators (e.g., *Ripk1*) causes resistance to immunotherapy. Panel A shows frequency histogram (top) and collapsed histograms (below) of enrichment or depletion (normalized as z scores) for all sgRNAs in GVAX+ PD-1 blockade-treated mice relative to $TCR\alpha^{-/-}$ mice, as in Figure 1. Red bars indicate the sgRNAs for the genes listed on the left. Representative flow plots show the frequencies of control or *Ripk1* null B16 cells for the conditions indicated (Panel B). Specifically, mixtures of *Ripk1* null tumors and control tumors (in a 1:10 ratio) were tested *in vitro*, in $TCR\alpha^{-/-}$ mice, or GVAX + PD-1 blockade-treated wild-type mice. The B16 cell numbers in immunotherapy-treated wild-type mice (treated with different sgRNAs) were compared relative to those in $TCR\alpha^{-/-}$ mice, the change in the ratios (log_2 normalized fold change) is shown in Panel C (mean and standard deviation; n = 8-10 mice per group). **** $p <$ 0.0001.

Detailed Description of the Invention

It has been determined herein that negative regulators of one or more biomarkers listed in Table 1, such as one or more regulators of TNF signaling and/or NF-κB activation (*e.g.*, RIPK1, BIRC2, TBK1, TRAF3, RNF31, RBCK1, OTULIN, TRAF6, TAB2, and TNIP1) can be used to increase interferon sensing by tumor cells and augment tumor immunity and immunotherapies. Thus, the instant disclosure provides at least a method of treating cancers, *e.g.*, those cancer types otherwise not responsive or weakly responsive to immunotherapies, with a combination of a negative regulator of one or more biomarkers listed in Table 1, such as one or more regulators of TNF signaling and/or NF-κB activation (*e.g.*, RIPK1, BIRC2, TBK1, TRAF3, RNF31, RBCK1, OTULIN, TRAF6, TAB2, and TNIP1), in combination with another immunotherapy. The results described herein are unexpected given that analyses of RIPK1 function has heretofore been largely confined to hematopoietic cells and not examined in cancer cells, as well as the fact that modulating sensitivity to immunotherapy is critical for immunotherapy effects rather than simply modulating interferon availability since interferon therapy is known to not significantly augment immunotherapy effects. Accordingly, the present invention provides exemplary RNA interfering agents inhibiting one or more biomarkers listed in Table 1, such as one or more regulators of TNF signaling and/or NF-κB activation (*e.g.*, RIPK1, BIRC2, TBK1, TRAF3, RNF31, RBCK1, OTULIN, TRAF6, TAB2, and TNIP1), which may be used in

the combination therapy and other methods described herein, such as agents that inhibit biomarker function and/or its ability to interact/bind to its substrates described herein, or by increasing its degradation and/or stability and/or interaction/binding to its inhibitors. Similarly, methods of screening for biomarker inhibitors and methods of diagnosing, prognosing, and monitoring cancer involving biomarker/immunotherapy combination therapies are provided.

I. Definitions

The articles "a" and "an" are used herein to refer to one or to more than one (*i.e.* to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

The term "altered amount" or "altered level" refers to increased or decreased copy number (*e.g.*, germline and/or somatic) of a biomarker nucleic acid, *e.g.*, increased or decreased expression level in a cancer sample, as compared to the expression level or copy number of the biomarker nucleic acid in a control sample. The term "altered amount" of a biomarker also includes an increased or decreased protein level of a biomarker protein in a sample, *e.g.*, a cancer sample, as compared to the corresponding protein level in a normal, control sample. Furthermore, an altered amount of a biomarker protein may be determined by detecting posttranslational modification such as methylation status of the marker, which may affect the expression or activity of the biomarker protein.

The amount of a biomarker in a subject is "significantly" higher or lower than the normal amount of the biomarker, if the amount of the biomarker is greater or less, respectively, than the normal level by an amount greater than the standard error of the assay employed to assess amount, and preferably at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200%, 300%, 350%, 400%, 500%, 600%, 700%, 800%, 900%, 1000% or than that amount. Alternately, the amount of the biomarker in the subject can be considered "significantly" higher or lower than the normal amount if the amount is at least about two, and preferably at least about three, four, or five times, higher or lower, respectively, than the normal amount of the biomarker. Such "significance" can also be applied to any other measured parameter described herein, such as for expression, inhibition, cytotoxicity, cell growth, and the like.

The term "altered level of expression" of a biomarker refers to an expression level or copy number of the biomarker in a test sample, *e.g.*, a sample derived from a patient suffering from cancer, that is greater or less than the standard error of the assay employed

to assess expression or copy number, and is preferably at least twice, and more preferably three, four, five or ten or more times the expression level or copy number of the biomarker in a control sample (*e.g.*, sample from a healthy subjects not having the associated disease) and preferably, the average expression level or copy number of the biomarker in several control samples. The altered level of expression is greater or less than the standard error of the assay employed to assess expression or copy number, and is preferably at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200%, 300%, 350%, 400%, 500%, 600%, 700%, 800%, 900%, 1000% or more times the expression level or copy number of the biomarker in a control sample (*e.g.*, sample from a healthy subjects not having the associated disease) and preferably, the average expression level or copy number of the biomarker in several control samples. In some embodiments, the level of the biomarker refers to the level of the biomarker itself, the level of a modified biomarker (*e.g.*, phosphorylated biomarker), or to the level of a biomarker relative to another measured variable, such as a control (*e.g.*, phosphorylated biomarker relative to an unphosphorylated biomarker).

The term "altered activity" of a biomarker refers to an activity of the biomarker which is increased or decreased in a disease state, *e.g.,* in a cancer sample, as compared to the activity of the biomarker in a normal, control sample. Altered activity of the biomarker may be the result of, for example, altered expression of the biomarker, altered protein level of the biomarker, altered structure of the biomarker, or, *e.g.,* an altered interaction with other proteins involved in the same or different pathway as the biomarker or altered interaction with transcriptional activators or inhibitors.

The term "altered structure" of a biomarker refers to the presence of mutations or allelic variants within a biomarker nucleic acid or protein, *e.g.,* mutations which affect expression or activity of the biomarker nucleic acid or protein, as compared to the normal or wild-type gene or protein. For example, mutations include, but are not limited to substitutions, deletions, or addition mutations. Mutations may be present in the coding or non-coding region of the biomarker nucleic acid.

Unless otherwise specified here within, the terms "antibody" and "antibodies" refers to antigen-binding portions adaptable to be expressed within cells as "intracellular antibodies." (Chen *et al.* (1994) *Human Gene Ther.* 5:595-601). Methods are well-known in the art for adapting antibodies to target (*e.g.*, inhibit) intracellular moieties, such as the use of single-chain antibodies (scFvs), modification of immunoglobulin VL domains for

hyperstability, modification of antibodies to resist the reducing intracellular environment, generating fusion proteins that increase intracellular stability and/or modulate intracellular localization, and the like. Intracellular antibodies can also be introduced and expressed in one or more cells, tissues or organs of a multicellular organism, for example for prophylactic and/or therapeutic purposes (*e.g.*, as a gene therapy) (see, at least PCT Publs. WO 08/020079, WO 94/02610, WO 95/22618, and WO 03/014960; U.S. Pat. No. 7,004,940; Cattaneo and Biocca (1997) *Intracellular Antibodies: Development and Applications* (Landes and Springer-Verlag publs.); Kontermann (2004) *Methods* 34:163-170; Cohen *et al.* (1998) *Oncogene* 17:2445-2456; Auf der Maur *et al.* (2001) *FEBS Lett.* 508:407-412; Shaki-Loewenstein *et al.* (2005) *J. Immunol. Meth.* 303:19-39).

Antibodies may be polyclonal or monoclonal; xenogeneic, allogeneic, or syngeneic; or modified forms thereof (*e.g.* humanized, chimeric, etc.). Antibodies may also be fully human. Preferably, antibodies of the present invention bind specifically or substantially specifically to a biomarker polypeptide or fragment thereof. The terms "monoclonal antibodies" and "monoclonal antibody composition", as used herein, refer to a population of antibody polypeptides that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of an antigen, whereas the term "polyclonal antibodies" and "polyclonal antibody composition" refer to a population of antibody polypeptides that contain multiple species of antigen binding sites capable of interacting with a particular antigen. A monoclonal antibody composition typically displays a single binding affinity for a particular antigen with which it immunoreacts.

Antibodies may also be "humanized", which is intended to include antibodies made by a non-human cell having variable and constant regions which have been altered to more closely resemble antibodies that would be made by a human cell. For example, by altering the non-human antibody amino acid sequence to incorporate amino acids found in human germline immunoglobulin sequences. The humanized antibodies of the present invention may include amino acid residues not encoded by human germline immunoglobulin sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*), for example in the CDRs. The term "humanized antibody", as used herein, also includes antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

The term "assigned score" refers to the numerical value designated for each of the biomarkers after being measured in a patient sample. The assigned score correlates to the absence, presence or inferred amount of the biomarker in the sample. The assigned score can be generated manually (*e.g.*, by visual inspection) or with the aid of instrumentation for image acquisition and analysis. In certain embodiments, the assigned score is determined by a qualitative assessment, for example, detection of a fluorescent readout on a graded scale, or quantitative assessment. In one embodiment, an "aggregate score," which refers to the combination of assigned scores from a plurality of measured biomarkers, is determined. In one embodiment the aggregate score is a summation of assigned scores. In another embodiment, combination of assigned scores involves performing mathematical operations on the assigned scores before combining them into an aggregate score. In certain, embodiments, the aggregate score is also referred to herein as the "predictive score."

The term "biomarker" refers to a measurable entity of the present invention that has been determined to be predictive of combinatorial therapy effects on a cancer using one or more inhibitors of TNF signaling and/or NF-κB activation (*e.g.*, RIPK1, BIRC2, TBK1, TRAF3, RNF31, RBCK1, OTULIN, TRAF6, TAB2, and TNIP1), in combination with an immunotherapy (*e.g.*, immune checkpoint inhibitors). Biomarkers can include, without limitation, nucleic acids and proteins, including those shown in the Tables, the Examples, the Figures, and otherwise described herein. As described herein, any relevant characteristic of a biomarker can be used, such as the copy number, amount, activity, location, modification (*e.g.*, phosphorylation), and the like.

A "blocking" antibody or an antibody "antagonist" is one which inhibits or reduces at least one biological activity of the antigen(s) it binds. In certain embodiments, the blocking antibodies or antagonist antibodies or fragments thereof described herein substantially or completely inhibit a given biological activity of the antigen(s).

The term "body fluid" refers to fluids that are excreted or secreted from the body as well as fluids that are normally not (*e.g.* amniotic fluid, aqueous humor, bile, blood and blood plasma, cerebrospinal fluid, cerumen and earwax, cowper's fluid or pre-ejaculatory fluid, chyle, chyme, stool, female ejaculate, interstitial fluid, intracellular fluid, lymph, menses, breast milk, mucus, pleural fluid, pus, saliva, sebum, semen, serum, sweat, synovial fluid, tears, urine, vaginal lubrication, vitreous humor, vomit).

The terms "cancer" or "tumor" or "hyperproliferative" refer to the presence of cells possessing characteristics typical of cancer-causing cells, such as uncontrolled proliferation,

immortality, metastatic potential, rapid growth and proliferation rate, and certain characteristic morphological features. Unless otherwise stated, the terms include metaplasias. In some embodiments, such cells exhibit such characteristics in part or in full due to the expression and activity of the signaling pathways regulated by one or more biomarkers listed in Table 1, such as one or more regulators of TNF signaling and/or NF-κB activation (*e.g.*, RIPK1, BIRC2, TBK1, TRAF3, RNF31, RBCK1, OTULIN, TRAF6, TAB2, and TNIP1) (*e.g.*, NF-kappaB Signaling pathway, MAP kinase pathway, JAK-STAT signaling pathway, or other signaling pathways involving receptor tyrosine kinases, non-receptor tyrosine kinases, Src family kinases, and/or signal transducer and activator of transcription (STAT) proteins). In some embodiments, the cancer cells described herein are not sensitive to at least one of immunotherapies. Such insensitivity, without limitation, may be related to the inactivation or decreased activation, compared to control cells (*e.g.*, normal and/or wild-type non-cancer cells, and/or cancer cells without this insensitivity to immunotherapies), of interferon signaling (*e.g.*, IFNγ signaling) in such cancer cells and/or other surrounding cells and/or cells localized near to such cancer cells. In some embodiments, the cancer cells are treatable with an agent capable of antagonizing one or more biomarkers listed in Table 1, such as one or more regulators of TNF signaling and/or NF-κB activation (*e.g.*, RIPK1, BIRC2, TBK1, TRAF3, RNF31, RBCK1, OTULIN, TRAF6, TAB2, and TNIP1). In some embodiments, the treatment with the agent antagonizing one or more biomarkers listed in Table 1 as described herein would increase IFNγ signaling in such cancer cells, compared to pre-treatment situations, or would restore IFNγ signaling in such cancer cells to at least comparable to the levels in control cells, so that such cancer cells would regain sensitivity to immunotherapies. The term "interferon signaling" or "IFNγ signaling" used herein refers to any cell signaling downstream and/or related to the interaction of interferon (*e.g.*, IFNγ) and their receptor(s). Some exemplary IFNγ cell signaling include, without limitation, the activation of macrophages and/or induction of Class II major histocompatibility complex (MHC) molecule expression, and/or activation of multiple immune effector genes through the Janus kinase (JAK)-STAT signaling pathway (e.g., through STAT1 transcription factor).

Cancer cells are often in the form of a tumor, but such cells may exist alone within an animal, or may be a non-tumorigenic cancer cell, such as a leukemia cell. As used herein, the term "cancer" includes premalignant as well as malignant cancers. Cancers include, but are not limited to, B cell cancer, *e.g.*, multiple myeloma, Waldenström's

macroglobulinemia, the heavy chain diseases, such as, for example, alpha chain disease, gamma chain disease, and mu chain disease, benign monoclonal gammopathy, and immunocytic amyloidosis, melanomas, breast cancer, lung cancer, bronchus cancer, colorectal cancer, prostate cancer, pancreatic cancer, stomach cancer, ovarian cancer, urinary bladder cancer, brain or central nervous system cancer, peripheral nervous system cancer, esophageal cancer, cervical cancer, uterine or endometrial cancer, cancer of the oral cavity or pharynx, liver cancer, kidney cancer, testicular cancer, biliary tract cancer, small bowel or appendix cancer, salivary gland cancer, thyroid gland cancer, adrenal gland cancer, osteosarcoma, chondrosarcoma, cancer of hematologic tissues, and the like. Other non-limiting examples of types of cancers applicable to the methods encompassed by the present invention include human sarcomas and carcinomas, *e.g.*, fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, colorectal cancer, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, liver cancer, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, bone cancer, brain tumor, testicular cancer, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, retinoblastoma; leukemias, *e.g.*, acute lymphocytic leukemia and acute myelocytic leukemia (myeloblastic, promyelocytic, myelomonocytic, monocytic and erythroleukemia); chronic leukemia (chronic myelocytic (granulocytic) leukemia and chronic lymphocytic leukemia); and polycythemia vera, lymphoma (Hodgkin's disease and non-Hodgkin's disease), multiple myeloma, Waldenstrom's macroglobulinemia, and heavy chain disease. In some embodiments, cancers are epithlelial in nature and include but are not limited to, bladder cancer, breast cancer, cervical cancer, colon cancer, gynecologic cancers, renal cancer, laryngeal cancer, lung cancer, oral cancer, head and neck cancer, ovarian cancer, pancreatic cancer, prostate cancer, or skin cancer. In other embodiments, the cancer is breast cancer, prostate cancer, lung cancer, or colon cancer. In still other

embodiments, the epithelial cancer is non-small-cell lung cancer, nonpapillary renal cell carcinoma, cervical carcinoma, ovarian carcinoma (*e.g.*, serous ovarian carcinoma), or breast carcinoma. The epithelial cancers may be characterized in various other ways including, but not limited to, serous, endometrioid, mucinous, clear cell, Brenner, or undifferentiated.

In certain embodiments, the cancer encompasses colorectal cancer (*e.g.*, colorectal carcinoma).

The term "colorectal cancer" as used herein, is meant to include cancer of cells of the intestinal tract below the small intestine (*e.g.*, the large intestine (colon), including the cecum, ascending colon, transverse colon, descending colon, and sigmoid colon, and rectum). Additionally, as used herein, the term "colorectal cancer" is meant to further include cancer of cells of the duodenum and small intestine (jejunum and ileum). Colorectal cancer also includes neoplastic diseases involving proliferation of a single clone of cells of the colon and includes adenocarcinoma and carcinoma of the colon whether in a primary site or metastasized.

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and ranks second in cancer mortality. Extensive genetic and genomic analysis of human CRC has uncovered germline and somatic mutations relevant to CRC biology and malignant transformation (Fearon *et al.* (1990) *Cell* 61, 759-767). These mutations have been linked to well-defined disease stages from aberrant crypt proliferation or hyperplasic lesions to benign adenomas, to carcinoma *in situ*, and finally to invasive and metastatic disease, thereby establishing a genetic paradigm for cancer initiation and progression. Genetic and genomic instability are catalysts for colon carcinogenesis (Lengauer *et al.* (1998) *Nature* 396:643-649). CRC can present with two distinct genomic profiles that have been termed (i) chromosomal instability neoplasia (CIN), characterized by rampant structural and numerical chromosomal aberrations driven in part by telomere dysfunction (Artandi *et al.* (2000) *Nature* 406:641-645; Fodde *et al.* (2001) *Nat. Rev. Cancer* 1:55-67; Maser and DePinho (2002) *Science* 297:565-569; Rudolph *et al.* (2001) *Nat. Genet.* 28:155-159) and mitotic aberrations (Lengauer *et al.* (1998) *Nature* 396:643-649) and (ii) microsatellite instability neoplasia (MIN), characterized by near diploid karyotypes with alterations at the nucleotide level due to mutations in mismatch repair (MMR) genes (Fishel *et al.* (1993) *Cell* 75:1027-1038; Ilyas *et al.* (1999) *Eur. J. Cancer* 35:335-351; Modrich (1991) *Annu. Rev. Genet.* 25:229-253; Parsons *et al.* (1995) *Science* 268:738-740; Parsons *et al.* (1993)

Cell 75:1227-1236). Germline MMR mutations are highly penetrant lesions which drive the MIN phenotype in hereditary nonpolyposis colorectal cancers, accounting for 1-5% of CRC cases (de la Chapelle (2004) *Nat. Rev. Cancer* 4:769-780; Lynch and de la Chapelle (1999) *J. Med. Genet.* 36:801-818; Umar *et al.* (2004) *Nat. Rev. Cancer* 4:153-158). While CIN and MIN are mechanistically distinct, their genomic and genetic consequences emphasize the requirement of dominant mutator mechanisms to drive intestinal epithelial cells towards a threshold of oncogenic changes needed for malignant transformation.

A growing number of genetic mutations have been identified and functionally validated in CRC pathogenesis. Activation of the WNT signaling pathway is an early requisite event for adenoma formation. Somatic alterations are present in *APC* in greater than 70% of nonfamilial sporadic cases and appear to contribute to genomic instability and induce the expression of *c-myc* and *Cyclin D1* (Fodde *et al.* (2001) *Nat. Rev. Cancer* 1:55-67), while activating *β-catenin* mutations represent an alternative means of WNT pathway deregulation in CRC (Morin (1997) *Science* 275:1787-1790). *K-Ras* mutations occur early in neoplastic progression and are present in approximately 50% of large adenomas (Fearon and Gruber (2001) Molecular abnormalities in colon and rectal cancer, ed. J. Mendelsohm, P.H., M. Israel, and L. Liotta, W.B. Saunders, Philadelphia). The *BRAF* serine/threonine kinase and *PIK3CA* lipid kinase are mutated in 5-18% and 28% of sporadic CRCs, respectively (Samuels *et al.* (2004) *Science* 304:554; Davies *et al.* (2002) *Nature* 417:949-954; Rajagopalan *et al.* (2002) *Nature* 418:934; Yuen *et al.* (2002) *Cancer Res.* 62:6451-6455). *BRAF* and *K-ras* mutations are mutually exclusive in CRC, suggesting over-lapping oncogenic activities (Davies *et al.* (2002) *Nature* 417:949-954; Rajagopalan *et al.* (2002) *Nature* 418:934). Mutations associated with CRC progression, specifically the adenoma-to-carcinoma transition, target the *TP53* and the TGF-β pathways (Markowitz *et al.* (2002) *Cancer Cell* 1:233-236). Greater than 50% of CRCs harbor *TP53* inactivating mutations (Fearon and Gruber (2001) Molecular abnormalities in colon and rectal cancer, ed. J. Mendelsohm, P.H., M. Israel, and L. Liotta, W.B. Saunders, Philadelphia) and 30% of cases possess *TGFβ-RII* mutations (Markowitz (2000) *Biochim. Biophys. Acta* 1470:M13-M20; Markowitz *et al.* (1995) *Science* 268:1336-1338). MIN cancers consistently inactivate *TGFβ-RII* by frameshift mutations, whereas CIN cancers target the pathway via inactivating somatic mutations in the *TGFβ-RII* kinase domain (15%) or in the downstream signaling components of the pathway, including *SMAD4* (15%) or *SMAD2* (5%) transcription factors (Markowitz (2000) *Biochim. Biophys. Acta* 1470:M13-M20). In some embodiments, the

colorectal cancer is microsatellite instable (MSI) colorectal cancer (Llosa *et al.* (2014) *Cancer Disc.* CD-14-0863; published online Oct. 30, 2014). MSI represents about 15% of sporadic CRC and about 5-6% of stage IV CRCs. MSI is caused by epigenetic silencing or mutation of DNA mismatch repair genes and typically presents with lower stage disease than microsatellite stable subset (MSS) CRC. MSI highly express immune checkpoints, such as PD-1, PD-L1, CTLA-4, LAG-3, and IDO. In other embodiments, the colorectal cancer is MSS CRC.

In certain embodiments, the cancer encompasses melanoma. The term "melanoma" as used herein, is generally meant to include cancers that develop from the pigment-containing cells, known as melanocytes, in the basal layer of the epidermis. Melanomas typically occur in the skin but may rarely occur in the mouth, intestines, or eye. In women they most commonly occur on the legs, while in men they are most common on the back. Sometimes they develop from a mole with concerning changes including an increase in size, irregular edges, change in color, itchiness, or skin breakdown. Thus, the term "melanoma" also includes cancers developing from these cells, tissues, and organs.

Melanomas are among the most dangerous forms of skin cancer and develop when unrepaired DNA damage to skin cells (most often caused by ultraviolet radiation from sunshine or tanning beds) triggers gene mutations that lead the skin cells to multiply rapidly and form malignant tumors. The primary cause of melanoma is ultraviolet light (UV) exposure in those with low levels of skin pigment. Melanomas often resemble moles; some develop from moles. Those with many moles, a history of affected family members, and who have poor immune function are at greater risk. A number of rare genetic defects such as xeroderma pigmentosum also increase risk (Azoury and Lange, 2014 *Surg Clin North Am*. 2014 94:945-962).

Melanoma can be divided into different types, including, at least, lentigo maligna, lentigo maligna melanoma, superficial spreading melanoma, acral lentiginous melanoma, mucosal melanoma, nodular melanoma, polypoid melanoma, desmoplastic melanoma, amelanotic melanoma, soft-tissue melanoma, melanoma with small nevus-like cells, melanoma with features of a Spitz nevus, uveal melanoma, etc. (see James, et al., 2006 *Andrews' Diseases of the Skin: clinical Dermatology.* Saunders Elsevier. pp. 694–9)

Diagnosis is by biopsy of any concerning skin lesion, including, at least, shave (tangential) biopsy, punch biopsy, incisional and excisional biopsies, "optical" biopsies (e.g., by reflectance confocal microscopy (RCM)), fine needle aspiration (FNA) biopsy,

surgical lymph node biopsy, sentinel lymph node biopsy, etc. In addition, visual inspection may also be used for diagnosis, such as a popular method for the signs and symptoms of melanoma as mnemonic "ABCDE": Asymmetrical skin lesion, Border of the lesion is irregular, Color: melanomas usually have multiple colors, Diameter: moles greater than 6 mm are more likely to be melanomas than smaller moles, and Enlarging: Enlarging or evolving. Another method as the "ugly duckling sign" is also known in the art (Mascaro and Mascaro, 1998 *Arch Dermatol.* 134: 1484-1485).

Treatment of melanoma includes surgery, chemotherapy (such as temozolomide, dacarbazine (also termed DTIC), etc.), radiation therapy, oncolytic virotherapy (e.g., see Forbes *et al.*, 2013 *Front. Genet.* 4:184), and immunotherapy (e.g., interleukin-2 (IL-2), interferon, etc.). Targeted therapies (*e.g.*, as in Maverakis *et al.*, 2015 *Acta Derm Venereol.* 95: 516-524) may include: 1) adoptive cell therapy (ACT) using TILs immune cells (tumor infiltrating lymphocytes) isolated from a person's own melanoma tumor). Cells are grown in large numbers in a laboratory and returned to the patient after a treatment that temporarily reduces normal T cells in the patient's body. TIL therapy following lymphodepletion can result in durable complete response in a variety of setups (Besser *et al.*, 2010 *Clin. Cancer Res.* 16:2646-2655); and 2) adoptive transfer of genetically altered (expressing T cell receptors (TCRs)) autologous lymphocytes into patient's lymphocytes, where the altered lymphocytes recognize and bind to the surface of melanoma cells and kill them. Other therapies include, at least, B-Raf inhibitors (such as vemurafenib, see Chapman *et al.*, 2011 *N. Engl. J. Med.* 364:2507-2516) and ipilimumab (alone or in combination with dacarbazine, see, e.g., Robert *et al.* (2011) *N. Engl. J. Med.* 364:2517-2526).

The term "coding region" refers to regions of a nucleotide sequence comprising codons which are translated into amino acid residues, whereas the term "noncoding region" refers to regions of a nucleotide sequence that are not translated into amino acids (*e.g.*, 5' and 3' untranslated regions).

The term "complementary" refers to the broad concept of sequence complementarity between regions of two nucleic acid strands or between two regions of the same nucleic acid strand. It is known that an adenine residue of a first nucleic acid region is capable of forming specific hydrogen bonds ("base pairing") with a residue of a second nucleic acid region which is antiparallel to the first region if the residue is thymine or uracil. Similarly, it is known that a cytosine residue of a first nucleic acid strand is capable

of base pairing with a residue of a second nucleic acid strand which is antiparallel to the first strand if the residue is guanine. A first region of a nucleic acid is complementary to a second region of the same or a different nucleic acid if, when the two regions are arranged in an antiparallel fashion, at least one nucleotide residue of the first region is capable of base pairing with a residue of the second region. Preferably, the first region comprises a first portion and the second region comprises a second portion, whereby, when the first and second portions are arranged in an antiparallel fashion, at least about 50%, and preferably at least about 75%, at least about 90%, or at least about 95% of the nucleotide residues of the first portion are capable of base pairing with nucleotide residues in the second portion. More preferably, all nucleotide residues of the first portion are capable of base pairing with nucleotide residues in the second portion.

The term "control" refers to any reference standard suitable to provide a comparison to the expression products in the test sample. In one embodiment, the control comprises obtaining a "control sample" from which expression product levels are detected and compared to the expression product levels from the test sample. Such a control sample may comprise any suitable sample, including but not limited to a sample from a control cancer patient (can be stored sample or previous sample measurement) with a known outcome; normal tissue or cells isolated from a subject, such as a normal patient or the cancer patient, cultured primary cells/tissues isolated from a subject such as a normal subject or the cancer patient, adjacent normal cells/tissues obtained from the same organ or body location of the cancer patient, a tissue or cell sample isolated from a normal subject, or a primary cells/tissues obtained from a depository. In another preferred embodiment, the control may comprise a reference standard expression product level from any suitable source, including but not limited to housekeeping genes, an expression product level range from normal tissue (or other previously analyzed control sample), a previously determined expression product level range within a test sample from a group of patients, or a set of patients with a certain outcome (for example, survival for one, two, three, four years, etc.) or receiving a certain treatment (for example, standard of care cancer therapy). It will be understood by those of skill in the art that such control samples and reference standard expression product levels can be used in combination as controls in the methods of the present invention. In one embodiment, the control may comprise normal or non-cancerous cell/tissue sample. In another preferred embodiment, the control may comprise an expression level for a set of patients, such as a set of cancer patients, or for a set of cancer patients receiving a certain

treatment, or for a set of patients with one outcome versus another outcome. In the former case, the specific expression product level of each patient can be assigned to a percentile level of expression, or expressed as either higher or lower than the mean or average of the reference standard expression level. In another preferred embodiment, the control may comprise normal cells, cells from patients treated with combination chemotherapy, and cells from patients having benign cancer. In another embodiment, the control may also comprise a measured value for example, average level of expression of a particular gene in a population compared to the level of expression of a housekeeping gene in the same population. Such a population may comprise normal subjects, cancer patients who have not undergone any treatment (*i.e.*, treatment naive), cancer patients undergoing standard of care therapy, or patients having benign cancer. In another preferred embodiment, the control comprises a ratio transformation of expression product levels, including but not limited to determining a ratio of expression product levels of two genes in the test sample and comparing it to any suitable ratio of the same two genes in a reference standard; determining expression product levels of the two or more genes in the test sample and determining a difference in expression product levels in any suitable control; and determining expression product levels of the two or more genes in the test sample, normalizing their expression to expression of housekeeping genes in the test sample, and comparing to any suitable control. In particularly preferred embodiments, the control comprises a control sample which is of the same lineage and/or type as the test sample. In another embodiment, the control may comprise expression product levels grouped as percentiles within or based on a set of patient samples, such as all patients with cancer. In one embodiment a control expression product level is established wherein higher or lower levels of expression product relative to, for instance, a particular percentile, are used as the basis for predicting outcome. In another preferred embodiment, a control expression product level is established using expression product levels from cancer control patients with a known outcome, and the expression product levels from the test sample are compared to the control expression product level as the basis for predicting outcome. As demonstrated by the data below, the methods of the present invention are not limited to use of a specific cut-point in comparing the level of expression product in the test sample to the control.

The "copy number" of a biomarker nucleic acid refers to the number of DNA sequences in a cell (*e.g.*, germline and/or somatic) encoding a particular gene product.

Generally, for a given gene, a mammal has two copies of each gene. The copy number can be increased, however, by gene amplification or duplication, or reduced by deletion. For example, germline copy number changes include changes at one or more genomic loci, wherein said one or more genomic loci are not accounted for by the number of copies in the normal complement of germline copies in a control (*e.g.*, the normal copy number in germline DNA for the same species as that from which the specific germline DNA and corresponding copy number were determined). Somatic copy number changes include changes at one or more genomic loci, wherein said one or more genomic loci are not accounted for by the number of copies in germline DNA of a control (*e.g.*, copy number in germline DNA for the same subject as that from which the somatic DNA and corresponding copy number were determined).

The "normal" copy number (*e.g.*, germline and/or somatic) of a biomarker nucleic acid or "normal" level of expression of a biomarker nucleic acid or protein is the activity/level of expression or copy number in a biological sample, *e.g.,* a sample containing tissue, whole blood, serum, plasma, buccal scrape, saliva, cerebrospinal fluid, urine, stool, and bone marrow, from a subject, *e.g.,* a human, not afflicted with cancer, or from a corresponding non-cancerous tissue in the same subject who has cancer.

As used herein, the term "costimulate" with reference to activated immune cells includes the ability of a costimulatory molecule to provide a second, non-activating receptor mediated signal (a "costimulatory signal") that induces proliferation or effector function. For example, a costimulatory signal can result in cytokine secretion, *e.g.,* in a T cell that has received a T cell-receptor-mediated signal. Immune cells that have received a cell-receptor mediated signal, *e.g.,* via an activating receptor are referred to herein as "activated immune cells."

The term "determining a suitable treatment regimen for the subject" is taken to mean the determination of a treatment regimen (*i.e.*, a single therapy or a combination of different therapies that are used for the prevention and/or treatment of the cancer in the subject) for a subject that is started, modified and/or ended based or essentially based or at least partially based on the results of the analysis according to the present invention. One example is starting an adjuvant therapy after surgery whose purpose is to decrease the risk of recurrence, another would be to modify the dosage of a particular chemotherapy. The determination can, in addition to the results of the analysis according to the present invention, be based on personal characteristics of the subject to be treated. In most cases,

the actual determination of the suitable treatment regimen for the subject will be performed by the attending physician or doctor.

The term "diagnosing cancer" includes the use of the methods, systems, and code of the present invention to determine the presence or absence of a cancer or subtype thereof in an individual. The term also includes methods, systems, and code for assessing the level of disease activity in an individual.

A molecule is "fixed" or "affixed" to a substrate if it is covalently or non-covalently associated with the substrate such that the substrate can be rinsed with a fluid (*e.g.* standard saline citrate, pH 7.4) without a substantial fraction of the molecule dissociating from the substrate.

The term "expression signature" or "signature" refers to a group of one or more coordinately expressed biomarkers related to a measured phenotype. For example, the genes, proteins, metabolites, and the like making up this signature may be expressed in a specific cell lineage, stage of differentiation, or during a particular biological response. The biomarkers can reflect biological aspects of the tumors in which they are expressed, such as the cell of origin of the cancer, the nature of the non-malignant cells in the biopsy, and the oncogenic mechanisms responsible for the cancer. Expression data and gene expression levels can be stored on computer readable media, *e.g.*, the computer readable medium used in conjunction with a microarray or chip reading device. Such expression data can be manipulated to generate expression signatures.

"Homologous" as used herein, refers to nucleotide sequence similarity between two regions of the same nucleic acid strand or between regions of two different nucleic acid strands. When a nucleotide residue position in both regions is occupied by the same nucleotide residue, then the regions are homologous at that position. A first region is homologous to a second region if at least one nucleotide residue position of each region is occupied by the same residue. Homology between two regions is expressed in terms of the proportion of nucleotide residue positions of the two regions that are occupied by the same nucleotide residue. By way of example, a region having the nucleotide sequence 5'-ATTGCC-3' and a region having the nucleotide sequence 5'-TATGGC-3' share 50% homology. Preferably, the first region comprises a first portion and the second region comprises a second portion, whereby, at least about 50%, and preferably at least about 75%, at least about 90%, or at least about 95% of the nucleotide residue positions of each of the

portions are occupied by the same nucleotide residue. More preferably, all nucleotide residue positions of each of the portions are occupied by the same nucleotide residue.

The term "immune cell" refers to cells that play a role in the immune response. Immune cells are of hematopoietic origin, and include lymphocytes, such as B cells and T cells; natural killer cells; myeloid cells, such as monocytes, macrophages, eosinophils, mast cells, basophils, and granulocytes.

The term "immunotherapy" or "immunotherapies" refer to any treatment that uses certain parts of a subject's immune system to fight diseases such as cancer. The subject's own immune system is stimulated (or suppressed), with or without administration of one or more agent for that purpose. Immunotherapies that are designed to elicit or amplify an immune response are referred to as "activation immunotherapies." Immunotherapies that are designed to reduce or suppress an immune response are referred to as "suppression immunotherapies." Any agent believed to have an immune system effect on the genetically modified transplanted cancer cells can be assayed to determine whether the agent is an immunotherapy and the effect that a given genetic modification has on the modulation of immune response. In some embodiments, the immunotherapy is cancer cell-specific. In some embodiments, immunotherapy can be "untargeted," which refers to administration of agents that do not selectively interact with immune system cells, yet modulates immune system function. Representative examples of untargeted therapies include, without limitation, chemotherapy, gene therapy, and radiation therapy.

Immunotherapy is one form of targeted therapy that may comprise, for example, the use of cancer vaccines and/or sensitized antigen presenting cells. For example, an oncolytic virus is a virus that is able to infect and lyse cancer cells, while leaving normal cells unharmed, making them potentially useful in cancer therapy. Replication of oncolytic viruses both facilitates tumor cell destruction and also produces dose amplification at the tumor site. They may also act as vectors for anticancer genes, allowing them to be specifically delivered to the tumor site. The immunotherapy can involve passive immunity for short-term protection of a host, achieved by the administration of pre-formed antibody directed against a cancer antigen or disease antigen (*e.g.*, administration of a monoclonal antibody, optionally linked to a chemotherapeutic agent or toxin, to a tumor antigen). For example, anti-VEGF and mTOR inhibitors are known to be effective in treating renal cell carcinoma. Immunotherapy can also focus on using the cytotoxic lymphocyte-recognized epitopes of cancer cell lines. Alternatively, antisense polynucleotides, ribozymes, RNA

interference molecules, triple helix polynucleotides and the like, can be used to selectively modulate biomolecules that are linked to the initiation, progression, and/or pathology of a tumor or cancer.

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In some embodiments, the immunotherapy described herein comprises at least one of immunogenic chemotherapies. The term "immunogenic chemotherapy" refers to any chemotherapy that has been demonstrated to induce immunogenic cell death, a state that is detectable by the release of one or more damage-associated molecular pattern (DAMP) molecules, including, but not limited to, calreticulin, ATP and HMGB1 (Kroemer *et al.* (2013), *Annu. Rev. Immunol.*, 31:51-72). Specific representative examples of consensus immunogenic chemotherapies include anthracyclines, such as doxorubicin and the platinum drug, oxaliplatin, 5'-fluorouracil, among others.

In some embodiments, immunotherapy comprises inhibitors of one or more immune checkpoints. The term "immune checkpoint" refers to a group of molecules on the cell surface of CD4+ and/or CD8+ T cells that fine-tune immune responses by down-modulating or inhibiting an anti-tumor immune response. Immune checkpoint proteins are well-known in the art and include, without limitation, CTLA-4, PD-1, VISTA, B7-H2, B7-H3, PD-L1, B7-H4, B7-H6, ICOS, HVEM, PD-L2, CD160, gp49B, PIR-B, KIR family receptors, TIM-1, TIM-3, TIM-4, LAG-3, GITR, 4-IBB, OX-40, BTLA, SIRP, CD47, CD48, 2B4 (CD244), B7.1, B7.2, ILT-2, ILT-4, TIGIT, HHLA2, butyrophilins, IDO, CD39, CD73 and A2aR (see, for example, WO 2012/177624). The term further encompasses biologically active protein fragment, as well as nucleic acids encoding full-length immune checkpoint proteins and biologically active protein fragments thereof. In some embodiment, the term further encompasses any fragment according to homology descriptions provided herein. In one embodiment, the immune checkpoint is PD-1.

Immune checkpoints and their sequences are well-known in the art and representative embodiments are described below. For example, the term "PD-1" refers to a member of the immunoglobulin gene superfamily that functions as a coinhibitory receptor having PD-L1 and PD-L2 as known ligands. PD-1 was previously identified using a subtraction cloning based approach to select for genes upregulated during TCR-induced activated T cell death. PD-1 is a member of the CD28/CTLA-4 family of molecules based on its ability to bind to PD-L1. Like CTLA-4, PD-1 is rapidly induced on the surface of T-cells in response to anti-CD3 (Agata *et al.* 25 (1996) *Int. Immunol.* 8:765). In contrast to CTLA-4, however, PD-1 is also induced on the surface of B-cells (in response to anti-IgM). PD-1 is also expressed on a subset of thymocytes and myeloid cells (Agata *et al.* (1996) *supra;* Nishimura *et al.* (1996) *Int. Immunol.* 8:773).

The nucleic acid and amino acid sequences of a representative human PD-1 biomarker is available to the public at the GenBank database under NM_005018.2 and NP_005009.2 and is shown in Table 1 (see also Ishida *et al.* (1992) 20 *EMBO J* 11:3887; Shinohara *et al.* (1994) *Genomics* 23:704; U.S. Patent 5,698,520). PD-1 has an extracellular region containing immunoglobulin superfamily domain, a transmembrane domain, and an intracellular region including an immunoreceptor tyrosine-based inhibitory motif (ITIM) (Ishida *et al.* (1992) *EMBO J.* 11:3887; Shinohara *et al.* (1994) *Genomics* 23:704; and U.S. Patent 5,698,520) and an immunoreceptor tyrosine-based switch motif (ITSM). These features also define a larger family of polypeptides, called the immunoinhibitory receptors, which also includes gp49B, PIR-B, and the killer inhibitory receptors (KIRs) (Vivier and Daeron (1997) *Immunol. Today* 18:286). It is often assumed that the tyrosyl phosphorylated ITIM and ITSM motif of these receptors interacts with SH2-domain containing phosphatases, which leads to inhibitory signals. A subset of these immunoinhibitory receptors bind to MHC polypeptides, for example the KIRs, and CTLA4 binds to B7-1 and B7-2. It has been proposed that there is a phylogenetic relationship between the MHC and B7 genes (Henry *et al.* (1999) *Immunol. Today* 20(6):285-8). Nucleic acid and polypeptide sequences of PD-1 orthologs in organisms other than humans are well-known and include, for example, mouse PD-1 (NM_008798.2 and NP_032824.1), rat PD-1 (NM_001106927.1 and NP_001100397.1), dog PD-1 (XM_543338.3 and XP_543338.3), cow PD-1 (NM_001083506.1 and NP_001076975.1), and chicken PD-1 (XM_422723.3 and XP_422723.2).

PD-1 polypeptides are inhibitory receptors capable of transmitting an inhibitory signal to an immune cell to thereby inhibit immune cell effector function, or are capable of promoting costimulation (*e.g.*, by competitive inhibition) of immune cells, *e.g.*, when present in soluble, monomeric form. Preferred PD-1 family members share sequence identity with PD-1 and bind to one or more B7 family members, *e.g.*, B7-1, B7-2, PD-1 ligand, and/or other polypeptides on antigen presenting cells.

The term "PD-1 activity," includes the ability of a PD-1 polypeptide to modulate an inhibitory signal in an activated immune cell, *e.g.*, by engaging a natural PD-1 ligand on an antigen presenting cell. Modulation of an inhibitory signal in an immune cell results in modulation of proliferation of, and/or cytokine secretion by, an immune cell. Thus, the term "PD-1 activity" includes the ability of a PD-1 polypeptide to bind its natural ligand(s), the ability to modulate immune cell costimulatory or inhibitory signals, and the ability to modulate the immune response.

The term "PD-1 ligand" refers to binding partners of the PD-1 receptor and includes both PD-L1 (Freeman *et al.* (2000*) J. Exp. Med.* 192:1027-1034) and PD-L2 (Latchman *et al.* (2001) *Nat. Immunol.* 2:261). At least two types of human PD-1 ligand polypeptides exist. PD-1 ligand proteins comprise a signal sequence, and an IgV domain, an IgC domain, a transmembrane domain, and a short cytoplasmic tail. Both PD-L1 (See Freeman *et al.* (2000) for sequence data) and PD-L2 (See Latchman *et al.* (2001) *Nat. Immunol.* 2:261 for sequence data) are members of the B7 family of polypeptides. Both PD-L1 and PD-L2 are expressed in placenta, spleen, lymph nodes, thymus, and heart. Only PD-L2 is expressed in pancreas, lung and liver, while only PD-L1 is expressed in fetal liver. Both PD-1 ligands are upregulated on activated monocytes and dendritic cells, although PD-L1 expression is broader. For example, PD-L1 is known to be constitutively expressed and upregulated to higher levels on murine hematopoietic cells (*e.g.*, T cells, B cells, macrophages, dendritic cells (DCs), and bone marrow-derived mast cells) and non-hematopoietic cells (*e.g.*, endothelial, epithelial, and muscle cells), whereas PD-L2 is inducibly expressed on DCs, macrophages, and bone marrow-derived mast cells (see Butte *et al.* (2007) *Immunity* 27:111).

PD-1 ligands comprise a family of polypeptides having certain conserved structural and functional features. The term "family" when used to refer to proteins or nucleic acid molecules, is intended to mean two or more proteins or nucleic acid molecules having a common structural domain or motif and having sufficient amino acid or nucleotide

sequence homology, as defined herein. Such family members can be naturally or non-naturally occurring and can be from either the same or different species. For example, a family can contain a first protein of human origin, as well as other, distinct proteins of human origin or alternatively, can contain homologues of non-human origin. Members of a family may also have common functional characteristics. PD-1 ligands are members of the B7 family of polypeptides. The term "B7 family" or "B7 polypeptides" as used herein includes costimulatory polypeptides that share sequence homology with B7 polypeptides, *e.g.*, with B7-1, B7-2, B7h (Swallow *et al.* (1999) *Immunity* 11:423), and/or PD-1 ligands (*e.g.*, PD-L1 or PD-L2). For example, human B7-1 and B7-2 share approximately 26% amino acid sequence identity when compared using the BLAST program at NCBI with the default parameters (Blosum62 matrix with gap penalties set at existence 11 and extension 1 (See the NCBI website). The term B7 family also includes variants of these polypeptides which are capable of modulating immune cell function. The B7 family of molecules share a number of conserved regions, including signal domains, IgV domains and the IgC domains. IgV domains and the IgC domains are art-recognized Ig superfamily member domains. These domains correspond to structural units that have distinct folding patterns called Ig folds. Ig folds are comprised of a sandwich of two β sheets, each consisting of anti-parallel β strands of 5-10 amino acids with a conserved disulfide bond between the two sheets in most, but not all, IgC domains of Ig, TCR, and MHC molecules share the same types of sequence patterns and are called the C1-set within the Ig superfamily. Other IgC domains fall within other sets. IgV domains also share sequence patterns and are called V set domains. IgV domains are longer than IgC domains and contain an additional pair of β strands.

Preferred B7 polypeptides are capable of providing costimulatory or inhibitory signals to immune cells to thereby promote or inhibit immune cell responses. For example, B7 family members that bind to costimulatory receptors increase T cell activation and proliferation, while B7 family members that bind to inhibitory receptors reduce costimulation. Moreover, the same B7 family member may increase or decrease T cell costimulation. For example, when bound to a costimulatory receptor, PD-1 ligand can induce costimulation of immune cells or can inhibit immune cell costimulation, *e.g.*, when present in soluble form. When bound to an inhibitory receptor, PD-1 ligand polypeptides can transmit an inhibitory signal to an immune cell. Preferred B7 family members include B7-1, B7-2, B7h, PD-L1 or PD-L2 and soluble fragments or derivatives thereof. In one

embodiment, B7 family members bind to one or more receptors on an immune cell, *e.g.*, CTLA4, CD28, ICOS, PD-1 and/or other receptors, and, depending on the receptor, have the ability to transmit an inhibitory signal or a costimulatory signal to an immune cell, preferably a T cell.

Modulation of a costimulatory signal results in modulation of effector function of an immune cell. Thus, the term "PD-1 ligand activity" includes the ability of a PD-1 ligand polypeptide to bind its natural receptor(s) (*e.g.* PD-1 or B7-1), the ability to modulate immune cell costimulatory or inhibitory signals, and the ability to modulate the immune response.

The term "PD-L1" refers to a specific PD-1 ligand. Two forms of human PD-L1 molecules have been identified. One form is a naturally occurring PD-L1 soluble polypeptide, *i.e.,* having a short hydrophilic domain and no transmembrane domain, and is referred to herein as PD-L1S. The second form is a cell-associated polypeptide, *i.e.,* having a transmembrane and cytoplasmic domain, referred to herein as PD-L1M. The nucleic acid and amino acid sequences of representative human PD-L1 biomarkers regarding PD-L1M are also available to the public at the GenBank database under NM_014143.3 and NP_054862.1. PD-L1 proteins comprise a signal sequence, and an IgV domain and an IgC domain. The signal sequence of PD-L1S contains about amino acid 1 to about amino acid 18. The signal sequence of PD-L1M contains about amino acid 1 to about amino acid 18. The IgV domain of PD-L1S contains about amino acid 19 to about amino acid 134 and the IgV domain of PD-L1M contains about amino acid 19 to about amino acid 134. The IgC domain of PD-L1S contains about amino acid 135 to about amino acid 227 and the IgC domain of PD-L1M contains about amino acid 135 to about amino acid 227. The hydrophilic tail of the PD-L1 exemplified in PD-L1S comprises a hydrophilic tail shown from about amino acid 228 to about amino acid 245. The PD-L1 polypeptide exemplified in PD-L1M comprises a transmembrane domain shown from about amino acids 239 to about amino acid 259 of PD-L1M and a cytoplasmic domain shown from about 30 amino acid 260 to about amino acid 290 of PD-L1M. In addition, nucleic acid and polypeptide sequences of PD-L1 orthologs in organisms other than humans are well-known and include, for example, mouse PD-L1 (NM_021893.3 and NP_068693.1), rat PD-L1 (NM_001191954.1 and NP_001178883.1), dog PD-L1 (XM_541302.3 and XP_541302.3), cow PD-L1 (NM_001163412.1 and NP_001156884.1), and chicken PD-L1 (XM_424811.3 and XP_424811.3).

The term "PD-L2" refers to another specific PD-1 ligand. PD-L2 is a B7 family member expressed on various APCs, including dendritic cells, macrophages and bone-marrow derived mast cells (Zhong *et al.* (2007) Eur. J. Immunol. 37:2405). APC-expressed PD-L2 is able to both inhibit T cell activation through ligation of PD-1 and costimulate T cell activation, through a PD-1 independent mechanism (Shin *et al.* (2005) J. Exp. Med. 201:1531). In addition, ligation of dendritic cell-expressed PD-L2 results in enhanced dendritic cell cytokine expression and survival (Radhakrishnan *et al.* (2003) *J. Immunol.* 37:1827; Nguyen *et al.* (2002) *J. Exp. Med.* 196:1393). The nucleic acid and amino acid sequences of representative human PD-L2 biomarkers are well-known in the art and are also available to the public at the GenBank database under NM_025239.3 and NP_079515.2. PD-L2 proteins are characterized by common structural elements. In some embodiments, PD-L2 proteins include at least one or more of the following domains: a signal peptide domain, a transmembrane domain, an IgV domain, an IgC domain, an extracellular domain, a transmembrane domain, and a cytoplasmic domain. For example, amino acids 1-19 of PD-L2 comprises a signal sequence. As used herein, a "signal sequence" or "signal peptide" serves to direct a polypeptide containing such a sequence to a lipid bilayer, and is cleaved in secreted and membrane bound polypeptides and includes a peptide containing about 15 or more amino acids which occurs at the N-terminus of secretory and membrane bound polypeptides and which contains a large number of hydrophobic amino acid residues. For example, a signal sequence contains at least about 10-30 amino acid residues, preferably about 15- 25 amino acid residues, more preferably about 18-20 amino acid residues, and even more preferably about 19 amino acid residues, and has at least about 35-65%, preferably about 38-50%, and more preferably about 40-45% hydrophobic amino acid residues (*e.g.*, valine, leucine, isoleucine or phenylalanine). In another embodiment, amino acid residues 220-243 of the native human PD-L2 polypeptide and amino acid residues 201-243 of the mature polypeptide comprise a transmembrane domain. As used herein, the term "transmembrane domain" includes an amino acid sequence of about 15 amino acid residues in length which spans the plasma membrane. More preferably, a transmembrane domain includes about at least 20, 25, 30, 35, 40, or 45 amino acid residues and spans the plasma membrane. Transmembrane domains are rich in hydrophobic residues, and typically have an alpha-helical structure. In a preferred embodiment, at least 50%, 60%, 70%, 80%, 90%, 95% or more of the amino acids of a transmembrane domain are hydrophobic, *e.g.*, leucines, isoleucines, tyrosines, or

tryptophans. Transmembrane domains are described in, for example, Zagotta, W. N. *et al.* (1996) Annu. Rev. Neurosci. 19: 235-263. In still another embodiment, amino acid residues 20-120 of the native human PD-L2 polypeptide and amino acid residues 1-101 of the mature polypeptide comprise an IgV domain. Amino acid residues 121- 219 of the native human PD-L2 polypeptide and amino acid residues 102-200 of the mature polypeptide comprise an IgC domain. As used herein, IgV and IgC domains are recognized in the art as Ig superfamily member domains. These domains correspond to structural units that have distinct folding patterns called Ig folds. Ig folds are comprised of a sandwich of two ß sheets, each consisting of antiparallel (3 strands of 5-10 amino acids with a conserved disulfide bond between the two sheets in most, but not all, domains. IgC domains of Ig, TCR, and MHC molecules share the same types of sequence patterns and are called the Cl set within the Ig superfamily. Other IgC domains fall within other sets. IgV domains also share sequence patterns and are called V set domains. IgV domains are longer than C-domains and form an additional pair of strands. In yet another embodiment, amino acid residues 1-219 of the native human PD-L2 polypeptide and amino acid residues 1-200 of the mature polypeptide comprise an extracellular domain. As used herein, the term "extracellular domain" represents the N-terminal amino acids which extend as a tail from the surface of a cell. An extracellular domain of the present invention includes an IgV domain and an IgC domain, and may include a signal peptide domain. In still another embodiment, amino acid residues 244-273 of the native human PD-L2 polypeptide and amino acid residues 225-273 of the mature polypeptide comprise a cytoplasmic domain. As used herein, the term "cytoplasmic domain" represents the C-terminal amino acids which extend as a tail into the cytoplasm of a cell. In addition, nucleic acid and polypeptide sequences of PD-L2 orthologs in organisms other than humans are well-known and include, for example, mouse PD-L2 (NM_021396.2 and NP_067371.1), rat PD-L2 (NM_001107582.2 and NP_001101052.2), dog PD-L2 (XM_847012.2 and XP_852105.2), cow PD-L2 (XM_586846.5 and XP_586846.3), and chimpanzee PD-L2 (XM_001140776.2 and XP_001140776.1).

The term "PD-L2 activity," "biological activity of PD-L2," or "functional activity of PD-L2," refers to an activity exerted by a PD-L2 protein, polypeptide or nucleic acid molecule on a PD-L2-responsive cell or tissue, or on a PD- L2 polypeptide binding partner, as determined *in vivo*, or *in vitro*, according to standard techniques. In one embodiment, a PD-L2 activity is a direct activity, such as an association with a PD-L2 binding partner. As

used herein, a "target molecule" or "binding partner" is a molecule with which a PD-L2 polypeptide binds or interacts in nature, such that PD-L2-mediated function is achieved. In an exemplary embodiment, a PD-L2 target molecule is the receptor RGMb. Alternatively, a PD-L2 activity is an indirect activity, such as a cellular signaling activity mediated by interaction of the PD- L2 polypeptide with its natural binding partner (*i.e.*, physiologically relevant interacting macromolecule involved in an immune function or other biologically relevant function), *e.g.*, RGMb. The biological activities of PD-L2 are described herein. For example, the PD-L2 polypeptides of the present invention can have one or more of the following activities: 1) bind to and/or modulate the activity of the receptor RGMb, PD-1, or other PD-L2 natural binding partners, 2) modulate intra-or intercellular signaling, 3) modulate activation of immune cells, *e.g.* , T lymphocytes, and 4) modulate the immune response of an organism, *e.g.*, a mouse or human organism.

"Anti-immune checkpoint therapy" refers to the use of agents that inhibit immune checkpoint nucleic acids and/or proteins. Inhibition of one or more immune checkpoints can block or otherwise neutralize inhibitory signaling to thereby upregulate an immune response in order to more efficaciously treat cancer. Exemplary agents useful for inhibiting immune checkpoints include antibodies, small molecules, peptides, peptidomimetics, natural ligands, and derivatives of natural ligands, that can either bind and/or inactivate or inhibit immune checkpoint proteins, or fragments thereof; as well as RNA interference, antisense, nucleic acid aptamers, etc. that can downregulate the expression and/or activity of immune checkpoint nucleic acids, or fragments thereof. Exemplary agents for upregulating an immune response include antibodies against one or more immune checkpoint proteins block the interaction between the proteins and its natural receptor(s); a non-activating form of one or more immune checkpoint proteins (*e.g.*, a dominant negative polypeptide); small molecules or peptides that block the interaction between one or more immune checkpoint proteins and its natural receptor(s); fusion proteins (*e.g.* the extracellular portion of an immune checkpoint inhibition protein fused to the Fc portion of an antibody or immunoglobulin) that bind to its natural receptor(s); nucleic acid molecules that block immune checkpoint nucleic acid transcription or translation; and the like. Such agents can directly block the interaction between the one or more immune checkpoints and its natural receptor(s) (*e.g.*, antibodies) to prevent inhibitory signaling and upregulate an immune response. Alternatively, agents can indirectly block the interaction between one or more immune checkpoint proteins and its natural receptor(s) to prevent inhibitory signaling

and upregulate an immune response. For example, a soluble version of an immune checkpoint protein ligand such as a stabilized extracellular domain can binding to its receptor to indirectly reduce the effective concentration of the receptor to bind to an appropriate ligand. In one embodiment, anti-PD-1 antibodies, anti-PD-L1 antibodies, and/or anti-PD-L2 antibodies, either alone or in combination, are used to inhibit immune checkpoints. These embodiments are also applicable to specific therapy against particular immune checkpoints, such as the PD-1 pathway (*e.g.*, anti-PD-1 pathway therapy, otherwise known as PD-1 pathway inhibitor therapy).

The term "RIPK1," also known as receptor-interacting serine/threonine-protein kinase 1 and RIP-1, refers to a member of a group of proteins functioning in a variety of cellular pathways including the NF-κB pathway and programmed necrotic cell death (necroptosis). This group of serine-threonine kinases transduce inflammatory and cell-death signals (programmed necrosis) following death receptors ligation, activation of pathogen recognition receptors (PRRs), and DNA damage, affecting embryonic development, tissue homeostasis, immunity and inflammation. RIPK1 is implicated in inflammatory and cell death signaling and its kinase activity is believed to drive RIPK3-mediated necroptosis (Kelliher *et al.* (1998) *Immunity* 8:297-303; Meylan *et al.* (2004) *Nature Immunol.*5:503-507; Christofferson *et al.* (2014) *Annu. Rev. Physiol.* 76:129-150). When the proinflammatory cytokine TNFα stimulates its receptor, TNFR1, RIPK1 regulates whether the cell lives by activating NF-κB or dies by apoptosis or necroptosis, two distinct pathways of programmed cell death that may be activated to eliminate unwanted cells. RIPK1 has an N-terminal Ser/Thr kinase domain and a C-terminal death domain. The kinase domain of RIPK1 is involved in regulating necroptosis, and the death domain regulates RIPK1 recruitment to the intracellular domain of TNFR1 (Stanger *et al.* (1995) *Cell* 81:513-523; Hsu *et al.* (1996) *Immunity* 4:387-396). RIPK1 interacts with RIP3 kinase, a downstream mediator of RIPK1 in the execution of necroptosis, via their RIP homotypic interaction motifs and activates NF-κB (Yu *et al.* (1999) *Curr. Biol.* 9:539-542; Li *et al.* (2012) *Cell* 150:339-350). Upon TNF-induced necrosis, the RIPK1-RIPK3 dimer further interacts with PGAM5 and MLKL, forming a complex leading to PGAM5 phosphorylation and increased PGAM5 phosphatase activity. RIPK1 also interacts with TNFRSF6 and TRADD via their death domains, thus recruited by TRADD to TNFRSF1A in a TNF-dependent process. RIPK1 further interacts (via kinase domain) with DAB2IP (via Ras-GAP domain), in a TNF-alpha-dependent manner. Other binding partners and/or

those RIPK1 interacts with include, *e.g.*, RNF216, EGFR, IKBKG, TRAF1, TRAF2, TRAF3, BNLF1, SQSTM1, MAVS/IPS1, ZFAND5, BIRC2/c-IAP1, BIRC3/c-IAP2, XIAP/BIRC4, ARHGEF2, RFFL, RNF34, TICAM1, CA11, CASP8, CFLAR, CRADD, RNF11, TNFRSF1A, and UBC (Liao *et al.* (2008) *Curr. Biol.* 18:641-649; Kataoka *et al.* (2000) *Curr. Biol.* 10:640-648; Ahmad *et al.* (1997) *Cancer Res.* 57:615-619; Shembade *et al.* (2009) *EMBO J.* 28:513-522; Chen *et al.* (2002) *J. Biol. Chem.* 277:15985-15991; Sanz *et al.* (1999) *EMBO J.* 18:3044-3053). Proteolytic processing of RIPK1, through both caspase-dependent and -independent mechanisms, triggers lethality that is dependent on the generation of one or more specific C-terminal cleavage product(s) of RIPk1 upon stress. For example, caspase-8 proteolytically cleaves RIPK1 during TNF-induced apoptosis (Chaudhary *et al.* (2000) *Oncogene* 19:4451-4460). Such cleavage abolishes NF-kappa-B activation and enhances pro-apoptotic signaling through the TRADD-FADD interaction. RIPK1 and RIPK3 undergo reciprocal auto- and trans-phosphorylation. Phosphorylation of Ser-161 on RIPK1 by RIPK3 is necessary for the formation of the necroptosis-inducing complex. RIPK1 can be ubiquitinated by Lys-11-, Lys-48-, Lys-63- and linear-linked type ubiquitin. Polyubiquitination with Lys-63-linked chains on by TRAF2 induces RIPK1 association with the IKK complex. Deubiquitination of Lys-63-linked chains and polyubiquitination with Lys-48-linked chains by TNFAIP3 leads to RIPK1 proteasomal degradation and consequently down-regulates TNF-alpha-induced NF-kappaB signaling. Lys-48-linked polyubiquitination by RFFL or RNF34 also promotes proteasomal degradation and negatively regulates TNF-alpha-induced NFkappa-B signaling. RIPK1 can also be polyubiquitinated with Lys-48 and Lys-63-linked chains by BIRC2/c-IAP1 and BIRC3/c-IAP2, leading to activation of NF-kappa-B (Bertrand *et al.* (2008) *Mol. Cell.* 30:689-700; Varfolomeev *et al.* (2008) *J. Biol. Chem.* 283:24295-24299).

The nucleic acid and amino acid sequences of a representative human RIPK1 is available to the public at the GenBank database (Gene ID 8738) and is shown in Table 1. Human RIPK1 isoforms include the longer isoform 1 (GenBank database numbers NM_003804.4 and NP_003795.2, encoded by the shorter transcript variant 1), and the shorter isoforms 2 (NM_001317061.1 and NP_001303990.1, encoded by a longer transcript variant 2, which contains two alternate exons in the 5' UTR, resulting in the use of a downstream start codon, compared to variant 1). The domain structure of RIPK1 polypeptide is well known and accessible in UniProtKB database under the accession number Q13546, including, in the order from the 5' terminus to the 3' terminus, a protein

kinase domain comprising, *e.g.*, amino acid positions 17-289 of NP_003795.2), an intermediate RIP homotypic interaction motif (RHIM) which is important for NF-kB activation and RHIM-dependent signaling and comprises, *e.g.*, amino acid positions 531-547 of NP_003795.2), and a death domain comprising, *e.g.*, amino acid 583-669 of NP_003795.2.

Nucleic acid and polypeptide sequences of RIPK1 orthologs in organisms other than humans are well-known and include, for example, chimpanzee (*Pan troglodytes*) RIPK1 (XM_016954826.1 and XP_016810315.1; XM_016954825.1 and XP_016810314.1; XM_016954824.1 and XP_016810313.1; XM_001160973.3 and XP_001160973.2; and XM_016954827.1 and XP_016810316.1), Rhesus monkey RIPK1 (XM_015135437.1 and XP_014990923.1; XM_015135438.1 and XP_014990924.1; XM_015135434.1 and XP_014990920.1; XM_001091986.3 and XP_001091986.1; XM_015135435.1 and XP_014990921.1; XM_015135436.1 and XP_014990922.1; and XM_015135439.1 and XP_014990925.1), dog RIPK1 (XM_005639981.2 and XP_005640038.1), mouse RIPK1 (NM_009068.3 and NP_033094.3), cattle RIPK1 (NM_001035012.1 and NP_001030184.1), Norway rat (*Rattus norvegicus*) RIPK1 (NM_001107350.1 and NP_001100820.1), chicken RIPK1 (NM_204402.2 and NP_989733.2), tropical clawed frog (*Xenopus tropicalis*) RIPK1 (NM_001079035.1 and NP_001072503.1), zebrafish (*Danio rerio*) RIPK1 (NM_199674.2 and NP_955968.2), zebrafish (*Danio rerio*) RIPK1 (NM_001043350.1 and NP_001036815.1),

The term "RIPK1 activity" includes the ability of a RIPK1 polypeptide (and its fragments, domains, and/or motifs thereof, discussed herein) to bind its substrate and/or catalyze serine/threonine-protein kinase activity. RIPK1 activity may also include one or more of functions, such as those disclosed herein in the NF-κB pathway and programmed necrotic cell death (necroptosis). For example, RIPK1 may interact with various proteins disclosed herein, such as RIP3 kinase, for its functions in signaling related to TNF-alpha, NFkappa-B, and/or necroptosis. RIPK1 may also be proteolyticly modified, such as being cleaved, ubiquitinated, deubiquitinated, or otherwise disclosed herein, for it functions.

The term "RIPK1 substrate(s)" refers to binding partners of a RIPK1 polypeptide (and its fragments, domains, and/or motifs thereof, discussed herein), *e.g.*, the proteins listed herein. Furthermore, RIPK1 substrates may refer to downstream members in the signaling pathways where RIPK1 has a functional role.

The term "RIPK1-regulated signaling pathway(s)" includes signaling pathways in which RIPK1 (and its fragments, domains, and/or motifs thereof, discussed herein) binds to at least one of its substrate, through which at least one cellular function and/or activity and/or cellular protein profiles is changed (*e.g.*, through RIP3 kinases or by being proteolyticly modified). In some embodiments, RIPK1 promotes ubiquitination and proteasome degradation for at least one of its substrates which bind to it. RIPK1-regulated signaling pathways include at least those described herein, such as TNFR1 pathway, regulation by c-FLIP, activated TLR4 signaling, apoptosis modulation and signaling, TNF signaling (reactome), Death Receptor signaling, NFkappa-B pathway, P38 MAPK signaling pathway, etc.

The term "RIPK1 inhibitor(s)" includes any natural or non-natural agent prepared, synthesized, manufactured, and/or purified by human that is capable of reducing, inhibiting, blocking, preventing, and/or that inhibits the ability of a RIPK1 polypeptide (and its fragments, domains, and/or motifs thereof, discussed herein). In one embodiment, such inhibitors may reduce or inhibit the binding/interaction between RIPK1 and its substrates or other binding partners. In another embodiment, such inhibitors may reduce or inhibit the catalytic function of RIPK1 as a serine/threonine-protein kinase. In still another embodiment, such inhibitors may increase or promote the turnover rate, reduce or inhibit the expression and/or the stability (*e.g.*, the half-life), and/or change the cellular localization of RIPK1, resulting in at least a decrease in RIPK1 levels and/or activity. Such inhibitors may be any molecule, including but not limited to small molecule compounds, antibodies or intrabodies, RNA interfering (RNAi) agents (including at least siRNAs, shRNAs, microRNAs (miRNAs), piwi, and other well-known agents). Such inhibitors may be specific to RIPK1 or also inhibit at least one of other serine/threonine-protein kinases. Small molecule inhibitory compounds for RIPK1 polypeptides are well known and commercially available (*e.g.*, Necrostatin-1 (5-(1H-Indol-3-ylmethyl)-3-methyl-2-thioxo-4-imidazolidinone, Cat. # 2324, Tocris, Bristol, UK) prevents osmotic nephrosis (Linkermann *et al.* (2013) *J Am Soc Nephrol.* 24:1545-1557; Cui *et al.* (2016) *Sci Rep.* 6, doi:10.1038/srep33803). In addition, GSK481 ((S)-5-benzyl-N-(5-methyl-4-oxo-2,3,4,5-tetrahydrobenzo[b][1,4]oxazepin-3-yl)isoxazole-3-carboxamide, Cat. # 1622849-58-4, APExBio, Bristol, UK) showed complete specificity for RIP1 kinase against all other tested kinases when profiled over both a P33 radiolabeled assay screen and inhibits S166 phosphorylation in wild-type human RIPK1 but not wild-type mouse RIPK1 (Harris *et al.*

(2016) *J Med Chem* 59:2163-2178)). RNA interference for RIPK1 polypeptides are well known and commercially available (*e.g.*, human or mouse shRNA (Cat. # TG320591, TL320591, TL501905, TF320591, TR320591, and TF501905) and siRNA (Cat. # SR418761, SR305755, and SR509132) products and human or mouse gene knockout kit via CRISPR (Cat. # KN216024 and KN314840) from Origene (Rockville, MD, siRNA/shRNA products (Cat. # sc-37387, sc-37388, sc-36426, sc-44326, sc-36427, sc-36428, and sc-36429) from Santa Cruz Biotechnology (Dallas, Texas), Ready-to-package AAV shRNA clones from Vigene Biosciences (Rockville, MD, etc.). Methods for detection, purification, and/or inhibition of RIPK1 (*e.g.*, by anti-RIPK1 antibodies) are also well known and commercially available (*e.g.*, multiple anti-RIPK1 antibodies from Origene (Cat. # AP00087PU-N, TA800325AM, and other about 70 antibodies), Cell Signaling Technology (Danvers, MA, Cat. # 3493, 14577, 4926, etc.), abcam (Cambridge, MA, Cat. # ab72139, ab202985, ab125072, ab178420, ab137451, etc.), R&D Systems (Minneapolis, MN, Cat. # MAB3585), Santa Cruz Biotechnology (Cat. # sc-133102), etc.). RIPK1 knockout mouse strains and cell lines are also well known and available at the International Mouse Strain Resource (IMSR) (*e.g.*, stain names *Ripk1*$^{tm1.1Vmd}$ and *Ripk1*^{tm1Led}).

The term "BIRC2," also known as baculoviral IAP repeat containing 2, c-IAP1, and apoptosis inhibitor 1, refers to a member of a family of proteins that inhibits apoptosis by binding to tumor necrosis factor receptor-associated factors TRAF1 and TRAF2, probably by interfering with activation of ICE-like proteases. BIRC2 inhibits apoptosis induced by serum deprivation and menadione, a potent inducer of free radicals. As a multi-functional protein, BIRC2 regulates not only caspases and apoptosis, but also inflammatory signaling and immunity, mitogenic kinase signaling, and cell proliferation, as well as cell invasion and metastasis. BIRC2 acts as an E3 ubiquitin-protein ligase regulating NF-kappa-B signaling and regulates both canonical and non-canonical NF-kappa-B signaling by acting in opposite directions: acts as a positive regulator of the canonical pathway and suppresses constitutive activation of non-canonical NF-kappa-B signaling. The target proteins for the E3 ubiquitin-protein ligase activity of BIRC2 include, at least RIPK1 (leading to activation of NF-κB, see Bertrand *et al.* (2008) *Mol. Cell.* 30:689-700; Varfolomeev *et al.* (2008) *J. Biol. Chem.* 283:24295-24299), RIPK2, RIPK3, RIPK4, CASP3, CASP7, CASP8, TRAF2, DIABLO/SMAC, MAP3K14/NIK, MAP3K5/ASK1, IKBKG/NEMO, IKBKE, and MXD1/MAD1. BIRC2 also functions as an E3 ubiquitin-protein ligase of the NEDD8 conjugation pathway, targeting effector caspases for neddylation and inactivation. BIRC2

is as an important regulator of innate immune signaling via regulation of Toll-like receptors (TLRs), Nodlike receptors (NLRs) and RIG-I like receptors (RLRs), collectively referred to as pattern recognition receptors (PRRs). BIRC2 protects cells from spontaneous formation of the ripoptosome, a large multi-protein complex that has the capability to kill cancer cells in a caspase-dependent and caspase-independent manner. BIRC2 suppresses ripoptosome formation by ubiquitinating RIPK1 and CASP8. BIRC2 also stimulates the transcriptional activity of E2F1 and plays a role in the modulation of the cell cycle. BIRC2 interacts with DIABLO/SMAC and with PRSS25 and such interactions inhibit apoptotic suppressor activity. BIRC2 also interacts with CASP9, TRAF2, E2F1, RIPK1, RIPK2, RIPK3, RIPK4, BIRC5/surviving, RAC1, TSGA10, ABHD17A, BIRC7, and USP19. BIRC2 functions in multiple pathways, including, at least, TNFR1 pathway (*e.g.*, DR2 signaling, TWEAK pathway, Fas signaling, TNF-alpha/NF-kB Signaling Pathway, etc.), apoptosis modulation and signaling (*e.g.*, death receptor signaling, apoptotic TNF-family pathways, etc.), activated Toll-like receptor 4 (TLR4) signaling (*e.g.*, MyD88-independent TLR3/TLR4 cascade), TNF signaling (REACTOME), regulation of activated PAK-2p34 by proteasome mediated degradation (*e.g.*, TNFR2 non-canonical NF-κB pathway), etc. BIRC2 is suggested to be related to lung cancers and lymphoma.

The nucleic acid and amino acid sequences of a representative human BIRC2 is available to the public at the GenBank database (Gene ID 329) and is shown in Table 1. Human BIRC2 isoforms include the predominant transcript variant 1 and the encoded longer isoform 1 (GenBank database numbers NM_001166.4 and NP_001157.1), a transcript variant 2, which differs in the 5' UTR compared to variant 1, and the encoded protein having the same sequence as isoform 1 (NM_001256163.1 and NP_001243092.1), and a transcript variant 3, having an alternate splice site in the 5' region resulting in a downstream AUG start codon compared to variant 1, and the encoded shorter isoform 2 (shorter at the N-terminus). The domain structure of BIRC2 polypeptide is well known and accessible in UniProtKB database under the accession number Q13490, including, in the order from the 5' terminus to the 3' terminus, three BIR domain comprising, *e.g.*, amino acid positions 46-113, 184-250, and 269-336 of NP_001157.1, a caspase activation and recruitment domain (CARD) domain comprising, *e.g.*, amino acid positions 453-543 of NP_001157.1, and a Really Interesting New Gene (RING)-type zinc finger comprising, *e.g.*, amino acid positions 571-606 of NP_001157.1.

Nucleic acid and polypeptide sequences of BIRC2 orthologs in organisms other than humans are well-known and include, for example, chimpanzee (*Pan troglodytes*) BIRC2 (XM_016921873.1 and XP_016777362.1, XM_001152603.5 and XP_001152603.1, XM_016921875.1 and XP_016777364.1, XM_016921874.1 and XP_016777363.1, and XM_001152534.3 and XP_001152534.1), Rhesus monkey BIRC2 (NM_001261321.1 and NP_001248250.1), dog BIRC2 (NM_001048023.1 and NP_001041488.1), mouse BIRC2 (NM_007465.3 and NP_031491.2, representing the transcript variant 1 and the encoded isoform 1, and NM_001291503.1 and NP_001278432.1, representing transcript variant 2 and the encoding protein having the same sequence as isoform 1), cattle BIRC2 (XM_015474583.1 and XP_015330069.1), Norway rat (*Rattus norvegicus*) BIRC2 (NM_021752.2 and NP_068520.2), chicken BIRC2 (NM_001007822.1 and NP_001007823.1), tropical clawed frog (*Xenopus tropicalis*) BIRC2 (NM_001005449.1 and NP_001005449.1), zebrafish (*Danio rerio*) BIRC2 (NM_194395.2 and NP_919376.1), and fruit fly Diap2 (NM_176182.2 and NP_788362.1, and NM_057779.5 and NP_477127.1).

The term "BIRC2 activity" includes the ability of a BIRC2 polypeptide (and its fragments, domains, and/or motifs thereof, discussed herein) to bind its substrate and/or biological activity. BIRC2 activity may also include one or more of functions, such as those disclosed herein in the NF-κB pathway and programmed necrotic cell death. For example, BIRC2 may interact with various proteins (*e.g.*, its ubiquitination substrates) disclosed herein for its functions in signaling related to TNF-alpha, NFkappa-B, and/or necroptosis. BIRC2 may also be proteolyticly modified, such as being cleaved, ubiquitinated, deubiquitinated, or otherwise disclosed herein, for it functions.

The term "BIRC2 substrate(s)" refers to binding partners of a BIRC2 polypeptide (and its fragments, domains, and/or motifs thereof, discussed herein), *e.g.*, the proteins listed herein. Furthermore, BIRC2 substrates may refer to downstream members in the signaling pathways where BIRC2 has a functional role.

The term "BIRC2-regulated signaling pathway(s)" includes signaling pathways in which BIRC2 (and its fragments, domains, and/or motifs thereof, discussed herein) binds to at least one of its substrate, through which at least one cellular function and/or activity and/or cellular protein profiles is changed (*e.g.*, through RIP3 kinases or by being proteolyticly modified). In some embodiments, BIRC2 promotes ubiquitination and proteasome degradation for at least one of its substrates which bind to it. BIRC2-regulated

signaling pathways include at least those described herein, such as TNFR1 pathway (*e.g.*, DR2 signaling, TWEAK pathway, Fas signaling, TNF-alpha/NF-kB Signaling Pathway, etc.), apoptosis modulation and signaling (*e.g.*, death receptor signaling, apoptotic TNF-family pathways, etc.), activated Toll-like receptor 4 (TLR4) signaling (*e.g.*, MyD88-independent TLR3/TLR4 cascade), TNF signaling (REACTOME), regulation of activated PAK-2p34 by proteasome mediated degradation (*e.g.*, TNFR2 non-canonical NF-κB pathway), etc.

The term "BIRC2 inhibitor(s)" includes any natural or non-natural agent prepared, synthesized, manufactured, and/or purified by human that is capable of reducing, inhibiting, blocking, preventing, and/or that inhibits the ability of a BIRC2 polypeptide (and its fragments, domains, and/or motifs thereof, discussed herein). In one embodiment, such inhibitors may reduce or inhibit the binding/interaction between BIRC2 and its substrates or other binding partners. In another embodiment, such inhibitors may reduce or inhibit the catalytic function of BIRC2 as a ubiquitin ligase. In still another embodiment, such inhibitors may increase or promote the turnover rate, reduce or inhibit the expression and/or the stability (*e.g.*, the half-life), and/or change the cellular localization of BIRC2, resulting in at least a decrease in BIRC2 levels and/or activity(*e.g.*, its ubiquitin ligase activity). Such inhibitors may be any molecule, including but not limited to small molecule compounds, antibodies or intrabodies, RNA interfering (RNAi) agents (including at least siRNAs, shRNAs, microRNAs (miRNAs), piwi, and other well-known agents). Such inhibitors may be specific to BIRC2 or also inhibit at least one of other E3 ubiquitin ligases. Small molecule inhibitory compounds for BIRC2 polypeptides are well known and commercially available (*e.g.*, GDC-0152, LCL161, Birinapant (TL32711), AT-406 (SM-406), BV6, etc.). RNA interference for BIRC2 polypeptides are well known and commercially available (*e.g.*, human or mouse shRNA (Cat. # TR314474, TF500114, TF710504, TG314474, etc.) and siRNA (Cat. # SR418175, SR300231, and SR509801) products and human or mouse gene knockout kit via CRISPR (Cat. # KN205373 and KN302170) from Origene (Rockville, MD), siRNA/shRNA products (Cat. # sc-29848 and sc-29849) and CRISPR products (Cat. # sc-419151) from Santa Cruz Biotechnology (Dallas, Texas), Ready-to-package AAV shRNA clones from Vigene Biosciences (Rockville, MD, Cat. # SH877067). Methods for detection, purification, and/or inhibition of BIRC2 (*e.g.*, by anti-BIRC2 antibodies) are also well known and commercially available (*e.g.*, multiple anti-BIRC2 antibodies from Origene (Cat. # TA307562, TA321297,

TA306169, etc.), Cell Signaling Technology (Danvers, MA, Cat. # 7065, 4952, etc.), abcam (Cambridge, MA, Cat. # ab108361, ab201588, ab196592, etc.), and Santa Cruz Biotechnology (Cat. # sc-271419, sc-7943, etc.). Human BIRC2 knockout cell lines are also well known and available from Horizon Discovery (Cambridge, UK, Cat. # HZGHC002590c010).

The term "TBK1," also known as TRAF family member-associated NFκB activator (TANK)-binding kinase 1 and NFκB-activating kinase, refers to a member of a family of serine/threonine protein kinases that plays an essential role in regulating inflammatory responses to foreign agents. Following activation of toll-like receptors by viral or bacterial components, TBK1 associates with TRAF3 and TANK and phosphorylates interferon regulatory factors (IRFs) IRF3 and IRF7 as well as DDX3X, resulting in subsequent homodimerization and nuclear translocation of the IRFs and leading to transcriptional activation of pro-inflammatory and antiviral genes including IFNA and IFNB. In order to establish such an antiviral state, TBK1 form several different complexes whose composition depends on the type of cell and cellular stimuli. Thus, several scaffolding molecules including FADD, TRADD, MAVS, AZI2, TANK or TBKBP1/SINTBAD can be recruited to the TBK1-containing-complexes. Under particular conditions, TBK1 functions as a NFκB effector by phosphorylating NFκB inhibitor alpha/NFKBIA, IKBKB or RELA to translocate NFκB to the nucleus. TBK1 restricts bacterial proliferation by phosphorylating the autophagy receptor OPTN/Optineurin on Ser-177, thus enhancing LC3 binding affinity and antibacterial autophagy. TBK1 also phosphorylates and activates AKT1. TBK1 is suggested to play a role in energy balance regulation by sustaining a state of chronic, low-grade inflammation in obesity, which leads to a negative impact on insulin sensitivity. TBK1 also attenuates retroviral budding by phosphorylating the endosomal sorting complex required for transport-I (ESCRT-I) subunit VPS37C. Other phosphorylation substrates of TBK1 include, at least Borna disease virus (BDV) P protein. TBK1 interacts with, at least, NCK1 (Chou and Hanafusa (1995) *J. Biol. Chem.* 270:7359-7364), TANK (Pomerantz and Baltimore (2000) *EMBO J.* 18:6694-6704; Bouwmeester *et al.* (2004) *Nat. Cell Biol.* 6:97-105), and TRAF2 (Bonnard *et al.* (2000) *EMBO J.* 19:4976-4985). Transcriptional factors activated upon TBK1 activation include IRF3, IRF7, and ZEB1 (Liu (2014) *Lab Invest.* 94:362-370). TBK1 is also suggested to interact OPTN, TBKBP1, IRF3, IRF7, and TANK. TBK1 functions in multiple pathways, including, at least, RIG-I/MDA5 mediated induction of IFN-alpha/beta pathways (*e.g.*, RIG-I-like

Receptor (RLR) signaling pathways, TRAF3-dependent IFR activation pathway, TRAF6-mediated IRF7 activation, cytosolic DNA-sensing pathway, etc.), activated TLR4 signaling (*e.g.*, MyD88-independent TLR3/TLR4 cascade, TRIF-mediated TLR3/TLR4 signaling, etc.), cytosolic sensors of pathogen-associated DNA (*e.g.*, ZBP1(DAI) mediated induction of type I IFNs, IRF3-mediated induction of type I IFN, STING mediated induction of host immune responses, STAT6-mediated induction of chemokines, etc.), Toll-like receptor signaling pathway, Influenza A or herpes simplex virus infection, Notch signaling pathway, NF-kB (NFκB) pathway, etc. TBK1 is suggested to be related to multiple diseases and disorders including, at least frontotemporal dementia and/or amyotrophic lateral sclerosis 1 and 4 (ftdals 1 and ftdals4), herpes simplex encephalitis, borna disease (enzootic encephalomyelitis), and crustacean allergy.

The nucleic acid and amino acid sequences of a representative human TBK1 is available to the public at the GenBank database (Gene ID 29110) and is shown in Table 1 (*e.g.*, NM_013254.3 and NP_037386.1). The domain structure of TBK1 polypeptide is well known and accessible in UniProtKB database under the accession number Q9UHD2, including, in the order from the 5' terminus to the 3' terminus, a protein kinase domain comprising, *e.g.*, amino acid positions 9-310 of NP_037386.1, and a ubiquitin-like domain comprising, *e.g.*, amino acid positions 309-385 of NP_037386.1.

Nucleic acid and polypeptide sequences of TBK1 orthologs in organisms other than humans are well-known and include, for example, chimpanzee (*Pan troglodytes*) TBK1 (XM_509194.4 and XP_509194.2), Rhesus monkey TBK1 (NM_001261193.1 and NP_001248122.1), dog TBK1 (XM_538266.5 and XP_538266.3), mouse TBK1 (NM_019786.4 and NP_062760.3), cattle TBK1 (NM_001192755.1 and NP_001179684.1), Norway rat (*Rattus norvegicus*) TBK1 (NM_001106786.1 and NP_001100256.1), chicken TBK1 (NM_001199558.1 and NP_001186487.1), tropical clawed frog (*Xenopus tropicalis*) TBK1 (NM_001142180.1 and NP_001135652.1), and zebrafish (*Danio rerio*) TBK1 (NM_001044748.2 and NP_001038213.2).

The term "TBK1 activity" includes the ability of a TBK1 polypeptide (and its fragments, domains, and/or motifs thereof, discussed herein) to bind its substrate and/or biological activity. TBK1 activity may also include one or more of functions, such as its serine/threonine protein kinase activity, and/or others disclosed herein in the NF-κB pathway and other related pathways. For example, TBK1 may interact with various proteins (*e.g.*, its substrates) disclosed herein for its functions in signaling. TBK1 may also

be proteolyticly modified, such as being cleaved, ubiquitinated, deubiquitinated, or otherwise disclosed herein, for it functions.

The term "TBK1 substrate(s)" refers to binding partners of a TBK1 polypeptide (and its fragments, domains, and/or motifs thereof, discussed herein), *e.g.*, the substrates described herein of TBK1 as a serine/threonine protein kinase. Furthermore, TBK1 substrates may refer to downstream members in the signaling pathways where TBK1 has a functional role.

The term "TBK1-regulated signaling pathway(s)" includes signaling pathways in which TBK1 (and its fragments, domains, and/or motifs thereof, discussed herein) binds to at least one of its substrate, through which at least one cellular function and/or activity and/or cellular protein profiles is changed. In some embodiments, TBK1 is a serine/threonine protein kinase and phosphorylates and activates its substrates. TBK1-regulated signaling pathways include at least those described herein, such as RIG-I/MDA5 mediated induction of IFN-alpha/beta pathways (*e.g.*, RIG-I-like Receptor (RLR) signaling pathways, TRAF3-dependent IFR activation pathway, TRAF6-mediated IRF7 activation, cytosolic DNA-sensing pathway, etc.), activated TLR4 signaling (*e.g.*, MyD88-independent TLR3/TLR4 cascade, TRIF-mediated TLR3/TLR4 signaling, etc.), cytosolic sensors of pathogen-associated DNA (*e.g.*, ZBP1(DAI) mediated induction of type I IFNs, IRF3-mediated induction of type I IFN, STING mediated induction of host immune responses, STAT6-mediated induction of chemokines, etc.), Toll-like receptor signaling pathway, Influenza A or herpes simplex virus infection, Notch signaling pathway, NF-kB (NFκB) pathway, etc.

The term "TBK1 inhibitor(s)" includes any natural or non-natural agent prepared, synthesized, manufactured, and/or purified by human that is capable of reducing, inhibiting, blocking, preventing, and/or that inhibits the ability of a TBK1 polypeptide (and its fragments, domains, and/or motifs thereof, discussed herein). In one embodiment, such inhibitors may reduce or inhibit the binding/interaction between TBK1 and its substrates or other binding partners. In another embodiment, such inhibitors may reduce or inhibit the catalytic function of TBK1 as a serine/threonine protein kinase. In still another embodiment, such inhibitors may increase or promote the turnover rate, reduce or inhibit the expression and/or the stability (*e.g.*, the half-life), and/or change the cellular localization of TBK1, resulting in at least a decrease in TBK1 levels and/or activity (*e.g.*, its serine/threonine protein kinase activity). Such inhibitors may be any molecule, including

but not limited to small molecule compounds, antibodies or intrabodies, RNA interfering (RNAi) agents (including at least siRNAs, shRNAs, microRNAs (miRNAs), piwi, and other well-known agents). Such inhibitors may be specific to TBK1 or also inhibit at least one of other serine/threonine-protein kinases. RNA interference for TBK1 polypeptides are well known and commercially available (*e.g.*, human, mouse, or rat shRNA (Cat. # TF320685, TL503203, TF705359, etc.) and siRNA (Cat. # SR309210, SR419607, SR505252, etc.) products and human or mouse gene knockout kit via CRISPR (Cat. # KN317271 and KN205238) from Origene (Rockville, MD, siRNA/shRNA products (Cat. # sc-39058 and sc-39059) and CRISPR products (Cat. # sc-425191 and sc-401066) from Santa Cruz Biotechnology (Dallas, Texas), Ready-to-package AAV shRNA clones from Vigene Biosciences (Rockville, MD, Cat. # SH866073). Methods for detection, purification, and/or inhibition of TBK1 (*e.g.*, by anti-TBK1 antibodies) are also well known and commercially available (*e.g.*, multiple anti-TBK1 antibodies from Origene (Cat. # TA336453, TA334468, TA320202, etc.), Cell Signaling Technology (Danvers, MA, Cat. # 3504, 3013, etc.), abcam (Cambridge, MA, Cat. # ab40676, ab109735, ab12116, etc.), and Santa Cruz Biotechnology (Cat. # sc-9910, sc-9085, sc-52957, etc.)). Human TBK1 knockout cell lines are also well known and available from Horizon Discovery (Cambridge, UK, Cat. # HZGHC000031c010).

The term "TRAF3," also known as TNF receptor-associated factor 3, LMP1-associated protein 1, CRAF1, and CAP1, refers to a member of a family of TNF receptor associated factor (TRAF) protein family. TRAF proteins associate with, and mediate the signal transduction from, members of the TNF receptor (TNFR) superfamily. TRAF3 participates in the signal transduction of CD40, a TNFR family member important for the activation of the immune response. TRAF3 is found to be a critical component of the lymphotoxin-beta receptor (LTbetaR) signaling complex, which induces NF-κB activation and cell death initiated by LTbeta ligation. Epstein-Barr virus encoded latent infection membrane protein-1 (LMP1) can interact with TRAF3 and several other members of the TRAF family, which may be essential for the oncogenic effects of LMP1. TRAF3 regulates pathways leading to the activation of NF-κB and MAP kinases, and plays a central role in the regulation of B-cell survival, including, at least, producing cytokines and interferon. TRAF3 is required for normal antibody isotype switching from IgM to IgG and plays a role in T-cell dependent immune responses and regulation of antiviral responses. TRAF3 is an essential constituent of several E3 ubiquitin-protein ligase

complexes. TRAF3 may have E3 ubiquitin-protein ligase activity and promote Lys-63-linked ubiquitination of target proteins. TRAF3 inhibits TRAF2-mediated activation of NF-κB and down-regulates proteolytic processing of NFKB2, and thereby inhibits non-canonical activation of NF-κB. TRAF3 also promotes ubiquitination and proteasomal degradation of MAP3K14.

TRAF3 can form heterotrimers with TRAF2 and TRAF5. TRAF3 interacts with, at least, TRAFD1, LTBR/TNFRSF3, TNFRSF4, TNFRSF5/CD40, TNFRSF8/CD30, TNFRSF13C, TICAM1, TNFRSF17/BCMA, TLR4, EDAR, MAP3K5, MAP3K14, TRAIP/TRIP, TDP2/TTRAP, TANK/ITRAF, TRAF3IP1, OTUB1, OTUB2, OTUD5, CARD14, RNF216, MAVS, OPTN, BIRC2, BIRC3, and TBK1. Its interaction with TNFRSF5/CD40 is modulated by TANK/ITRAF, which competes for the same binding site. TRAF3 is also identified in a complex with TRAF2, MAP3K14 and BIRC3. Upon exposure to bacterial lipopolysaccharide (LPS), TRAF3 is recruited to a transient complex containing TLR4, TRAF3, TRAF6, IKBKG, MAP3K7, MYD88, TICAM1, BIRC2, BIRC3 and UBE2N. TRAF3 also interacts (via RING-type zinc finger domain) with SRC and interacts (via MATH domain) with PTPN22, which promotes TRAF3 polyubiquitination. TRAF3 functions in multiple pathways, including, at least, activated TLR4 signaling (*e.g.*, MyD88-independent TLR3/TLR4 cascade, TLR3 and TLR4 cascade, etc.), RIG-I/MDA5 mediated induction of IFN-alpha/beta pathways (*e.g.*, RIG-I-like Receptor (RLR) signaling pathways, TRAF3-dependent IRF activation pathway, etc.), RANK signaling in osteoclasts (*e.g.*, APRIL pathway, BAFF in B-cell signaling, etc.), TNFR1 pathway (TWEAK pathway, GITR pathway, etc.), NF-κB signaling, apoptosis and autophagy, etc. TRAF3 is suggested to be related to multiple diseases and disorders including, at least, herpes simplex encephalitis 3, herpes simplex meningo-encephalitis, splenic marginal zone lymphoma, etc.

The nucleic acid and amino acid sequences of a representative human TRAF3 is available to the public at the GenBank database (Gene ID 7187) and is shown in Table 1 (*e.g.*, NM_145725.2 and NP_663777.1, representing the longest transcript variant 1 and the longest isoform 1, NM_145726.2 and NP_663778.1, representing the transcript variant 2 (lacking an in-frame coding segment compared to variant 1) and the encoded isoform 2 (lacking an internal region compared to isoform 1), NM_003300.3 and NP_003291.2, representing the transcript variant 3 (differing in the 5'UTR compared to variant 1) and the encoded isoform 1 (*i.e.*, having the same sequence as isoform 1), and NM_001199427.1 and NP_001186356.1, representing the transcript variant 4 (differing in the 5'UTR and

lacking an in-frame coding segment compared to variant 1) and the encoded isoform 3 (lacking an internal region compared to isoform 1). The domain structure of TRAF3 polypeptide is well known and accessible in UniProtKB database under the accession number Q13114, including, in the order from the 5' terminus to the 3' terminus, a RING-type zinc finger domain comprising, *e.g.*, amino acid positions 68-77 of NP_663777.1, two TRAF-type zinc finger domains comprising, *e.g.*, amino acid positions 135-190 and 191-249 of NP_663777.1, a coiled coil region comprising, *e.g.*, amino acid positions 267-338 of NP_663777.1, and a meprin and TRAF-C homology (MATH) domain comprising, *e.g.*, amino acid positions 415-560 of NP_663777.1.

Nucleic acid and polypeptide sequences of TRAF3 orthologs in organisms other than humans are well-known and include, for example, chimpanzee (*Pan troglodytes*) TRAF3 (XM_016926751.1 and XP_016782240.1, XM_016926752.1 and XP_016782241.1, XM_016926754.1 and XP_016782243.1, and XM_016926753.1 and XP_016782242.1), Rhesus monkey TRAF3 (XM_015144530.1 and XP_015000016.1, XM_001082535.3 and XP_001082535.3, XM_015144532.1 and XP_015000018.1, XM_015144534.1 and XP_015000020.1, XM_015144531.1 and XP_015000017.1, and XM_015144529.1 and XP_015000015.1), dog TRAF3 (XM_849522.4 and XP_854615.1, and XM_003435066.3 and XP_003435114.1), mouse TRAF3 (NM_011632.3 and NP_035762.2, representing the transcript variant 1 and the encoded longer isoform a, and NM_001286122.1 and NP_001273051.1, representing the transcript variant 3 (lacking an internal exon in the 5' UTR and an in-frame exon in the coding region, compared to variant 1) and the encoded shorter isoform b (lacking an internal segment, compared to isoform a)), cattle TRAF3 (NM_001205586.1 and NP_001192515.1), Norway rat (*Rattus norvegicus*) TRAF3 (NM_001108724.1 and NP_001102194.1), chicken TRAF3 (XM_004936344.2 and XP_004936401.1, XM_004936341.2 and XP_004936398.1, XM_015287823.1 and XP_015143309.1, XM_015287819.1 and XP_015143305.1, XM_015287818.1 and XP_015143304.1, XM_004936343.2 and XP_004936400.1, XM_015287825.1 and XP_015143311.1, XM_015287827.1 and XP_015143313.1, XM_015287820.1 and XP_015143306.1, XM_015287821.1 and XP_015143307.1, XM_015287822.1 and XP_015143308.1, and XM_015287826.1 and XP_015143312.1), tropical clawed frog (*Xenopus tropicalis*) TRAF3 (XM_002937990.4 and XP_002938036.2), and zebrafish (*Danio rerio*) TRAF3 (NM_001003513.1 and NP_001003513.1).

The term "TRAF3 activity" includes the ability of a TRAF3 polypeptide (and its fragments, domains, and/or motifs thereof, discussed herein) to bind its substrate and/or biological activity. TRAF3 activity may also include one or more of functions, such as forming E3 ubiquitin-protein ligase complexes, and/or others disclosed herein in the NF-κB pathway and other related pathways. For example, TRAF3 may interact with various proteins disclosed herein for its functions in signaling. TRAF3 may also be proteolyticly modified, such as being cleaved, ubiquitinated, deubiquitinated, or otherwise disclosed herein, for its functions.

The term "TRAF3 substrate(s)" refers to binding partners of a TRAF3 polypeptide (and its fragments, domains, and/or motifs thereof, discussed herein), *e.g.*, the binding partners described herein of TRAF3 for multiple signal transduction pathways. Furthermore, TRAF3 substrates may refer to downstream members in the signaling pathways where TRAF3 has a functional role.

The term "TRAF3-regulated signaling pathway(s)" includes signaling pathways in which TRAF3 (and its fragments, domains, and/or motifs thereof, discussed herein) binds to at least one of its substrate, through which at least one cellular function and/or activity and/or cellular protein profiles is changed. TRAF3-regulated signaling pathways include at least those described herein, such as activated TLR4 signaling (*e.g.*, MyD88-independent TLR3/TLR4 cascade, TLR3 and TLR4 cascade, etc.), RIG-I/MDA5 mediated induction of IFN-alpha/beta pathways (*e.g.*, RIG-I-like Receptor (RLR) signaling pathways, TRAF3-dependent IRF activation pathway, etc.), RANK signaling in osteoclasts (*e.g.*, APRIL pathway, BAFF in B-cell signaling, etc.), TNFR1 pathway (TWEAK pathway, GITR pathway, etc.), NF-κB signaling, apoptosis and autophagy, etc.

The term "TRAF3 inhibitor(s)" includes any natural or non-natural agent prepared, synthesized, manufactured, and/or purified by human that is capable of reducing, inhibiting, blocking, preventing, and/or that inhibits the ability of a TRAF3 polypeptide (and its fragments, domains, and/or motifs thereof, discussed herein). In one embodiment, such inhibitors may reduce or inhibit the binding/interaction between TRAF3 and its substrates or other binding partners. In still another embodiment, such inhibitors may increase or promote the turnover rate, reduce or inhibit the expression and/or the stability (*e.g.*, the half-life), and/or change the cellular localization of TRAF3, resulting in at least a decrease in TRAF3 levels and/or activity. Such inhibitors may be any molecule, including but not limited to small molecule compounds, antibodies or intrabodies, RNA interfering (RNAi)

agents (including at least siRNAs, shRNAs, microRNAs (miRNAs), piwi, and other well-known agents). Such inhibitors may be specific to TRAF3 or also inhibit at least one of other TRAF family members and TNF receptor (TNFR) superfamily members. RNA interference for TRAF3 polypeptides are well known and commercially available (*e.g.*, human, mouse, or rat shRNA (Cat. # TL502320 and TG300876) and siRNA (Cat. # SR304928 and SR417046) products and human or mouse gene knockout kit via CRISPR (Cat. # KN318123 and KN210417) from Origene (Rockville, MD), siRNA/shRNA products (Cat. # sc-29510, sc-36712, and sc-44277) and CRISPR products (Cat. # sc-423494 and sc-400473) from Santa Cruz Biotechnology (Dallas, Texas), Ready-to-package AAV shRNA clones from Vigene Biosciences (Rockville, MD, Cat. # SH872930 and SH800612). Methods for detection, purification, and/or inhibition of TRAF3 (*e.g.*, by anti-TRAF3 antibodies) are also well known and commercially available (*e.g.*, multiple anti-TRAF3 antibodies from Origene (Cat. # TA322870, TA322871, TA336438, etc.), Cell Signaling Technology (Danvers, MA, Cat. # 4729), abcam (Cambridge, MA, Cat. # ab36988, ab155298, etc.), and Santa Cruz Biotechnology (Cat. # sc-949, sc-6933, sc-1828, etc.)). Human TRAF3 knockout cell lines are also well known and available from Horizon Discovery (Cambridge, UK, Cat. # HZGHC002608c001).

The term "RNF31," also known as RING finger protein 31, HOIL-1-interacting protein, ZIBRA, and HOIP, refers to a member of a family of proteins containing RING-type zinc fingers, a motif present in a variety of functionally distinct proteins and known to be involved in protein-DNA and protein-protein interactions. RNF31 is an E3 ubiquitin-protein ligase component of the linear ubiquitin chain assembly complex (LUBAC), which conjugates linear (Met-1-linked) polyubiquitin chains to substrates and plays a key role in NF-κB activation and regulation of inflammation. LUBAC conjugates linear polyubiquitin to IKBKG and RIPK1 and is involved in activation of the canonical NF-κB and the JNK signaling pathways. Linear ubiquitination mediated by the LUBAC complex interferes with TNF-induced cell death and thereby prevents inflammation. LUBAC is proposed to be recruited to the TNF-R1 signaling complex (TNF-RSC) following polyubiquitination of TNF-RSC components by BIRC2 and/or BIRC3 and to conjugate linear polyubiquitin to IKBKG and possibly other components contributing to the stability of the complex. Together with OTULIN, the LUBAC complex regulates the canonical Wnt signaling during angiogenesis. RNF31 binds polyubiquitin of different linkage types. LUBAC comprises SHARPIN, RBCK1 and RNF31, with a molecular weight of about 600 kDa, suggesting a

heteromultimeric assembly of its subunits. RNF31 associates with the TNF-R1 signaling complex (TNF-RSC) in a stimulation-dependent manner. RNF31 also interacts (via the PUB domain) with OTULIN (via the PIM motif) and interacts (via the PUB domain) with VCP (via the PIM motif). RNF31 also interacts with CYLD and MUSK. RNF31 functions in multiple pathways, including, at least, TNF signaling (REACTOME, *e.g.*, death receptor signaling, TNFR1-induced NF-κB pathway), Toll-Like receptor signaling pathways (*e.g.*, NOD-like receptor signaling pathway), ubiquitination cascade pathway, GPCR signaling, etc. RNF31 is suggested to be related to multiple diseases and disorders including, at least polyglucosan body myopathy 1 with or without immunodeficiency, otulipenia (*e.g.*, autoinflammation, panniculitis and dermatosis syndrome), glycogen storage disease iv (*e.g.*, amylopectinosis), hepatitis c virus infection, etc.

The nucleic acid and amino acid sequences of a representative human RNF31 is available to the public at the GenBank database (Gene ID 55072) and is shown in Table 1 (*e.g.*, NM_017999.4 and NP_060469.4, representing the longer transcript variant 1 and the encoded longer isoform 1, and NM_001310332.1 and NP_001297261.1, representing the shorter transcript variant 2 (using an alternate first exon and an alternate splice site in a 5' exon compared to variant 1, resulting in the use of a downstream translation initiation site) and the encoded shorter isoform 2 (having a distinct N-terminus)). The domain structure of RNF31 polypeptide is well known and accessible in UniProtKB database under the accession number Q9UHD2, including, in the order from the 5' terminus to the 3' terminus, a PNGase/UBA or UBX (PUB) domain comprising, *e.g.*, amino acid positions 71-142 of NP_060469.4, and a UBA domain comprising, *e.g.*, amino acid positions 564-615 of NP_060469.4. Other regions include, for example, a polyubiquitin-binding region comprising, *e.g.*, amino acid positions 1-485 of NP_060469.4, a region interacting with RBCK1 comprising, *e.g.*, amino acid positions 563-616 of NP_060469.4, and an linear ubiquitin chain determining domain (LDD) region comprising, *e.g.*, amino acid positions 910-1072 of NP_060469.4. Multiple zinc finger domains on RNF31 include three RanBP2-type zinc finger domains comprising, *e.g.*, amino acid positions 299-329, 350-379, and 409-438 of NP_060469.4, one RING-type zinc finger comprising, *e.g.*, amino acid positions 699-747 of NP_060469.4, one IBR-type zinc finger comprising, *e.g.*, amino acid positions 779-841 of NP_060469.4, and another RING-type zinc finger comprising, *e.g.*, amino acid positions 860-909 of NP_060469.4. The PUB domain of RNF31 mediates interaction with the PIM motifs of VCP and RNF31, with a strong preference for RNF31. The RanBP2-

type zinc fingers mediate the specific interaction with ubiquitin. The UBA domain mediates association with RBCK1/HOIL1 via interaction with its UBL domain. RING 1 and IBR zinc-fingers catalyze the first step transfer of ubiquitin from the E2 onto RING 2, to transiently form a HECT-like covalent thioester intermediate (Smit *et al.* (2012) *EMBO J.* 31:3833-3844). The LDD domain mediates the final transfer of ubiquitin from RING 2 onto the N-terminus of a target ubiquitin.

Nucleic acid and polypeptide sequences of RNF31 orthologs in organisms other than humans are well-known and include, for example, chimpanzee (*Pan troglodytes*) RNF31 (XM_001166671.4 and XP_001166671.2, XM_009427566.2 and XP_009425841.2, and XM_009427568.2 and XP_009425843.2), Rhesus monkey RNF31 (XM_001112195.3 and XP_001112195.1, and XM_015143429.1 and XP_014998915.1), dog RNF31 (XM_005623255.2 and XP_005623312.1, XM_005623256.2 and XP_005623313.1, XM_537383.5 and XP_537383.2, and XM_005623257.2 and XP_005623314.1), mouse RNF31 (NM_194346.2 and NP_919327.2), Norway rat (*Rattus norvegicus*) RNF31 (NM_001108868.1 and NP_001102338.2), and tropical clawed frog (*Xenopus tropicalis*) RNF31 (NM_001097175.1 and NP_001090644.1).

The term "RNF31 activity" includes the ability of a RNF31 polypeptide (and its fragments, domains, and/or motifs thereof, discussed herein) to bind its substrate and/or biological activity. RNF31 activity may also include one or more of functions, such as its E3 ubiquitin ligase activity, and/or others disclosed herein in the NF-κB pathway and other related pathways. For example, RNF31 may interact with various proteins (*e.g.*, its ubiquitination substrates) disclosed herein for its functions in signaling. RNF31 may also be proteolyticly modified, such as being cleaved, ubiquitinated, deubiquitinated, or otherwise disclosed herein, for it functions.

The term "RNF31 substrate(s)" refers to binding partners of a RNF31 polypeptide (and its fragments, domains, and/or motifs thereof, discussed herein), *e.g.*, the substrates described herein of RNF31 as an E3 ubiquitin ligase. Furthermore, RNF31 substrates may refer to downstream members in the signaling pathways where RNF31 has a functional role.

The term "RNF31-regulated signaling pathway(s)" includes signaling pathways in which RNF31 (and its fragments, domains, and/or motifs thereof, discussed herein) binds to at least one of its substrate, through which at least one cellular function and/or activity and/or cellular protein profiles is changed. In some embodiments, RNF31 is an E3

ubiquitin ligase and promotes ubiquitination of its substrates. RNF31-regulated signaling pathways include at least those described herein, such as TNF signaling (REACTOME, *e.g.*, death receptor signaling, TNFR1-induced NF-κB pathway), Toll-Like receptor signaling pathways (*e.g.*, NOD-like receptor signaling pathway), ubiquitination cascade pathway, GPCR signaling, etc. RNF31 is suggested to be related to multiple diseases and disorders including, at least polyglucosan body myopathy 1 with or without immunodeficiency, otulipenia (*e.g.*, autoinflammation, panniculitis and dermatosis syndrome), glycogen storage disease iv (*e.g.*, amylopectinosis), hepatitis c virus infection, etc.

The term "RNF31 inhibitor(s)" includes any natural or non-natural agent prepared, synthesized, manufactured, and/or purified by human that is capable of reducing, inhibiting, blocking, preventing, and/or that inhibits the ability of a RNF31 polypeptide (and its fragments, domains, and/or motifs thereof, discussed herein). In one embodiment, such inhibitors may reduce or inhibit the binding/interaction between RNF31 and its substrates or other binding partners. In another embodiment, such inhibitors may reduce or inhibit the catalytic function of RNF31 as an E3 ubiquitin ligase. In still another embodiment, such inhibitors may increase or promote the turnover rate, reduce or inhibit the expression and/or the stability (*e.g.*, the half-life), and/or change the cellular localization of RNF31, resulting in at least a decrease in RNF31 levels and/or activity (*e.g.*, its E3 ubiquitin ligase activity). Such inhibitors may be any molecule, including but not limited to small molecule compounds, antibodies or intrabodies, RNA interfering (RNAi) agents (including at least siRNAs, shRNAs, microRNAs (miRNAs), piwi, and other well-known agents). Such inhibitors may be specific to RNF31 or also inhibit at least one of other E3 ubiquitin ligases. RNA interference for RNF31 polypeptides are well known and commercially available (*e.g.*, human, mouse, or rat shRNA (Cat. # TF320708, TF508054, TF707395, etc.) and siRNA (Cat. # SR421932 and SR310467) products and human or mouse gene knockout kit via CRISPR (Cat. # KN314948 and KN204117) from Origene (Rockville, MD), siRNA/shRNA products (Cat. # sc-92101 and sc-153046) and CRISPR products (Cat. # sc-435274 and sc-412436) from Santa Cruz Biotechnology (Dallas, Texas), Ready-to-package AAV shRNA clones from Vigene Biosciences (Rockville, MD, Cat. # SH898682). Methods for detection, purification, and/or inhibition of RNF31 (*e.g.*, by anti-RNF31 antibodies) are also well known and commercially available (*e.g.*, anti-RNF31 antibodies from Origene (Cat. # TA329873 and TA302821), R&D System (Minneapolis, MN, Cat. #

MAB8039 and AF8039), abcam (Cambridge, MA, Cat. # ab40676, ab109735, ab12116, etc.), and Novus Biologicals (Littleton, CO; Cat. # AF8039, MAB8039, NBP2-27290, etc.)). Human RNF31 knockout cell lines are also well known and available from Horizon Discovery (Cambridge, UK, Cat. # HZGHC004529c011).

The term "RBCK1," also known as RanBP2-type and C3HC4-type zinc finger-containing protein 1, Hepatitis B Virus X-Associated Protein 4, RING Finger Protein 54, HOIL-1, and RNF54, refers to a member of a family of proteins containing multiple types of zinc fingers, a motif present in a variety of functionally distinct proteins and known to be involved in protein-DNA and protein-protein interactions. Like RNF31, RBCK1 is also an E3 ubiquitin-protein ligase component of the linear ubiquitin chain assembly complex (LUBAC), which conjugates linear (Met-1-linked) polyubiquitin chains to substrates and plays a key role in NF-κB activation and regulation of inflammation. LUBAC conjugates linear polyubiquitin to IKBKG and RIPK1 and is involved in activation of the canonical NF-κB and the JNK signaling pathways. Linear ubiquitination mediated by the LUBAC complex interferes with TNF-induced cell death and thereby prevents inflammation. LUBAC is proposed to be recruited to the TNF-R1 signaling complex (TNF-RSC) following polyubiquitination of TNF-RSC components by BIRC2 and/or BIRC3 and to conjugate linear polyubiquitin to IKBKG and possibly other components contributing to the stability of the complex. Together with OTULIN, the LUBAC complex regulates the canonical Wnt signaling during angiogenesis. LUBAC comprises SHARPIN, RBCK1 and RNF31, with a molecular weight of about 600 kDa, suggesting a heteromultimeric assembly of its subunits. RBCK1 promotes ubiquitination of oxidized IREB2, which requires both heme and oxygen. RBCK1 also promotes ubiquitination of TAB2 and IRF3 and their degradation by the proteasome. RBCK1 interacts with beta-I-type (PRKCB1), zeta-type protein kinase C (PRKCZ), UBE2L3, PRKCH, EYA1, TAB2, TAB3, MAP3K7, TRAF6, RIPK1, and IRF3. RBCK1 interacts with the HBV pX/HBx protein, which is required to activate transcription of the viral genome. RBCK1 associates with the TNF-R1 signaling complex (TNF-RSC) in a stimulation-dependent manner. RBCK1 functions in multiple pathways, including, at least, TNF signaling (REACTOME, *e.g.*, death receptor signaling, TNFR1-induced NF-κB pathway), Toll-Like receptor signaling pathways (*e.g.*, NOD-like receptor signaling pathway), GPCR signaling, class I MHC mediated antigen processing and presentation (*e.g.*, antigen processing through ubiquitination and proteasome degradation), adaptive immune system, etc. RBCK1 is suggested to be related to multiple

diseases and disorders including, at least, polyglucosan body myopathy 1 with or without immunodeficiency, branchiootic syndrome, and glycogen storage disease iv (*e.g.*, amylopectinosis), etc.

The nucleic acid and amino acid sequences of a representative human RBCK1 is available to the public at the GenBank database (Gene ID 10616) and is shown in Table 1 (*e.g.*, NM_006462.5 and NP_006453.1, representing transcript variant 1 and the encoded isoform 1, NM_031229.3 and NP_112506.2, representing transcript variant 2 and the encoded longest isoform 2, NM_001323956.1 and NP_001310885.1, representing transcript variant 3 and the encoded isoform 3, NM_001323958.1 and NP_001310887.1, representing transcript variant 4 and the encoded isoform 3, and NM_001323960.1 and NP_001310889.1, representing transcript variant 5 and the encoded isoform 4). The domain structure of RBCK1 polypeptide is well known and accessible in UniProtKB database under the accession number Q9BYM8, including, in the order from the 5' terminus to the 3' terminus, a ubiquitin-like (UBL) domain comprising, *e.g.*, amino acid positions 55-119 of NP_112506.2, and a coiled coil domain comprising, *e.g.*, amino acid positions 233-261 of NP_112506.2. A region for RBCK1 interacting with TAB2 comprises, *e.g.*, amino acid positions 1-270 of NP_112506.2. A region for RBCK1 interacting with IRF3 comprises, *e.g.*, amino acid positions 1-220 of NP_112506.2. A region for RBCK1 interacting with RNF31 comprises, *e.g.*, amino acid positions 69-131 of NP_112506.2. Multiple zinc finger domains on RBCK1 include one RanBP2-type zinc finger comprising, *e.g.*, amino acid positions 193-222 of NP_112506.2, two RING-type zinc fingers comprising, *e.g.*, amino acid positions 282-327 and 437-463 of NP_112506.2, and one IBR-type zinc finger comprising, *e.g.*, amino acid positions 362-411 of NP_112506.2. The RanBP2-type zinc finger, also called Npl4 zinc finger (NZF), mediates binding to 'Met-1'-linked polyubiquitins. The UBL domain mediates association with RNF31 via interaction with its UBA domain (Ikeda *et al.* (2011) *Nature* 471:637-641).

Nucleic acid and polypeptide sequences of RBCK1 orthologs in organisms other than humans are well-known and include, for example, chimpanzee (*Pan troglodytes*) RBCK1 (XM_001152050.5 and XP_001152050.1, XM_016937253.1 and XP_016792742.1, XM_009436647.2 and XP_009434922.1, XM_016937254.1 and XP_016792743.1, XM_016937252.1 and XP_016792741.1, and XM_016937255.1 and XP_016792744.1), Rhesus monkey RBCK1 (NM_001266297.1 and NP_001253226.1), dog RBCK1 (XM_542942.5 and XP_542942.4), mouse RBCK1 (NM_001083921.1 and

NP_001077390.1, representing the longer transcript variant 1 and the encoded protein, and NM_019705.3 and NP_062679.2, representing the shorter transcript variant 2 (differing in the 5' UTR compared to variant 1) and the encoded same protein), cattle RBCK1 (NM_001075161.1 and NP_001068629.1), pig RBCK1 (XM_021078286.1 and XP_020933945.1, and XM_021078287.1 and XP_020933946.1), Norway rat (*Rattus norvegicus*) RBCK1 (NM_021764.1 and NP_068532.2), and tropical clawed frog (*Xenopus tropicalis*) RBCK1 (NM_001097175.1 and NP_001090644.1), and zebrafish RBCK1 (NM_001002168.1 and NP_001002168.1).

The term "RBCK1 activity" includes the ability of a RBCK1 polypeptide (and its fragments, domains, and/or motifs thereof, discussed herein) to bind its substrate and/or biological activity. RBCK1 activity may also include one or more of functions, such as its E3 ubiquitin ligase activity, and/or others disclosed herein in the NF-κB pathway and other related pathways. For example, RBCK1 may interact with various proteins (*e.g.*, its ubiquitination substrates) disclosed herein for its functions in signaling. RBCK1 may also be proteolyticly modified, such as being cleaved, ubiquitinated, deubiquitinated, or otherwise disclosed herein, for it functions.

The term "RBCK1 substrate(s)" refers to binding partners of a RBCK1 polypeptide (and its fragments, domains, and/or motifs thereof, discussed herein), *e.g.*, the substrates described herein of RBCK1 as an E3 ubiquitin ligase. Furthermore, RBCK1 substrates may refer to downstream members in the signaling pathways where RBCK1 has a functional role.

The term "RBCK1-regulated signaling pathway(s)" includes signaling pathways in which RBCK1 (and its fragments, domains, and/or motifs thereof, discussed herein) binds to at least one of its substrate, through which at least one cellular function and/or activity and/or cellular protein profiles is changed. In some embodiments, RBCK1 is an E3 ubiquitin ligase and promotes ubiquitination of its substrates. RBCK1-regulated signaling pathways include at least those described herein, such as TNF signaling (REACTOME, *e.g.*, death receptor signaling, TNFR1-induced NF-κB pathway), Toll-Like receptor signaling pathways (*e.g.*, NOD-like receptor signaling pathway), GPCR signaling, class I MHC mediated antigen processing and presentation (*e.g.*, antigen processing through ubiquitination and proteasome degradation), adaptive immune system, etc.

The term "RBCK1 inhibitor(s)" includes any natural or non-natural agent prepared, synthesized, manufactured, and/or purified by human that is capable of reducing, inhibiting,

blocking, preventing, and/or that inhibits the ability of a RBCK1 polypeptide (and its fragments, domains, and/or motifs thereof, discussed herein). In one embodiment, such inhibitors may reduce or inhibit the binding/interaction between RBCK1 and its substrates or other binding partners. In another embodiment, such inhibitors may reduce or inhibit the catalytic function of RBCK1 as an E3 ubiquitin ligase. In still another embodiment, such inhibitors may increase or promote the turnover rate, reduce or inhibit the expression and/or the stability (*e.g.*, the half-life), and/or change the cellular localization of RBCK1, resulting in at least a decrease in RBCK1 levels and/or activity (*e.g.*, its E3 ubiquitin ligase activity). Such inhibitors may be any molecule, including but not limited to small molecule compounds, antibodies or intrabodies, RNA interfering (RNAi) agents (including at least siRNAs, shRNAs, microRNAs (miRNAs), piwi, and other well-known agents). Such inhibitors may be specific to RBCK1 or also inhibit at least one of other E3 ubiquitin ligases. RNA interference for RBCK1 polypeptides are well known and commercially available (*e.g.*, human, mouse, or rat shRNA (Cat. # TR309920, TF502567, TF710513, etc.) and siRNA (Cat. # SR307231, SR416003, and SR503965) products and human or mouse gene knockout kit via CRISPR (Cat. # KN203906 and KN314537) from Origene (Rockville, MD), siRNA/shRNA products (Cat. # sc-61446 and sc-61447) and CRISPR products (Cat. # sc-423985 and sc-402044) from Santa Cruz Biotechnology (Dallas, Texas), Ready-to-package AAV shRNA clones from Vigene Biosciences (Rockville, MD, Cat. # SH865441 and SH819112). Methods for detection, purification, and/or inhibition of RBCK1 (*e.g.*, by anti-RBCK1 antibodies) are also well known and commercially available (*e.g.*, anti-RBCK1 antibodies from Origene (Cat. # AP11958PU-N), abcam (Cambridge, MA, Cat. # ab38540, ab219955, ab108479, etc.), Novus Biologicals (Littleton, CO; Cat. # NBP1-88301, H00010616-M01, NBP2-59048, etc.), and Santa Cruz Biotechnology (sc-365523, sc-367525, sc-49718, etc.)). Human RBCK1 knockout cell lines are also well known and available from Horizon Discovery (Cambridge, UK, Cat. # HZGHC10616).

The term "OTULIN," also known as OTU (Ovarian Tumor) domain-containing deubiquitinase with linear linkage specificity, ubiquitin thioesterase, Gumby, AIPDS, GUM, and FAM105B, refers to a member of a group of ubiquitin isopeptidases in the peptidase C65 family. OTULIN specifically recognizes and removes M1(Met1)-linked, or linear, ubiquitin chains from protein substrates, while linear ubiquitin chains are known to regulate the NF-kappa B signaling pathway in the context of immunity and inflammation. Mutations in *Otulin* cause a potentially fatal autoinflammatory syndrome in human patients.

As a deubiquitinase, OTULIN acts as a regulator of angiogenesis and innate immune response. OTULIN also facilitates thiol-dependent hydrolysis of ester, thioester, amide, peptide and isopeptide bonds formed by the C-terminal Gly of ubiquitin (Mevissen *et al.* (2013) *Cell* 154:169-184). OTULIN associates with the LUBAC complex via direct interaction with RNF31 and counteracts its action by cleaving linear polyubiquitin chains to substrates (Keusekotten *et al.* (2013) *Cell* 153:1312-1326). Thus, OTULIN acts as a negative regulator of NF-κB by counteracting activity of the LUBAC complex and maintaining homeostasis of the LUBAC complex by restricting autoubiquination of the LUBAC complex subunit RNF31. OTULIN is required during angiogenesis, craniofacial and neuronal development by regulating the canonical Wnt signaling together with the LUBAC complex. Some reports show that OTULIN function is restricted to homeostasis of the LUBAC complex, because it is not stably associated with TNF or NOD2 receptor signaling complexes (RSCs). However, further report have shown that it plays active roles in receptor signaling. For example, OTULIN acts as a key negative regulator of inflammation by restricting spontaneous inflammation and maintaining immune homeostasis. In myeloid cell, OTULIN is required to prevent unwarranted secretion of cytokines leading to inflammation and autoimmunity by restricting linear polyubiquitin formation. OTULIN plays a key role in innate immune response by restricting linear polyubiquitin formation on RIPK2 in response to NOD2 stimulation, probably to limit NOD2-dependent proinflammatory signaling. OTULIN interacts with RNF31 (through the PIM motif of OTULIN and the PUB domain of RNF31), segment polarity protein disheveled homolog DVL2, transmembrane protein 239, protein FAM168A, Matrix protein 2, OTU domain-containing protein 1, OTU domain-containing protein 7B, and DAZ-associated protein 2 (Damgaard *et al.* (2016) *Cell* 166:1215-1230). OTULIN functions in multiple pathways, including, at least, TNF signaling (REACTOME, *e.g.*, death receptor signaling, TNFR1 signaling), NF-κB pathway, ubiquitination cascade pathway, metabolism of proteins (*e.g.*, post-translational protein modification), GPCR signaling, etc. OTULIN is suggested to be related to multiple diseases and disorders including, at least, otulipenia (*e.g.*, autoinflammation, panniculitis and dermatosis syndrome (AIPDS)), and panniculitis, etc.

The nucleic acid and amino acid sequences of a representative human OTULIN is available to the public at the GenBank database (Gene ID 90268) and is shown in Table 1 (*e.g.*, NM_138348.5 and NP_612357.4). The domain structure of OTULIN polypeptide is

well known and accessible in UniProtKB database under the accession number Q96BN8, including, an OTU-like cysteine protease domain comprising, *e.g.*, amino acid positions 118-346 of NP_612357.4. In addition, OTULIN comprises multiple linear diubiquitin binding motifs comprising, *e.g.*, amino acid positions 95-96, 124-126, 255-259, 283-289, and 336-338 of NP_612357.4. A PIM motif locates at, *e.g.*, amino acid positions 52-57 of NP_612357.4, responsible for binding to the PUB domain of RNF31 (Schaeffer *et al.* (2014) *Mol. Cell* 54:349-361).

Nucleic acid and polypeptide sequences of OTULIN orthologs in organisms other than humans are well-known and include, for example, chimpanzee (*Pan troglodytes*) OTULIN (XM_009448807.2 and XP_009447082.2, and XM_517640.6 and XP_517640.3), Rhesus monkey OTULIN (NM_001193800.1 and NP_001180729.1), dog OTULIN (XM_005639659.2 and XP_005639716.1, XM_843160.4 and XP_848253.2, XM_014110329.1 and XP_013965804.1, and XM_005639660.2 and XP_005639717.1), mouse OTULIN (NM_001013792.2 and NP_001013814.2), cattle OTULIN (NM_001100328.1 and NP_001093798.1), chicken OTULIN (XM_015282228.1 and XP_015137714.1, XM_004935111.2 and XP_004935168.2, XM_015282238.1 and XP_015137724.1, XM_015282229.1 and XP_015137715.1, XM_015282239.1 and XP_015137725.1, XM_015282236.1 and XP_015137722.1, XM_015282233.1 and XP_015137719.1, XM_015282230.1 and XP_015137716.1, XM_004935105.2 and XP_004935162.2, XM_004935104.2 and XP_004935161.2, XM_003640779.3 and XP_003640827.3, XM_015282235.1 and XP_015137721.1, XM_015282237.1 and XP_015137723.1, XM_015282231.1 and XP_015137717.1, and XM_015282232.1 and XP_015137718.1), Norway rat (*Rattus norvegicus*) OTULIN (NM_001302889.1 and NP_001289818.1), and tropical clawed frog (*Xenopus tropicalis*) OTULIN (XM_018094965.1 and XP_017950454.1, XM_004915327.3 and XP_004915384.1, XM_004915328.3 and XP_004915385.1, XM_004915329.3 and XP_004915386.1, XM_004915330.3 and XP_004915387.1, XM_018094966.1 and XP_017950455.1, XM_002933121.4 and XP_002933167.3, and XM_004915325.3 and XP_004915382.2), and zebrafish OTULIN (NM_001166015.1 and NP_001159487.1).

The term "OTULIN activity" includes the ability of an OTULIN polypeptide (and its fragments, domains, and/or motifs thereof, discussed herein) to bind its substrate and/or biological activity. OTULIN activity may also include one or more of functions, such as its deubiquitinase activity, and/or others disclosed herein in the NF-κB pathway and other

related pathways. For example, OTULIN may interact with various proteins (*e.g.*, its deubiquitination substrates) disclosed herein for its functions in signaling. OTULIN may also be proteolyticly modified, such as being cleaved, ubiquitinated, deubiquitinated, acetylated, phosphorylated, or otherwise disclosed herein, for it functions.

The term "OTULIN substrate(s)" refers to binding partners of an OTULIN polypeptide (and its fragments, domains, and/or motifs thereof, discussed herein), *e.g.*, the substrates described herein of OTULIN as a deubiquitinase. Furthermore, OTULIN substrates may refer to downstream members in the signaling pathways where OTULIN has a functional role.

The term "OTULIN-regulated signaling pathway(s)" includes signaling pathways in which OTULIN (and its fragments, domains, and/or motifs thereof, discussed herein) binds to at least one of its substrate, through which at least one cellular function and/or activity and/or cellular protein profiles is changed. In some embodiments, OTULIN is a deubiquitinase and removes ubiquitin from and/or restricts linear polyubiquitination formation on its substrates. OTULIN-regulated signaling pathways include at least those described herein, such as TNF signaling (REACTOME, *e.g.*, death receptor signaling, TNFR1 signaling), NF-κB pathway, ubiquitination cascade pathway, metabolism of proteins (*e.g.*, post-translational protein modification), GPCR signaling, etc.

The term "OTULIN inhibitor(s)" includes any natural or non-natural agent prepared, synthesized, manufactured, and/or purified by human that is capable of reducing, inhibiting, blocking, preventing, and/or that inhibits the ability of an OTULIN polypeptide (and its fragments, domains, and/or motifs thereof, discussed herein). In one embodiment, such inhibitors may reduce or inhibit the binding/interaction between OTULIN and its substrates or other binding partners. In another embodiment, such inhibitors may reduce or inhibit the catalytic function of OTULIN as a deubiquitinase. In still another embodiment, such inhibitors may increase or promote the turnover rate, reduce or inhibit the expression and/or the stability (*e.g.*, the half-life), and/or change the cellular localization of OTULIN, resulting in at least a decrease in OTULIN levels and/or activity(*e.g.*, its deubiquitinase activity). Such inhibitors may be any molecule, including but not limited to small molecule compounds, antibodies or intrabodies, RNA interfering (RNAi) agents (including at least siRNAs, shRNAs, microRNAs (miRNAs), piwi, and other well-known agents). Such inhibitors may be specific to OTULIN or also inhibit at least one of other deubiquitinase. RNA interference for OTULIN polypeptides are well known and commercially available

(*e.g.*, human or mouse shRNA (Cat. # TL304698 and TG517394) and siRNA (Cat. # SR313974 and SR405655) products and human or mouse gene knockout kit via CRISPR (Cat. # KN312688 and KN224840) from Origene (Rockville, MD, siRNA/shRNA products (Cat. # sc-91772 and sc-141659) and CRISPR products (Cat. # sc-407676 and sc-436617) from Santa Cruz Biotechnology (Dallas, Texas), Ready-to-package AAV shRNA clones from Vigene Biosciences (Rockville, MD, Cat. # SH811464). Methods for detection, purification, and/or inhibition of OTULIN (*e.g.*, by anti-OTULIN antibodies) are also well known and commercially available (*e.g.*, anti-OTULIN antibodies from Origene (Cat. # TA335406), abcam (Cambridge, MA, Cat. # ab151117, ab114137, ab182598, etc.), Novus Biologicals (Littleton, CO; Cat. # NBP2-14722, etc.), and Cell Signaling Technology (Danvers, MA, Cat. # 14127)). Human OTULIN knockout cell lines are also well known and available from Horizon Discovery (Cambridge, UK, Cat. # HZGHC90268).

The term "TRAF6," also known as TNF receptor-associated factor 6, RNF85, and Interleukin-1 signal transducer, refers to a member of the TNF receptor associated factor (TRAF) protein family. TRAF proteins associate with, and mediate the signal transduction from, members of the TNF receptor (TNFR) superfamily. TRAF6 participates in the signal from members of the TNFR superfamily as well as the Toll/IL-1 family, such as CD40, TNFSF11/RANCE and IL-1. TRAF6 interacts with various protein kinases including IRAK1/IRAK, SRC, and PKCzeta, which provides a link between distinct signaling pathways. TRAF6 functions as a signal transducer in the NF-κB pathway that activates IκB kinase (IKK) in response to proinflammatory cytokines. The interaction of TRAF6 with UBE2N/UBC13, and UBE2V1/UEV1A, which are ubiquitin conjugating enzymes catalyzing the formation of polyubiquitin chains, has been found to be required for IKK activation. TRAF6 also interacts with the transforming growth factor (TGF) beta receptor complex and is required for Smad-independent activation of the JNK and p38 kinases. TRAF6 has an amino terminal RING domain which is followed by four zinc-finger motifs, a central coiled-coil region and a highly conserved carboxyl terminal domain, known as the TRAF-C domain. Two alternatively spliced transcript variants, encoding an identical protein, have been reported. TRAF6 is an E3 ubiquitin ligase that, together with UBE2N and UBE2V1, mediates the synthesis of Lys-63-linked-polyubiquitin chains conjugated to proteins, such as IKBKG, IRAK1, AKT1 and AKT2. TRAF6 also mediates ubiquitination of free/unanchored polyubiquitin chain that leads to MAP3K7 activation. TRAF6 leads to the activation of NF-kappa-B and JUN and may be essential for the formation of functional

osteoclasts. TRAF6 seems to also play a role in dendritic cells (DCs) maturation and/or activation. TRAF6 represses c-Myb-mediated transactivation in B-lymphocytes. TRAF6 is an adapter protein that seems to play a role in signal transduction initiated via TNF receptor, IL-1 receptor and IL-17 receptor. TRAF6 regulates osteoclast differentiation by mediating the activation of adapter protein complex 1 (AP-1) and NF-κB, in response to RANK-L stimulation. Together with MAP3K8, TRAF6 mediates CD40 signals that activate ERK in B-cells and macrophages, and thus may play a role in the regulation of immunoglobulin production. TRAF6 can form homotrimers and homooligomers. The N-terminal region of TRAF6 is dimeric, while the C-terminal region is trimeric. Upon IL1B treatment, TRAF6 forms a complex with PELI1, IRAK1, IRAK4 and MYD88, which then recruits MAP3K7/TAK1, TAB1 and TAB2 to mediate NF-κB activation. Direct binding of SMAD6 to PELI1 prevents the complex formation and hence negatively regulates IL1R-TLR signaling and eventually NF-κB-mediated gene expression. TRAF6 binds to TNFRSF5/CD40, TNFRSF11A/RANK, NGFR, TNFRSF17, IRAK2, IRAK3, RIPK2, MAP3K1, MAP3K5, MAP3K14, CSK, TRAF, TRAF-interacting protein TRIP, and TNF receptor associated protein TDP2. TRAF6 also interacts with IL17R, SQSTM1 bridging NTRK1 and NGFR and forms a ternary complex with SQSTM1 and PRKCZ. TRAF6 further interacts with PELI2 and PELI3 and binds UBE2V1, TAX1BP1, ZNF675, ARRB1, ARRB2, MAP3K7, and TAB1/MAP3K7IP1 during IL-1 signaling. TRAF6 also interacts with UBE2N, TGFBR1, HDAC1, RANGAP1, AKT1, AKT2, and AKT3, NUMBL, RBCK1, TRAF3IP2, LIMD1, RSAD2/viperin, EIF2AK2/PKR (via the kinase catalytic domain), ZFAND5, IL1RL1, TRAFD1, AJUBA, MAVS/IPS1, WDR34 (via WD domains), IFIT3 (via N-terminus), TICAM2, CARD14, CD40, MAP3K8 (which is required for ERK activation), TICAM1 (this interaction is enhanced in the presence of WDFY1), TANK (which increases in response to DNA damage), USP10 (this interaction increases in response to DNA damage), and ZC3H12A (this interaction increases in response to DNA damage and is stimulated by TANK). TRAF6 functions in multiple pathways, including, at least, activated TLR4 signaling (*e.g.*, MyD88-independent TLR3/TLR4 cascade, TLR2, TLR3, TLR4, TLR 7/8, TLR 9, and TLR10 cascade, MAP kinase activation in TLR cascade, JNK (c-Jun kinases) phosphorylation and activation mediated by activated human TAK1, etc.), Toll comparative pathway (*e.g.*, TLR-TRIF pathway, IL-1 pathway, and iNOS signaling), RANK signaling in osteoclasts (*e.g.*, APRIL pathway, BAFF in B-cell signaling, etc.), and p75 NTR receptor-mediated signaling (*e.g.*, cell death signaling via NRAGE,

NRIF and NADE, NRIF signals cell death from the nucleus, NF-κB activation, etc.). TRAF6 is suggested to be related to multiple diseases and disorders including, at least, ectodermal dysplasia 10a, hypohidrotic/hair/nail type, autosomal dominant, incontinentia pigmenti (*e.g.*, bloch-sulzberger syndrome, bloch-siemens syndrome, etc.), immunodeficiency with hyper-igm, type 3 (*e.g.*, CD40 deficiency, hyper igm syndrome 3, etc.), toxoplasmosis, ectodermal dysplasia 10b, hypohidrotic/hair/tooth type, autosomal recessive (*e.g.*, x-linked hypohidrotic ectodermal dysplasia), etc.

The nucleic acid and amino acid sequences of a representative human TRAF6 is available to the public at the GenBank database (Gene ID 7189) and is shown in Table 1 (*e.g.*, NM_145803.2 and NP_665802.1, representing the longest transcript variant 1 and the encoded protein, and NM_004620.3 and NP_004611.1, representing the shorter transcript variant 2 (lacking a segment in the 5' UTR, compared to variant 1) and the encoded same protein). The domain structure of TRAF6 polypeptide is well known and accessible in UniProtKB database under the accession number Q9Y4K3, including, in the order from the 5' terminus to the 3' terminus, a RING-type zinc finger domain comprising, *e.g.*, amino acid positions 70-109 of NP_665802.1, two TRAF-type zinc finger domains comprising, *e.g.*, amino acid positions 150-202 and 203-259 of NP_665802.1, a coiled coil region comprising, *e.g.*, amino acid positions 288-348 of NP_665802.1, and a meprin and TRAF-C homology (MATH) domain comprising, *e.g.*, amino acid positions 350-499 of NP_665802.1. The N-terminal fragment of TRAF6 (comprising, *e.g.*, amino acid positions 1-354 of NP_665802.1) is capable of interacting with TAX1BP1 and the C-terminal fragment (comprising, *e.g.*, amino acid positions 355-522 of NP_665802.1) is capable of interacting with TANK.

Nucleic acid and polypeptide sequences of TRAF6 orthologs in organisms other than humans are well-known and include, for example, chimpanzee (*Pan troglodytes*) TRAF6 (XM_001154136.5 and XP_001154136.1, and XM_016920687.1 and XP_016776176.1), Rhesus monkey TRAF6 (NM_001135796.1 and NP_001129268.1), dog TRAF6 (XM_003432322.3 and XP_003432370.1), mouse TRAF6 (NM_009424.3 and NP_033450.2, representing the longer transcript variant 1 and the encoded protein, and NM_001303273.1 and NP_001290202.1, representing the shorter transcript variant 2 (lacking an exon in the 5' UTR, compared to variant 1) and the encoded same protein), cattle TRAF6 (NM_001034661.2 and NP_001029833.1), Norway rat (*Rattus norvegicus*) TRAF6 (NM_001107754.2 and NP_001101224.1), chicken TRAF6 (XM_004941548.2 and

XP_004941605.1, XM_004941547.2 and XP_004941604.1, XM_004941546.2 and XP_004941603.1, XM_004941545.2 and XP_004941602.1, and XM_015287208.1 and XP_015142694.1), tropical clawed frog (*Xenopus tropicalis*) TRAF6 (NM_001008161.2 and NP_001008162.2), fruit fly TRAF6 (NM_078525.4 and NP_511080.2), and zebrafish (*Danio rerio*) TRAF6 (NM_001044752.1 and NP_001038217.1).

The term "TRAF6 activity" includes the ability of a TRAF6 polypeptide (and its fragments, domains, and/or motifs thereof, discussed herein) to bind its substrate and/or biological activity. TRAF6 activity may also include one or more of functions, such as E3 ubiquitin-protein ligase activity, and/or others disclosed herein in the NF-κB pathway and other related pathways. For example, TRAF6 may interact with various proteins disclosed herein for its functions in signaling. TRAF6 may also be proteolyticly modified, such as being cleaved, ubiquitinated, deubiquitinated, acetylated, phosphorylated, or otherwise disclosed herein, for its functions.

The term "TRAF6 substrate(s)" refers to binding partners of a TRAF6 polypeptide (and its fragments, domains, and/or motifs thereof, discussed herein), *e.g.*, the binding partners described herein of TRAF6 for multiple signal transduction pathways. Furthermore, TRAF6 substrates may refer to downstream members in the signaling pathways where TRAF6 has a functional role.

The term "TRAF6-regulated signaling pathway(s)" includes signaling pathways in which TRAF6 (and its fragments, domains, and/or motifs thereof, discussed herein) binds to at least one of its substrate, through which at least one cellular function and/or activity and/or cellular protein profiles is changed. TRAF6-regulated signaling pathways include at least those described herein, such as activated TLR4 signaling (*e.g.*, MyD88-independent TLR3/TLR4 cascade, TLR2, TLR3, TLR4, TLR 7/8, TLR 9, and TLR10 cascade, MAP kinase activation in TLR cascade, JNK (c-Jun kinases) phosphorylation and activation mediated by activated human TAK1, etc.), Toll comparative pathway (*e.g.*, TLR-TRIF pathway, IL-1 pathway, and iNOS signaling), RANK signaling in osteoclasts (*e.g.*, APRIL pathway, BAFF in B-cell signaling, etc.), and p75 NTR receptor-mediated signaling (*e.g.*, cell death signaling via NRAGE, NRIF and NADE, NRIF signals cell death from the nucleus, NF-κB activation, etc.).

The term "TRAF6 inhibitor(s)" includes any natural or non-natural agent prepared, synthesized, manufactured, and/or purified by human that is capable of reducing, inhibiting, blocking, preventing, and/or that inhibits the ability of a TRAF6 polypeptide (and its

fragments, domains, and/or motifs thereof, discussed herein). In one embodiment, such inhibitors may reduce or inhibit the binding/interaction between TRAF6 and its substrates or other binding partners. In still another embodiment, such inhibitors may increase or promote the turnover rate, reduce or inhibit the expression and/or the stability (*e.g.*, the half-life), and/or change the cellular localization of TRAF6, resulting in at least a decrease in TRAF6 levels and/or activity. Such inhibitors may be any molecule, including but not limited to small molecule compounds, antibodies or intrabodies, RNA interfering (RNAi) agents (including at least siRNAs, shRNAs, microRNAs (miRNAs), piwi, and other well-known agents). Such inhibitors may be specific to TRAF6 or also inhibit at least one of other TRAF family members and TNF receptor (TNFR) superfamily members or related ubiquitin ligases. RNA interference for TRAF6 polypeptides are well known and commercially available (*e.g.*, human, mouse, or rat shRNA (Cat. # TG515356, TF300871, and TF706303) and siRNA (Cat. # SR416822, SR510754, and SR322090) products and human or mouse gene knockout kit via CRISPR (Cat. # KN206042 and KN318129) from Origene (Rockville, MD), siRNA/shRNA products (Cat. # sc-36717, sc-44329, sc-36718, etc.) and CRISPR products (Cat. # sc-423497) from Santa Cruz Biotechnology (Dallas, Texas), Ready-to-package AAV shRNA clones from Vigene Biosciences (Rockville, MD, Cat. # SH874771). Methods for detection, purification, and/or inhibition of TRAF6 (*e.g.*, by anti-TRAF6 antibodies) are also well known and commercially available (*e.g.*, multiple anti-TRAF6 antibodies from Origene (Cat. # TA321662, TA352399, AM26628AF-N, etc.), Cell Signaling Technology (Danvers, MA, Cat. # 8028), abcam (Cambridge, MA, Cat. # ab33915, ab94720, etc.), and Santa Cruz Biotechnology (Cat. # sc-33897, sc-8409, sc-33895, etc.)). Human TRAF6 knockout cell lines are also well known and available from Horizon Discovery (Cambridge, UK, Cat. # HZGHC003466c008).

The term "TAB2," also known as TGF-Beta Activated Kinase 1/MAP3K7 Binding Protein 2, Mitogen-Activated Protein Kinase Kinase Kinase 7-Interacting Protein 2, CHTD2, and MAP3K7IP2, refers to a member of a family of activators of MAP3K7/TAK1, which is required for the IL-1 induced activation of NF-κB and MAPK8/JNK. TAB2 forms a kinase complex with TRAF6, MAP3K7 and TAB1, thus serving as an adaptor that links MAP3K7 and TRAF6. TAB2, along with TAB1 and MAP3K7, also participates in the signal transduction induced by TNFSF11/RANKL through the activation of the receptor activator of NF-κB (TNFRSF11A/RANK), which may regulate the development and function of osteoclasts. Studies of the related mouse protein indicate that TAB2 functions

to protect against liver damage caused by chemical stressors. Mutations in *Tab2* cause congenital heart defects, multiple types, 2 (CHTD2) (Thienpont *et al.* (2010) *Am J Hum Genet.* 86:839-849). TAB2 binds to Lys-63-linked polyubiquitin chains and promotes autophosphorylation of MAP3K7 at Thr-187. TAB2 also interacts with NCOR1 and HDAC3 to form a ternary complex and interacts (via C-terminal) with NUMBL (via PTB domain) and with WDR34 (via WD domains). TAB2 has been shown to interact with RBCK1, TRAF6, TRIM5, HDAC3, MAP3K7IP1, MAP3K7IP3, MAP3K7, NFKB1, NUMBL, Nuclear receptor co-repressor 1, and TRAF2 (Baek *et al.* (2002) *Cell* 110:55-67; Li *et al.* (2009) *Mol. Cell* 33:30-42). TAB2 functions in multiple pathways, including, at least, activated TLR4 signaling (*e.g.*, MyD88-independent TLR3/TLR4 cascade, TLR2, TLR3, TLR4, TLR 7/8, TLR 9, and TLR10 cascade, MAP kinase activation in TLR cascade, JNK (c-Jun kinases) phosphorylation and activation mediated by activated human TAK1, etc.), Toll-like receptor signaling pathway (*e.g.*, NOD-like receptor signaling pathways, IL-1 family signaling pathways, Mucin expression in CF via TLRs, EGFR signaling pathways, etc.), TRAF pathway, bacterial infections in CF airways (*e.g.*, IL-1 beta-dependent CFTR expression, immune response TLR signaling pathways, etc.), NF-κB signaling, etc. TAB2 is related to multiple diseases and disorders including, at least, congenital heart defects, nonsyndromic, 2, left ventricular outflow tract obstruction (*e.g.*, aortic valve disease 2, coarctation of the aorta, hypoplastic left heart syndrome, etc.), pulmonary valve disease, ectodermal dysplasia 10b, hypohidrotic/hair/tooth type, autosomal recessive (*e.g.*, xhed, cst syndrome, etc.), etc.

The nucleic acid and amino acid sequences of a representative human TAB2 is available to the public at the GenBank database (Gene ID 23118) and is shown in Table 1 (*e.g.*, NM_015093.5 and NP_055908.1, representing the longest transcript variant 1 and the encoded longer isoform a, NM_001292034.2 and NP_001278963.1, representing the transcript variant 3 (differing in the 5' UTR compared to variant 1) and the encoded same isoform a, and NM_001292035.2 and NP_001278964.1, representing the transcript variant 4 (containing an alternate 5' exon structure, and thus differing in the 5' UTR and initiating translation at an alternate start codon, compared to variant 1) and the encoded shorter isoform b (having a distinct N-terminus)). The domain structure of TAB2 polypeptide is well known and accessible in UniProtKB database under the accession number Q9NYJ8, including, in the order from the 5' terminus to the 3' terminus, a CUE domain capable of binding ubiquitin comprising, *e.g.*, amino acid positions 8-51 of NP_055908.1, a coiled coil

region comprising, *e.g.*, amino acid positions 532-619 of NP_055908.1, and a RanBP2-type zinc finger domain capable of binding to two consecutive 'Lys-63'-linked ubiquitins comprising, *e.g.*, amino acid positions 663-693 of NP_055908.1.

Nucleic acid and polypeptide sequences of TAB2 orthologs in organisms other than humans are well-known and include, for example, chimpanzee (*Pan troglodytes*) TAB2 (XM_016956449.1 and XP_016811938.1, XM_009452188.2 and XP_009450463.2, XM_016956450.1 and XP_016811939.1, and XM_016956448.1 and XP_016811937.1), Rhesus monkey TAB2 (NM_001257790.2 and NP_001244719.1), dog TAB2 (XM_005615502.2 and XP_005615559.1, XM_014112730.1 and XP_013968205.1, XM_541145.5 and XP_541145.2, and XM_005615503.2 and XP_005615560.1), mouse TAB2 (NM_138667.3 and NP_619608.1), cattle TAB2 (NM_001192372.1 and NP_001179301.1), Norway rat (*Rattus norvegicus*) TAB2 (NM_001012062.1 and NP_001012062.1), chicken TAB2 (XM_015284276.1 and XP_015139762.1, XM_004935601.2 and XP_004935658.1, XM_419660.3 and XP_419660.1, XM_015284275.1 and XP_015139761.1), and tropical clawed frog (*Xenopus tropicalis*) TAB2 (NM_001097294.1 and NP_001090763.1).

The term "TAB2 activity" includes the ability of a TAB2 polypeptide (and its fragments, domains, and/or motifs thereof, discussed herein) to bind its substrate and/or biological activity. TAB2 activity may also include one or more of functions, such as activating kinases and promoting autophosphorylation, and/or others disclosed herein in the NF-κB pathway and other related pathways. For example, TAB2 may interact with various proteins disclosed herein for its functions in signaling. TAB2 may also be proteolyticly modified, such as being cleaved, ubiquitinated, deubiquitinated, acetylated, phosphorylated, or otherwise disclosed herein, for its functions.

The term "TAB2 substrate(s)" refers to binding partners of a TAB2 polypeptide (and its fragments, domains, and/or motifs thereof, discussed herein), *e.g.*, the binding partners described herein of TAB2 for multiple signal transduction pathways. Furthermore, TAB2 substrates may refer to downstream members in the signaling pathways where TAB2 has a functional role.

The term "TAB2-regulated signaling pathway(s)" includes signaling pathways in which TAB2 (and its fragments, domains, and/or motifs thereof, discussed herein) binds to at least one of its substrate, through which at least one cellular function and/or activity and/or cellular protein profiles is changed. TAB2-regulated signaling pathways include at

least those described herein, such as activated TLR4 signaling (*e.g.*, MyD88-independent TLR3/TLR4 cascade, TLR2, TLR3, TLR4, TLR 7/8, TLR 9, and TLR10 cascade, MAP kinase activation in TLR cascade, JNK (c-Jun kinases) phosphorylation and activation mediated by activated human TAK1, etc.), Toll-like receptor signaling pathway (*e.g.*, NOD-like receptor signaling pathways, IL-1 family signaling pathways, Mucin expression in CF via TLRs, EGFR signaling pathways, etc.), TRAF pathway, bacterial infections in CF airways (*e.g.*, IL-1 beta-dependent CFTR expression, immune response TLR signaling pathways, etc.), NF-κB Signaling, etc.

The term "TAB2 inhibitor(s)" includes any natural or non-natural agent prepared, synthesized, manufactured, and/or purified by human that is capable of reducing, inhibiting, blocking, preventing, and/or that inhibits the ability of a TAB2 polypeptide (and its fragments, domains, and/or motifs thereof, discussed herein). In one embodiment, such inhibitors may reduce or inhibit the binding/interaction between TAB2 and its substrates or other binding partners. In still another embodiment, such inhibitors may increase or promote the turnover rate, reduce or inhibit the expression and/or the stability (*e.g.*, the half-life), and/or change the cellular localization of TAB2, resulting in at least a decrease in TAB2 levels and/or activity. Such inhibitors may be any molecule, including but not limited to small molecule compounds, antibodies or intrabodies, RNA interfering (RNAi) agents (including at least siRNAs, shRNAs, microRNAs (miRNAs), piwi, and other well-known agents). Such inhibitors may be specific to TAB2 or also inhibit at least one of other related proteins. RNA interference for TAB2 polypeptides are well known and commercially available (*e.g.*, human, mouse, or rat shRNA (Cat. # TL316993, TF504187, and TF701571) and siRNA (Cat. # SR308018, SR419201, and SR509887) products and human or mouse gene knockout kit via CRISPR (Cat. # KN207721 and KN317118) from Origene (Rockville, MD), siRNA/shRNA products (Cat. # sc-41049, sc-41050, etc.) and CRISPR products (Cat. # sc-401596) from Santa Cruz Biotechnology (Dallas, Texas), Ready-to-package AAV shRNA clones from Vigene Biosciences (Rockville, MD, Cat. # SH888308). Methods for detection, purification, and/or inhibition of TAB2 (*e.g.*, by anti-TAB2 antibodies) are also well known and commercially available (*e.g.*, multiple anti-TAB2 antibodies from Origene (Cat. # AM06537SU-N, TA330285, TA319843, etc.), Cell Signaling Technology (Danvers, MA, Cat. # 3745 and 3744), abcam (Cambridge, MA, Cat. # ab172412, ab140201, etc.), and Santa Cruz Biotechnology (Cat. # sc-20756, sc-11851, sc-

398188, etc.)). Human TAB2 knockout cell lines are also well known and available from Horizon Discovery (Cambridge, UK, Cat. # HZGHC002592c005).

The term "TNIP1," also known as TNFAIP3-interacting protein 1, NAF1, Nip40-1, and A20-Binding Inhibitor of NF-κB Activation 1, refers to a member of a family of A20-binding proteins which plays a role in autoimmunity and tissue homeostasis through the regulation of NF-κB activation. TNIP1 inhibits NF-κB activation and TNF-induced NF-κB-dependent gene expression by regulating A20/TNFAIP3-mediated deubiquitination of IKBKG, thus linking A20/TNFAIP3 to ubiquitinated IKBKG. TNIP1 also regulates EGF-induced ERK1/ERK2 signaling pathway and blocks MAPK3/MAPK1 nuclear translocation and MAPK1-dependent transcription. TNIP1 increases cell surface CD4(T4) antigen expression. TNIP1 is involved in the anti-inflammatory response of macrophages and positively regulates TLR-induced activation of CEBPB. TNIP1 is also involved in the prevention of autoimmunity and leukocyte integrin activation during inflammation (mediated by association with SELPLG and dependent on phosphorylation by SRC-family kinases). TNIP1 interacts with HIV-1 matrix protein and is packaged into virions, while its overexpression inhibits viral replication. TNIP1 may regulate matrix nuclear localization, both nuclear import of PIC (Preintegration complex) and export of GAG polyprotein and viral genomic RNA during virion production. In case of infection, TNIP1 promotes association of IKBKG with Shigella flexneri E3 ubiquitin-protein ligase ipah9.8 p, which in turn promotes polyubiquitination and proteasome degradation of IKBKG and perturbs NF-κB activation during bacterial infection. TNIP1 interacts with TNFAIP3 (Heyninck *et al.* (1999) *J. Cell Biol.* 145:1471-1482) and MAPK1 (Zhang *et al.* (2002) *Biochem. Biophys. Res. Commun.* 297:17-23). TNIP1 also interacts with polyubiquitinated IKBKG and facilitates TNFAIP3-mediated de-ubiquitination of NEMO/IKBKG. TNIP1 further interacts with SELPLG, PIK3CD, IRAK1 (polyubiquitinated), MYD88 (indicative for participation in an activated TLR-signaling complex). TNIP1 functions in multiple pathways, including, at least, NF-κB signaling, TRAF pathway (*e.g.*, 14-3-3 induced apoptosis), deubiquitination and protein metabolism, IL-23 signaling pathway (*e.g.*, angiopoietin-TIE2 signaling), etc. TNIP1 is related to multiple diseases and disorders including, at least, psoriatic arthritis, chronic intestinal vascular insufficiency, HIV-1 infection/AIDS, rheumatoid arthritis, and systemic lupus erythematosus.

The nucleic acid and amino acid sequences of a representative human TNIP1 is available to the public at the GenBank database (Gene ID 10318) and is shown in Table 1.

Multiple transcript variants and resulting proteins of TNIP1 include, *e.g.*, NM_001252385.1 and NP_001239314.1, representing the longest transcript variant 1 and the encoded isoform 1, NM_001252386.1 and NP_001239315.1, representing the transcript variant 2 and the encoded isoform 2 (with a shorter N-terminus and a distinct C-terminus compared to isoform 1), NM_001252390.1 and NP_001239319.1, representing the transcript variant 3 (differing in the 5' UTR and using an alternate splice site in the 3' coding region, compared to variant 1) and the encoded longest isoform 3, NM_001252391.1 and NP_001239320.1, representing the transcript variant 4 (differing in the 5' UTR and using an alternate splice site in the 3' coding region compared to variant 1) and the encoded same isoform 3, NM_006058.4 and NP_006049.3, representing the transcript variant 5 (using an alternate splice site in the 3' coding region compared to variant 1) and the encoded same isoform 3, NM_001252392.1 and NP_001239321.1, representing the transcript variant 6 (using an alternate splice site in the 3' coding region compared to variant 1) and the encoded isoform 4 (longer and having a distinct C-terminus, compared to isoform 1), NM_001252393.1 and NP_001239322.1, representing the transcript variant 7 (using two alternate splice sites in the 3' coding region, compared to variant 1) and the encoded same isoform 4, NM_001258454.1 and NP_001245383.1, representing the transcript variant 8 (differing in the 5' UTR compared to variant 1) and the encoded same isoform 3, NM_001258455.1 and NP_001245384.1, representing the transcript variant 9 (lacking an exon and using an alternate splice site in the 3' coding region which results in a frameshift compared to variant 1) and the encoded isoform 5 (shorter and having a distinct C-terminus compared to isoform 1), and NM_001258456.1 and NP_001245385.1, representing the transcript variant 10 (lacking two exons in the 3' coding region which results in a frameshift compared to variant 1) and the encoded isoform 6 (having a distinct C-terminus). The domain structure of TNIP1 polypeptide is well known and accessible in UniProtKB database under the accession number Q15025, including, in the order from the 5' terminus to the 3' terminus, a domain capable of binding NEF comprising, *e.g.*, amino acid positions 94-412 of NP_006049.3, a domain capable of binding Shigella flexneri ipah9.8 comprising, *e.g.*, amino acid positions 351-367 of NP_006049.3, a domain for inhibitory activity of TNF-induced NF-κB activation comprising, *e.g.*, amino acid positions 431-588 of NP_006049.3, a ubiquitin-binding domain (UBD) comprising, *e.g.*, amino acid positions 452-510 of NP_006049.3, and a nuclear localization signal comprising, *e.g.*, amino acid positions 524-

530 of NP_006049.3. TNIP1 contains three coiled coil regions comprising, *e.g.*, amino acid positions 20-73, 196-258, and 294-535 of NP_006049.3.

Nucleic acid and polypeptide sequences of TNIP1 orthologs in organisms other than humans are well-known and include, for example, chimpanzee (*Pan troglodytes*) TNIP1 (XM_003310917.4 and XP_003310965.1, XM_016954075.1 and XP_016809564.1, XM_016954073.1 and XP_016809562.1, XM_016954074.1 and XP_016809563.1, XM_003310918.4 and XP_003310966.1, XM_001167619.5 and XP_001167619.1, XM_518040.6 and XP_518040.3, XM_016954076.1 and XP_016809565.1, and XM_016954077.1 and XP_016809566.1), Rhesus monkey TNIP1 (XM_002804583.2 and XP_002804629.1, XM_015141244.1 and XP_014996730.1, XM_015141246.1 and XP_014996732.1, XM_001109469.3 and XP_001109469.2, XM_001109428.3 and XP_001109428.1, XM_015141243.1 and XP_014996729.1, and XM_015141245.1 and XP_014996731.1), dog TNIP1 (XM_546296.5 and XP_546296.3, and XM_014113057.1 and XP_013968532.1), mouse TNIP1 (NM_021327.4 and NP_067302.2, representing the transcript variant 1 and the encoded longest isoform 1, NM_001199275.2 and NP_001186204.1, representing the transcript variant 2 (containing an alternate exon in the 5' UTR, compared to variant 1) and the encoded same isoform 1, NM_001199276.2 and NP_001186205.1, representing the transcript variant 3 (differing in the 5' UTR, lacking a portion of the 5' coding region, and initiating translation at a downstream in-frame start codon, compared to variant 1) and the encoded isoform 2 (having a shorter N-terminus, compared to isoform 1), NM_001271455.1 and NP_001258384.1, representing the transcript variant 4 (differing in the 5' UTR, lacking a portion of the 5' coding region, and initiating translation at a downstream in-frame start codon, compared to variant 1) and the encoded same isoform 2, and NM_001271456.1 and NP_001258385.1, representing the transcript variant 5 (differing in the 5' UTR and using an alternate splice site in the 3' coding region, which results in a frameshift, compared to variant 1) and the encoded isoform 3 (having a distinct C-terminus, compared to isoform 1)), cattle TNIP1 (NM_001024554.3 and NP_001019725.2), Norway rat (*Rattus norvegicus*) TNIP1 (NM_001108826.1 and NP_001102296.1), chicken TNIP1 (XM_004944944.2 and XP_004945001.1, XM_004944943.2 and XP_004945000.1, and XM_003642060.3 and XP_003642108.2), tropical clawed frog (*Xenopus tropicalis*) TNIP1 (NM_001079235.1 and NP_001072703.1), and zebrafish TNIP1 (NM_001079952.1 and NP_001073421.1).

The term "TNIP1 activity" includes the ability of a TNIP1 polypeptide (and its fragments, domains, and/or motifs thereof, discussed herein) to bind its substrate and/or biological activity. TNIP1 activity may also include one or more of functions, such as promoting deubiquitination, inhibiting NF-κB activation and gene expression, and/or others disclosed herein in the NF-κB pathway and other related pathways. For example, TNIP1 may interact with various proteins disclosed herein for its functions in signaling. TNIP1 may also be proteolyticly modified, such as being cleaved, ubiquitinated, deubiquitinated, acetylated, phosphorylated, or otherwise disclosed herein, for its functions.

The term "TNIP1 substrate(s)" refers to binding partners of a TNIP1 polypeptide (and its fragments, domains, and/or motifs thereof, discussed herein), *e.g.*, the binding partners described herein of TNIP1 for multiple signal transduction pathways. Furthermore, TNIP1 substrates may refer to downstream members in the signaling pathways where TNIP1 has a functional role.

The term "TNIP1-regulated signaling pathway(s)" includes signaling pathways in which TNIP1 (and its fragments, domains, and/or motifs thereof, discussed herein) binds to at least one of its substrate, through which at least one cellular function and/or activity and/or cellular protein profiles is changed. TNIP1-regulated signaling pathways include at least those described herein, such as NF-κB signaling, TRAF pathway (*e.g.*, 14-3-3 induced apoptosis), deubiquitination and protein metabolism, IL-23 signaling pathway (*e.g.*, angiopoietin-TIE2 signaling), etc.

The term "TNIP1 inhibitor(s)" includes any natural or non-natural agent prepared, synthesized, manufactured, and/or purified by human that is capable of reducing, inhibiting, blocking, preventing, and/or that inhibits the ability of a TNIP1 polypeptide (and its fragments, domains, and/or motifs thereof, discussed herein). In one embodiment, such inhibitors may reduce or inhibit the binding/interaction between TNIP1 and its substrates or other binding partners. In still another embodiment, such inhibitors may increase or promote the turnover rate, reduce or inhibit the expression and/or the stability (*e.g.*, the half-life), and/or change the cellular localization of TNIP1, resulting in at least a decrease in TNIP1 levels and/or activity. Such inhibitors may be any molecule, including but not limited to small molecule compounds, antibodies or intrabodies, RNA interfering (RNAi) agents (including at least siRNAs, shRNAs, microRNAs (miRNAs), piwi, and other well-known agents). Such inhibitors may be specific to TNIP1 or also inhibit at least one of other related proteins. RNA interference for TNIP1 polypeptides are well known and

commercially available (*e.g.*, human, mouse, or rat shRNA (Cat. # TF308719, TF512490, and TF707355) and siRNA (Cat. # SR306991 and SR418640) products and human or mouse gene knockout kit via CRISPR (Cat. # KN204210 and KN318006) from Origene (Rockville, MD), siRNA/shRNA products (Cat. # sc-92019 and sc-140779) and CRISPR products (Cat. # sc-425441) from Santa Cruz Biotechnology (Dallas, Texas), Ready-to-package AAV shRNA clones from Vigene Biosciences (Rockville, MD, Cat. # SH886816). Methods for detection, purification, and/or inhibition of TNIP1 (*e.g.*, by anti-TNIP1 antibodies) are also well known and commercially available (*e.g.*, anti-TNIP1 antibodies from Origene (Cat. # AP54318PU-N and TA351831), Cell Signaling Technology (Danvers, MA, Cat. # 4664), abcam (Cambridge, MA, Cat. # ab207584, ab70152, etc.), and Santa Cruz Biotechnology (Cat. # sc-134660)). Human TNIP1 knockout cell lines are also well known and available from Horizon Discovery (Cambridge, UK, Cat. # HZGHC10318).

The term "immune response" includes T cell mediated and/or B cell mediated immune responses. Exemplary immune responses include T cell responses, *e.g.*, cytokine production and cellular cytotoxicity. In addition, the term immune response includes immune responses that are indirectly effected by T cell activation, *e.g.*, antibody production (humoral responses) and activation of cytokine responsive cells, *e.g.*, macrophages.

The term "immunotherapeutic agent" can include any molecule, peptide, antibody or other agent which can stimulate a host immune system to generate an immune response to a tumor or cancer in the subject. Various immunotherapeutic agents are useful in the compositions and methods described herein.

The term "inhibit" includes the decrease, limitation, or blockage, of, for example a particular action, function, or interaction. In some embodiments, cancer is "inhibited" if at least one symptom of the cancer is alleviated, terminated, slowed, or prevented. As used herein, cancer is also "inhibited" if recurrence or metastasis of the cancer is reduced, slowed, delayed, or prevented.

The term "interaction", when referring to an interaction between two molecules, refers to the physical contact (*e.g.*, binding) of the molecules with one another. Generally, such an interaction results in an activity (which produces a biological effect) of one or both of said molecules.

An "isolated protein" refers to a protein that is substantially free of other proteins, cellular material, separation medium, and culture medium when isolated from cells or

produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. An "isolated" or "purified" protein or biologically active portion thereof is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the antibody, polypeptide, peptide or fusion protein is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of a biomarker polypeptide or fragment thereof, in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly produced. In one embodiment, the language "substantially free of cellular material" includes preparations of a biomarker protein or fragment thereof, having less than about 30% (by dry weight) of non-biomarker protein (also referred to herein as a "contaminating protein"), more preferably less than about 20% of non-biomarker protein, still more preferably less than about 10% of non-biomarker protein, and most preferably less than about 5% non-biomarker protein. When antibody, polypeptide, peptide or fusion protein or fragment thereof, *e.g.*, a biologically active fragment thereof, is recombinantly produced, it is also preferably substantially free of culture medium, *i.e.*, culture medium represents less than about 20%, more preferably less than about 10%, and most preferably less than about 5% of the volume of the protein preparation.

As used herein, the term "isotype" refers to the antibody class (*e.g.*, IgM, IgG1, IgG2C, and the like) that is encoded by heavy chain constant region genes.

As used herein, the term "K_D" is intended to refer to the dissociation equilibrium constant of a particular antibody-antigen interaction. The binding affinity of antibodies of the disclosed invention may be measured or determined by standard antibody-antigen assays, for example, competitive assays, saturation assays, or standard immunoassays such as ELISA or RIA.

A "kit" is any manufacture (*e.g.* a package or container) comprising at least one reagent, *e.g.* a probe or small molecule, for specifically detecting and/or affecting the expression of a marker of the present invention. The kit may be promoted, distributed, or sold as a unit for performing the methods of the present invention. The kit may comprise one or more reagents necessary to express a composition useful in the methods of the present invention. In certain embodiments, the kit may further comprise a reference standard, *e.g.*, a nucleic acid encoding a protein that does not affect or regulate signaling pathways controlling cell growth, division, migration, survival or apoptosis. One skilled in

the art can envision many such control proteins, including, but not limited to, common molecular tags (*e.g.*, green fluorescent protein and beta-galactosidase), proteins not classified in any of pathway encompassing cell growth, division, migration, survival or apoptosis by GeneOntology reference, or ubiquitous housekeeping proteins. Reagents in the kit may be provided in individual containers or as mixtures of two or more reagents in a single container. In addition, instructional materials which describe the use of the compositions within the kit can be included.

The term "neoadjuvant therapy" refers to a treatment given before the primary treatment. Examples of neoadjuvant therapy can include chemotherapy, radiation therapy, and hormone therapy. For example, in treating breast cancer, neoadjuvant therapy can allows patients with large breast cancer to undergo breast-conserving surgery.

The "normal" level of expression of a biomarker is the level of expression of the biomarker in cells of a subject, *e.g.*, a human patient, not afflicted with a cancer. An "over-expression" or "significantly higher level of expression" of a biomarker refers to an expression level in a test sample that is greater than the standard error of the assay employed to assess expression, and is preferably at least 10%, and more preferably 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 times or more higher than the expression activity or level of the biomarker in a control sample (*e.g.*, sample from a healthy subject not having the biomarker associated disease) and preferably, the average expression level of the biomarker in several control samples. A "significantly lower level of expression" of a biomarker refers to an expression level in a test sample that is at least 10%, and more preferably 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 times or more lower than the expression level of the biomarker in a control sample (*e.g.*, sample from a healthy subject not having the biomarker associated disease) and preferably, the average expression level of the biomarker in several control samples.

An "over-expression" or "significantly higher level of expression" of a biomarker refers to an expression level in a test sample that is greater than the standard error of the assay employed to assess expression, and is preferably at least 10%, and more preferably 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 times

or more higher than the expression activity or level of the biomarker in a control sample (*e.g.*, sample from a healthy subject not having the biomarker associated disease) and preferably, the average expression level of the biomarker in several control samples. A "significantly lower level of expression" of a biomarker refers to an expression level in a test sample that is at least 10%, and more preferably 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 times or more lower than the expression level of the biomarker in a control sample (*e.g.*, sample from a healthy subject not having the biomarker associated disease) and preferably, the average expression level of the biomarker in several control samples.

The term "pre-determined" biomarker amount and/or activity measurement(s) may be a biomarker amount and/or activity measurement(s) used to, by way of example only, evaluate a subject that may be selected for a particular treatment, evaluate a response to a treatment such as inhibitors of one or more biomarkers listed in Table 1, in combination with an immunotherapy, and/or evaluate the disease state. A pre-determined biomarker amount and/or activity measurement(s) may be determined in populations of patients with or without cancer. The pre-determined biomarker amount and/or activity measurement(s) can be a single number, equally applicable to every patient, or the pre-determined biomarker amount and/or activity measurement(s) can vary according to specific subpopulations of patients. Age, weight, height, and other factors of a subject may affect the pre-determined biomarker amount and/or activity measurement(s) of the individual. Furthermore, the pre-determined biomarker amount and/or activity can be determined for each subject individually. In one embodiment, the amounts determined and/or compared in a method described herein are based on absolute measurements. In another embodiment, the amounts determined and/or compared in a method described herein are based on relative measurements, such as ratios (*e.g.*, serum biomarker normalized to the expression of housekeeping or otherwise generally constant biomarker). The pre-determined biomarker amount and/or activity measurement(s) can be any suitable standard. For example, the pre-determined biomarker amount and/or activity measurement(s) can be obtained from the same or a different human for whom a patient selection is being assessed. In one embodiment, the pre-determined biomarker amount and/or activity measurement(s) can be obtained from a previous assessment of the same patient. In such a manner, the progress of the selection of the patient can be monitored over time. In addition, the control

can be obtained from an assessment of another human or multiple humans, *e.g.*, selected groups of humans, if the subject is a human. In such a manner, the extent of the selection of the human for whom selection is being assessed can be compared to suitable other humans, *e.g.,* other humans who are in a similar situation to the human of interest, such as those suffering from similar or the same condition(s) and/or of the same ethnic group.

The term "predictive" includes the use of a biomarker nucleic acid and/or protein status, *e.g.,* over- or under- activity, emergence, expression, growth, remission, recurrence or resistance of tumors before, during or after therapy, for determining the likelihood of response of a cancer to inhibitors of one or more biomarkers listed in Table 1, in combination with an immunotherapy (*e.g.*, treatment with a combination of an inhibitor of one or more biomarkers listed in Table 1 and an immunotherapy, such as an immune checkpoint inhibitor). Such predictive use of the biomarker may be confirmed by, *e.g.,* (1) increased or decreased copy number (*e.g.,* by FISH, FISH plus SKY, single-molecule sequencing, *e.g.*, as described in the art at least at J. Biotechnol., 86:289-301, or qPCR), overexpression or underexpression of a biomarker nucleic acid (*e.g.,* by ISH, Northern Blot, or qPCR), increased or decreased biomarker protein (*e.g.,* by IHC), or increased or decreased activity, *e.g.,* in more than about 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 100%, or more of assayed human cancers types or cancer samples; (2) its absolute or relatively modulated presence or absence in a biological sample, *e.g.,* a sample containing tissue, whole blood, serum, plasma, buccal scrape, saliva, cerebrospinal fluid, urine, stool, or bone marrow, from a subject, *e.g.* a human, afflicted with cancer; (3) its absolute or relatively modulated presence or absence in clinical subset of patients with cancer (*e.g.*, those responding to a particular inhibitor of one or more biomarkers listed in Table 1, in combination with an immunotherapy or those developing resistance thereto).

The term "pre-malignant lesions" as described herein refers to a lesion that, while not cancerous, has potential for becoming cancerous. It also includes the term "pre-malignant disorders" or "potentially malignant disorders." In particular this refers to a benign, morphologically and/or histologically altered tissue that has a greater than normal risk of malignant transformation, and a disease or a patient's habit that does not necessarily alter the clinical appearance of local tissue but is associated with a greater than normal risk of precancerous lesion or cancer development in that tissue (leukoplakia, erythroplakia, erytroleukoplakia lichen planus (lichenoid reaction) and any lesion or an area which

histological examination showed atypia of cells or dysplasia. In one embodiment, a metaplasia is a pre-malignant lesion.

The terms "prevent," "preventing," "prevention," "prophylactic treatment," and the like refer to reducing the probability of developing a disease, disorder, or condition in a subject, who does not have, but is at risk of or susceptible to developing a disease, disorder, or condition.

The term "probe" refers to any molecule which is capable of selectively binding to a specifically intended target molecule, for example, a nucleotide transcript or protein encoded by or corresponding to a biomarker nucleic acid. Probes can be either synthesized by one skilled in the art, or derived from appropriate biological preparations. For purposes of detection of the target molecule, probes may be specifically designed to be labeled, as described herein. Examples of molecules that can be utilized as probes include, but are not limited to, RNA, DNA, proteins, antibodies, and organic molecules.

The term "prognosis" includes a prediction of the probable course and outcome of cancer or the likelihood of recovery from the disease. In some embodiments, the use of statistical algorithms provides a prognosis of cancer in an individual. For example, the prognosis can be surgery, development of a clinical subtype of cancer (*e.g.*, solid tumors, such as esophageal cancer and gastric cancer), development of one or more clinical factors, or recovery from the disease.

The term "response to immunotherapy" or "response to inhibitors of one or more biomarkers/immunotherapy combination therapy" relates to any response of the hyperproliferative disorder (*e.g.*, cancer) to an anti-cancer agent, such as an an inhibitor of one or more biomarkers listed in Table 1, and an immunotherapy, preferably to a change in tumor mass and/or volume after initiation of neoadjuvant or adjuvant therapy. Hyperproliferative disorder response may be assessed, for example for efficacy or in a neoadjuvant or adjuvant situation, where the size of a tumor after systemic intervention can be compared to the initial size and dimensions as measured by CT, PET, mammogram, ultrasound or palpation. Responses may also be assessed by caliper measurement or pathological examination of the tumor after biopsy or surgical resection. Response may be recorded in a quantitative fashion like percentage change in tumor volume or in a qualitative fashion like "pathological complete response" (pCR), "clinical complete remission" (cCR), "clinical partial remission" (cPR), "clinical stable disease" (cSD), "clinical progressive disease" (cPD) or other qualitative criteria. Assessment of

hyperproliferative disorder response may be done early after the onset of neoadjuvant or adjuvant therapy, *e.g.*, after a few hours, days, weeks or preferably after a few months. A typical endpoint for response assessment is upon termination of neoadjuvant chemotherapy or upon surgical removal of residual tumor cells and/or the tumor bed. This is typically three months after initiation of neoadjuvant therapy. In some embodiments, clinical efficacy of the therapeutic treatments described herein may be determined by measuring the clinical benefit rate (CBR). The clinical benefit rate is measured by determining the sum of the percentage of patients who are in complete remission (CR), the number of patients who are in partial remission (PR) and the number of patients having stable disease (SD) at a time point at least 6 months out from the end of therapy. The shorthand for this formula is CBR=CR+PR+SD over 6 months. In some embodiments, the CBR for a particular cancer therapeutic regimen is at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, or more. Additional criteria for evaluating the response to cancer therapies are related to "survival," which includes all of the following: survival until mortality, also known as overall survival (wherein said mortality may be either irrespective of cause or tumor related); "recurrence-free survival" (wherein the term recurrence shall include both localized and distant recurrence); metastasis free survival; disease free survival (wherein the term disease shall include cancer and diseases associated therewith). The length of said survival may be calculated by reference to a defined start point (*e.g.*, time of diagnosis or start of treatment) and end point (*e.g.*, death, recurrence or metastasis). In addition, criteria for efficacy of treatment can be expanded to include response to chemotherapy, probability of survival, probability of metastasis within a given time period, and probability of tumor recurrence. For example, in order to determine appropriate threshold values, a particular cancer therapeutic regimen can be administered to a population of subjects and the outcome can be correlated to biomarker measurements that were determined prior to administration of any cancer therapy. The outcome measurement may be pathologic response to therapy given in the neoadjuvant setting. Alternatively, outcome measures, such as overall survival and disease-free survival can be monitored over a period of time for subjects following cancer therapy for which biomarker measurement values are known. In certain embodiments, the doses administered are standard doses known in the art for cancer therapeutic agents. The period of time for which subjects are monitored can vary. For example, subjects may be monitored for at least 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 55, or 60 months. Biomarker measurement threshold

values that correlate to outcome of a cancer therapy can be determined using well-known methods in the art, such as those described in the Examples section.

The term "resistance" refers to an acquired or natural resistance of a cancer sample or a mammal to a cancer therapy (*i.e.*, being nonresponsive to or having reduced or limited response to the therapeutic treatment), such as having a reduced response to a therapeutic treatment by 25% or more, for example, 30%, 40%, 50%, 60%, 70%, 80%, or more, to 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 15-fold, 20-fold or more. The reduction in response can be measured by comparing with the same cancer sample or mammal before the resistance is acquired, or by comparing with a different cancer sample or a mammal that is known to have no resistance to the therapeutic treatment. A typical acquired resistance to chemotherapy is called "multidrug resistance." The multidrug resistance can be mediated by P-glycoprotein or can be mediated by other mechanisms, or it can occur when a mammal is infected with a multi-drug-resistant microorganism or a combination of microorganisms. The determination of resistance to a therapeutic treatment is routine in the art and within the skill of an ordinarily skilled clinician, for example, can be measured by cell proliferative assays and cell death assays as described herein as "sensitizing." In some embodiments, the term "reverses resistance" means that the use of a second agent in combination with a primary cancer therapy (*e.g.*, chemotherapeutic or radiation therapy) is able to produce a significant decrease in tumor volume at a level of statistical significance (*e.g.*, $p<0.05$) when compared to tumor volume of untreated tumor in the circumstance where the primary cancer therapy (*e.g.*, chemotherapeutic or radiation therapy) alone is unable to produce a statistically significant decrease in tumor volume compared to tumor volume of untreated tumor. This generally applies to tumor volume measurements made at a time when the untreated tumor is growing log rhythmically.

The terms "response" or "responsiveness" refers to an anti-cancer response, *e.g.* in the sense of reduction of tumor size or inhibiting tumor growth. The terms can also refer to an improved prognosis, for example, as reflected by an increased time to recurrence, which is the period to first recurrence censoring for second primary cancer as a first event or death without evidence of recurrence, or an increased overall survival, which is the period from treatment to death from any cause. To respond or to have a response means there is a beneficial endpoint attained when exposed to a stimulus. Alternatively, a negative or detrimental symptom is minimized, mitigated or attenuated on exposure to a stimulus. It will be appreciated that evaluating the likelihood that a tumor or subject will exhibit a

favorable response is equivalent to evaluating the likelihood that the tumor or subject will not exhibit favorable response (*i.e.*, will exhibit a lack of response or be non-responsive).

An "RNA interfering agent" as used herein, is defined as any agent which interferes with or inhibits expression of a target biomarker gene by RNA interference (RNAi). Such RNA interfering agents include, but are not limited to, nucleic acid molecules including RNA molecules which are homologous to the target biomarker gene of the present invention, or a fragment thereof, short interfering RNA (siRNA), and small molecules which interfere with or inhibit expression of a target biomarker nucleic acid by RNA interference (RNAi).

"RNA interference (RNAi)" is an evolutionally conserved process whereby the expression or introduction of RNA of a sequence that is identical or highly similar to a target biomarker nucleic acid results in the sequence specific degradation or specific post-transcriptional gene silencing (PTGS) of messenger RNA (mRNA) transcribed from that targeted gene (*see* Coburn and Cullen (2002) *J. Virol.* 76:9225), thereby inhibiting expression of the target biomarker nucleic acid. In one embodiment, the RNA is double stranded RNA (dsRNA). This process has been described in plants, invertebrates, and mammalian cells. In nature, RNAi is initiated by the dsRNA-specific endonuclease Dicer, which promotes processive cleavage of long dsRNA into double-stranded fragments termed siRNAs. siRNAs are incorporated into a protein complex that recognizes and cleaves target mRNAs. RNAi can also be initiated by introducing nucleic acid molecules, *e.g.,* synthetic siRNAs or RNA interfering agents, to inhibit or silence the expression of target biomarker nucleic acids. As used herein, "inhibition of target biomarker nucleic acid expression" or "inhibition of marker gene expression" includes any decrease in expression or protein activity or level of the target biomarker nucleic acid or protein encoded by the target biomarker nucleic acid. The decrease may be of at least 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% or more as compared to the expression of a target biomarker nucleic acid or the activity or level of the protein encoded by a target biomarker nucleic acid which has not been targeted by an RNA interfering agent.

The term "sample" used for detecting or determining the presence or level of at least one biomarker is typically brain tissue, cerebrospinal fluid, whole blood, plasma, serum, saliva, urine, stool (*e.g.*, feces), tears, and any other bodily fluid (*e.g.*, as described above under the definition of "body fluids"), or a tissue sample (*e.g.*, biopsy) such as a small intestine, colon sample, or surgical resection tissue. In certain instances, the method of the

present invention further comprises obtaining the sample from the individual prior to detecting or determining the presence or level of at least one marker in the sample.

The term "sensitize" means to alter cancer cells or tumor cells in a way that allows for more effective treatment of the associated cancer with a cancer therapy (*e.g.*, anti-immune checkpoint, chemotherapeutic, and/or radiation therapy). In some embodiments, normal cells are not affected to an extent that causes the normal cells to be unduly injured by the therapies. An increased sensitivity or a reduced sensitivity to a therapeutic treatment is measured according to a known method in the art for the particular treatment and methods described herein below, including, but not limited to, cell proliferative assays (Tanigawa N, Kern D H, Kikasa Y, Morton D L, Cancer Res 1982; 42: 2159-2164), cell death assays (Weisenthal L M, Shoemaker R H, Marsden J A, Dill P L, Baker J A, Moran E M, Cancer Res 1984; 94: 161-173; Weisenthal L M, Lippman M E, Cancer Treat Rep 1985; 69: 615-632; Weisenthal L M, In: Kaspers G J L, Pieters R, Twentyman P R, Weisenthal L M, Veerman A J P, eds. Drug Resistance in Leukemia and Lymphoma. Langhorne, P A: Harwood Academic Publishers, 1993: 415-432; Weisenthal L M, Contrib Gynecol Obstet 1994; 19: 82-90). The sensitivity or resistance may also be measured in animal by measuring the tumor size reduction over a period of time, for example, 6 month for human and 4-6 weeks for mouse. A composition or a method sensitizes response to a therapeutic treatment if the increase in treatment sensitivity or the reduction in resistance is 25% or more, for example, 30%, 40%, 50%, 60%, 70%, 80%, or more, to 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 15-fold, 20-fold or more, compared to treatment sensitivity or resistance in the absence of such composition or method. The determination of sensitivity or resistance to a therapeutic treatment is routine in the art and within the skill of an ordinarily skilled clinician. It is to be understood that any method described herein for enhancing the efficacy of a cancer therapy can be equally applied to methods for sensitizing hyperproliferative or otherwise cancerous cells (*e.g.*, resistant cells) to the cancer therapy.

"Short interfering RNA" (siRNA), also referred to herein as "small interfering RNA" is defined as an agent which functions to inhibit expression of a target biomarker nucleic acid, *e.g.,* by RNAi. An siRNA may be chemically synthesized, may be produced by *in vitro* transcription, or may be produced within a host cell. In one embodiment, siRNA is a double stranded RNA (dsRNA) molecule of about 15 to about 40 nucleotides in length, preferably about 15 to about 28 nucleotides, more preferably about 19 to about 25 nucleotides in length, and more preferably about 19, 20, 21, or 22 nucleotides in length,

and may contain a 3' and/or 5' overhang on each strand having a length of about 0, 1, 2, 3, 4, or 5 nucleotides. The length of the overhang is independent between the two strands, *i.e.*, the length of the overhang on one strand is not dependent on the length of the overhang on the second strand. Preferably the siRNA is capable of promoting RNA interference through degradation or specific post-transcriptional gene silencing (PTGS) of the target messenger RNA (mRNA).

In another embodiment, an siRNA is a small hairpin (also called stem loop) RNA (shRNA). In one embodiment, these shRNAs are composed of a short (*e.g.*, 19-25 nucleotide) antisense strand, followed by a 5-9 nucleotide loop, and the analogous sense strand. Alternatively, the sense strand may precede the nucleotide loop structure and the antisense strand may follow. These shRNAs may be contained in plasmids, retroviruses, and lentiviruses and expressed from, for example, the pol III U6 promoter, or another promoter (*see, e.g.,* Stewart, *et al.* (2003) *RNA* Apr;9(4):493-501 incorporated by reference herein).

RNA interfering agents, *e.g.,* siRNA molecules, may be administered to a patient having or at risk for having cancer, to inhibit expression of a biomarker gene which is overexpressed in cancer and thereby treat, prevent, or inhibit cancer in the subject.

The term "small molecule" is a term of the art and includes molecules that are less than about 1000 molecular weight or less than about 500 molecular weight. In one embodiment, small molecules do not exclusively comprise peptide bonds. In another embodiment, small molecules are not oligomeric. Exemplary small molecule compounds which can be screened for activity include, but are not limited to, peptides, peptidomimetics, nucleic acids, carbohydrates, small organic molecules (*e.g.*, polyketides) (Cane *et al.* (1998) *Science* 282:63), and natural product extract libraries. In another embodiment, the compounds are small, organic non-peptidic compounds. In a further embodiment, a small molecule is not biosynthetic.

The term "specific binding" refers to antibody binding to a predetermined antigen. Typically, the antibody binds with an affinity (K_D) of approximately less than 10^{-7} M, such as approximately less than 10^{-8} M, 10^{-9} M or 10^{-10} M or even lower when determined by surface plasmon resonance (SPR) technology in a BIACORE® assay instrument using an antigen of interest as the analyte and the antibody as the ligand, and binds to the predetermined antigen with an affinity that is at least 1.1-, 1.2-, 1.3-, 1.4-, 1.5-, 1.6-, 1.7-, 1.8-, 1.9-, 2.0-, 2.5-, 3.0-, 3.5-, 4.0-, 4.5-, 5.0-, 6.0-, 7.0-, 8.0-, 9.0-, or 10.0-fold or greater

than its affinity for binding to a non-specific antigen (*e.g.*, BSA, casein) other than the predetermined antigen or a closely-related antigen. The phrases "an antibody recognizing an antigen" and "an antibody specific for an antigen" are used interchangeably herein with the term "an antibody which binds specifically to an antigen." Selective binding is a relative term refering to the ability of an antibody to discriminate the binding of one antigen over another.

The term "subject" refers to any healthy animal, mammal or human, or any animal, mammal or human afflicted with a cancer, *e.g.*, brain, lung, ovarian, pancreatic, liver, breast, prostate, and/or colorectal cancers, melanoma, multiple myeloma, and the like. The term "subject" is interchangeable with "patient."

The term "survival" includes all of the following: survival until mortality, also known as overall survival (wherein said mortality may be either irrespective of cause or tumor related); "recurrence-free survival" (wherein the term recurrence shall include both localized and distant recurrence); metastasis free survival; disease free survival (wherein the term disease shall include cancer and diseases associated therewith). The length of said survival may be calculated by reference to a defined start point (*e.g.* time of diagnosis or start of treatment) and end point (*e.g.* death, recurrence or metastasis). In addition, criteria for efficacy of treatment can be expanded to include response to chemotherapy, probability of survival, probability of metastasis within a given time period, and probability of tumor recurrence.

The term "synergistic effect" refers to the combined effect of two or more anti-cancer agents (*e.g.*, inhibitors of one or more biomarkers listed in Table 1, in combination with an immunotherapy) can be greater than the sum of the separate effects of the anti-cancer agents/therapies alone.

The term "T cell" includes $CD4^+$ T cells and $CD8^+$ T cells. The term T cell also includes both T helper 1 type T cells and T helper 2 type T cells. The term "antigen presenting cell" includes professional antigen presenting cells (*e.g.*, B lymphocytes, monocytes, dendritic cells, Langerhans cells), as well as other antigen presenting cells (*e.g.*, keratinocytes, endothelial cells, astrocytes, fibroblasts, and oligodendrocytes).

The term "therapeutic effect" refers to a local or systemic effect in animals, particularly mammals, and more particularly humans, caused by a pharmacologically active substance. The term thus means any substance intended for use in the diagnosis, cure, mitigation, treatment or prevention of disease or in the enhancement of desirable physical

or mental development and conditions in an animal or human. The phrase "therapeutically-effective amount" means that amount of such a substance that produces some desired local or systemic effect at a reasonable benefit/risk ratio applicable to any treatment. In certain embodiments, a therapeutically effective amount of a compound will depend on its therapeutic index, solubility, and the like. For example, certain compounds discovered by the methods of the present invention may be administered in a sufficient amount to produce a reasonable benefit/risk ratio applicable to such treatment.

The terms "therapeutically-effective amount" and "effective amount" as used herein means that amount of a compound, material, or composition comprising a compound of the present invention which is effective for producing some desired therapeutic effect in at least a sub-population of cells in an animal at a reasonable benefit/risk ratio applicable to any medical treatment. Toxicity and therapeutic efficacy of subject compounds may be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD_{50} and the ED_{50}. Compositions that exhibit large therapeutic indices are preferred. In some embodiments, the LD_{50} (lethal dosage) can be measured and can be, for example, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, 1000% or more reduced for the agent relative to no administration of the agent. Similarly, the ED_{50} (*i.e.*, the concentration which achieves a half-maximal inhibition of symptoms) can be measured and can be, for example, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, 1000% or more increased for the agent relative to no administration of the agent. Also, Similarly, the IC_{50} (*i.e.*, the concentration which achieves half-maximal cytotoxic or cytostatic effect on cancer cells) can be measured and can be, for example, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, 1000% or more increased for the agent relative to no administration of the agent. In some embodiments, cancer cell growth in an assay can be inhibited by at least about 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or even 100%. In another embodiment, at least about a 10% , 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or even 100% decrease in a solid malignancy can be achieved.

A "transcribed polynucleotide" or "nucleotide transcript" is a polynucleotide (*e.g.* an mRNA, hnRNA, a cDNA, or an analog of such RNA or cDNA) which is complementary

to or homologous with all or a portion of a mature mRNA made by transcription of a biomarker nucleic acid and normal post-transcriptional processing (*e.g.* splicing), if any, of the RNA transcript, and reverse transcription of the RNA transcript.

As used herein, the term "unresponsiveness" includes refractivity of cancer cells to therapy or refractivity of therapeutic cells, such as immune cells, to stimulation, *e.g.*, stimulation via an activating receptor or a cytokine. Unresponsiveness can occur, *e.g.*, because of exposure to immunosuppressants or exposure to high doses of antigen. As used herein, the term "anergy" or "tolerance" includes refractivity to activating receptor-mediated stimulation. Such refractivity is generally antigen-specific and persists after exposure to the tolerizing antigen has ceased. For example, anergy in T cells (as opposed to unresponsiveness) is characterized by lack of cytokine production, *e.g.*, IL-2. T cell anergy occurs when T cells are exposed to antigen and receive a first signal (a T cell receptor or CD-3 mediated signal) in the absence of a second signal (a costimulatory signal). Under these conditions, reexposure of the cells to the same antigen (even if reexposure occurs in the presence of a costimulatory polypeptide) results in failure to produce cytokines and, thus, failure to proliferate. Anergic T cells can, however, proliferate if cultured with cytokines (*e.g.*, IL-2). For example, T cell anergy can also be observed by the lack of IL-2 production by T lymphocytes as measured by ELISA or by a proliferation assay using an indicator cell line. Alternatively, a reporter gene construct can be used. For example, anergic T cells fail to initiate IL-2 gene transcription induced by a heterologous promoter under the control of the 5' IL-2 gene enhancer or by a multimer of the AP1 sequence that can be found within the enhancer (Kang *et al.* (1992) *Science* 257:1134).

There is a known and definite correspondence between the amino acid sequence of a particular protein and the nucleotide sequences that can code for the protein, as defined by the genetic code (shown below). Likewise, there is a known and definite correspondence between the nucleotide sequence of a particular nucleic acid and the amino acid sequence encoded by that nucleic acid, as defined by the genetic code.

GENETIC CODE

Alanine (Ala, A)	GCA, GCC, GCG, GCT
Arginine (Arg, R)	AGA, ACG, CGA, CGC, CGG, CGT
Asparagine (Asn, N)	AAC, AAT
Aspartic acid (Asp, D)	GAC, GAT
Cysteine (Cys, C)	TGC, TGT

Glutamic acid (Glu, E)	GAA, GAG
Glutamine (Gln, Q)	CAA, CAG
Glycine (Gly, G)	GGA, GGC, GGG, GGT
Histidine (His, H)	CAC, CAT
Isoleucine (Ile, I)	ATA, ATC, ATT
Leucine (Leu, L)	CTA, CTC, CTG, CTT, TTA, TTG
Lysine (Lys, K)	AAA, AAG
Methionine (Met, M)	ATG
Phenylalanine (Phe, F)	TTC, TTT
Proline (Pro, P)	CCA, CCC, CCG, CCT
Serine (Ser, S)	AGC, AGT, TCA, TCC, TCG, TCT
Threonine (Thr, T)	ACA, ACC, ACG, ACT
Tryptophan (Trp, W)	TGG
Tyrosine (Tyr, Y)	TAC, TAT
Valine (Val, V)	GTA, GTC, GTG, GTT
Termination signal (end)	TAA, TAG, TGA

An important and well-known feature of the genetic code is its redundancy, whereby, for most of the amino acids used to make proteins, more than one coding nucleotide triplet may be employed (illustrated above). Therefore, a number of different nucleotide sequences may code for a given amino acid sequence. Such nucleotide sequences are considered functionally equivalent since they result in the production of the same amino acid sequence in all organisms (although certain organisms may translate some sequences more efficiently than they do others). Moreover, occasionally, a methylated variant of a purine or pyrimidine may be found in a given nucleotide sequence. Such methylations do not affect the coding relationship between the trinucleotide codon and the corresponding amino acid.

In view of the foregoing, the nucleotide sequence of a DNA or RNA encoding a biomarker nucleic acid (or any portion thereof) can be used to derive the polypeptide amino acid sequence, using the genetic code to translate the DNA or RNA into an amino acid sequence. Likewise, for polypeptide amino acid sequence, corresponding nucleotide sequences that can encode the polypeptide can be deduced from the genetic code (which, because of its redundancy, will produce multiple nucleic acid sequences for any given amino acid sequence). Thus, description and/or disclosure herein of a nucleotide sequence

which encodes a polypeptide should be considered to also include description and/or disclosure of the amino acid sequence encoded by the nucleotide sequence. Similarly, description and/or disclosure of a polypeptide amino acid sequence herein should be considered to also include description and/or disclosure of all possible nucleotide sequences that can encode the amino acid sequence.

Finally, nucleic acid and amino acid sequence information for the loci and biomarkers of the present invention (*e.g.*, biomarkers listed in Tables 1 and 2) are well-known in the art and readily available on publicly available databases, such as the National Center for Biotechnology Information (NCBI). For example, exemplary nucleic acid and amino acid sequences derived from publicly available sequence databases are provided below and include, for example, PCT Publ. WO 2014/022759, which is incorporated herein in its entirety by this reference.

Table 1

SEQ ID NO: 1 Human RIPK1 Variant 1 cDNA Sequence (NM_003804.4, CDS region from position 233-2248)

```
   1 agactgctcg tcaagtgtgg gaaaagctcc gtggcgtcac aagctactat ataaaaggcg
  61 gtgcccgccg gggccgagtg ggagtccgcg gcgagcgcag cagcagggcc cggtcctgcg
 121 cctcgggagt cggcgtccag gctcggagcg cgacacggag actaggtggc agggtacagc
 181 tctgccgggg ggggaaaaag tggtaccatt ttgggcgttc ttgagcttca gaatgcaacc
 241 agacatgtcc ttgaatgtca ttaagatgaa atccagtgac ttcctggaga gtgcagaact
 301 ggacagcgga ggctttggga aggtgtctct gtgtttccac agaacccagg gactcatgat
 361 catgaaaaca gtgtacaagg ggcccaactg cattgagcac aacgaggccc tcttggagga
 421 ggcgaagatg atgaacagac tgagacacag ccgggtggtg aagctcctgg gcgtcatcat
 481 agaggaaggg aagtactccc tggtgatgga gtacatggag aagggcaacc tgatgcacgt
 541 gctgaaagcc gagatgagta ctccgctttc tgtaaaagga aggataattt tggaaatcat
 601 tgaaggaatg tgctacttac atggaaaagg cgtgatacac aaggacctga agcctgaaaa
 661 tatccttgtt gataatgact tccacattaa gatcgcagac ctcggccttg cctcctttaa
 721 gatgtggagc aaactgaata atgaagagca caatgagctg agggaagtgg acggcaccgc
 781 taagaagaat ggcggcaccc tctactacat ggcgcccgag cacctgaatg acgtcaacgc
 841 aaagcccaca gagaagtcgg atgtgtacag ctttgctgta gtactctggg cgatatttgc
 901 aaataaggag ccatatgaaa atgctatctg tgagcagcag ttgataatgt gcataaaatc
 961 tgggaacagg ccagatgtgg atgacatcac tgagtactgc ccaagagaaa ttatcagtct
1021 catgaagctc tgctgggaag cgaatccgga agctcggccg acatttcctg gcattgaaga
1081 aaaatttagg ccttttttatt taagtcaatt agaagaaagt gtagaagagg acgtgaagag
1141 tttaaagaaa gagtattcaa acgaaaatgc agttgtgaag agaatgcagt ctcttcaact
1201 tgattgtgtg gcagtacctt caagccggtc aaattcagcc acagaacagc ctggttcact
1261 gcacagttcc cagggacttg ggatgggtcc tgtggaggag tcctggtttg ctccttccct
1321 ggagcaccca caagaagaga atgagcccag cctgcagagt aaactccaag acgaagccaa
1381 ctaccatctt tatggcagcc gcatggacag gcagacgaaa cagcagccca gacagaatgt
1441 ggcttacaac agagaggagg aaaggagacg cagggtctcc catgaccctt ttgcacagca
1501 aagaccttac gagaattttc agaatacaga gggaaaaggc actgcttatt ccagtgcagc
1561 cagtcatggt aatgcagtgc accagccctc agggctcacc agccaacctc aagtactgta
1621 tcagaacaat ggattatata gctcacatgg ctttggaaca agaccactgg atccaggaac
1681 agcaggtccc agagtttggt acaggccaat tccaagtcat atgcctagtc tgcataatat
1741 cccagtgcct gagaccaact atctaggaaa tacacccacc atgccattca gctccttgcc
1801 accaacagat gaatctataa aatataccat atacaatagt actggcattc agattggagc
1861 ctacaattat atggagattg gtgggacgag ttcatcacta ctagacagca caaatacgaa
1921 cttcaaagaa gagccagctg ctaagtacca agctatcttt gataatacca ctagtctgac
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2101 actgaaagaa aaggtttacc agatgctcca aaagtgggtg atgagggaag gcataaaggg
2161 agccacggtg gggaagctgg cccaggcgct ccaccagtgt tccaggatcg accttctgag
2221 cagcttgatt tacgtcagcc agaactaacc ctggatgggc tacggcagct gaagtggacg
2281 cctcacttag tggataaccc cagaaagttg gctgcctcag agcattcaga attctgtcct
2341 cactgatagg ggttctgtgt ctgcagaaat tttgtttcct gtacttcata gctggagaat
2401 ggggaaagaa atctgcagca aaggggtctc actctgttgc caggctggtc tcaaacttct
2461 ggactcaagt gatcctcccg cctcggcctt ccaaagtgct gggatatcag gcactgagcc
2521 actgcgccca gccaacaatc cgctctgagg aaagcgtaag caggaagacc tcttaatggc
2581 atagcaccaa taaaaaaatg actcctagtt gtgtttggaa agggagagaa gagatgtctg
2641 aggaaggtca tgttctttca gcttatggca tttcctagag ttttgttgaa gcaagaagaa
2701 aaactcagag aatataaaat caacttttaa aattgtgtgc tctcttcttc acgtaggctc
2761 ctgttaaaaa caaagtgcag tcagattcta agccctgttc agagacttcg tggatcacag
2821 ctgcagctca ccgccacatc acaggatccg ttaacgttaa tacccaatac tctgtcagcc
2881 actgtaggct ctaagaacca cgtgcagtct tcagcccatt aaattatcga ttatttttta
2941 atgaattgaa tttatattga gtcttcaaat taactgaatg gatttaaagg ggtaccaagg
3001 agggggggaaa catcagaatt tcccaggcag ttgttgcaag gaattggtac taaccgtgac
3061 tacaacaaaa attcttgatt gacttttaaa gttatttcct ggcattctgg taccttcacc
3121 cagcctgagt gccctggaga gggaacagga aatgctgatc tctacccctg ggtgagacca
3181 gaacctcagg gctgatactg ttgagtggct tcctcggttt actctgtgta ctgtgaaagt
3241 attttcatat tttttctgtg tgccagagtg aaaaaggaca gcttctgagt gtggtaattg
3301 tgcctctagc acccagcctt tcaaagccca cctgaaacct gggggtggat gaaagaacta
3361 gaatagaaga ctgaagctgg gtaggccgct cagtgtccac tggcattttg ctaaaccgac
3421 aaggaaggct gtgtgcttag ctctccccag agggagggcg agaagggtgt ggtgatggtc
3481 aatctggctg tcggaacaga ttctggtgtc ttgggctgat aacagtgttg ttgattctga
3541 ttgtgaatcc cctcaactct agcagacaca tacacacccc tgaaatgggg ctgcagagca
3601 ggctgtctca gccttgccac tgtcggcatc tcggcctggg taattctgtt gtggggactg
3661 tcctgttcct tgtaggatgt ttagtagcat ccctgccccc acctactaga tgccaggggc
3721 actgttctcc ccagcccccc gccccagttg tgacaatagt ctctaaacat tgtcaaatgg
3781 tccaaggaaa ggggaaaatt gccccggttg agaagagcac tgctgtaaag taatgagcct
3841 cggctctcct gtctgcacct gtccggttac tacttggcca ccacgcagcc ttggctccta
3901 cagcccaaaa gggagaatgg agggaggctc caggctttgc tggaggggcc tgggtgagtt
3961 ctgtttgctc cttgtaccac catccaaatg gtgttatcaa atctcttaga ttccaaagag
4021 gttgaataat taatgttcaa aggcaagagg gcaaggcatt ttttaacact ttttaaaata
4081 aaaatttata ccacaa
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SEQ ID NO: 2 Human RIPK1 isoform 1 Amino Acid Sequence (NP_003795.2)

```
   1 mqpdmslnvi kmkssdfles aeldsggfgk vslcfhrtqg lmimktvykg pnciehneal
  61 leeakmmnrl rhsrvvkllg viieegkysl vmeymekgnl mhvlkaemst plsvkgriil
 121 eiiegmcylh gkgvihkdlk penilvdndf hikiadlgla sfkmwsklnn eehnelrevd
 181 gtakknggtl yymapehlnd vnakpteksd vysfavvlwa ifankepyen aiceqqlimc
 241 iksgnrpdvd diteycprei islmklcwea npearptfpg ieekfrpfyl sqleesveed
 301 vkslkkeysn enavvkrmqs lqldcvavps srsnsateqp gslhssqglg mgpveeswfa
 361 pslehpqeen epslqsklqd eanyhlygsr mdrqtkqqpr qnvaynreee rrrrvshdpf
 421 aqqrpyenfq ntegkgtays saashgnavh qpsgltsqpq vlyqnnglys shgfgtrpld
 481 pgtagprvwy rpipshmpsl hnipvpetny lgntptmpfs slpptdesik ytiynstgiq
 541 igaynymeig gtssslldst ntnfkeepaa kyqaifdntt sltdkhldpi renlgkhwkn
 601 carklgftqs qideidhdye rdglkekvyq mlqkwvmreg ikgatvgkla qalhqcsrid
 661 llssliyvsq n
```

SEQ ID NO: 3 Human RIPK1 Variant 2 cDNA Sequence (NM_001317061.1, CDS region from position 1000-2526)

```
   1 agactgctcg tcaagtgtgg gaaaagctcc gtggcgtcac aagctactat ataaaaggcg
  61 gtgcccgccg gggccgagtg ggagtccgcg gcgagcgcag cagcagggcc cggtcctgcg
 121 cctcgggagt cggcgtccag gctcggagcg cgacacggag actaggtggc aggaaagaag
 181 gcccataggt gctgctgtat gagcttcctc cgctagacag agctggcccc agcctttaga
 241 tgaggtgtag aaggctggtt acccgggtca cctgtagccg gccactgtgc aacaccatgg
 301 gcagcctagg agtttccaga gtccccctctc cccaagggta cagctctgcc gggggggggaa
 361 aaagtggtac cattttgggc gttcttgagc ttcagaatgc aaccagacat gtccttgaat
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 421 gtcattaaga tgaaatccag tgacttcctg gagagtgcag aactggacag cggaggcttt
 481 gggaaggtgt ctctgtgttt ccacagaacc cagggactca tgatcatgaa aacagtgtac
 541 aaggggccca actgcattga gcacaacgag gccctcttgg aggaggcgaa gatgatgaac
 601 agactgagac acagccgggt ggtgaagctc ctgggcgtca tcatagagga agggaagtac
 661 tccctggtga tggagtacat ggagaagggc aacctgatgc acgtgctgaa agccgagatg
 721 agtactccgc tttctgtaaa aggaaggata attttggaaa tcattgaagg aatgtgctac
 781 ttacatggaa aaggcgtgat acacaaggac ctgaagcctg aaaatatcct tgttgataat
 841 gacttccaca ttaaggttca ggctacagct caaaacaagc aacactgcag tccttatcct
 901 cagtgtgtct ttgggatcct tggacacttg catccatggg cctctggacg ccatttgggg
 961 aaatagaaga tcgcagacct cggccttgcc tcctttaaga tgtggagcaa actgaataat
1021 gaagagcaca atgagctgag ggaagtggac ggcaccgcta agaagaatgg cggcaccctc
1081 tactacatgg cgcccgagca cctgaatgac gtcaacgcaa agcccacaga gaagtcggat
1141 gtgtacagct ttgctgtagt actctgggcg atatttgcaa ataaggagcc atatgaaaat
1201 gctatctgtg agcagcagtt gataatgtgc ataaaatctg ggaacaggcc agatgtggat
1261 gacatcactg agtactgccc aagagaaatt atcagtctca tgaagctctg ctgggaagcg
1321 aatccggaag ctcggccgac atttcctggc attgaagaaa aatttaggcc tttttattta
1381 agtcaattag aagaaagtgt agaagaggac gtgaagagtt taaagaaaga gtattcaaac
1441 gaaaatgcag ttgtgaagag aatgcagtct cttcaacttg attgtgtggc agtaccttca
1501 agccggtcaa attcagccac agaacagcct ggttcactgc acagttccca gggacttggg
1561 atgggtcctg tggaggagtc ctggtttgct ccttccctgg agcacccaca agaagagaat
1621 gagcccagcc tgcagagtaa actccaagac gaagccaact accatcttta tggcagccgc
1681 atggacaggc agacgaaaca gcagcccaga cagaatgtgg cttacaacag agaggaggaa
1741 aggagacgca gggtctccca tgacccтttt gcacagcaaa gaccttacga gaattttcag
1801 aatacagagg gaaaaggcac tgcttattcc agtgcagcca gtcatggtaa tgcagtgcac
1861 cagccctcag ggctcaccag ccaacctcaa gtactgtatc agaacaatgg attatatagc
1921 tcacatggct ttggaacaag accactggat ccaggaacag caggtcccag agtttggtac
1981 aggccaattc caagtcatat gcctagtctg cataatatcc cagtgcctga gaccaactat
2041 ctaggaaata cacccaccat gccattcagc tccttgccac caacagatga atctataaaa
2101 tataccatat acaatagtac tggcattcag attggagcct acaattatat ggagattggt
2161 gggacgagtt catcactact agacagcaca aatacgaact tcaaagaaga gccagctgct
2221 aagtaccaag ctatctttga taataccact agtctgacgg ataaacacct ggacccaatc
2281 agggaaaatc tgggaaagca ctggaaaaac tgtgcccgta aactgggctt cacacagtct
2341 cagattgatg aaattgacca tgactatgag cgagatggac tgaaagaaaa ggtttaccag
2401 atgctccaaa agtgggtgat gagggaaggc ataaagggag ccacggtggg gaagctggcc
2461 caggcgctcc accagtgttc caggatcgac cttctgagca gcttgattta cgtcagccag
2521 aactaaccct ggatgggcta cggcagctga agtggacgcc tcacttagtg gataacccca
2581 gaaagttggc tgcctcagag cattcagaat tctgtcctca ctgatagggg ttctgtgtct
2641 gcagaaattt tgtttcctgt acttcatagc tggagaatgg ggaaagaaat ctgcagcaaa
2701 ggggtctcac tctgttgcca ggctggtctc aaacttctgg actcaagtga tcctcccgcc
2761 tcggccttcc aaagtgctgg gatatcaggc actgagccac tgcgcccagc caacaatccg
2821 ctctgaggaa agcgtaagca ggaagacctc ttaatggcat agcaccaata aaaaaatgac
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3121 aggatccgtt aacgttaata cccaatactc tgtcagccac tgtaggctct aagaaccacg
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3241 cttcaaatta actgaatgga tttaaagggg taccaaggag gggggaaaca tcagaatttc
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3361 cttttaaagt tatttcctgg cattctggta ccttcaccca gcctgagtgc cctggagagg
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3601 aaagcccacc tgaaacctgg gggtggatga aagaactaga atagaagact gaagctgggt
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3781 ctggtgtctt gggctgataa cagtgttgtt gattctgatt gtgaatcccc tcaactctag
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4021 cccagttgtg acaatagtct ctaaacattg tcaaatggtc caaggaaagg ggaaaattgc
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4201 ggaggctcca ggctttgctg gaggggcctg ggtgagttct gtttgctcct tgtaccacca
4261 tccaaatggt gttatcaaat ctcttagatt ccaaagaggt tgaataatta atgttcaaag
4321 gcaagagggc aaggcatttt ttaacacttt ttaaaataaa aatttatacc acaa
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SEQ ID NO: 4 Human RIPK1 isoform 2 Amino Acid Sequence (NP_001303990.1)

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   1 mwsklnneeh nelrevdgta kknggtlyym apehlndvna kpteksdvys favvlwaifa
  61 nkepyenaic eqqlimciks gnrpdvddit eycpreiisl mklcweanpe arptfpgiee
 121 kfrpfylsql eesveedvks lkkeysnena vvkrmqslql dcvavpssrs nsateqpgsl
 181 hssqglgmgp veeswfapsl ehpqeeneps lqsklqdean yhlygsrmdr qtkqqprqnv
 241 aynreeerrr rvshdpfaqq rpyenfqnte gkgtayssaa shgnavhqps gltsqpqvly
 301 qnnglysshg fgtrpldpgt agprvwyrpi pshmpslhni pvpetnylgn tptmpfsslp
 361 ptdesikyti ynstgiqiga ynymeiggts sslldstntn fkeepaakyq aifdnttslt
 421 dkhldpiren lgkhwkncar klgftqsqid eidhdyerdg lkekvyqmlq kwvmregikg
 481 atvgklaqal hqcsridlls sliyvsqn
```

SEQ ID NO: 5 Mouse RIPK1 cDNA Sequence (NM_009068.3, CDS region from position 137-2107)

```
   1 cgaaaagcgc ggaacttgct gtcatctagc gggaggttgg actcttcttg aggtcgtttt
  61 agctcaagtc gagactgaag gacacagcac taagcaagaa ccaaaagtgg tgtgttggag
 121 attctgagca atcaaaatgc aaccagacat gtccttggac aatattaaga tggcatccag
 181 tgacctgctg gagaagacag acctagacag cggaggcttc gggaaggtgt ccttgtgtta
 241 ccacagaagc catggatttg tcatcctgaa aaaagtatac acagggccca accgcgctga
 301 gtacaatgag gttctcttgg aagaggggaa gatgatgcac agactgagac acagtcgagt
 361 ggtgaagcta ctgggcatca tcatagaaga agggaactat tcgctggtga tggagtacat
 421 ggagaagggc aacctgatgc acgtgctaaa gacccagata gatgtcccac tttcattgaa
 481 aggaaggata atcgtggagg ccatagaagg catgtgctac ttacatgaca aaggtgtgat
 541 acacaaggac ctgaagcctg agaatatcct cgttgatcgt gactttcaca ttaagatagc
 601 cgatcttggt gtggcttcct ttaagacatg gagcaaactg actaaggaga aagacaacaa
 661 gcagaaagaa gtgagcagca ccactaagaa gaacaatggt ggtacccttt actacatggc
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2281 cagaacatgc aagcataaag accgctgaat gaatggtcgg tccatgacca gtaggaggaa
2341 aaaaattaaa aatacagtgt attgagtttt caaagggaga gaagatgttg ggggaaggtg
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2401 gccttcgttc agcttgtgtc atagtcatca cttaggttat ttgctcagtt tcctgtggtt
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2761 agctcaggta ctggctgtca ttgcagagct gctgctggtt ctggctgctg taaaaagtct
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3121 gctagggtag aagactgaag ccgcctacac cagccacagt cactgttagg atgggctgca
3181 cagccgcttt ggccttgttg gccattctgg cactcattgg cacttcatcc tcctttgttg
3241 ggctatcctg tactcagtag gatatttggg aacattcctg gcctccatct gcaagatgcc
3301 aacagtacaa gtcaacctca caacacccac caggtgtgag aaaaataact agatattgct
3361 acatgttctt tggagggact ctcctgtaac gttatttccc ccatccccag acaaggtttc
3421 tcagttctcg ctgtcctgga actcactttg tagaccagat tggtctccca attcagagat
3481 ctgcctgcct ctgcctcctg agtgctggga ataaaggcat gtgccaccac cacccagctt
3541 cctgctgtaa gtttatatgc cttacttctc ctgtcacaca ggctaggcca tcattaacag
3601 catggaacat gcacagtgga agggaattgg agcagggaag gttccaggct ttcctgaagg
3661 agcctgcata gtctttaaaa aatgttttct ttttaacttc taatgctgtt ttaagaagac
3721 agaattagaa actgcttcta gtggagtttt aaggagaata aaatagcaag ggaggccaga
3781 gctggttttg gaattgcagt agcaatcagg gtcctagtat taaaatcatg tctgttagtg
3841 ttcagtaggg gaagacttgt gtgtccactg ctgaagatag gtgcttcaga tccaggcctg
3901 gattgtgacc acagactttc agatcttttc caggctcacc actgacagcc agacaatggc
3961 ttggcacctc gagttgtggc tgagcctgta tgagttgaag gatggtcagt agatgaagcc
4021 gtttactctc atggtgtgtg cccttgtaca agagatctgg gcgcgtgaca caaagccagt
4081 cattgacagg ctagacccaa agacctttaa gcactcccat ttgcttccgc tgtatcacaa
4141 taactgatga ctttgtagtc agtggtacct gctccgaaca agtacgtgga gtgtgggagt
4201 ccctctggga gcccagcgtg gaattagaac acctctttaa agccacctcc taagccgagc
4261 agaatgcagt ggactggggt acaagtcaga cctgagtgtc attgtccaca tcatggaaaa
4321 acaagatggc caccagaaca ctgaaggcct gaggagacta accctgttcc cagaactcag
4381 agttctgttt ggttctgttt ttaataagat aacctttctt taatgtatac atatgcatat
4441 acatatatat ttgtacttta aatacagagt actgaataaa atttatgtga ctataaaaaa
4501 aaaaaaaaaa aa
```

SEQ ID NO: 6 Mouse RIPK1 Amino Acid Sequence (NP_033094.3)

```
   1 mqpdmsldni kmassdllek tdldsggfgk vslcyhrshg fvilkkvytg pnraeynevl
  61 leegkmmhrl rhsrvvkllg iiieegnysl vmeymekgnl mhvlktqidv plslkgriiv
 121 eaiegmcylh dkgvihkdlk penilvdrdf hikiadlgva sfktwskltk ekdnkqkevs
 181 sttkknnggt lyymapehln dinakpteks dvysfgivlw aifakkepye nvicteqfvi
 241 ciksgnrpnv eeileycpre iislmercwq aipedrptfl gieeefrpfy lshfeeyvee
 301 dvaslkkeyp dqspvlqrmf slqhdcvplp psrsnseqpg slhssqglqm gpveeswfss
 361 speypqdend rsvqaklqee asyhafgifa ekqtkpqprq neaynreeer krrvshdpfa
 421 qqrareniks agarghsdps ttsrgiavqq lswpatqtvw nnglynqhgf gttgtgvwyp
 481 pnlsqmysty ktpvpetnip gstptmpyfs gpvaddliky tifnssgiqi gnhnymdvgl
 541 nsqppnntck eestsrhqai fdnttsltde hlnpirenlg rqwkncarkl gftesqidei
 601 dhdyerdglk ekvyqmlqkw lmregtkgat vgklaqalhq ccridllnhl irasqs
```

SEQ ID NO: 7 Human BIRC2 Transcript Variant 1 cDNA Sequence (NM_001166.4, CDS region from position 1453-3309)

```
   1 aacgctggtc ctcggccggg cgcgctgacg tcatcgtgcg tcagagtgag cccggatggg
  61 gcggcgggct tcgggagcgc ccgggctgat ccgagccgag cgggccgtat ctccttgtcg
 121 gcgccgctga ttcccggctc tgcggaggcc tctaggcagc cgcgcagctt ccgtgtttgc
 181 tgcgcccgca ctgcgattta caaccctgaa gaatctccct atccctattt tgtccccctg
 241 cagtaataaa tcccattatg gagatctcga aactttataa agggatatag tttgaattct
 301 atggagtgta attttgtgta tgaattatat ttttaaaaca ttgaagagtt ttcagaaaga
 361 aggctagtag agttgattac tgatacttta tgctaagcag tacttttttg gtagtacaat
 421 attttgttag gcgtttctga taacactaga aaggacaagt tttatcttgt gataaattga
```

```
 481 ttaatgttta caacatgact gataattata gctgaatagt ccttaaatga tgaacaggtt
 541 atttagtttt taaatgcagt gtaaaaagtg tgctgtggaa attttatggc taactaagtt
 601 tatggagaaa ataccttcag ttgatcaaga ataatagtgg tatacaaagt taggaagaaa
 661 gtcaacatga tgctgcagga aatggaaaca aatacaaatg atatttaaca aagatagagt
 721 ttacagtttt tgaactttaa gccaaattca tttgacatca agcactatag caggcacagg
 781 ttcaacaaag cttgtgggta ttgacttccc ccaaaagttg tcagctgaag taatttagcc
 841 cacttaagta aatactatga tgataagctg tgtgaactta gcttttaaat agtgtgacca
 901 tatgaaggtt ttaattactt ttgtttattg gaataaaatg agatttttg ggttgtcatg
 961 ttaaagtgct tatagggaaa gaagcctgca tataattttt taccttgtgg cataatcagt
1021 aattggtctg ttattcaggc ttcatagctt gtaaccaaat ataaataaaa ggcataattt
1081 aggtattcta tagttgctta gaattttgtt aatataaatc tctgtgaaaa atcaaggagt
1141 tttaatattt tcagaagtgc atccaccttt cagggcttta agttagtatt actcaagatt
1201 atgaacaaat agcacttagg ttacctgaaa gagttactac aaccccaaag agttgtgttc
1261 taagtagtat cttggtaatt cagagagata ctcatcctac ctgaatataa actgagataa
1321 atccagtaaa gaaagtgtag taaattctac ataagagtct atcattgatt tctttttgtg
1381 gtaaaaatct tagttcatgt gaagaaattt catgtgaatg ttttagctat caaacagtac
1441 tgtcacctac tcatgcacaa aactgcctcc caaagacttt tcccaggtcc ctcgtatcaa
1501 aacattaaga gtataatgga agatagcacg atcttgtcag attggacaaa cagcaacaaa
1561 caaaaaatga agtatgactt ttcctgtgaa ctctacagaa tgtctacata ttcaactttc
1621 cccgccgggg tgcctgtctc agaaaggagt cttgctcgtg ctggttttta ttatactggt
1681 gtgaatgaca aggtcaaatg cttctgttgt ggcctgatgc tggataactg gaaactagga
1741 gacagtccta ttcaaaagca taaacagcta tatcctagct gtagctttat tcagaatctg
1801 gtttcagcta gtctgggatc cacctctaag aatacgtctc caatgagaaa cagttttgca
1861 cattcattat ctcccacctt ggaacatagt agcttgttca gtggttctta ctccagcctt
1921 tctccaaacc ctcttaattc tagagcagtt gaagacatct cttcatcgag gactaacccc
1981 tacagttatg caatgagtac tgaagaagcc agatttctta cctaccatat gtggccatta
2041 acttttttgt caccatcaga attggcaaga gctggttttt attatatagg acctggagat
2101 agggtagcct gctttgcctg tggtgggaag ctcagtaact gggaaccaaa ggatgatgct
2161 atgtcagaac accggaggca ttttcccaac tgtccatttt tggaaaattc tctagaaact
2221 ctgaggttta gcatttcaaa tctgagcatg cagacacatg cagctcgaat gagaacattt
2281 atgtactggc catctagtgt tccagttcag cctgagcagc ttgcaagtgc tggtttttat
2341 tatgtgggtc gcaatgatga tgtcaaatgc ttttgttgtg atggtggctt gaggtgttgg
2401 gaatctggag atgatccatg ggtagaacat gccaagtggt ttccaaggtg tgagttcttg
2461 atacgaatga aaggccaaga gtttgttgat gagattcaag gtagatatcc tcatcttctt
2521 gaacagctgt tgtcaacttc agataccact ggagaagaaa atgctgaccc accaattatt
2581 cattttggac ctggagaaag ttcttcagaa gatgctgtca tgatgaatac acctgtggtt
2641 aaatctgcct tggaaatggg ctttaataga gacctggtga aacaaacagt tcaaagtaaa
2701 atcctgacaa ctggagagaa ctataaaaca gttaatgata ttgtgtcagc acttcttaat
2761 gctgaagatg aaaaaagaga agaggagaag gaaaaacaag ctgaagaaat ggcatcagat
2821 gatttgtcat taattcggaa gaacagaatg gctctctttc aacaattgac atgtgtgctt
2881 cctatcctgg ataatctttt aaaggccaat gtaattaata aacaggaaca tgatattatt
2941 aaacaaaaaa cacagatacc tttacaagcg agagaactga ttgataccat tttggttaaa
3001 ggaaatgctg cggccaacat cttcaaaaac tgtctaaaag aaattgactc tacattgtat
3061 aagaacttat ttgtggataa gaatatgaag tatattccaa cagaagatgt ttcaggtctg
3121 tcactggaag aacaattgag gaggttgcaa gaagaacgaa cttgtaaagt gtgtatggac
3181 aaagaagttt ctgttgtatt tattccttgt ggtcatctgg tagtatgcca ggaatgtgcc
3241 ccttctctaa gaaaatgccc tatttgcagg ggtataatca agggtactgt tcgtacattt
3301 ctctcttaaa gaaaaatagt ctatatttta acctgcataa aaaggtcttt aaaatattgt
3361 tgaacacttg aagccatcta aagtaaaaag ggaattatga gtttttcaat tagtaacatt
3421 catgttctag tctgctttgg tactaataat cttgtttctg aaaagatggt atcatatatt
3481 taatcttaat ctgtttattt acaagggaag atttatgttt ggtgaactat attagtatgt
3541 atgtgtacct aagggagtag tgtcactgct tgttatgcat catttcagga gttactggat
3601 ttgttgttct ttcagaaagc tttgaatact aaattatagt gtagaaaaga actggaaacc
3661 aggaactctg gagttcatca gagttatggt gccgaattgt ctttggtgct tttcacttgt
3721 gttttaaaat aaggattttt ctcttatttc tccccctagt ttgtgagaaa catctcaata
3781 aagtgcttta aaaagaaaaa aaaaaaaaaa aaa1
```

SEQ ID NO: 8 Human BIRC2 Transcript Variant 2 cDNA Sequence (NM_001256163.1, CDS region from position 1730-3586)

```
   1 gcggtgagtg ctgctccttc cgggctcggg cggcgtgggg cgggtgggga cgcgagggcc
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  61 cgcgggggcc cacttccctg atgtggcggc gaacgaggaa ggacggggcc tgaggccctt
 121 cggccaaggg tcgagggtcg ccgggggctc tctgctttct actctcgcca aggttttatt
 181 ggattcggaa gccccaactt cgagacttgc agtcaaagcg atttttaaaa tgacttgttt
 241 tcaagcctct ggccgccgcc cactcttctg gcccttggac tttgaccaag atgttttctc
 301 gcagtttttg caaggtttta aacttagccc tcggcgttct tttaatgtaa tacattgaaa
 361 cgaagatatt tcggtggcgg cgatatttca tatttcatag ttgccactgc gctctgtcat
 421 tccagtagtc gtctgttgtg tattgtgaga agcaactctg ggaaattatt ggatttacaa
 481 ccctgaagaa tctccctatc cctattttgt ccccctgcag taataaatcc cattatggag
 541 atctcgaaac tttataaagg gatatagttt gaattctatg gagtgtaatt ttgtgtatga
 601 attatatttt taaaacattg aagagttttc agaaagaagg ctagtagagt tgattactga
 661 tactttatgc taagcagtac ttttttggta gtacaatatt ttgttaggcg tttctgataa
 721 cactagaaag gacaagtttt atcttgtgat aaattgatta atgtttacaa catgactgat
 781 aattatagct gaatagtcct taaatgatga acaggttatt tagtttttaa atgcagtgta
 841 aaaagtgtgc tgtggaaatt ttatggctaa ctaagtttat ggagaaaata ccttcagttg
 901 atcaagaata atagtggtat acaaagttag gaagaaagtc aacatgatgc tgcaggaaat
 961 ggaaacaaat acaaatgata tttaacaaag atagagttta cagttttga actttaagcc
1021 aaattcattt gacatcaagc actatagcag gcacaggttc aacaaagctt gtgggtattg
1081 acttccccca aaagttgtca gctgaagtaa tttagcccac ttaagtaaat actatgatga
1141 taagctgtgt gaacttagct tttaaatagt gtgaccatat gaaggtttta attacttttg
1201 tttattggaa taaaatgaga ttttttgggt tgtcatgtta aagtgcttat agggaaagaa
1261 gcctgcatat aatttttac cttgtggcat aatcagtaat tggtctgtta ttcaggcttc
1321 atagcttgta accaaatata aataaaaggc ataatttagg tattctatag ttgcttagaa
1381 ttttgttaat ataaatctct gtgaaaaatc aaggagtttt aatattttca gaagtgcatc
1441 caccttttcag ggctttaagt tagtattact caagattatg aacaaatagc acttaggtta
1501 cctgaaagag ttactacaac cccaaagagt tgtgttctaa gtagtatctt ggtaattcag
1561 agagatactc atcctacctg aatataaact gagataaatc cagtaaagaa agtgtagtaa
1621 attctacata agagtctatc attgatttct ttttgtggta aaaatcttag ttcatgtgaa
1681 gaaatttcat gtgaatgttt tagctatcaa acagtactgt cacctactca tgcacaaaac
1741 tgcctcccaa agacttttcc caggtccctc gtatcaaaac attaagagta taatggaaga
1801 tagcacgatc ttgtcagatt ggacaaacag caacaaacaa aaaatgaagt atgacttttc
1861 ctgtgaactc tacagaatgt ctacatattc aactttcccc gccggggtgc ctgtctcaga
1921 aaggagtctt gctcgtgctg gtttttatta tactggtgtg aatgacaagg tcaaatgctt
1981 ctgttgtggc ctgatgctgg ataactggaa actaggagac agtcctattc aaaagcataa
2041 acagctatat cctagctgta gctttattca gaatctggtt tcagctagtc tgggatccac
2101 ctctaagaat acgtctccaa tgagaaacag ttttgcacat tcattatctc ccaccttgga
2161 acatagtagc ttgttcagtg gttcttactc cagcctttct ccaaaccctc ttaattctag
2221 agcagttgaa gacatctctt catcgaggac taacccctac agttatgcaa tgagtactga
2281 agaagccaga tttcttacct accatatgtg gccattaact tttttgtcac catcagaatt
2341 ggcaagagct ggtttttatt atataggacc tggagatagg gtagcctgct ttgcctgtgg
2401 tgggaagctc agtaactggg aaccaaagga tgatgctatg tcagaacacc ggaggcattt
2461 tcccaactgt ccatttttgg aaaattctct agaaactctg aggtttagca tttcaaatct
2521 gagcatgcag acacatgcag ctcgaatgag aacatttatg tactggccat ctagtgttcc
2581 agttcagcct gagcagcttg caagtgctgg tttttattat gtgggtcgca atgatgatgt
2641 caaatgcttt tgttgtgatg gtggcttgag gtgttgggaa tctggagatg atccatgggt
2701 agaacatgcc aagtggtttc caaggtgtga gttcttgata cgaatgaaag gccaagagtt
2761 tgttgatgag attcaaggta gatatcctca tcttcttgaa cagctgttgt caacttcaga
2821 taccactgga gaagaaaatg ctgacccacc aattattcat tttggacctg gagaaagttc
2881 ttcagaagat gctgtcatga tgaatacacc tgtggttaaa tctgccttgg aaatgggctt
2941 taatagagac ctggtgaaac aaacagttca aagtaaaatc ctgacaactg gagagaacta
3001 taaaacagtt aatgatattg tgtcagcact tcttaatgct gaagatgaaa aaagagaaga
3061 ggagaaggaa aaacaagctg aagaaatggc atcagatgat ttgtcattaa ttcggaagaa
3121 cagaatggct ctctttcaac aattgacatg tgtgcttcct atcctggata atcttttaaa
3181 ggccaatgta attaataaac aggaacatga tattattaaa caaaaaacac agataccttt
3241 acaagcgaga gaactgattg ataccatttt ggttaaagga aatgctgcgg ccaacatctt
3301 caaaaactgt ctaaaagaaa ttgactctac attgtataag aacttatttg tggataagaa
3361 tatgaagtat attccaacag aagatgtttc aggtctgtca ctggaagaac aattgaggag
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3481 tccttgtggt catctggtag tatgccagga atgtgcccct tctctaagaa aatgccctat
3541 ttgcaggggt ataatcaagg gtactgttcg tacatttctc tcttaaagaa aaatagtcta
3601 tattttaacc tgcataaaaa ggtctttaaa atattgttga acacttgaag ccatctaaag
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3721 taataatctt gtttctgaaa agatggtatc atatatttaa tcttaatctg tttatttaca
3781 agggaagatt tatgtttggt gaactatatt agtatgtatg tgtacctaag ggagtagtgt
3841 cactgcttgt tatgcatcat ttcaggagtt actggatttg ttgttctttc agaaagcttt
3901 gaatactaaa ttatagtgta gaaaagaact ggaaaccagg aactctggag ttcatcagag
3961 ttatggtgcc gaattgtctt tggtgctttt cacttgtgtt ttaaaataag gatttttctc
4021 ttatttctcc ccctagtttg tgagaaacat ctcaataaag tgctttaaaa agaaaaaaaa
4081 aaaaaaaa
```

SEQ ID NO: 9 Human BIRC2 Isoform 1 Amino Acid Sequence (NP_001157.1)

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   1 mhktasqrlf pgpsyqniks imedstilsd wtnsnkqkmk ydfscelyrm stystfpagv
  61 pvserslara gfyytgvndk vkcfccglml dnwklgdspi qkhkqlypsc sfiqnlvsas
 121 lgstskntsp mrnsfahsls ptlehsslfs gsysslspnp lnsravedis ssrtnpysya
 181 msteearflt yhmwpltfls pselaragfy yigpgdrvac facggklsnw epkddamseh
 241 rrhfpncpfl ensletlrfs isnlsmqtha armrtfmywp ssvpvqpeql asagfyyvgr
 301 nddvkcfccd gglrcwesgd dpwvehakwf prceflirmk gqefvdeiqg ryphlleqll
 361 stsdttgeen adppiihfgp gesssedavm mntpvvksal emgfnrdlvk qtvqskiltt
 421 genyktvndi vsallnaede kreeekekqa eemasddlsl irknrmalfq qltcvlpild
 481 nllkanvink qehdiikqkt qiplqareli dtilvkgnaa anifknclke idstlyknlf
 541 vdknmkyipt edvsglslee qlrrlqeert ckvcmdkevs vvfipcghlv vcqecapslr
 601 kcpicrgiik gtvrtfls
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SEQ ID NO: 10 Human BIRC2 Transcript Variant 3 cDNA Sequence (NM_001256166.1, CDS region from position 197-1906)

```
   1 aacgctggtc ctcggccggg cgcgctgacg tcatcgtgcg tcagagtgag cccggatggg
  61 gcggcgggct tcgggagcgc ccgggctgat ccgagccgag cgggccgtat ctccttgtcg
 121 gcgccgctga ttcccggctc tgcggaggcc tctaggcagc cgcgcagctt ccgtgtttgc
 181 tgcgcccgca ctgcgaatgt ctacatattc aactttcccc gccggggtgc ctgtctcaga
 241 aaggagtctt gctcgtgctg gtttttatta tactggtgtg aatgacaagg tcaaatgctt
 301 ctgttgtggc ctgatgctgg ataactggaa actaggagac agtcctattc aaaagcataa
 361 acagctatat cctagctgta gctttattca gaatctggtt tcagctagtc tgggatccac
 421 ctctaagaat acgtctccaa tgagaaacag ttttgcacat tcattatctc ccaccttgga
 481 acatagtagc ttgttcagtg gttcttactc cagcctttct ccaaaccctc ttaattctag
 541 agcagttgaa gacatctctt catcgaggac taacccctac agttatgcaa tgagtactga
 601 agaagccaga tttcttacct accatatgtg gccattaact tttttgtcac catcagaatt
 661 ggcaagagct ggtttttatt atataggacc tggagatagg gtagcctgct ttgcctgtgg
 721 tgggaagctc agtaactggg aaccaaagga tgatgctatg tcagaacacc ggaggcattt
 781 tcccaactgt ccatttttgg aaaattctct agaaactctg aggtttagca tttcaaatct
 841 gagcatgcag acacatgcag ctcgaatgag aacatttatg tactggccat ctagtgttcc
 901 agttcagcct gagcagcttg caagtgctgg tttttattat gtgggtcgca atgatgatgt
 961 caaatgcttt tgttgtgatg gtggcttgag gtgttgggaa tctggagatg atccatgggt
1021 agaacatgcc aagtggtttc caaggtgtga gttcttgata cgaatgaaag gccaagagtt
1081 tgttgatgag attcaaggta gatatcctca tcttcttgaa cagctgttgt caacttcaga
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1261 taatagagac ctggtgaaac aaacagttca aagtaaaatc ctgacaactg gagagaacta
1321 taaaacagtt aatgatattg tgtcagcact tcttaatgct gaagatgaaa aaagagaaga
1381 ggagaaggaa aaacaagctg aagaaatggc atcagatgat ttgtcattaa ttcggaagaa
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1501 ggccaatgta attaataaac aggaacatga tattattaaa caaaaaacac agataccttt
1561 acaagcgaga gaactgattg ataccatttt ggttaaagga aatgctgcgg ccaacatctt
1621 caaaaactgt ctaaaagaaa ttgactctac attgtataag aacttatttg tggataagaa
1681 tatgaagtat attccaacag aagatgtttc aggtctgtca ctggaagaac aattgaggag
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1801 tccttgtggt catctggtag tatgccagga atgtgcccct tctctaagaa aatgccctat
1861 ttgcaggggt ataatcaagg gtactgttcg tacatttctc tcttaaagaa aaatagtcta
1921 tattttaacc tgcataaaaa ggtctttaaa atattgttga acacttgaag ccatctaaag
1981 taaaaaggga attatgagtt tttcaattag taacattcat gttctagtct gctttggtac
2041 taataatctt gtttctgaaa agatggtatc atatatttaa tcttaatctg tttatttaca
2101 agggaagatt tatgtttggt gaactatatt agtatgtatg tgtacctaag ggagtagtgt
2161 cactgcttgt tatgcatcat ttcaggagtt actggatttg ttgttctttc agaaagcttt
```

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2221 gaatactaaa ttatagtgta gaaaagaact ggaaaccagg aactctggag ttcatcagag
2281 ttatggtgcc gaattgtctt tggtgctttt cacttgtgtt ttaaaataag gatttttctc
2341 ttatttctcc ccctagtttg tgagaaacat ctcaataaag tgctttaaaa agaaaaaaaa
2401 aaaaaaaa
```

SEQ ID NO: 11 Human BIRC2 Isoform 2 Amino Acid Sequence (NP_001243095.1)

```
   1 mstystfpag vpvserslar agfyytgvnd kvkcfccglm ldnwklgdsp iqkhkqlyps
  61 csfiqnlvsa slgstsknts pmrnsfahsl sptlehsslf sgsysslspn plnsravedi
 121 sssrtnpysy amsteearfl tyhmwpltfl spselaragf yyigpgdrva cfacggklsn
 181 wepkddamse hrrhfpncpf lensletlrf sisnlsmqth aarmrtfmyw pssvpvqpeq
 241 lasagfyyvg rnddvkcfcc dgglrcwesg ddpwvehakw fprceflirm kgqefvdeiq
 301 gryphlleql lstsdttgee nadppiihfg pgesssedav mmntpvvksa lemgfnrdlv
 361 kqtvqskilt tgenyktvnd ivsallnaed ekreeekekq aeemasddls lirknrmalf
 421 qqltcvlpil dnllkanvin kqehdiikqk tqiplqarel idtilvkgna aanifknclk
 481 eidstlyknl fvdknmkyip tedvsglsle eqlrrlqeer tckvcmdkev svvfipcghl
 541 vvcqecapsl rkcpicrgii kgtvrtfls
```

SEQ ID NO: 12 Mouse BIRC2 Transcript Variant 1 cDNA Sequence (NM_007465.3, CDS region from position 970-2808)

```
   1 ttttgggaag gctgcggatc aacaatcagg cgcctctgct tccagggcgc cggggcctg
  61 actcggtgat tgatagtcct caagagtctt catgttctct tatgtttttc tagaaatcag
 121 cccactcatg gacatctgaa catttctata agacgctgcg gtttgctttg cagtgtgttc
 181 ttgtgtatga ttagttatat aaaatacgaa gttttcaaaa agaaggctag tgcaacagaa
 241 aagctttgct aaaacagatt cttagttatt tgaggtaaca aaagaaagcc atgtcttgaa
 301 ttgattcgtt cttaattata acagacttat agtggaaagg gccttaaaca caggcggact
 361 ttataaaatg cagtcttagg tttatgtgca aaatactgtc tgttgaccag atgtattcac
 421 atgatatata cagagtcaag gtggtgatat agaagattta acagtgaggg agttaacagt
 481 ctgtgcttta agcgcagttc ctttacagtg aatactgtag tcttaataga cctgagctga
 541 ctgctgcagt tgatgtaacc cactttagag aatactgtat gacatcttct ctaaggaaaa
 601 ccagctgcag acttcactca gttcctttca tttcatagga aaaggagtag ttcagatgtc
 661 atgtttaagt ccttataagg gaaaagagcc tgaatatatg ccctagtacc taggcttcat
 721 aactagtaat aagaagttag ttatgggtaa atagatctca ggttacccag aagagttcat
 781 gtgaccccca aagagtccta actagtgtct tggcaagtga gacagatttg tcctgtgagg
 841 gtgtcaattc accagtccaa gcagaagaca atgaatctat ccagtcaggt gtctgtggtg
 901 gagatctagt gtcaagtggt gagaaacttc atctggaagt ttaagcggtc agaaatacta
 961 ttactactca tggacaaaac tgtctcccag agactcggcc aaggtacctt acaccaaaaa
1021 cttaaacgta taatggagaa gagcacaatc ttgtcaaatt ggacaaagga gagcgaagaa
1081 aaaatgaagt ttgacttttc gtgtgaactc taccgaatgt ctacatattc agcttttccc
1141 aggggagttc ctgtctcaga gaggagtctg gctcgtgctg gcttttatta tacaggtgtg
1201 aatgacaaag tcaagtgctt ctgctgtggc ctgatgttgg ataactggaa acaaggggac
1261 agtcctgttg aaaagcacag acagttctat cccagctgca gctttgtaca gactctgctt
1321 tcagccagtc tgcagtctcc atctaagaat atgtctcctg tgaaaagtag atttgcacat
1381 tcgtcacctc tggaacgagg tggcattcac tccaacctgt gctctagccc tcttaattct
1441 agagcagtgg aagacttctc atcaaggatg gatccctgca gctatgccat gagtacagaa
1501 gaggccagat ttcttactta cagtatgtgg cctttaagtt ttctgtcacc agcagagctg
1561 gccagagctg gcttctatta catagggcct ggagacaggg tggcctgttt tgcctgtggt
1621 gggaaactga gcaactggga accaaaggat gatgctatgt cagagcaccg cagacatttt
1681 ccccactgtc catttctgga aaatacttca gaaacacaga ggtttagtat atcaaatcta
1741 agtatgcaga cacactctgc tcgattgagg acatttctgt actggccacc tagtgttcct
1801 gttcagcccg agcagcttgc aagtgctgga ttctattacg tggatcgcaa tgatgatgtc
1861 aagtgctttt gttgtgatgg tggcttgaga tgttgggaac ctggagatga cccctggata
1921 gaacacgcca aatggtttcc aaggtgtgag ttcttgatac ggatgaaggg tcaggagttt
1981 gttgatgaga ttcaagctag atatcctcat cttcttgagc agctgttgtc cacttcagac
2041 accccaggag aagaaaatgc tgaccctaca gagacagtgg tgcattttgg ccctggagaa
2101 agttcggaag atgtcgtcat gatgagcacg cctgtggtta aagcagcctt ggaaatgggc
2161 ttcagtagga gcctggtgag acagacggtt cagcggcaga tcctggccac tggtgagaac
2221 tacaggaccg tcaatgatat tgtctcagta cttttgaatg ctgaagatga gagaagagaa
2281 gaggagaagg aaagacagac tgaagagatg gcatcaggtg acttatcact gattcggaag
2341 aatagaatgg ccctctttca acagttgaca catgtccttc ctatcctgga taatcttctt
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2401 gaggccagtg taattacaaa acaggaacat gatattatta gacagaaaac acagataccc
2461 ttacaagcaa gagagcttat tgacaccgtt ttagtcaagg gaaatgctgc agccaacatc
2521 ttcaaaaact ctctgaagga aattgactcc acgttatatg aaaacttatt tgtggaaaag
2581 aatatgaagt atattccaac agaagacgtt tcaggcttgt cattggaaga gcagttgcgg
2641 agattacaag aagaacgaac ttgcaaagtg tgtatggaca gagaggtttc tattgtgttc
2701 attccgtgtg gtcatctagt agtctgccag gaatgtgccc cttctctaag gaagtgcccc
2761 atctgcaggg ggacaatcaa ggggactgtg cgcacatttc tctcatgagt gaagaatggt
2821 ctgaaagtat tgttggacat cagaagctgt cagaacaaag aatgaactac tgatttcagc
2881 tcttcagcag gacattctac tctctttcaa gattagtaat cttgctttat gaagggtagc
2941 attgtatatt taagcttagt ctgttgcaag ggaaggtcta tgctgttgag ctacaggact
3001 gtgtctgttc cagagcagga gttgggatgc ttgctgtatg tccttcagga cttcttggat
3061 ttggaatttg tgaaagcttt ggattcaggt gatgtggagc tcagaaatcc tgaaaccagt
3121 ggctctggta ctcagtagtt agggtaccct gtgcttcttg gtgcttttcc tttctggaaa
3181 ataaggattt ttctgctact ggtaaatatt ttctgtttgt gagaaatata ttaaagtgtt
3241 tcttttaaag gcgtgcatca ttgtagtgtg tgcagggatg tatgcaggca aaacactgtg
3301 tatataataa ataaatcttt ttaaaaagtg tta
```

SEQ ID NO: 13 Mouse BIRC2 Transcript Variant 2 cDNA Sequence (NM_001291503.1, CDS region from position 1014-2852)

```
   1 cagtcaaccc aggctagtct cgaatttgcg gcaatcctcc tgcctccaat cgttctaggt
  61 gctgggatta ctggtgtgca gcacctcggc tgtctcttca gattttctgc agattgatag
 121 tcctcaagag tcttcatgtt ctcttatgtt tttctagaaa tcagcccact catggacatc
 181 tgaacatttc tataagacgc tgcggtttgc tttgcagtgt gttcttgtgt atgattagtt
 241 atataaaata cgaagttttc aaaaagaagg ctagtgcaac agaaaagctt tgctaaaaca
 301 gattcttagt tatttgaggt aacaaaagaa agccatgtct tgaattgatt cgttcttaat
 361 tataacagac ttatagtgga aagggcctta aacacaggcg gactttataa aatgcagtct
 421 taggtttatg tgcaaaatac tgtctgttga ccagatgtat tcacatgata tatacagagt
 481 caaggtggtg atatagaaga tttaacagtg agggagttaa cagtctgtgc tttaagcgca
 541 gttcctttac agtgaatact gtagtcttaa tagacctgag ctgactgctg cagttgatgt
 601 aacccacttt agagaatact gtatgacatc ttctctaagg aaaaccagct gcagacttca
 661 ctcagttcct ttcatttcat aggaaaagga gtagttcaga tgtcatgttt aagtccttat
 721 aagggaaaag agcctgaata tatgccctag tacctaggct tcataactag taataagaag
 781 ttagttatgg gtaaatagat ctcaggttac ccagaagagt tcatgtgacc cccaaagagt
 841 cctaactagt gtcttggcaa gtgagacaga tttgtcctgt gagggtgtca attcaccagt
 901 ccaagcagaa gacaatgaat ctatccagtc aggtgtctgt ggtggagatc tagtgtcaag
 961 tggtgagaaa cttcatctgg aagtttaagc ggtcagaaat actattacta ctcatggaca
1021 aaactgtctc ccagagactc ggccaaggta ccttacacca aaaacttaaa cgtataatgg
1081 agaagagcac aatcttgtca aattggacaa aggagagcga agaaaaaatg aagtttgact
1141 tttcgtgtga actctaccga atgtctacat attcagcttt tcccagggga gttcctgtct
1201 cagagaggag tctggctcgt gctggctttt attatacagg tgtgaatgac aaagtcaagt
1261 gcttctgctg tggcctgatg ttggataact ggaaacaagg ggacagtcct gttgaaaagc
1321 acagacagtt ctatcccagc tgcagctttg tacagactct gctttcagcc agtctgcagt
1381 ctccatctaa gaatatgtct cctgtgaaaa gtagatttgc acattcgtca cctctggaac
1441 gaggtggcat tcactccaac ctgtgctcta gccctcttaa ttctagagca gtggaagact
1501 tctcatcaag gatggatccc tgcagctatg ccatgagtac agaagaggcc agatttctta
1561 cttacagtat gtggccttta agttttctgt caccagcaga gctggccaga gctggcttct
1621 attacatagg gcctggagac agggtggcct gttttgcctg tggtgggaaa ctgagcaact
1681 gggaaccaaa ggatgatgct atgtcagagc accgcagaca ttttccccac tgtccatttc
1741 tggaaaatac ttcagaaaca cagaggttta gtatatcaaa tctaagtatg cagacacact
1801 ctgctcgatt gaggacattt ctgtactggc cacctagtgt tcctgttcag cccgagcagc
1861 ttgcaagtgc tggattctat tacgtggatc gcaatgatga tgtcaagtgc ttttgttgtg
1921 atggtggctt gagatgttgg gaacctggag atgacccctg gatagaacac gccaaatggt
1981 ttccaaggtg tgagttcttg atacggatga agggtcagga gtttgttgat gagattcaag
2041 ctagatatcc tcatcttctt gagcagctgt tgtccacttc agacacccca ggagaagaaa
2101 atgctgaccc tacagagaca gtggtgcatt ttggccctgg agaaagttcg gaagatgtcg
2161 tcatgatgag cacgcctgtg gttaaagcag ccttggaaat gggcttcagt aggagcctgg
2221 tgagacagac ggttcagcgg cagatcctgg ccactggtga gaactacagg accgtcaatg
2281 atattgtctc agtacttttg aatgctgaag atgagagaag agaagaggag aaggaaagac
2341 agactgaaga gatggcatca ggtgacttat cactgattcg gaagaataga atggccctct
2401 ttcaacagtt gacacatgtc cttcctatcc tggataatct tcttgaggcc agtgtaatta
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2461 caaaacagga acatgatatt attagacaga aaacacagat acccttacaa gcaagagagc
2521 ttattgacac cgttttagtc aagggaaatg ctgcagccaa catcttcaaa aactctctga
2581 aggaaattga ctccacgtta tatgaaaact tatttgtgga aaagaatatg aagtatattc
2641 caacagaaga cgtttcaggc ttgtcattgg aagagcagtt gcggagatta caagaagaac
2701 gaacttgcaa agtgtgtatg gacagagagg tttctattgt gttcattccg tgtggtcatc
2761 tagtagtctg ccaggaatgt gccccttctc taaggaagtg ccccatctgc agggggacaa
2821 tcaaggggac tgtgcgcaca tttctctcat gagtgaagaa tggtctgaaa gtattgttgg
2881 acatcagaag ctgtcagaac aaagaatgaa ctactgattt cagctcttca gcaggacatt
2941 ctactctctt tcaagattag taatcttgct ttatgaaggg tagcattgta tatttaagct
3001 tagtctgttg caagggaagg tctatgctgt tgagctacag gactgtgtct gttccagagc
3061 aggagttggg atgcttgctg tatgtccttc aggacttctt ggatttggaa tttgtgaaag
3121 ctttggattc aggtgatgtg gagctcagaa atcctgaaac cagtggctct ggtactcagt
3181 agttagggta ccctgtgctt cttggtgctt ttcctttctg gaaaataagg atttttctgc
3241 tactggtaaa tattttctgt ttgtgagaaa tatattaaag tgtttctttt aaaggcgtgc
3301 atcattgtag tgtgtgcagg gatgtatgca ggcaaaacac tgtgtatata ataaataaat
3361 ctttttaaaa agtgtta
```

SEQ ID NO: 14 Mouse BIRC2 Amino Acid Sequence (NP_001278432.1)

```
   1 mdktvsqrlg qgtlhqklkr imekstilsn wtkeseekmk fdfscelyrm stysafprgv
  61 pvserslara gfyytgvndk vkcfccglml dnwkqgdspv ekhrqfypsc sfvqtllsas
 121 lqspsknmsp vksrfahssp lerggihsnl csplnsrav edfssrmdpc syamsteear
 181 fltysmwpls flspaelara gfyyigpgdr vacfacggkl snwepkddam sehrrhfphc
 241 pflentsetq rfsisnlsmq thsarlrtfl ywppsvpvqp eqlasagfyy vdrnddvkcf
 301 ccdgglrcwe pgddpwieha kwfprcefli rmkgqefvde iqaryphlle qllstsdtpg
 361 eenadptetv vhfgpgesse dvvmmstpvv kaalemgfsr slvrqtvqrq ilatgenyrt
 421 vndivsvlln aederreeek erqteemasg dlslirknrm alfqqlthvl pildnlleas
 481 vitkqehdii rqktqiplqa relidtvlvk gnaaanifkn slkeidstly enlfveknmk
 541 yiptedvsgl sleeqlrrlq eertckvcmd revsivfipc ghlvvcqeca pslrkcpicr
 601 gtikgtvrtf ls
```

SEQ ID NO: 15 Human TBK1 cDNA Sequence (NM_013254.3, CDS region from position 160-2349)

```
   1 gcacccgcac cggcgcgccg gccgtcggtc acgtggcctc cggccagggc ttgcgaagcc
  61 ggaagtgtcc tgagtctcga ggaggccgcg ggagcccgcc ggcggtggcg cggcggagac
 121 ccggctggta taacaagagg attgcctgat ccagccaaga tgcagagcac ttctaatcat
 181 ctgtggcttt tatctgatat tttaggccaa ggagctactg caaatgtctt tcgtggaaga
 241 cataagaaaa ctggtgattt atttgctatc aaagtattta ataacataag cttccttcgt
 301 ccagtggatg ttcaaatgag agaatttgaa gtgttgaaaa aactcaatca caaaaatatt
 361 gtcaaattat ttgctattga agaggagaca acaacaagac ataaagtact tattatggaa
 421 ttttgtccat gtgggagttt atacactgtt ttagaagaac cttctaatgc ctatggacta
 481 ccagaatctg aattcttaat tgttttgcga gatgtggtgg gtggaatgaa tcatctacga
 541 gagaatggta tagtgcaccg tgatatcaag ccaggaaata tcatgcgtgt tataggggaa
 601 gatggacagt ctgtgtacaa actcacagat tttggtgcag ctagagaatt agaagatgat
 661 gagcagtttg tttctctgta tggcacagaa gaatatttgc accctgatat gtatgagaga
 721 gcagtgctaa gaaaagatca tcagaagaaa tatggagcaa cagttgatct ttggagcatt
 781 ggggtaacat tttaccatgc agctactgga tcactgccat ttagaccctt tgaagggcct
 841 cgtaggaata aagaagtgat gtataaaata attacaggaa agccttctgg tgcaatatct
 901 ggagtacaga aagcagaaaa tggaccaatt gactggagtg gagacatgcc tgtttcttgc
 961 agtctttctc ggggtcttca ggttctactt acccctgttc ttgcaaacat ccttgaagca
1021 gatcaggaaa agtgttgggg ttttgaccag ttttttgcag aaactagtga tatacttcac
1081 cgaatggtaa ttcatgtttt ttcgctacaa caaatgacag ctcataagat ttatattcat
1141 agctataata ctgctactat atttcatgaa ctggtatata aacaaaccaa aattatttct
1201 tcaaatcaag aacttatcta cgaagggcga cgcttagtct tagaacctgg aaggctggca
1261 caacatttcc ctaaaactac tgaggaaaac cctatatttg tagtaagccg ggaacctctg
1321 aataccatag gattaatata tgaaaaaatt tccctcccta aagtacatcc acgttatgat
1381 ttagacgggg atgctagcat ggctaaggca ataacagggg ttgtgtgtta tgcctgcaga
1441 attgccagta ccttactgct ttatcaggaa ttaatgcgaa aggggatacg atggctgatt
1501 gaattaatta aagatgatta caatgaaact gttcacaaaa agacagaagt tgtgatcaca
1561 ttggatttct gtatcagaaa cattgaaaaa actgtgaaag tatatgaaaa gttgatgaag
```

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1621 atcaacctgg aagcggcaga gttaggtgaa atttcagaca tacacaccaa attgttgaga
1681 ctttccagtt ctcagggaac aatagaaacc agtcttcagg atatcgacag cagattatct
1741 ccaggtggat cactggcaga cgcatgggca catcaagaag gcactcatcc gaaagacaga
1801 aatgtagaaa aactacaagt cctgttaaat tgcatgacag agatttacta tcagttcaaa
1861 aaagacaaag cagaacgtag attagcttat aatgaagaac aaatccacaa atttgataag
1921 caaaaactgt attaccatgc cacaaaagct atgacgcact ttacagatga atgtgttaaa
1981 aagtatgagg cattttttgaa taagtcagaa gaatggataa gaaagatgct tcatcttagg
2041 aaacagttat tatcgctgac taatcagtgt tttgatattg aagaagaagt atcaaaatat
2101 caagaatata ctaatgagtt acaagaaact ctgcctcaga aaatgtttac agcttccagt
2161 ggaatcaaac ataccatgac cccaatttat ccaagttcta acacattagt agaaatgact
2221 cttggtatga agaaattaaa ggaagagatg gaaggggtgg ttaaagaact tgctgaaaat
2281 aaccacattt tagaaaggtt tggctcttta accatggatg gtggccttcg caacgttgac
2341 tgtctttagc tttctaatag aagtttaaga aaagtttccg tttgcacaag aaaataacgc
2401 ttgggcatta aatgaatgcc tttatagata gtcacttgtt tctacaattc agtatttgat
2461 gtggtcgtgt aaatatgtac aatattgtaa atacataaaa aatatacaaa tttttggctg
2521 ctgtgaagat gtaattttat cttttaacat ttataattat atgaggaaat ttgacctcag
2581 tgatcacgag aagaaagcca tgaccgacca atatgttgac atactgatcc tctactctga
2641 gtggggctaa ataagttatt ttctctgacc gcctactgga aatattttta agtggaacca
2701 aaataggcat ccttacaaat caggaagact gacttgacac gtttgtaaat ggtagaacgg
2761 tggctactgt gagtggggag cagaaccgca ccactgttat actgggataa caatttttt
2821 gagaaggata aagtggcatt attttatttt acaaggtgcc cagatcccag ttatccttgt
2881 atccatgtaa tttcagatga attattaagc aaacatttta aagtgaattc attattaaaa
2941 actattcatt tttttccttt ggccataaat gtgtaattgt cattaaaatt ctaaggtcat
3001 ttcaactgtt ttaagctgta tatttcttta attctgctta ctatttcatg gaaaaaaata
3061 aatttctcaa ttttaatgta aagagttaaa aaaaaaaa
```

SEQ ID NO: 16 Human TBK1 Amino Acid Sequence (NP_037386.1)

```
   1 mqstsnhlwl lsdilgqgat anvfrgrhkk tgdlfaikvf nnisflrpvd vqmrefevlk
  61 klnhknivkl faieeetttr hkvlimefcp cgslytvlee psnayglpes eflivlrdvv
 121 ggmnhlreng ivhrdikpgn imrvigedgq svykltdfga areleddeqf vslygteeyl
 181 hpdmyeravl rkdhqkkyga tvdlwsigvt fyhaatgslp frpfegprrn kevmykiitg
 241 kpsgaisgvq kaengpidws gdmpvscsls rglqvlltpv lanileadqe kcwgfdqffa
 301 etsdilhrmv ihvfslqqmt ahkiyihsyn tatifhelvy kqtkiissnq eliyegrrlv
 361 lepgrlaqhf pktteenpif vvsreplnti gliyekislp kvhprydldg dasmakaitg
 421 vvcyacrias tlllyqelmr kgirwlieli kddynetvhk ktevvitldf cirniektvk
 481 vyeklmkinl eaaelgeisd ihtkllrlss sqgtietslq didsrlspgg sladawahqe
 541 gthpkdrnve klqvllncmt eiyyqfkkdk aerrlaynee qihkfdkqkl yyhatkamth
 601 ftdecvkkye aflnkseewi rkmlhlrkql lsltnqcfdi eeevskyqey tnelqetlpq
 661 kmftassgik htmtpiypss ntlvemtlgm kklkeemegv vkelaennhi lerfgsltmd
 721 gglrnvdcl
```

SEQ ID NO: 17 Mouse TBK1 cDNA Sequence (NM_019786.4, CDS region from position 160-2349)

```
   1 tcggtcacgt gctccgtggc ccgggctggc gaagccggaa gtagcctggg gcgcgagaag
  61 gcccgggagc cgcgggctgt acgcggcgga cactcgcggg catacatgca aatctcttct
 121 tccccccttat cgtgaggaga agcgcctgga caagccgaga tgcagagcac ctccaaccat
 181 ctgtggctcc tgtctgatat cctaggccag ggggccactg caaatgtctt ccgaggaagg
 241 cataagaaaa ctggtgatct ctatgctgtc aaagtattta ataacataag cttccttcgc
 301 ccagtggatg ttcaaatgag agaatttgaa gtgttaaaaa aactcaatca caaaaacatt
 361 gtcaagttat ttgctattga agaggagaca acaacaagac ataaagtgct tattatggag
 421 ttttgtccct gtgggagttt atacactgtt ctagaggagc cgtccaatgc gtatggactt
 481 ccagaatcag aatttctcat tgtcttacga gatgtggtgg gcgggatgaa tcatctccga
 541 gagaacggca tagtgcaccg agatatcaag ccaggcaaca tcatgcgcgt catagggggag
 601 gacggccagt ctgtgtacaa actcacggat ttcggcgccg ctcgagagct ggaggacgat
 661 gagcagtttg tgtctctgta cggcacagaa gagtacctgc atccggacat gtatgaaagg
 721 gcagtgctaa gaaaggacca tcagaagaag tacggggcta ccgttgatct gtggagtgtt
 781 ggagtgacat tctaccatgc agccacgggg tcgctgccgt ttagaccctt cgaggggcct
 841 cggaggaaca aagaagtaat gtataaaata atcactggga agccgtctgg tgcaatatct
 901 ggagtacaga aagcagaaaa cggaccaatt gactggagtg gagacatgcc tctctcctgt
 961 agtctttctc agggtcttca ggcactgctt accccagttc ttgcaaacat acttgaagct
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1021 gatcaggaga agtgctgggg ttttgaccag ttctttgcag agaccagtga tgtgcttcac
1081 cgaatggtga tccatgtctt ctcgctacaa cacatgacgg cgcataagat ttacattcac
1141 agctataaca ctgctgctgt gttccatgaa ctggtctata aacaaaccaa gattgtttcc
1201 tcaaatcaag aacttatcta cgaaggacga cgcttagtcc tagaactcgg acgactagcc
1261 cagcattttc ctaaaaccac agaggaaaat cctatctttg tcacgagccg ggaacaactc
1321 aataccgtag gactgagata tgaaaaaatt tccctcccta aaatacatcc acgctatgat
1381 ctggatgggg acgccagcat ggccaaggca gtgacggggg ttgtgtgcta cgcctgcaga
1441 actgccagta ccctgctgct ctatcaagaa ttaatgcgaa agggggtacg gtggctggtt
1501 gaactggtta aggatgatta caacgagacc gtccacaaga agacggaggt agtgatcaca
1561 ctggatttct gcatcaggaa cattgagaag actgtgaaag tgtatgagaa gttgatgaag
1621 gtcaacctgg aagccgcaga gctgggtgag atttcagaca tacacaccaa gctgctgaga
1681 ctttccagtt ctcagggaac aatagaaagc agtcttcagg acatcagcag caggctgtct
1741 ccaggggget tgctggccga cacctgggca catcaagaag gcacgcatcc aagagacagg
1801 aatgtagaaa aactgcaggt cctgttgaac tgcatcacag agatttacta tcagttcaaa
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SEQ ID NO: 18 Mouse TBK1 Amino Acid Sequence (NP_062760.3)

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SEQ ID NO: 19 Human TRAF3 Transcript Variant 1 cDNA Sequence (NM_145725.2, CDS region from position 354-2060)

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SEQ ID NO: 20 Human TRAF3 Transcript Variant 3 cDNA Sequence (NM_003300.3, CDS region from position 215-1921)

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3721 gccagctggg ccgtgcacgt cagactgcct gcctcggctc tccccgtggc cgcgcgggga
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SEQ ID NO: 21 Human TRAF3 Isoform 1 Amino Acid Sequence (NP_663777.1)

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 181 ksqvpmialq khedtdcpcv vvscphkcsv qtllrselsa hlsecvnaps tcsfkrygcv
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 301 eierqkemlr nneskilhlq rvidsqaekl keldkeirpf rqnweeadsm kssveslqnr
 361 vtelesvdks agqvarntgl lesqlsrhdq mlsvhdirla dmdlrfqvle tasyngvliw
 421 kirdykrrkq eavmgktlsl ysqpfytgyf gykmcarvyl ngdgmgkgth lslffvimrg
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 541 tvlengtyik ddtifikviv dtsdlpdp
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SEQ ID NO: 22 Human TRAF3 Transcript Variant 2 cDNA Sequence (NM_145726.2, CDS region from position 354-1985)

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 121 gggagcagaa cgctgcggac cgcggcggag gacgcgcccg gcgcccctga gccggccgag
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 421 ctgaccgcag tgctgggacg ccagtttttg tccctgaaca aggaggttac aaggaaaagt
 481 ttgtgaagac cgtggaggac aagtacaagt gtgagaagtg ccacctggtg ctgtgcagcc
 541 cgaagcagac cgagtgtggg caccgcttct gcgagagctg catggcggcc ctgctgagct
 601 cttcaagtcc aaaatgtaca gcgtgtcaag agagcatcgt taaagataag gtgtttaagg
 661 ataattgctg caagagagaa attctggctc ttcagatcta ttgtcggaat gaaagcagag
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7561 gtatttattt gtgttttgag gtcttgcaat gtttttgtgt ttctgatgct aataactaaa
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SEQ ID NO: 23 Human TRAF3 Isoform 2 Amino Acid Sequence (NP_663778.1)

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 421 ytgyfgykmc arvylngdgm gkgthlslff vimrgeydal lpwpfkqkvt lmlmdqgssr
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 541 pdp
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SEQ ID NO: 24 Human TRAF3 Transcript Variant 4 cDNA Sequence (NM_001199427.1, CDS region from position 215-1672)

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SEQ ID NO: 27 Mouse TRAF3 Isoform a Amino Acid Sequence (NP_035762.2)

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 241 qgtnqqikah eassavqhvn llkewsnsle kkvsllqnes veknksiqsl hnqicsfeie
 301 ierqkemlrn neskilhlqr vidsqaeklk eldkeirpfr qnweeadsmk ssveslqnrv
 361 telesvdksa gqaarntgll esqlsrhdqm lsvhdirlad mdlrfqvlet asyngvliwk
 421 irdykrrkqe avmgktlsly sqpfytgyfg ykmcarvyln gdgmgkgthl slffvimrge
 481 ydallpwpfk qkvtlmlmdq gssrrhlgda fkpdpnsssf kkptgemnia sgcpvfvaqt
 541 vlengtyikd dtifikvivd tsdlpdp
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SEQ ID NO: 28 Mouse TRAF3 Transcript Variant 2 cDNA Sequence (NM_001286122.1, CDS region from position 251-1879)

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 301 acccctaaag ctgcagcctg atcgcggcgc agggtccgtg ctcgtgccgg agcaaggagg
 361 ctacaaggag aagtttgtga agacggtgga agacaagtac aagtgcgaga agtgccgcct
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SEQ ID NO: 29 Mouse TRAF3 Isoform b Amino Acid Sequence (NP_001273051.1)

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 421 tgyfgykmca rvylngdgmg kgthlslffv imrgeydall pwpfkqkvtl mlmdqgssrr
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 541 dp
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SEQ ID NO: 30 Human RNF31 Transcript Variant 1 cDNA Sequence (NM_017999.4, CDS region from position 250-3468)

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3181 cccaatgggc tcagggacga agcttgtggc aaggaaactc cagctggcta tgccggcctg
3241 tgccaggcac actacaaaga gtatcttgtg agcctcatca atgcccactc gctggaccca
3301 gccaccttgt atgaggtgga agagctggag acggccactg agcgctacct gcacgtacgc
3361 ccccagcctt tggctggaga ggatcccccct gcttaccagg cccgcttgtt acagaagctg
3421 acagaagagg taccccttggg acagagtatc ccccgcaggc ggaagtagct gagggcaagg
3481 gtcccgatga gggtcccatg gcctgctccc tcaggaacag ctccagcacc aataaagagg
3541 catcttacca cccaggaaaa aaaaaaaaaa a
```

SEQ ID NO: 31 Human RNF31 Isoform 1 Amino Acid Sequence (NP_060469.4)

```
   1 mpgeeeeraf lvareelasa lrrdsgqafs leqlrpllas slplaarylq ldaarlvrcn
  61 ahgeprnyln tlstalnile kygrnllspq rprywrgvkf nnpvfrstvd avqggrdvlr
 121 lygyteeqpd glsfpegqee pdehqvatvt levlllrtel slllqnthpr qqaleqlled
 181 kveddmlqls efdpllreia pgplttpsvp gstpgpcflc gsapgtlhcp sckqalcpac
 241 dhlfhghpsr ahhlrqtlpg vlqgthlsps lpasaqprpq stsllalgds slsspnpasa
 301 hlpwhcaaca mlnepwavlc vacdrprgck glglgtegpq gtgglepdla rgrwacqsct
 361 feneaaavlc sicerprlaq ppslvvdsrd agiclqplqq gdallasaqs qvwycihctf
 421 cnsspgwvcv mcnrtsspip aqhaprpyas slekgppkpg pprrlsaplp sscgdpekqr
 481 qdkmreeglq lvsmiregea agacpeeifs alqysgtevp lqwlrselpy vlemvaelag
 541 qqdpglgafs cqearrawld rhgnldeave ecvrtrrrkv qelqslgfgp eegslqalfq
 601 hggdvsralt elqrqrlepf rqrlwdsgpe ptpswdgpdk qslvrrllav yalpswgrae
 661 lalsllqetp rnyelgdvve avrhsqdraf lrrllaqeca vcgwalphnr mqaltscect
 721 icpdcfrqhf tialkekhit dmvcpacgrp dltddtqlls yfstldiqlr eslepdayal
 781 fhkkltegvl mrdpkflwca qcsfgfiyer eqleatcpqc hqtfcvrckr qweeqhrgrs
 841 cedfqnwkrm ndpeyqaqgl amylqengid cpkckfsyal arggcmhfhc tqcrhqfcsg
 901 cynafyaknk cpepncrvkk slhghhprdc lfylrdwtal rlqkllqdnn vmfnteppag
 961 aravpgggcr vieqkevpng lrdeacgket pagyaglcqa hykeylvsli nahsldpatl
1021 yeveeletat erylhvrpqp lagedppayq arllqkltee vplgqsiprr rk
```

SEQ ID NO: 32 Human RNF31 Transcript Variant 2 cDNA Sequence (NM_001310332.1, CDS region from position 359-3124)

```
   1 atagctaggg ccaactggaa gtccagggtt gggccccgaa actacctcaa caccctgtcc
  61 acggctctga acatcctgga gaaatacggc cgcaaccttc tcagccctca gcggcctcgg
 121 tactggcgtg gtgtcaagtt taataaccct gtctttcgca gcacggtgga tgctgtgcag
 181 gggggccgag atgtgctgcg attatatggc tacacagagg agcaaccaga tgggttgagc
 241 ttccccgaag ggcaggagga gccagatgag caccaggttg ctacagtcac actggaagta
```

```
 301 ctgctgcttc ggacagagct cagcctgcta ttgcaggtga gatgctcctc tagtcttgat
 361 ggacttatgc accagggctg gggagcccag cctaactcaa aatactcatc caagacagca
 421 ggcactggag cagctgttgg aagacaaggt tgaagatgat atgctgcagc tttcagaatt
 481 tgacccccta ttgagagaga ttgctcctgg ccccctcacc acaccctctg tcccaggctc
 541 cactcctggt ccctgcttcc tctgtggttc tgccccaggc acactgcact gcccatcctg
 601 taaacaggcc ctgtgtccag cctgtgacca cctgttccat ggacacccat cccgtgctca
 661 tcacctccgc cagaccctgc ctggggtcct gcagggtacc cacctgagcc ccagtttacc
 721 tgcctcagcc caaccacggc cccagtcgac ctccctgctg gccctgggag acagctctct
 781 ttcttccccc aatcctgcaa gtgctcattt gccctggcac tgtgctgcct gtgccatgct
 841 aaatgagcct tgggcagtgc tctgtgtggc ctgtgatcgg ccccgaggct gtaaggggtt
 901 ggggttggga actgagggtc cccaaggaac tggaggccta gaacctgatc ttgcacgggg
 961 tcggtgggcc tgccagagct gtacctttga gaatgaggca gctgctgtgc tatgttccat
1021 atgtgagcga cctcggctgg cccagcctcc cagcttggtg gtggattccc gagatgctgg
1081 catttgcctg caacccccttc agcaggggga tgctttgctg gcctctgccc agagtcaagt
1141 ctggtactgt attcactgta ccttctgcaa ctcgagccct ggctgggtgt gtgttatgtg
1201 caaccggact agtagcccca ttccagcaca acatgccccc cggccctatg ccagctcttt
1261 ggaaaaggga ccccccaagc ctgggccccc acgacgcctt agtgcccccc tgcccagttc
1321 ctgtggagat cctgagaagc agcgccaaga caagatgcgg gaagaaggcc tccagctagt
1381 gagcatgatc cgggaagggg aagccgcagg tgcctgtcca gaggagatct tctcggctct
1441 gcagtactcg ggcactgagg tgcctctgca gtggttgcgc tcagaactgc cctacgtcct
1501 ggagatggtg gctgagctgg ctggacagca ggaccctggg ctgggtgcct tttcctgtca
1561 ggaggcccgg agagcctggc tggatcgtca tggcaacctt gatgaagctg tggaggagtg
1621 tgtgaggacc aggcgaagga aggtgcagga gctccagtct ctaggctttg ggcctgagga
1681 ggggtctctc caggcattgt tccagcacgg aggtgatgtg tcacgggccc tgactgagct
1741 acagcgccaa cgcctagagc ccttccgcca gcgcctctgg gacagtggcc ctgagcccac
1801 cccttcctgg gatgggccag acaagcagag cctggtcagg cggcttttgg cagtctacgc
1861 actccccagc tggggccggg cagagctggc actgtcactg ctgcaggaga cacccaggaa
1921 ctatgagttg ggggatgtgg tagaagctgt gaggcacagc caggaccggg ccttcctgcg
1981 ccgcttgctt gcccaggagt gtgccgtgtg tggctgggcc ctgccccaca accggatgca
2041 ggccctgact tcctgtgagt gcaccatctg tcctgactgc ttccgccagc acttcaccat
2101 cgccttgaag gagaagcaca tcacagacat ggtgtgccct gcctgtggcc gccccgacct
2161 caccgatgac acacagttgc tcagctactt ctctaccctt gacatccagc ttcgcgagag
2221 cctagagcca gatgcctatg cgttgttcca taagaagctg accgagggtg tgctgatgcg
2281 ggaccccaag ttcttgtggt gtgcccagtg ctcctttggc ttcatatatg agcgtgagca
2341 gctggaggca acttgtcccc agtgtcacca gaccttctgt gtgcgctgca agcgccagtg
2401 ggaggagcag caccgaggtc ggagctgtga ggacttccag aactggaaac gcatgaacga
2461 cccagaatac caggcccagg gcctagcaat gtatcttcag gaaaacggca ttgactgccc
2521 caaatgcaag ttctcgtacg ccctggcccg aggaggctgc atgcactttc actgtaccca
2581 gtgccgccac cagttctgca gcggctgcta caatgccttt tacgccaaga ataaatgtcc
2641 agagcctaac tgcagggtga aaaagtccct gcacggccac caccctcgag actgcctctt
2701 ctacctgcgg gactggactg ctctccggct tcagaagctg ctacaggaca ataacgtcat
2761 gtttaataca gagcctccag ctggggcccg ggcagtccct ggaggcggct gccgagtgat
2821 agagcagaag gaggttccca atgggctcag ggacgaagct tgtggcaagg aaactccagc
2881 tggctatgcc ggcctgtgcc aggcacacta caaagagtat cttgtgagcc tcatcaatgc
2941 ccactcgctg gacccagcca ccttgtatga ggtggaagag ctggagacgg ccactgagcg
3001 ctacctgcac gtacgccccc agcctttggc tggagaggat ccccctgctt accaggcccg
3061 cttgttacag aagctgacag aagaggtacc cttgggacag agtatccccc gcaggcggaa
3121 gtagctgagg gcaagggtcc cgatgagggt cccatggcct gctccctcag gaacagctcc
3181 agcaccaata aagaggcatc ttaccaccca ggaaaaaaaa aaaaaaa
```

SEQ ID NO: 33 Human RNF31 Isoform 2 Amino Acid Sequence (NP_001297261.1)

```
   1 mdlctragep sltqnthprq qaleqlledk veddmlqlse fdpllreiap gplttpsvpg
  61 stpgpcflcg sapgtlhcps ckqalcpacd hlfhghpsra hhlrqtlpgv lqgthlspsl
 121 pasaqprpqs tsllalgdss lsspnpasah lpwhcaacam lnepwavlcv acdrprgckg
 181 lglgtegpqg tgglepdlar grwacqsctf eneaaavlcs icerprlaqp pslvvdsrda
 241 giclqplqqg dallasaqsq vwycihctfc nsspgwvcvm cnrtsspipa qhaprpyass
 301 lekgppkpgp prrlsaplps scgdpekqrq dkmreeglql vsmiregeaa gacpeeifsa
 361 lqysgtevpl qwlrselpyv lemvaelagq qdpglgafsc qearrawldr hgnldeavee
 421 cvrtrrrkvq elqslgfgpe egslqalfqh ggdvsralte lqrqrlepfr qrlwdsgpep
```

```
 481 tpswdgpdkq slvrrllavy alpswgrael alsllqetpr nyelgdvvea vrhsqdrafl
 541 rrllaqecav cgwalphnrm qaltscecti cpdcfrqhft ialkekhitd mvcpacgrpd
 601 ltddtqllsy fstldiqlre slepdayalf hkkltegvlm rdpkflwcaq csfgfiyere
 661 qleatcpqch qtfcvrckrq weeqhrgrsc edfqnwkrmn dpeyqaqgla mylqengidc
 721 pkckfsyala rggcmhfhct qcrhqfcsgc ynafyaknkc pepncrvkks lhghhprdcl
 781 fylrdwtalr lqkllqdnnv mfnteppaga ravpgggcrv ieqkevpngl rdeacgketp
 841 agyaglcqah ykeylvslin ahsldpatly eveeletate rylhvrpqpl agedppayqa
 901 rllqklteev plgqsiprrr k
```

SEQ ID NO: 34 Mouse RNF31 cDNA Sequence (NM_194346.2, CDS region from position 95-3295)

```
   1 gcggtggctt aagtgacccg ggctgctgtg gcgccgcgcg cacctgcgcc agcccctgga
  61 gggcagagaa gctgagcctg tctccagtct caggatgccg ggagacgagg agcgaggctt
 121 cctggcggcc cgcgaggagc tggcgagcgc cctgaggtgg gattctgcgc aggtttttcc
 181 cctggagcag ctcatgccgc ttctggccac ctctctgcca ccagccgccc gctacctgca
 241 gctggacgcc ggacgcttgg tccgctgcaa cgctcatggg gagcctcgaa actacctcaa
 301 caccctatcc acggccctga acatcctgga aaaatatggt cgcaacctcc tcagcccgca
 361 gcggccccgg tattggcgct cagtgaagtt taataacccc gtctttcgca gcacggtgga
 421 tgctgtgcag ggtggccggg atgtactacg gttgtatggc tatactgagg agcgcccaga
 481 tggattgagt ttccccgaag ggcaggagga accagatgaa taccaggttg ctgttgtcac
 541 actagaagta ctgctgcttc gcaccgagct cagtttgctg ttgcagaata ctcatcccag
 601 acagaatgca ctggaccagc tgctaagaga gagcgttgaa gatggtatgc tgcagctttc
 661 agagtttcac ccccttctga gggagattgt tcctggcccc cgcccctctg cccaaggctc
 721 cactcctggt ccctgtttcc tctgtggttc tgccccaggc acactgcact gtccagcctg
 781 taaccaagtc tcgtgcccag cttgtgacat tttgttccat gggcatccgt cccgtgcaca
 841 tcaccttcgc caagccctgc ctgggtccca ccagactgcc agcctgagct ctagtttacc
 901 tgcctcgtcc caaccacggc ccccctcctc ctccttggcc ctgggagata gctctctttc
 961 ttcccctgac cctgcaaatg cctgtctgcc ctggcattgt cttacctgtg ccacactaaa
1021 tgagccttgg gcagtgttct gtgcagtctg tagtcagccc aaaggctgca aggtgccggg
1081 aatagagggt tcccatggaa ccgggggcct agaacctgag cctgcacggg atcaatgggc
1141 ctgccagagc tgtacctttg agaatgaggc agcagctgtg ctatgcgcca tatgtgagcg
1201 acctcggctg gcccagcctc ccagcttggt ggtggattcc catgatgctg gtgtttgcca
1261 acagtccctt aagcaggagg atcctttgct caccgctgcc cagcctcagg tgtggtactg
1321 tgaccattgt accttctgca attcaggccc tgtctgggtg tgtgccatgt gcaaccgaac
1381 ccgagacccc atccctacac agcctgccct ccagtcctat cccagctctt tggaaaaggg
1441 acgcccaaag ccagggtcct cacaacacct tggttcctcc ctgcctgctt cctgtggaga
1501 cccagagaaa caacgccaag ataagatgcg gaaggaaggt ctccagctcg tgagcatgat
1561 ccaggaagga gaaactgcgg gtgccagtcc agaagaggtc ttctcagctc tccaatactc
1621 aggcacagag gtgcccctcc agtggttgcg ttcagagctg tcctacgtcc tggagatggt
1681 ggctgagctt gctggacaac aggatccaga gctgggggcc ttttcctgtc aggaagcccg
1741 gaaagcctgg cttgatcgcc atggcaacct ggatgaagct gtagaggagt gtgtgagggc
1801 caggaggagg aaggtgcacg agctgcagtc cctgggcttt gggcctaagg aagggtcact
1861 acaggcattg ttccagcatg ggggtgacgt ggctcgggcc ctgactgagt tacagcgcca
1921 gcgcctggag cccttccatc agcgcctatg ggacagagac cctgaaccca ctccctgctg
1981 ggatgggctg gacagacaga gcttggtcag acgccttctg gccgtctaca cactccccag
2041 ctggggccga gcagagctgg cgctggcgct gctgcaggag acacccagga actatgagtt
2101 gttggacgtg gtggaggctg tgaggcacag ccaggaccgg gcctttctgc gtcgactgct
2161 tgcccaggaa tgtgctgtgt gcgggtgggc ccttccccgc aaccggatgc aggccctgat
2221 ctcctgtgag tgcaccatat gtcccgaatg cttccgccaa cacttcacca ttgccctgaa
2281 ggagaagcac atcacagaca tggtgtgccc tgcctgtggc cgccctgacc tcactgatga
2341 cgctcagtta ctcagctact tctccaccct tgacatccag ctcagagaga gcctagaccc
2401 cgatgcatat gccctgtttc acaagaagct gaccgaggct gtgcttatgc gagaccccaa
2461 gttcttgtgg tgcgcccagt gttcctttgg cttcatctat gaacgcgaac agctggaggc
2521 gacgtgtccc cagtgtcacc agaccttctg tgtgcgctgc aagcgccagt gggaggagca
2581 gcacagagga cggagttgtg aggatttcca gaactggaaa cgcaccaatg acccagagta
2641 ccaggctcaa ggcttggcca tgtaccttca ggaaaacggc attgactgtc cgaaatgcaa
2701 gttctcgtac gcactggccc ggggaggctg catgcacttc cactgcacgc agtgtcgaca
2761 ccagttctgc agtggctgct acaacgcctt ttacgccaag aataaatgtc cagaccctaa
2821 ctgcaaggtg aaaaagtccc tgcatggcca ccaccctcga gactgcctct tctacctacg
2881 ggactggact gctgcccgcc tccagaaact gttgcaggac aataatgtca tgtttaatac
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2941 agagcctcca gctgggacac gggcagtccc tggaggggc tgcagagtga tggagcagaa
3001 ggaggtccat agtgggttca gggatgaagc ttgcggcaag gaaactccac ctggctatgc
3061 cggcctatgt caggcacact acaaagagta tctcgtgagc ctcatcaatg cccattcact
3121 ggacccagct accttgtatg aagtggagga gctggagaca gccactattc gctacctaca
3181 tttagctcct cagcccgcgg atggagagga tctgcctgct taccaggccc ggctattaca
3241 gaagctgaga gaagaggtac ccttgggaca gagtattgcc cgcagaagaa agtagtagca
3301 gagagccggg tcctgatggg acttcctgac ccaggcctca gcagcagttc cagcaccaat
3361 aaagaggcat cttatggcct aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa
3421 aaaaaaaaaa aaaaaaaaaa aaa
```

SEQ ID NO: 35 Mouse RNF31 Amino Acid Sequence (NP_919327.2)

```
   1 mpgdeergfl aareelasal rwdsaqvfpl eqlmpllats lppaarylql dagrlvrcna
  61 hgeprnylnt lstalnilek ygrnllspqr prywrsvkfn npvfrstvda vqggrdvlrl
 121 ygyteerpdg lsfpegqeep deyqvavvtl evlllrtels lllqnthprq naldqllres
 181 vedgmlqlse fhpllreivp gprpsaqgst pgpcflcgsa pgtlhcpacn qvscpacdil
 241 fhghpsrahh lrqalpgshq taslssslpa ssqprppsss lalgdsslss pdpanaclpw
 301 hcltcatlne pwavfcavcs qpkgckvpgi egshgtggle pepardqwac qsctfeneaa
 361 avlcaicerp rlaqppslvv dshdagvcqq slkqedpllt aaqpqvwycd hctfcnsgpv
 421 wvcamcnrtr dpiptqpalq sypsslekgr pkpgssqhlg sslpascgdp ekqrqdkmrk
 481 eglqlvsmiq egetagaspe evfsalqysg tevplqwlrs elsyvlemva elagqqdpel
 541 gafscqeark awldrhgnld eaveecvrar rrkvhelqsl gfgpkegslq alfqhggdva
 601 raltelqrqr lepfhqrlwd rdpeptpcwd gldrqslvrr llavytlpsw graelalall
 661 qetprnyell dvveavrhsq draflrrlla qecavcgwal prnrmqalis cecticpecf
 721 rqhftialke khitdmvcpa cgrpdltdda qllsyfstld iqlresldpd ayalfhkklt
 781 eavlmrdpkf lwcaqcsfgf iyereqleat cpqchqtfcv rckrqweeqh rgrscedfqn
 841 wkrtndpeyq aqglamylqe ngidcpkckf syalarggcm hfhctqcrhq fcsgcynafy
 901 aknkcpdpnc kvkkslhghh prdclfylrd wtaarlqkll qdnnvmfnte ppagtravpg
 961 ggcrvmeqke vhsgfrdeac gketppgyag lcqahykeyl vslinahsld patlyeveel
1021 etatirylhl apqpadgedl payqarllqk lreevplgqs iarrrk
```

SEQ ID NO: 36 Human RBCK1 Transcript Variant 1 cDNA Sequence (NM_006462.5, CDS region from position 690-2096)

```
   1 ccccgcctgc ggcccagctc cttcccgcgg ctctgcgatg cggcccgcag ggtgacccgg
  61 gcgggagtcc ggggacccgc gatcagcccc ggaggacggg gtggggtcgc cccaaacagg
 121 agcgccggga ccgctgggac cccgcactcg gcgtccgccg ccgccgggta gccgggcagt
 181 ggaggtcccg gatgaggcga caatttttcc ggccccccct cccagtcccg ccccacttcc
 241 ggggccgcca ctttcacttt ctcttccgcc gaagccgctc cccttgcgaa gaactggggc
 301 ctcccgggag gagagagggc tttgccttga aacccgggac gccaggggcg ctcccgcaag
 361 tgggggtcct ccgggacttg gaacgccccg gctgggtggt gtccgggcgt cctttccccg
 421 cttcttccca cctcggctgg tcccgtttcc tcctgcgccc agtgcggacc tgtctcggcg
 481 cccgctgccc tctcaccgcc ccacgcagga tcccggcctg gtcaccgggc agtgtgatgc
 541 ttcccgactg ccgcggggac agcgaggcac acacagggct tgggccgcgc cggaggccac
 601 acggcctggc tgagttgctc ctggtctccc gcctctccca ggcgacccgg aggtagcatt
 661 tcccaggagg cacggtcccc cccaggggga tgggcacagc cacgccagat ggacgagaag
 721 accaagaaag gctgtgggtg agcgtggagg atgctcagat gcacaccgtc accatctggc
 781 tcacagtgcg ccctgatatg acagtggcgt ctctcaagga catggttttt ctggactatg
 841 gcttcccacc agtcttgcag cagtgggtga ttgggcagcg gctggcacga gaccaggaga
 901 ccctgcactc ccatggggtg cggcagaatg gggacagtgc ctacctctat ctgctgtcag
 961 cccgcaacac ctccctcaac cctcaggagc tgcagcggga gcggcagctg cggatgctgg
1021 aagatctggg cttcaaggac ctcacgctgc agccgcgggg ccctctggag ccaggccccc
1081 caaagcccgg ggtcccccag gaacccggac gggggcagcc agatgcagtg cctgagcccc
1141 caccggtggg ctggcagtgc cccgggtgca ccttcatcaa caagcccacg cggcctggct
1201 gtgagatgtg ctgccgggcg cgccccgagg cctaccaggt ccccgcctca taccagcccg
1261 acgaggagga gcgagcgcgc ctggcgggcg aggaggaggc gctgcgtcag taccagcagc
1321 ggaagcagca gcagcaggag gggaactacc tgcagcacgt ccagctggac cagaggagcc
1381 tggtgctgaa cacggagccc gccgagtgcc ccgtgtgcta ctcggtgctg gcgcccggcg
1441 aggccgtggt gctgcgtgag tgtctgcaca ccttctgcag ggagtgcctg cagggcacca
1501 tccgcaacag ccaggaggcg gaggtctcct gccccttcat tgacaacacc tactcgtgct
1561 cgggcaagct gctggagagg gagatcaagg cgctcctgac ccctgaggat taccagcgat
1621 ttctagacct gggcatctcc attgctgaaa accgcagtgc cttcagctac cattgcaaga
```

```
1681 ccccagattg caagggatgg tgcttctttg aggatgatgt caatgagttc acctgccctg
1741 tgtgtttcca cgtcaactgc ctgctctgca aggccatcca tgagcagatg aactgcaagg
1801 agtatcagga ggacctggcc ctgcgggctc agaacgatgt ggctgcccgg cagacgacag
1861 agatgctgaa ggtgatgctg cagcagggcg aggccatgcg ctgcccccag tgccagatcg
1921 tggtacagaa gaaggacggc tgcgactgga tccgctgcac cgtctgccac accgagatct
1981 gctgggtcac caagggccca cgctggggcc ctgggggccc aggagacacc agcgggggct
2041 gccgctgcag ggtaaatggg attccttgcc acccaagctg tcagaactgc cactgagcta
2101 aagatggtgg ggccacatgc tgacccagcc ccacatccac attctgttag aatgtagctc
2161 agggagcttc gtggacggcc ttgcttgctg tagcgttgta ggggccctgc ctgcactgcg
2221 gttgtccacg gtcacatctg ccccagtgcc tttgtccttc ccttggggct tgccggccag
2281 acttctctcc cctgcggctc ccacctctgc ctgaccccag ccttaaacat agcccctggc
2341 cagaggcctt gctgggtgga gcctctgtgt gactccatac tcctcccacc acaacactca
2401 tctgtcaaac accaagcact ctcagcctcc ccgccttcag ctgtcagctt tctggggcta
2461 acttctctgc ctttgtggtt ggaggcctga ggcctcttgg aactcttgct aacctgttca
2521 gagccaggaa ggagactgca cagttttgaa agcacagccc gtcaggtccg gctctgcgtc
2581 tccctctctg cagcctgtgt aagctattat aattaaaatg gttttccggg aagggatgag
2641 tgtgatgtcc ttgagaggaa atgaatgtcc tggcctggga ctctacacac aggcaggatc
2701 ctgaggtctc tgggaactgc atcagaaagt tgacttgtca gtccatctgt ggtagaatga
2761 ggctgtgact gagcactggg acctttctac cagatgtgga ccccatgccc agcctcaggg
2821 gcaaggatgc tcttgggtca ccgtcagcca ggacaggtgg agtgtgcagt gtgtcaagtc
2881 tgcagagaag gatgggctta ggggcgggag gggaagtctt gccactcctg ctccctttg
2941 acctctcagc aggcatctag ggttggcagg tagatagttc aagaaggaac gaagctgctg
3001 cagttgaggg gtggggttgt ccatcctatt ttctcgtctc aagcaagatg gcacagtatc
3061 gattcagcag tatttactag aacccactct gtgctggtcg gaggttacta agacagggtc
3121 ctgggatgtt cattctctaa gtctttcctc cgctctgtga cccaccctcc ttccccttt
3181 gagatctggt atttgatgcc caacacattg tccacgctgt gacgtgacca tcatcatagc
3241 aggcagaggg cgcctctgct gctgaaggcc tgtgattttg tggggaaggg cctgttctag
3301 caactggaaa ggcactgcca cctgccgttg gatgccagga ctcaagagct ggccccagtc
3361 actgtgcgca gagctgtctg agaatgtgtg agtggactgg gtccttcggc actgcctgca
3421 ttggctcagg gcagtcaacc gtcgcagagg atgaggggca cactcaggca gcctccccgg
3481 ccctggaggc agaaaggccc aggcagaacc actgactggg aggaaacaga aaaagcagag
3541 gagagccagg ctgcaggcgt gtggatggga ccagctcagg cagacgctgt ctcataccca
3601 ctctcccctc tcttgccagg gcctggcctg gtgtctctca ggagcctggg catgagacaa
3661 aagcagagat tgttctcttg tggtaccaca ggctgtaacc agtccaccca gtgttgtttt
3721 agaaatttaa atcggttgcc catcttttta aattggcaac atcgtttacc acattaaaat
3781 ctagatgccc tgcttctctt gaaaaaaaaa aaaaaaaa
```

SEQ ID NO: 37 Human RBCK1 Isoform 1 Amino Acid Sequence (NP_006453.1)

```
   1 mgtatpdgre dqerlwvsve daqmhtvtiw ltvrpdmtva slkdmvfldy gfppvlqqwv
  61 igqrlardqe tlhshgvrqn gdsaylylls arntslnpqe lqrerqlrml edlgfkdltl
 121 qprgplepgp pkpgvpqepg rgqpdavpep ppvgwqcpgc tfinkptrpg cemccrarpe
 181 ayqvpasyqp deeerarlag eeealrqyqq rkqqqqegny lqhvqldqrs lvlntepaec
 241 pvcysvlapg eavvlreclh tfcreclqgt irnsqeaevs cpfidntysc sgkllereik
 301 alltpedyqr fldlgisiae nrsafsyhck tpdckgwcff eddvneftcp vcfhvncllc
 361 kaiheqmnck eyqedlalra qndvaarqtt emlkvmlqqg eamrcpqcqi vvqkkdgcdw
 421 irctvchtei cwvtkgprwg pggpgdtsgg crcrvngipc hpscqnch
```

SEQ ID NO: 38 Human RBCK1 Transcript Variant 2 cDNA Sequence (NM_031229.3, CDS region from position 709-2241)

```
   1 ccccgcctgc ggcccagctc cttcccgcgg ctctgcgatg cggcccgcag ggtgacccgg
  61 gcgggagtcc ggggacccgc gatcagcccc ggaggacggg gtggggtcgc cccaaacagg
 121 agcgccggga ccgctgggac cccgcactcg gcgtccgccg ccgccgggta gccgggcagt
 181 ggaggtcccg gatgaggcga caatttttcc ggcccccccc cccagtcccg ccccacttcc
 241 ggggccgcca ctttcacttt ctcttccgcc gaagccgctc cccttgcgaa gaactggggc
 301 ctcccgggag gagagagggc tttgccttga aacccgggac gccaggggcg ctcccgcaag
 361 tgggggtcct ccgggacttg gaacgccccg gctgggtggt gtccgggcgt cctttccccg
 421 cttcttccca cctcggctgg tcccgtttcc tcctgcgccc agtgcggacc tgtctcggcg
 481 cccgctgccc tctcaccgcc ccacgcagga tcccggcctg gtcaccgggc agtgtgatgc
 541 ttcccgactg ccgcggggac agcgaggcac acacagggct tgggccgcgc cggaggccac
 601 acggcctggc tgagttgctc ctggtctccc gcctctccca ggcgacccgg aggtagcatt
```

```
 661 tcccaggagg cacggtcccc cccaggggga tgggcacagc cacgccagat ggacgagaag
 721 accaagaaag cagaggaaat ggccctgagc ctcacccgag cagtggcggg cggggatgaa
 781 caggtggcaa tgaagtgtgc catctggctg gcagagcaac gggtgccccc gagtgtgcaa
 841 ctgaagcctg aggtctcccc aacgcaggac atcaggctgt gggtgagcgt ggaggatgct
 901 cagatgcaca ccgtcaccat ctggctcaca gtgcgccctg atatgacagt ggcgtctctc
 961 aaggacatgg ttttttctgga ctatggcttc ccaccagtct tgcagcagtg ggtgattggg
1021 cagcggctgg cacgagacca ggagaccctg cactcccatg gggtgcggca gaatggggac
1081 agtgcctacc tctatctgct gtcagcccgc aacacctccc tcaaccctca ggagctgcag
1141 cgggagcggc agctgcggat gctggaagat ctgggcttca aggacctcac gctgcagccg
1201 cggggccctc tggagccagg ccccccaaag cccggggtcc cccaggaacc cggacggggg
1261 cagccagatg cagtgcctga gcccccaccg gtgggctggc agtgccccgg gtgcaccttc
1321 atcaacaagc ccacgcggcc tggctgtgag atgtgctgcc gggcgcgccc cgaggcctac
1381 caggtccccg cctcatacca gcccgacgag gaggagcgag cgcgcctggc gggcgaggag
1441 gaggcgctgc gtcagtacca gcagcggaag cagcagcagc aggaggggaa ctacctgcag
1501 cacgtccagc tggaccagag gagcctggtg ctgaacacgg agcccgccga gtgccccgtg
1561 tgctactcgg tgctggcgcc cggcgaggcc gtggtgctgc gtgagtgtct gcacaccttc
1621 tgcagggagt gcctgcaggg caccatccgc aacagccagg aggcggaggt ctcctgcccc
1681 ttcattgaca acacctactc gtgctcgggc aagctgctgg agagggagat caaggcgctc
1741 ctgacccctg aggattacca gcgatttcta gacctgggca tctccattgc tgaaaaccgc
1801 agtgccttca gctaccattg caagacccca gattgcaagg gatggtgctt ctttgaggat
1861 gatgtcaatg agttcacctg ccctgtgtgt ttccacgtca actgcctgct ctgcaaggcc
1921 atccatgagc agatgaactg caaggagtat caggaggacc tggccctgcg ggctcagaac
1981 gatgtggctg cccggcagac gacagagatg ctgaaggtga tgctgcagca gggcgaggcc
2041 atgcgctgcc cccagtgcca gatcgtggta cagaagaagg acggctgcga ctggatccgc
2101 tgcaccgtct gccacaccga gatctgctgg gtcaccaagg gcccacgctg gggccctggg
2161 ggcccaggag acaccagcgg gggctgccgc tgcagggtaa atgggattcc ttgccaccca
2221 agctgtcaga actgccactg agctaaagat ggtggggcca catgctgacc cagccccaca
2281 tccacattct gttagaatgt agctcaggga gcttcgtgga cggccttgct tgctgtagcg
2341 ttgtaggggc cctgcctgca ctgcggttgt ccacggtcac atctgcccca gtgcctttgt
2401 ccttcccttg gggcttgccg gccagacttc tctcccctgc ggctcccacc tctgcctgac
2461 cccagcctta aacatagccc ctggccagag gccttgctgg gtggagcctc tgtgtgactc
2521 catactcctc ccaccacaac actcatctgt caaacaccaa gcactctcag cctccccgcc
2581 ttcagctgtc agctttctgg ggctaacttc tctgcctttg tggttggagg cctgaggcct
2641 cttggaactc ttgctaacct gttcagagcc aggaaggaga ctgcacagtt ttgaaagcac
2701 agcccgtcag gtccggctct gcgtctccct ctctgcagcc tgtgtaagct attataatta
2761 aaatggtttt ccgggaaggg atgagtgtga tgtccttgag aggaaatgaa tgtcctggcc
2821 tgggactcta cacacaggca ggatcctgag gtctctggga actgcatcag aaagttgact
2881 tgtcagtcca tctgtggtag aatgaggctg tgactgagca ctgggacctt tctaccagat
2941 gtggacccca tgcccagcct caggggcaag gatgctcttg ggtcaccgtc agccaggaca
3001 ggtggagtgt gcagtgtgtc aagtctgcag agaaggatgg gcttaggggc gggaggggaa
3061 gtcttgccac tcctgctccc ttttgacctc tcagcaggca tctagggttg gcaggtagat
3121 agttcaagaa ggaacgaagc tgctgcagtt gaggggtggg gttgtccatc ctatttttctc
3181 gtctcaagca agatggcaca gtatcgattc agcagtattt actagaaccc actctgtgct
3241 ggtcggaggt tactaagaca gggtcctggg atgttcattc tctaagtctt tcctccgctc
3301 tgtgacccac cctccttccc cttttgagat ctggtatttg atgcccaaca cattgtccac
3361 gctgtgacgt gaccatcatc atagcaggca gagggcgcct ctgctgctga aggcctgtga
3421 ttttgtgggg aagggcctgt tctagcaact ggaaaggcac tgccacctgc cgttggatgc
3481 caggactcaa gagctggccc cagtcactgt gcgcagagct gtctgagaat gtgtgagtgg
3541 actgggtcct tcggcactgc ctgcattggc tcagggcagt caaccgtcgc agaggatgag
3601 gggcacactc aggcagcctc cccggccctg gaggcagaaa ggcccaggca gaaccactga
3661 ctgggaggaa acagaaaaag cagaggagag ccaggctgca ggcgtgtgga tgggaccagc
3721 tcaggcagac gctgtctcat acccactctc ccctctcttg ccagggcctg gcctggtgtc
3781 tctcaggagc ctgggcatga gacaaaagca gagattgttc tcttgtggta ccacaggctg
3841 taaccagtcc acccagtgtt gttttagaaa tttaaatcgg ttgcccatct ttttaaattg
3901 gcaacatcgt ttaccacatt aaaatctaga tgccctgctt ctcttgaaaa aaaaaaaaaa
3961 aaa
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SEQ ID NO: 39 Human RBCK1 Isoform 2 Amino Acid Sequence (NP_006453.1)

```
   1 mdektkkaee malsltrava ggdeqvamkc aiwlaeqrvp lsvqlkpevs ptqdirlwvs
  61 vedaqmhtvt iwltvrpdmt vaslkdmvfl dygfppvlqq wvigqrlard qetlhshgvr
 121 qngdsaylyl lsarntslnp qelqrerqlr mledlgfkdl tlqprgplep gppkpgvpqe
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 181 pgrgqpdavp epppvgwqcp gctfinkptr pgcemccrar peayqvpasy qpdeeerarl
 241 ageeealrqy qqrkqqqqeg nylqhvqldq rslvlntepa ecpvcysvla pgeavvlrec
 301 lhtfcreclq gtirnsqeae vscpfidnty scsgkllere ikalltpedy qrfldlgisi
 361 aenrsafsyh cktpdckgwc ffeddvneft cpvcfhvncl lckaiheqmn ckeyqedlal
 421 raqndvaarq ttemlkvmlq qgeamrcpqc qivvqkkdgc dwirctvcht eicwvtkgpr
 481 wgpggpgdts ggcrcrvngi pchpscqnch
```

SEQ ID NO: 40 Human RBCK1 Transcript Variant 3 cDNA Sequence (NM_001323956.1, CDS region from position 1058-2080)

```
   1 ccccgcctgc ggcccagctc cttcccgcgg ctctgcgatg cggcccgcag ggtgacccgg
  61 gcgggagtcc ggggacccgc gatcagcccc ggaggacggg gtggggtcgc cccaaacagg
 121 agcgccggga ccgctgggac cccgcactcg gcgtccgccg ccgccgggta gccgggcagt
 181 ggaggtcccg gatgaggcga caatttttcc ggccccccct cccagtcccg ccccacttcc
 241 ggggccgcca ctttcacttt ctcttccgcc gaagccgctc cccttgcgaa gaactggggc
 301 ctcccgggag gagagagggc tttgccttga aacccgggac gccaggggcg ctcccgcaag
 361 tgggggtcct ccgggacttg gaacgccccg gctgggtggt gtccgggcgt cctttccccg
 421 cttcttccca cctcggctgg tcccgtttcc tcctgcgccc agtgcggacc tgtctcggcg
 481 cccgctgccc tctcaccgcc ccacgcagga tcccggcctg gtcaccgggc agtgtgatgc
 541 ttcccgactg ccgcggggac agcgaggcac acacagggct tgggccgcgc cggaggccac
 601 acggcctggc tgagttgctc ctggtctccc gcctctccca ggcgacccgg aggtagcatt
 661 tcccaggagg cacggtcccc cccaggggga tgggcacagc cacgccagat ggacgagaag
 721 accaagaaag cagaggaaat ggccctgagc ctcacccgag cagtggcggg cggggatgaa
 781 caggtggcaa tgaagtgtgc catctggctg gcagagcaac gggtgccccc gagtgtgcaa
 841 ctgaagcctg aggtctcccc aacgcaggac atcaggctgt gggtgagcgt ggaggatgct
 901 cagatgcaca ccgtcaccat ctggctcaca gtgcgccctg atatgacagt ggcgtctctc
 961 aaggacatgg tttttctgga ctatggcttc ccaccagtct tgcagcagtg ggtgattggg
1021 cagcggctgg cacgagacca ggagaccctg cactcccatg gggtgcggca gaatggggac
1081 agtgcctacc tctatctgct gtcagcccgc aacacctccc tcaaccctca ggagctgcag
1141 cgggagcggc agctgcggat gctggaagat ctgggcttca aggacctcac gctgcagccg
1201 cggggccctc tggagccagg ccccccaaag cccggggtcc cccaggaacc cggacggggg
1261 cagccagatg cagtgcctga gcccccaccg gtgggctggc agtgccccgg gtgcaccttc
1321 atcaacaagc ccacgcggcc tggctgtgag atgtgctgcc gggcgcgccc cgaggcctac
1381 caggtccccg cctcatacca gcccgacgag gaggagcgag cgcgcctggc gggcgaggag
1441 gaggcgctgc gtcagtacca gcagggagtg cctgcagggc accatccgca acagccagga
1501 ggcggaggtc tcctgccccc tcattgacaa cacctactcg tgctcgggca agctgctgga
1561 gagggagatc aaggcgctcc tgacccctga ggattaccag cgatttctag acctgggcat
1621 ctccattgct gaaaaccgca gtgccttcag ctaccattgc aagaccccag attgcaaggg
1681 atggtgcttc tttgaggatg atgtcaatga gttcacctgc cctgtgtgtt tccacgtcaa
1741 ctgcctgctc tgcaaggcca tccatgagca gatgaactgc aaggagtatc aggaggacct
1801 ggccctgcgg gctcagaacg atgtggctgc ccggcagacg acagagatgc tgaaggtgat
1861 gctgcagcag ggcgaggcca tgcgctgccc ccagtgccag atcgtggtac agaagaagga
1921 cggctgcgac tggatccgct gcaccgtctg ccacaccgag atctgctggg tcaccaaggg
1981 cccacgctgg ggccctgggg gcccaggaga caccagcggg ggctgccgct gcagggtaaa
2041 tgggattcct tgccacccaa gctgtcagaa ctgccactga gctaaagatg gtggggccac
2101 atgctgaccc agccccacat ccacattctg ttagaatgta gctcagggag cttcgtggac
2161 ggccttgctt gctgtagcgt tgtaggggcc ctgcctgcac tgcggttgtc cacggtcaca
2221 tctgccccag tgcctttgtc cttcccttgg ggcttgccgg ccagacttct ctcccctgcg
2281 gctcccacct ctgcctgacc ccagccttaa acatagcccc tggccagagg ccttgctggg
2341 tggagcctct gtgtgactcc atactcctcc caccacaaca ctcatctgtc aaacaccaag
2401 cactctcagc ctccccgcct tcagctgtca gctttctggg gctaacttct ctgcctttgt
2461 ggttggaggc ctgaggcctc ttggaactct tgctaacctg ttcagagcca ggaaggagac
2521 tgcacagttt tgaaagcaca gcccgtcagg tccggctctg cgtctccctc tctgcagcct
2581 gtgtaagcta ttataattaa aatggttttc cgggaaggga tgagtgtgat gtccttgaga
2641 ggaaatgaat gtcctggcct gggactctac acacaggcag gatcctgagg tctctgggaa
2701 ctgcatcaga aagttgactt gtcagtccat ctgtggtaga atgaggctgt gactgagcac
2761 tgggaccttt ctaccagatg tggaccccat gcccagcctc aggggcaagg atgctcttgg
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2881 cttaggggcg ggaggggaag tcttgccact cctgctccct tttgacctct cagcaggcat
2941 ctagggttgg caggtagata gttcaagaag gaacgaagct gctgcagttg aggggtgggg
3001 ttgtccatcc tattttctcg tctcaagcaa gatggcacag tatcgattca gcagtattta
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3061 ctagaaccca ctctgtgctg gtcggaggtt actaagacag ggtcctggga tgttcattct
3121 ctaagtcttt cctccgctct gtgacccacc ctccttcccc ttttgagatc tggtatttga
3181 tgcccaacac attgtccacg ctgtgacgtg accatcatca tagcaggcag agggcgcctc
3241 tgctgctgaa ggcctgtgat tttgtgggga agggcctgtt ctagcaactg gaaaggcact
3301 gccacctgcc gttggatgcc aggactcaag agctggcccc agtcactgtg cgcagagctg
3361 tctgagaatg tgtgagtgga ctgggtcctt cggcactgcc tgcattggct cagggcagtc
3421 aaccgtcgca gaggatgagg ggcacactca ggcagcctcc ccggccctgg aggcagaaag
3481 gcccaggcag aaccactgac tgggaggaaa cagaaaaagc agaggagagc caggctgcag
3541 gcgtgtggat gggaccagct caggcagacg ctgtctcata cccactctcc cctctcttgc
3601 cagggcctgg cctggtgtct ctcaggagcc tgggcatgag acaaaagcag agattgttct
3661 cttgtggtac cacaggctgt aaccagtcca cccagtgttg ttttagaaat ttaaatcggt
3721 tgcccatctt tttaaattgg caacatcgtt taccacatta aaatctagat gccctgcttc
3781 tcttgaaaaa aaaaaaaaaa aa
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SEQ ID NO: 41 Human RBCK1 Transcript Variant 4 cDNA Sequence (NM_001323958.1, CDS region from position 913-1935)

```
   1 ccccgcctgc ggcccagctc cttcccgcgg ctctgcgatg cggcccgcag ggtgacccgg
  61 gcgggagtcc ggggacccgc gatcagcccc ggaggacggg gtggggtcgc cccaaacagg
 121 agcgccggga ccgctgggac cccgcactcg gcgtccgccg ccgccgggta gccgggcagt
 181 ggaggtcccg gatgaggcga caatttttcc ggccccccct cccagtcccg ccccacttcc
 241 ggggccgcca ctttcacttt ctcttccgcc gaagccgctc cccttgcgaa gaactggggc
 301 ctcccgggag gagagagggc tttgccttga aacccgggac gccaggggcg ctcccgcaag
 361 tgggggtcct ccgggacttg gaacgccccg gctgggtggt gtccgggcgt cctttccccg
 421 cttcttccca cctcggctgg tcccgtttcc tcctgcgccc agtgcggacc tgtctcggcg
 481 cccgctgccc tctcaccgcc ccacgcagga tcccggcctg gtcaccgggc agtgtgatgc
 541 ttcccgactg ccgcggggac agcgaggcac acacagggct tgggccgcgc cggaggccac
 601 acggcctggc tgagttgctc ctggtctccc gcctctccca ggcgacccgg aggtagcatt
 661 tcccaggagg cacggtcccc cccaggggga tgggcacagc cacgccagat ggacgagaag
 721 accaagaaag gctgtgggtg agcgtggagg atgctcagat gcacaccgtc accatctggc
 781 tcacagtgcg ccctgatatg acagtggcgt ctctcaagga catggttttt ctggactatg
 841 gcttcccacc agtcttgcag cagtgggtga ttgggcagcg gctggcacga gaccaggaga
 901 ccctgcactc ccatggggtg cggcagaatg gggacagtgc ctacctctat ctgctgtcag
 961 cccgcaacac ctccctcaac cctcaggagc tgcagcggga gcggcagctg cggatgctgg
1021 aagatctggg cttcaaggac ctcacgctgc agccgcgggg ccctctggag ccaggccccc
1081 caaagcccgg ggtcccccag gaacccggac gggggcagcc agatgcagtg cctgagcccc
1141 caccggtggg ctggcagtgc cccgggtgca ccttcatcaa caagcccacg cggcctggct
1201 gtgagatgtg ctgccgggcg cgccccgagg cctaccaggt ccccgcctca taccagcccg
1261 acgaggagga gcgagcgcgc ctggcgggcg aggaggaggc gctgcgtcag taccagcagg
1321 gagtgcctgc agggcaccat ccgcaacagc caggaggcgg aggtctcctg ccccttcatt
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1501 ttcagctacc attgcaagac cccagattgc aagggatggt gcttctttga ggatgatgtc
1561 aatgagttca cctgccctgt gtgtttccac gtcaactgcc tgctctgcaa ggccatccat
1621 gagcagatga actgcaagga gtatcaggag gacctggccc tgcgggctca gaacgatgtg
1681 gctgcccggc agacgacaga gatgctgaag gtgatgctgc agcagggcga ggccatgcgc
1741 tgcccccagt gccagatcgt ggtacagaag aaggacggct gcgactggat ccgctgcacc
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1861 ggagacacca gcgggggctg ccgctgcagg gtaaatggga ttccttgcca cccaagctgt
1921 cagaactgcc actgagctaa agatggtggg gccacatgct gacccagccc cacatccaca
1981 ttctgttaga atgtagctca gggagcttcg tggacggcct tgcttgctgt agcgttgtag
2041 gggccctgcc tgcactgcgg ttgtccacgg tcacatctgc cccagtgcct ttgtccttcc
2101 cttggggctt gccggccaga cttctctccc ctgcggctcc cacctctgcc tgaccccagc
2161 cttaaacata gcccctggcc agaggccttg ctgggtggag cctctgtgtg actccatact
2221 cctcccacca caacactcat ctgtcaaaca ccaagcactc tcagcctccc cgccttcagc
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2341 actcttgcta acctgttcag agccaggaag gagactgcac agttttgaaa gcacagcccg
2401 tcaggtccgg ctctgcgtct ccctctctgc agcctgtgta agctattata attaaaatgg
2461 ttttccggga agggatgagt gtgatgtcct tgagaggaaa tgaatgtcct ggcctgggac
2521 tctacacaca ggcaggatcc tgaggtctct gggaactgca tcagaaagtt gacttgtcag
2581 tccatctgtg gtagaatgag gctgtgactg agcactggga cctttctacc agatgtggac
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2641 cccatgccca gcctcagggg caaggatgct cttgggtcac cgtcagccag gacaggtgga
2701 gtgtgcagtg tgtcaagtct gcagagaagg atgggcttag gggcgggagg ggaagtcttg
2761 ccactcctgc tccctttga cctctcagca ggcatctagg gttggcaggt agatagttca
2821 agaaggaacg aagctgctgc agttgagggg tggggttgtc catcctattt tctcgtctca
2881 agcaagatgg cacagtatcg attcagcagt atttactaga acccactctg tgctggtcgg
2941 aggttactaa gacagggtcc tgggatgttc attctctaag tctttcctcc gctctgtgac
3001 ccaccctcct tccccttttg agatctggta tttgatgccc aacacattgt ccacgctgtg
3061 acgtgaccat catcatagca ggcagagggc gcctctgctg ctgaaggcct gtgattttgt
3121 ggggaagggc ctgttctagc aactggaaag gcactgccac ctgccgttgg atgccaggac
3181 tcaagagctg gccccagtca ctgtgcgcag agctgtctga gaatgtgtga gtggactggg
3241 tccttcggca ctgcctgcat tggctcaggg cagtcaaccg tcgcagagga tgaggggcac
3301 actcaggcag cctccccggc cctggaggca gaaaggccca ggcagaacca ctgactggga
3361 ggaaacagaa aaagcagagg agagccaggc tgcaggcgtg tggatgggac cagctcaggc
3421 agacgctgtc tcatacccac tctcccctct cttgccaggg cctggcctgg tgtctctcag
3481 gagcctgggc atgagacaaa agcagagatt gttctcttgt ggtaccacag gctgtaacca
3541 gtccacccag tgttgtttta gaaatttaaa tcggttgccc atctttttaa attggcaaca
3601 tcgtttacca cattaaaatc tagatgccct gcttctcttg aaaaaaaaaa aaaaaaa
```

SEQ ID NO: 42 Human RBCK1 Isoform 3 Amino Acid Sequence (NP_006453.1)

```
   1 mgcgrmgtvp tsiccqpatp pstlrscsgs gscgcwkiwa srtsrcsrga lwsqapqspg
  61 sprnpdggsq mqclsphrwa gsapgapsst sprglavrca agraprptrs pphtsptrrs
 121 erawrarrrr cvstsreclq gtirnsqeae vscpfidnty scsgkllere ikalltpedy
 181 qrfldlgisi aenrsafsyh cktpdckgwc ffeddvneft cpvcfhvncl lckaiheqmn
 241 ckeyqedlal raqndvaarq ttemlkvmlq qgeamrcpqc qivvqkkdgc dwirctvcht
 301 eicwvtkgpr wgpggpgdts ggcrcrvngi pchpscqnch
```

SEQ ID NO: 43 Human RBCK1 Transcript Variant 5 cDNA Sequence (NM_001323960.1, CDS region from position 709-1026)

```
   1 ccccgcctgc ggcccagctc cttcccgcgg ctctgcgatg cggcccgcag ggtgacccgg
  61 gcgggagtcc ggggacccgc gatcagcccc ggaggacggg gtggggtcgc cccaaacagg
 121 agcgccggga ccgctgggac cccgcactcg gcgtccgccg ccgccgggta gccgggcagt
 181 ggaggtcccg gatgaggcga caatttttcc ggccccccct cccagtcccg ccccacttcc
 241 ggggccgcca ctttcacttt ctcttccgcc gaagccgctc cccttgcgaa gaactggggc
 301 ctcccgggag gagagagggc tttgccttga aacccgggac gccaggggcg ctcccgcaag
 361 tgggggtcct ccgggacttg gaacgccccg gctgggtggt gtccgggcgt cctttccccg
 421 cttcttccca cctcggctgg tcccgtttcc tcctgcgccc agtgcggacc tgtctcggcg
 481 cccgctgccc tctcaccgcc ccacgcagga tcccggcctg gtcaccgggc agtgtgatgc
 541 ttcccgactg ccgcggggac agcgaggcac acacagggct tgggccgcgc cggaggccac
 601 acggcctggc tgagttgctc ctggtctccc gcctctccca ggcgacccgg aggtagcatt
 661 tcccaggagg cacggtcccc cccaggggga tgggcacagc cacgccagat ggacgagaag
 721 accaagaaag cagaggaaat ggccctgagc ctcacccgag cagtggcggg cggggatgaa
 781 caggtggcaa tgaagtgtgc catctggctg gcagagcaac gggtgcccct gagtgtgcaa
 841 ctgaagcctg aggtctcccc aacgcaggac atcagattcc tcatggtgca aaatggccat
 901 tccagctcca tccagccatc acatcacagg aggaagggaa gaaagacacc cctccacact
 961 cttctaaaga gcatagctca aaaattgtac acacttcttc cgttaattcc tgtggaccag
1021 aactgattcc cacagctaca gttcagcttg aggggagact gtatagccaa gatattcagc
1081 tagaattcag gggttcgctt ggtaagggaa gggaagagaa tggatactgt cggtctgtgc
1141 tccaggagac ttaaactcaa tgctgaaaca ctttgcacaa tgcctggcgt gttatgcact
1201 caataataaa cattagtgtc tatcgttaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa
1261 aaaaaaaaaa aaaa
```

SEQ ID NO: 44 Human RBCK1 Isoform 4 Amino Acid Sequence (NP_001310889.1)

```
   1 mdektkkaee malsltrava ggdeqvamkc aiwlaeqrvp lsvqlkpevs ptqdirflmv
  61 qnghsssiqp shhrrkgrkt plhtllksia qklytllpli pvdqn
```

SEQ ID NO: 45 Mouse RBCK1 Transcript Variant 1 cDNA Sequence (NM_001083921.1, CDS region from position 371-1897)

```
   1 cgctgctcgc gctgtcttcc gggtagcgcg agcctccggg ctggggttcc ggacgctagg
```

```
  61 gcgcccgcgc cggctggctg gccggcctcc ctccgtccct cgcttttggg tcgtggttac
 121 tcatcgccct ttgcagactt ggctcggggc ctccttcgct gtccgtgtcc ctcgcggggc
 181 cccattggtt cccagtgccc ggcctcgggc cctgggcagt gtgatgctgc ccgagtgcgg
 241 actggaacac acgcacgagg ccggctaggg cagagttgct tctaccttcc cgctctctcc
 301 caggttacct caaagtagcg ttttccggaa gcagcagccc tttctgaggg gatgggcgca
 361 gccaggccag atggacgaga agaccaagaa agcagaggag atggccctga gccttgcccg
 421 ggcagtggct ggcggagatg aacaggctgc tatcaagtat gccacctggc tggcagagca
 481 gagggtgccc ctcagggtgc aagtaaaacc cgaggtctcc ccaacacagg acatcaggct
 541 ctgtgtgagt gtggaggatg cgtacatgca caccgtcacc atttggctca cagtacgccc
 601 ggatatgaca gtggcctccc ttaaggacat ggtattcctg gactatggct tcccacctag
 661 cctgcagcag tgggtggttg gacagaggct ggcacgagac caggagacct tgcattcaca
 721 cggcattcgg cggaatggag acggtgccta tctctacctg ctgtcagccc gcaacacgtc
 781 actcaaccca caagagctgc agcggcagcg gcaactgcga atgttggaag atttgggctt
 841 caaggacctc acccttcagt cacgggggcc cttggaacct gtccttccga agcccaggac
 901 caaccaggag ccgggacagc cagatgcagc accggagtca ccaccggtgg gctggcagtg
 961 ccctggctgc actttcatca acaaacccac acggcctggg tgtgagatgt gctgtcgtgc
1021 aaggcctgag acctaccaga tacctgcttc ataccagcct gacgaggaag agcgagcacg
1081 cctggccggc gaggaggagg cgctgcgcca gtaccagcag cggaaacagc agcagcagga
1141 ggggaactac ctgcagcacg tgcagctgga gcagaggagc ctggtgctga acaccgaacc
1201 tactgagtgc cctgtgtgct actcagtgct ggcacccggc gaggccgtgg tgctgcgtga
1261 gtgtctgcac accttctgca gggagtgcct gcagggcacc atccgaaaca gccaggaggc
1321 ggaggtggct tgccccttca ttgacagcac ctactcatgc cccggcaagc tgctggagag
1381 agagatccgg gcgctcctgt cccctgagga ctaccagcgt ttcctggatc tgggtgtgtc
1441 catcgcagag aaccgaagca ccttgagcta ccactgcaag acccccgact gcaggggttg
1501 gtgcttcttt gaggatgatg tcaacgagtt cacctgtcct gtgtgcaccc gtgtcaactg
1561 cctgctctgc aaggccatcc atgagcacat gaattgcagg gagtaccaag acgacctggc
1621 cctgcgggct cagaatgatg tggctgcccg gcagacgaca gagatgctaa aggtaatgct
1681 gcagcagggc gaggccatgc actgcccaca gtgccggatt gtggtgcaga agaaggacgg
1741 ctgtgactgg atccgctgta cagtctgcca cactgagatc tgctgggtca ccaagggccc
1801 acgctggggc ccaggggccc caggggacac cagtggtggc tgccgctgcc gggtcaatgg
1861 gattccatgc cacccaagct gtcaaaactg ccactaagtc gacgatggtg gctctcatac
1921 tgacccagcc ccacatgtat agcagtgaac agcagggcta ggcaagggac tggcctggtt
1981 tggccccata cctttaggct tggtaaggga gaaagcccga gctgtactgc agtctgattg
2041 ctgtgttaca aggacccagc tgagctgatg tcattcgcac tgcctctgtt gcttctggct
2101 gaccagacct catggccctg cactagacac ccgaccctta ctagccacca gccttaatta
2161 aacacggcct tggcctaagg cctggctggc tagtgctctg agtgactcta ttttccctga
2221 gcttgctccc tgaggaccca ccacagcctc atcctttgcc ttgctggctt tctgggcacg
2281 tcttaaagct gatgtgaatc catccagggc ctaggtagat tggtaagtca ggaaacagcc
2341 catggggttc agctctgcat cctcctcttt aagtgaatta aagtggtttt cccagaaaaa
2401 aaaaaaaaaa
```

SEQ ID NO: 46 Mouse RBCK1 Transcript Variant 2 cDNA Sequence (NM_019705.3, CDS region from position 212-1738)

```
   1 gcctggagtc ggccgggtgg gggagagggg ggggatcatg ggtccctaga ccaagtgact
  61 ctggtccgcc caaccaagtg tggcgcgaaa gtgccaggag cagtctagag gtctgcccca
 121 gttggtgacc ctgcagagtc tcgggttacc tcaaagtagc gttttccgga agcagcagcc
 181 ctttctgagg ggatgggcgc agccaggcca gatggacgag aagaccaaga aagcagagga
 241 gatggccctg agccttgccc gggcagtggc tggcggagat gaacaggctg ctatcaagta
 301 tgccacctgg ctggcagagc agagggtgcc cctcagggtg caagtaaaac ccgaggtctc
 361 cccaacacag gacatcaggc tctgtgtgag tgtggaggat gcgtacatgc acaccgtcac
 421 catttggctc acagtacgcc cggatatgac agtggcctcc cttaaggaca tggtattcct
 481 ggactatggc ttcccaccta gcctgcagca gtgggtggtt ggacagaggc tggcacgaga
 541 ccaggagacc ttgcattcac acggcattcg gcggaatgga gacggtgcct atctctacct
 601 gctgtcagcc cgcaacacgt cactcaaccc acaagagctg cagcggcagc ggcaactgcg
 661 aatgttggaa gatttgggct tcaaggacct cacccttcag tcacgggggc ccttggaacc
 721 tgtccttccg aagcccagga ccaaccagga gccgggacag ccagatgcag caccggagtc
 781 accaccggtg ggctggcagt gccctggctg cactttcatc aacaaaccca cacggcctgg
 841 gtgtgagatg tgctgtcgtg caaggcctga gacctaccag atacctgctt cataccagcc
 901 tgacgaggaa gagcgagcac gcctggccgg cgaggaggag gcgctgcgcc agtaccagca
 961 gcggaaacag cagcagcagg aggggaacta cctgcagcac gtgcagctgg agcagaggag
```

```
1021 cctggtgctg aacaccgaac ctactgagtg ccctgtgtgc tactcagtgc tggcacccgg
1081 cgaggccgtg gtgctgcgtg agtgtctgca caccttctgc agggagtgcc tgcagggcac
1141 catccgaaac agccaggagg cggaggtggc ttgccccttc attgacagca cctactcatg
1201 ccccggcaag ctgctggaga gagagatccg ggcgctcctg tcccctgagg actaccagcg
1261 tttcctggat ctgggtgtgt ccatcgcaga gaaccgaagc accttgagct accactgcaa
1321 gacccccgac tgcaggggtt ggtgcttctt tgaggatgat gtcaacgagt tcacctgtcc
1381 tgtgtgcacc cgtgtcaact gcctgctctg caaggccatc catgagcaca tgaattgcag
1441 ggagtaccaa gacgacctgg ccctgcgggc tcagaatgat gtggctgccc ggcagacgac
1501 agagatgcta aaggtaatgc tgcagcaggg cgaggccatg cactgcccac agtgccggat
1561 tgtggtgcag aagaaggacg gctgtgactg gatccgctgt acagtctgcc acactgagat
1621 ctgctgggtc accaagggcc cacgctgggg cccagggggc ccaggggaca ccagtggtgg
1681 ctgccgctgc cgggtcaatg ggattccatg ccacccaagc tgtcaaaact gccactaagt
1741 cgacgatggt ggctctcata ctgacccagc cccacatgta tagcagtgaa cagcagggct
1801 aggcaaggga ctggcctggt ttggccccat acctttaggc ttggtaaggg agaaagcccg
1861 agctgtactg cagtctgatt gctgtgttac aaggacccag ctgagctgat gtcattcgca
1921 ctgcctctgt tgcttctggc tgaccagacc tcatggccct gcactagaca cccgaccctt
1981 actagccacc agccttaatt aaacacggcc ttggcctaag gcctggctgg ctagtgctct
2041 gagtgactct attttccctg agcttgctcc ctgaggaccc accacagcct catcctttgc
2101 cttgctggct ttctgggcac gtcttaaagc tgatgtgaat ccatccaggg cctaggtaga
2161 ttggtaagtc aggaaacagc ccatggggtt cagctctgca tcctcctctt taagtgaatt
2221 aaagtggttt tcccagaaaa aaaaaaaaaa a1
```

SEQ ID NO: 47 Mouse RBCK1 Amino Acid Sequence (NP_001077390.1)

```
   1 mdektkkaee malslarava ggdeqaaiky atwlaeqrvp lrvqvkpevs ptqdirlcvs
  61 vedaymhtvt iwltvrpdmt vaslkdmvfl dygfppslqq wvvgqrlard qetlhshgir
 121 rngdgaylyl lsarntslnp qelqrqrqlr mledlgfkdl tlqsrgplep vlpkprtnqe
 181 pgqpdaapes ppvgwqcpgc tfinkptrpg cemccrarpe tyqipasyqp deeerarlag
 241 eeealrqyqq rkqqqqegny lqhvqleqrs lvlnteptec pvcysvlapg eavvlreclh
 301 tfcreclqgt irnsqeaeva cpfidstysc pgkllereir allspedyqr fldlgvsiae
 421 qndvaarqtt emlkvmlqqg eamhcpqcri vvqkkdgcdw irctvchtei cwvtkgprwg
 481 pggpgdtsgg crcrvngipc hpscqnch
```

SEQ ID NO: 48 Human OTULIN cDNA Sequence (NM_138348.5, CDS region from position 182-1240)

```
   1 cacctcctcg cgcagggtca gaggccgtgg ggcgggccac ggtgacgcgc gcggaagcgc
  61 tctgcgggcc ctcggaaacc gccccggcgg ctgagaggct gcggccactg cctggcaccc
 121 cgacgggagg ggctccggat cgttcggagc cggctgaacc ccttcggccg cgagcgaccg
 181 catgagtcgg gggactatgc cccagcccga agcgtggcca ggcgcgagct gcgccgagac
 241 gccggcgcgg gaggcggcgg ccacggcgcg ggacggcggg aaggcggcgg ccagcgggca
 301 gccgcggccc gagatgcagt gcccggccga gcatgaggag gacatgtacc gtgctgcaga
 361 tgaaatagaa aaggagaaag aattgcttat acatgaaaga ggggcatcag aaccgagatt
 421 aagcgtagct cctgaaatgg atatcatgga ctactgcaaa aaagaatgga gaggaaatac
 481 acagaaagca acgtgtatga aaatgggcta tgaagaggtt tctcagaagt tcacctccat
 541 acggcgagtc cgtggtgata attactgtgc actgagggcc acgctgttcc aggccatgag
 601 ccaggctgtg gggctgccgc cctggctgca ggacccggag ctcatgctgt taccagaaaa
 661 actcataagc aaatacaact ggatcaagca atggaaactt ggactgaaat ttgatgggaa
 721 gaatgaggac ctggttgata aaattaaaga gtcccttact ctgctgagga agaagtgggc
 781 aggcttggct gaaatgagaa ctgctgaagc aagacagata gcttgtgatg aactattcac
 841 aaatgaggcg gaggaatata gcctctatga agctgtaaaa tttctaatgc taaacagagc
 901 cattgaacta tataatgata aagagaaagg aaaggaagta ccatttttct ctgtgcttct
 961 gtttgctcgg gacacatcaa atgacccagg acagcttctg aggaaccacc tcaaccaggt
1021 gggacacact ggtggtcttg aacaggttga aatgttcctt cttgcctatg ctgtgcgcca
1081 caccatccag gtgtaccggc tctccaagta caacacggaa gaattcatca cagtctaccc
1141 caccgaccca cccaaggact ggccagtggt aacgctcatt gctgaggacg atcggcacta
1201 taacatcccc gtcagagtgt gtgaggagac cagtctatga gagacgcatg ctcctgacag
1261 cctggcgacg tggcgaagat gcacaggtgg ctcctgggct tgggctgcag gtttgggggt
1321 ctctaagaac aatctctgag aagaaccctt gggcccctgg gagccaagtt ggacaggatg
1381 tcctgaagac tagcttttga taagagaaat taaccaagtc tttcccctca tctatgatgc
1441 aatatatttc agtgggggcc ttcagagcac acctgttgga cggtgcaaac catatcttct
1501 ccagaaggca aatacttttg tatcagagga aactcagttt tggagaggaa tatgttcttt
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1561 atgtctcaaa tcaaaactct ctctaatggt aaactggctt ctaatttttt taagtacagt
1621 attttttttt ccccttttagt agtaacgggt ttctatagat cttcctatac agtctgcttt
1681 aactcaggac cttgagatta tgagactgac gtgctgccca ctgcactgag ggggcttcta
1741 acagtctgct ttaagtggta taattctggg atagatctgt tactggcata gtcatgacaa
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1921 tgggttattt caaggcattt ttgaagactg gaggagcgta ggagggagtg gtgagggaac
1981 ctagaacttc ttgctccttg tgactatgac agatgtctga tgcccccagc gcactggagt
2041 ctggggcttg gtgcaggtgg tgccccatca gtgtggacag acactacttg cctttgtggt
2101 ttagtgttgg aagctttaat tattctacag ttccatagat tcacaatttt atagccaaac
2161 agtttgtatc cacctgcttt cagaggagga accaaaggcc aaattacttt gaggtagggc
2221 ttagctggac ctgggttttc ctatgctctt ttcatgctgt gttggagggt gtgtccactg
2281 ccagatctgt gtaacccgtc tgggtcaggg gatgagtgac agcaaaccat cttaaaattt
2341 ttcatgagta catttaagcg gaagcatacg ggaatatgag tgcaaaagtg tggctgagcc
2401 gcgtatgccc ttgattggtt ttgggaagcc tgagggaggc agccttcctg gctatgagcc
2461 atcgcctgcc caatcaggct aagggtggtg actggggtgg tgaaggggca gctctgctga
2521 gcatggtctg ccttatggcc tgaattgtcc tcaaggggtg tggactgcag atggtgttca
2581 catgaaccgg agacatcact ctttaggatt ctactggcag cccctgaatt ggctcaacgt
2641 ttgtggaggt ggtatttccc tgaagtactg agctttgttt tataataatt aaaatcctta
2701 tttggtccaa tttaatatag tttagaagct attttttttg aggcaaaccg tttttgaaaa
2761 tgtaaatttt gtttttaatt aaaaataaag tcttagttaa gaaaactcag gatgcacaaa
2821 actattcaga ctgttacttt agctttctcc cattacctca tcagtaatat tcaccacatt
2881 ttgctaaata tttattgatt agagtgttga aatcaaattc tgctctaaat gtgtgtgaat
2941 atgttggaga ggctttgtgt tcttcactgt gaaatgcaat tgtgccttga ataagaaggt
3001 acctagaagc caaattaaag taataatgac ttcttattgg ctttgatttt tcattgcagt
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3121 ctcgaaacat ttcgatcata catacatagc agtagagatc tgtgcccttc aggtacattg
3181 aatctgacca tcagtttata tatgtcattg aattttaaga atactcatgt taataatagt
3241 catctatcct tgcattttga aactgttcta atcttagtga acttgaattg gatttctggg
3301 taaaagaatg tgtttctttt atgttgctta tgtccgaagg ccttgtcaga atctgtcaga
3361 ctcttgttta ggtttagtgt gatcatggcg tcagagaagc aaagctttca aataaatagt
3421 acttcaggaa atagaaatga ttgaccaact ttaaaaataa ttttttttta attgcaatat
3481 gcagcttcag ttgcccagaa tcttagttcc gtttctcatt cttggtcttg agctggtcag
3541 gtgacatcag cagattagaa gttgaatgga gattaagtgg attcaggagg atgttccact
3601 tagagcagtc ttcaaaatga taaggtgttc tagaagaaag gaatgtagta ggaactatac
3661 tatgcctaac tttctatccc agagtgtctt gcaagagttt aggagttttg gaccctgtgt
3721 attggcagaa aagttatctc catcttaagc aggcatgact tttatacctg tgagctcatt
3781 taaggtgcat ttaaacctaa aataatttcc ctgtattatg cttcatggga ttaacactgc
3841 ttttccagaa cattttcaga ttccctcct tacatcctga gctccttctg tatatacatc
3901 tgttgatttt atccatccac aaggaacaat gatagtcaca ttagagaaca agaaaccagt
3961 aatacatggt ctctaactga tgattcgggc ctggatttga ttgaaagtgt ttgcagttcc
4021 tcttccgtag aatacagagt ggatgaaaat gttttcaatg cacagaacag gatgaatcct
4081 tttttcttta tttagcgatt tacacttttg ttactctatt atatattcag ttagtgtctg
4141 ataagatttt ctttgcttaa ggagaacgga cattgccttg gtatgttttt ttttttttt
4201 ccctccactt ttggagctta tcaggtaaaa atctcaagcc acatgaattg ttaacacctc
4261 tgttgggaaa agcctttgtg agtttttatg tacttggtct ttgtttttgt tattcatcct
4321 gtgtcctccc tcttcccgat gtgctgtttt acctaggagt tagtctgctt tctgaggatc
4381 ttttagagag aggctgtgaa gtgctgaatc acctttaatg atacagcact tctgccatct
4441 cagcatctac ataggactta catagacttc ctgaatgtgt cttcttcaga tactaaagta
4501 cagttggatc attttcttat ctccttttct taagcagtac tttgcaggta ctccccttg
4561 aaagccagaa gcataaacca ttggggaatc ttaacttgta gacatgcagt aaaagaaatg
4621 catttatgta agatctgtga gtacttaaaa agaaagccct cagtgtgtgt gaagtgaatg
4681 tgaaatgtgt gtgaaataca tagaattccc aaatagttta gcaaaggcag ggcgcaatat
4741 caagtaattt aaaaatggtc caaggaactg taagaaggag gaactaattc tagaataaat
4801 gttaaaatgc cattcaagaa caaaaccaca gatgccatac agacctcctg tgcttaagtt
4861 atagaagaat aaaaatctga atgaatggaa ggccttacgt gtatacagtt tacaaattcc
4921 tatttctaaa atttaagtcc cttatttaac agaagtatgt attttaatgc ttaactgtct
4981 cgggaaacct catttgtgac atcatctaag gggatgggaa gactagggag ccagtgccac
5041 gttgaacaga acagtggttt agtgaatgtg tgaggaaaga catgggcaac tgattattaa
5101 tgtttttgta attcagttta taacttggaa ccaatgaaaa gcaacaaaac taaactggtt
5161 tgacagcctg ccacttctgg catttcctgt aagtcactag cagtaggtgt gaggtgggct
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5221 tgcccatgac caggaggggt gtgtgtgtgt gtgtgcatgt gtgtatatgc gtgttggtct
5281 gcagtcacag catacctta tgtgcatgtg tcctcgcagc ttgggactca gcagtattct
5341 gggagggtgg aggtgaactg tcccatgtat tgtattatat atttttgag atggggtctt
5401 gctctgttgc ccaggctgga gtgcagtggt gcgatctcag ctcactgcaa cttttgcctc
5461 ctggttcaag cagttctcct gcctcagcct gccaaatagc tgggattaca ggtgtgtacc
5521 accactccca gctaatttt gtatttttag tagagatggg gttttaccat gttggccagg
5581 ctggtctcga gctcctggcc tcaggtgatc cacctgcttt ggcctcccaa agtgctgaga
5641 ttacaggcgt gaactaccgc gcctggcccc atgtattgta ttttttcag gttatattga
5701 aatctactac caggaatgtc ggaatgggtt ttggtatgta taatggaaat agatagagtg
5761 gttaagtcta gaaacacata cattaattgt attgaaatgt tatatcaata catcatttat
5821 gatgtgtgtg tggtcccaga cctcatggcc accagtttgt ttaagcattg tgaatgcttt
5881 ttaatagcat tcattagcat taatggagga ggacactgtg ttttctcaat taatctcatt
5941 gatttgtttg gtataagttt gggtcagaaa tgaaactgcc aaaacatcga tcagtacaag
6001 gaagggacac agggcttaaa atgtccacag tcttggcagt ggacttggca gttctcccag
6061 taagcagaag tacttgagct taattctgaa cttcaaagta atattttata cttaatttta
6121 ggagttttca tttacatatt gaaaaatgcc ttgactgtat tcacataaat ggtgctaaaa
6181 cattgtaccc cttataagaa ctgcagcaat ccacagtaat gttggttact tctgagtatt
6241 tgataaagga acaaagtcaa aatgaatgta tttaataagc ttctttctca tttccattgt
6301 ttttataaaa atattttggt attgttgcct gcattttagc cacttctaac tttttgtatt
6361 atgaatttgg agaggataac aagccaactt tagaccctct gccagtggcg atggtgtttt
6421 tcttccatgg gtaagccatt gtgaatggag gctggtggag cagcacactg tgtgaacctg
6481 gcagcgctca tcttggcttg tttagcagtg cctctgttta caccataaga tgttccgcat
6541 gtgtccaaaa tttcgcctgc tctgaaaaac atgcccccga ggctctgtgc agtttggggc
6601 cttggttccc acctgcagat gcggtcagtt gcctggctcc tggggccaga gtttcctctg
6661 ttacttgttg agtctcttgt cttcactgtc agaagctgaa ctgacttggg ggctttgctg
6721 ttgatccact ttagcaaacc ctgctgcaga ggactgtaaa aacaaataac taaaaataaa
6781 cttagaaaat acaactcaga agcctggctc tgttgctgtg gtcaaatgct tgcctttgag
6841 gactttggca gattgtgcta ttacattggg ctctaacttt ctggcacccg caaggcagac
6901 ctatgatgtc cacatggctg actcttgacc cctggccatt gtgagcagag gaatcaaggg
6961 tttagggagg cagtattggg cagtgtggaa gagttagatg agatgaagca gttagcacag
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7081 cttgggtctg gattcaacag cattcccgct gcccactaaa agggtgggct tggacagttc
7141 ctgaacctcc ctaaatctgt ttcttcatct ttaccacaca cagtctgtct cagtggtggt
7201 gtggattaag tgaggtgcta gcacttatta ggggaagatc ctccagacct gcaaagcagg
7261 gctgcagccc caattctgac ccagccctgc tgtgtgtttg ccgcaaagag gtttggtgtg
7321 tgcgagacat ttctttatga accatttaca ctgctggaaa tgtacccact aatgtgatgg
7381 caagaacttc aacaaattgc ttatgaatca ggctctcaat ggaagaagtt aatcctctga
7441 agaggggaa gactggactt tcagaagtat gttggggata gagaatgaag tttgagtcag
7501 cagagctctg ccttcctgtt tgtcaaagaa ctagagtctg ggagtcttgc aagatggagc
7561 tgggcttctg aagaacccag tgggtaggta ctgggcatcc attagacggc agtgctaagt
7621 cggtcatgtg cagcagaaag acctccccac tgtgaacaac aaaccacttt cctcctccag
7681 tggcccattc atttggattg atacactttt tcagtgtcag aaatgcactt tctccctcta
7741 cttgtctgaa ttacgatgct ctgctcaact ctgtggacag tgtttcttga atttcctttt
7801 ccaccgcctt ttcccactca agaaagtgga gagaaaaaca gggatgcagg ctgtgggtct
7861 tgtggaagcc ctttacaacc attaaataaa gaacaccagc tgcattatgt gtgtttagaa
7921 cgagaagttg tttgtacagt atttttctat tgaccgcttc cgtcttgcct gaaacctggg
7981 cattctttct gtagtttctc tgaattttct gtctcacctc ctcaccccta cccttgcctt
8041 tt
```

SEQ ID NO: 49 Human OTULIN Amino Acid Sequence (NP_612357.4)

```
   1 msrgtmpqpe awpgascaet pareaaatar dggkaaasgq prpemqcpae heedmyraad
  61 eiekekelli hergaseprl svapemdimd yckkewrgnt qkatcmkmgy eevsqkftsi
 121 rrvrgdnyca lratlfqams qavglppwlq dpelmllpek liskynwikq wklglkfdgk
 181 nedlvdkike sltllrkkwa glaemrtaea rqiacdelft neaeeyslye avkflmlnra
 241 ielyndkekg kevpffsvll fardtsndpg qllrnhlnqv ghtggleqve mfllayavrh
 301 tiqvyrlsky nteefitvyp tdppkdwpvv tliaeddrhy nipvrvceet sl
```

SEQ ID NO: 50 Mouse OTULIN cDNA Sequence (NM_001013792.2, CDS region from position 85-1143)

```
   1 aagcgctctg cggacctcgc ggggtgcggg gcagcgcagc cacggctccg gacggcggga
```

```
  61 cggcagggcc cggcgtggcc gcggatgagt cggggaacca tgccccagcc cggagcgtgg
 121 cccggtgcaa gctgcgccga gacgccggcg cgcgaggccg gggccgcggc gcgggacggc
 181 gggaaggtga cggccggcgc gcagccccgg gccgcgacgc gatgcccggc cgagcacgag
 241 gaggacatgt accgtgctgc agatgaaata gaaaaggaga aagaattgct aatacatgaa
 301 agagggatat cagaacccag gttaagtgtg gctcctgaaa tggatatcat ggactactgc
 361 aaaaaagaat ggagaggaaa tactcagaaa gccacatgta tgaaaaaggg ctatgaggaa
 421 gtgtctcaga aattcacttc cataaggcga gtccgtggtg ataactactg tgcactgagg
 481 gccacactgt tccaggccat gagccagctg gccgagctgc ccccctggct gcaggacttg
 541 gagctcatac tgttaccaga gaagctgata aacaagtata cctggatcaa gcagtggaaa
 601 cttggactga aatttgatgg gaagagtgag gacctggttg aaaaaattaa ggaatccctt
 661 gccctgctaa ggaagaagtg ggtaagcctg gctgcaatga aaactgctga agcgaggcag
 721 acagcttgtg atgagctgtt caccaacgaa gaggaggagt acagcctcta cgaagctgtg
 781 aagttcctga tgttaaacag agccatcgaa ctgtacgatg acaaggagaa gggaaaggaa
 841 gtgccgttct tctctgtgct cttgtttgcc cgagacacat ccaatgaccc tgaacagctc
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 961 cttcttgcct atgctgtccg ccacagcatc cgggtgtacc ggctgtccaa gtataacaca
1021 gaggagttca tcacagtcta ccccactgat ccccccaagg actggccaat ggtgaccctc
1081 atagctgagg atgatcggca ctataacatc cctgtcagag tgtgtgagga gaccagtgtg
1141 tgagggacac acatgtgact gccaagagat gcgcatgtgc agctcctcct ctgggggcct
1201 cagatctgca ggtctccaag gacaatctgg gagaactctc gggcccctgg agccaacttt
1261 ggaccattat gttctgaaga ctagctgttg ataatgagaa ttaattagcc aagccttcac
1321 tccttgttct gttatgcagt gtgttccact gggggcttca gcacgtgcct ttggaaggtg
1381 caaactatgt ctccataagg cggatgcttt tgggtcaaag aaaactgttt ggaaaggaat
1441 ctgtccaatg aaagttctga cggtaaactg gttttacttc tctttttaaa a
```

SEQ ID NO: 51 Mouse OTULIN Amino Acid Sequence (NP_001013814.2)

```
   1 msrgtmpqpg awpgascaet pareagaaar dggkvtagaq praatrcpae heedmyraad
  61 eiekekelli hergiseprl svapemdimd yckkewrgnt qkatcmkkgy eevsqkftsi
 121 rrvrgdnyca lratlfqams qlaelppwlq dlelillpek linkytwikq wklglkfdgk
 181 sedlvekike slallrkkwv slaamktaea rqtacdelft neeeeyslye avkflmlnra
 241 ielyddkekg kevpffsvll fardtsndpe qllrnhlnqv ghtggleqve mfllayavrh
 301 sirvyrlsky nteefitvyp tdppkdwpmv tliaeddrhy nipvrvceet sv
```

SEQ ID NO: 52 Human TRAF6 Transcript Variant 1 cDNA Sequence (NM_145803.2, CDS region from position 382-1950)

```
   1 ctcctccccg gcgcgctccc tgcccctcgc tccccgcagc cagcagagaa ggcggaagca
  61 gtggcgtccg cagctggggc ttggcctgcg ggcggccagc gaaggtggcg aaggctccca
 121 ctggatccag agtttgccgt ccaagcagcc tcgtctcggc gcgcagtgtc tgtgtccgtc
 181 ctctaccagc gccttggctg agcggagtcg tgcggttggt gggggagccc tgccctcctg
 241 gttcggcctc cccgcgcact agaacgatca tgaacttctg aagggaccca gctttctttg
 301 tgtgctccaa gtgatttgca caaataataa tatatatatt tattgaagga gagaatcaga
 361 gcaagtgata atcaagttac tatgagtctg ctaaactgtg aaaacagctg tggatccagc
 421 cagtctgaaa gtgactgctg tgtggccatg gccagctcct gtagcgctgt aacaaaagat
 481 gatagtgtgg gtggaactgc cagcacgggg aacctctcca gctcatttat ggaggagatc
 541 cagggatatg atgtagagtt tgacccaccc ctggaaagca agtatgaatg ccccatctgc
 601 ttgatggcat tacgagaagc agtgcaaacg ccatgcggcc ataggttctg caaagcctgc
 661 atcataaaat caataaggga tgcaggtcac aaatgtccag ttgacaatga aatactgctg
 721 gaaaatcaac tatttccaga caattttgca aaacgtgaga ttctttctct gatggtgaaa
 781 tgtccaaatg aaggttgttt gcacaagatg gaactgagac atcttgagga tcatcaagca
 841 cattgtgagt ttgctcttat ggattgtccc caatgccagc gtcccttcca aaaattccat
 901 attaatattc acattctgaa ggattgtcca aggagacagg tttcttgtga caactgtgct
 961 gcatcaatgg catttgaaga taaagagatc catgaccaga actgtccttt ggcaaatgtc
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1261 aatttccagg aaactattca ccagttagag ggtcgccttg taagacaaga ccatcaaatc
1321 cgggagctga ctgctaaaat ggaaactcag agtatgtatg taagtgagct caaacgaacc
1381 attcgaaccc ttgaggacaa agttgctgaa atcgaagcac agcagtgcaa tggaatttat
1441 atttggaaga ttggcaactt tggaatgcat ttgaaatgtc aagaagagga gaaacctgtt
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1681 attcttgatc agtctgaagc acctgtaagg caaaaccacg aagagataat ggatgccaaa
1741 ccagagctgc ttgctttcca gcgacccaca atcccacgga acccaaaagg ttttggctat
1801 gtaactttta tgcatctgga agccctaaga caaagaactt tcattaagga tgacacatta
1861 ttagtgcgct gtgaggtctc cacccgcttt gacatgggta gccttcggag ggagggtttt
1921 cagccacgaa gtactgatgc aggggtatag cttgccctca cttgctcaaa aacaactacc
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2281 caagagtact aaacttttaa taataatgga ttttccttaa aacttcagtc tttttgtagt
2341 attatatgta atatattaaa agtgaaaatc actaccgcct tgtgctagtg ccctcgagaa
2401 gagttattgc tctagaaagt tgagttctca tttttttaac ctgttataga tttcagagga
2461 tttgaaccat aatccttgga aaacttaagt tctcattcac cccagttttt cctccaggtt
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2761 agctctgcat atgtcttcat ttgtttaatg cttacaagac aggagatgca cacatacaat
2821 cagcaacata aaaattaaaa gtgacccaag tagtcagcgc atgtggcatc tcattggtgg
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3721 ataattgtca agttccattt aaaaactgaa cagtagtatt tttgtatttg cgtagaaaaa
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5281 ctattaccgt ttttacacca gtaagtctta gataaactaa gcatgcttat gaattatgta
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5641 gaatcgcttg aacccgggaa gcacaggttg cagtgagcca agatcatgcc actgcacccc
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7321 tgacttttga aaattgaaag acatccagtg gtcttatcac aaagcctgct tttcctcaga
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7681 aatacaactg aattgctggg ctgacttaag attgaaggac tccattttag taagtagaga
7741 agtgtgacct ttctcaaccc aggttgtgaa tgtggattca cacttatctc aaaaaggcac
7801 ctggagtttt aactttatgt catgtctcag tactggttgc aaggtatgac caaaagtgtt
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7921 acccccctccg cactgttact acttccttac cagccctatg tactgcatca atgtctacaa
7981 gaaagcactc ttcattaaaa tgaaatatat atattaaaat aaaaaaaaaa aaaaaaaaaa1
```

SEQ ID NO: 53 Human TRAF6 Transcript Variant 2 cDNA Sequence (NM_004620.3, CDS region from position 289-1857)

```
   1 ctcctccccg gcgcgctccc tgcccctcgc tccccgcagc cagcagagaa ggcggaagca
  61 gtggcgtccg cagctggggc ttggcctgcg ggcggccagc gaaggtggcg aaggctccca
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 421 ctctccagct catttatgga ggagatccag ggatatgatg tagagtttga cccacccctg
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5641 ctcctaaaac cccagtttcc ttcagtgcag gctccaggag agggcagaga ccccattctg
5701 gtcactgctg aacccctgtt tttagcatac tgtgcatggg cctggccaat agtcacaagc
5761 tttaatggga gccagggcag aagctgactg gctgctgggt agcctacttg tcatgtaagt
5821 cagttggtaa agtgagagtg ttctttttc tgcttttctc ccgggacttt gctactgcag
5881 ttctcaaaca tggaagtgag tttaagacct agtgaacacc tcccacctag gatctgcagt
5941 gacattgggt gtgctctgat ttaatgcttc tatcatgtaa attctaattt ctccttaagg
6001 ctgttcaatc ctgaaataat taaacaactt gaagttgtat aaaattctcc ttggaaactt
6061 gtgatatttt attgtaattt atcttgtagc ttctgcttta tgccaactta aaatttgtgg
6121 aaatgttgtg aggaacttta ctcttatgtc tttgtctaca ggagtatttt tataaaggat
6181 ttatttgc
```

SEQ ID NO: 56 Mouse TRAF6 Transcript Variant 2 cDNA Sequence (NM_001303273.1, CDS region from position 224-1816)

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   1 tagcgagctg agaaggcgga agcagcggcg gccgcggctg gggctgaggc tccggccgtc
  61 ggcggacgca gcagccgcgg cccacgagcc gggagtttgg cgtcggagcc acttggtctc
 121 ggagtgccgt gtatgtaggc gacgcggcgc agcccgggga agccttccca gttggttgtg
 181 aagtctcagc gtgtacgatc gatcgactga caacagagct actatgagtc tcttaaactg
 241 tgagaacagc tgcgggtcca gccagtcgtc cagtgactgc tgcgctgcca tggccgcctc
 301 ctgcagcgct gcagtgaaag atgacagcgt gagtggctct gccagcaccg ggaacctctc
 361 cagctccttc atggaggaga tccagggcta cgatgtggag tttgacccac ctctggagag
 421 caagtatgag tgtcccatct gcttgatggc tttacgggaa gcagtgcaaa caccatgtgg
 481 ccacaggttc tgcaaagcct gcatcatcaa atccataagg gatgcagggc acaagtgccc
 541 agttgacaat gaaatactgc tggaaaatca actgtttccc gacaattttg caaagcgaga
 601 gattctttcc ctgacggtaa agtgcccaaa taaaggctgt ttgcaaaaga tggaactgag
 661 acatctcgag gatcatcaag tacattgtga atttgctcta gtgaattgtc cccagtgcca
 721 acgtcctttc cagaagtgcc aggttaatac acacattatt gaggattgtc ccaggaggca
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 841 aagctgtcct ctggcaaata tcatctgtga atactgtggt acaatcctca tcagagaaca
 901 gatgcctaat cattatgatc tggactgccc aacagctcca atcccttgca cattcagtgt
 961 ttttggctgt catgaaaaga tgcagaggaa tcacttggca cgacacttgc aagagaatac
1021 ccagttgcac atgagactgt tggcccaggc tgttcataat gttaaccttg ctttgcgtcc
1081 gtgcgatgcc gcctctccat cccggggatg tcgtccagag gacccaaatt atgaggaaac
1141 tatcaaacag ttggagagtc gcctagtaag acaggaccat cagatccggg agctgactgc
1201 caaaatggaa actcagagta tgtacgtggg cgagctcaaa cggaccattc ggaccctgga
1261 ggacaaggtt gccgaaatgg aagcacagca gtgtaacggg atctacattt ggaagattgg
1321 caactttggg atgcacttga aatcccaaga agaggaaaga cctgttgtca tccatagccc
1381 tggattctac acaggcagac ctgggtacaa gctgtgcatg cgcctgcatc ttcagttacc
1441 gacagctcag cgctgtgcaa actatatatc ccttttttgtc cacacaatgc aaggagaata
1501 tgacagccac ctcccctggc ccttccaggg tacaatacgc cttacaattc tcgaccagtc
1561 tgaagcactt ataaggcaaa accacgaaga ggtcatggac gccaaaccag aactgcttgc
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1681 cctggaagcc ttaagacagg gaaccttcat taaggatgat acattactag tgcgctgtga
1741 agtctctacc cgctttgaca tgggtggcct tcggaaggag ggtttccagc cacgaagtac
1801 tgatgcgggg gtgtagcgtc catgtacttg tgttcaaaaa ctaggaacca tatgggaaaa
1861 ccgtgccttc catgcctggc cccagtaaac aatgttcaaa caagcagtgg gagaggtgta
1921 aggcctagca gcagatgtca tcagtgaggt cacgagccac ttcttactgt taacaaatac
1981 ctgaggcagt tcccatggga acctacatgt cccctgtatc ttcaaaacgt caacatttga
2041 agggcctgtg gctcatctgt ctgtcagggt acccccttcac tgtgcttcca tgggctattt
2101 tgtccgtgta ctttactgta aaaaaggcca gacttagcgt gctgcagctc aatcgtttaa
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2221 gaaaatcacc actgcctgtg ctggtgccag tagagaagtt accgctctgg tgttgagttc
2281 tcatttagtt gactcctgtg aatttcagag gctttgaacc atgatccctg gaaggcttaa
2341 gttctcaagt actccctcct ctatagttca ctaaggatcc agggactggt ttaaccctta
2401 cttagtgtga atgtattgtc cactgaacac caagcatccc ccactacttt cctgttttga
2461 aatatgctcc aggcggcctc ttcccagtct gtaagaccgc ggtcatgtgc ttgccaactg
2521 ctgagtgtta ctgccatgga acctttcttg tctgtcccgt gcagcttggt ttccacagcc
2581 ggttgcatat cttctgttgc ttgcaaacac aaaatcacca gcccaaacga gtgatttagc
2641 tcactagcca ttaaatggca tctcgtggat gatgacagca actcttacag ccaggaaact
2701 tcagcccctc ttaactagct tttgatttag cttataaggt taattgaaat aaaattgatt
2761 tttctcaagg ggttggagaa ttggctcagt ggttaagagc cttggctgct cttccagagg
2821 atccccagtc tgtaactcca gttccagggc atctgacacc ctcatacaga cactcatgca
2881 ggcaaaacac cagtgcacat aaaattaaac aaacaaataa ataaataaat tgatttcctc
2941 aaaacagaat ttattggaac ttgggaaatt gtaggtacct gagagatgcc taaaccaagg
3001 ttggctatca cggttgtgtg gacactcagc ttgagtggtg gctttgtcca gctcagtaga
3061 ggttctgatc tgtgacccta atgtggagag gtgactgtcg tgctgctgtg tatttgttaa
3121 tgtcctgtac atatacagta ctttggagtc tagttctcag ggagccctac gactagttag
3181 agcctttgta aggaagcaga ggggatcctc tcctgctgtt tacacaagat cagctatgtg
3241 ttctggtggt aagaaaggca tccgtgcctt cagctgaatc agagacccga gcagtgctct
3301 gacctgccct gttcccagag aacgctcaga gcctccacca aggagtctgt ttctcagctg
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3421 aggtgagggg aagaccttgt cccctaccat tatcacaagc tcatcacagg tctcttctgt
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3541 aacttctgcc taaggagcac tgcatggtct tatactgtag ttgtttccca gtggagtaat
3601 aaatgtgggc ttgtttgttg tttctttaaa gcaagcagta gctgtgtcta tatttattta
3661 gaaattgcct gaagaagatt actcaactat ttgaagactt attttctata tgcctttctt
3721 aatttttta tgttatatgt caccacaaaa atatgaactc cccaaccccc tctccgtttt
3781 ttgaaaaagg aaatgacatt taagaacttc ctcatcagat ttctcttttt aaaatatctg
3841 tattaggaag agcagtcgtt tcctgccgtg gttttgactt tttttaaaaa aaactctaac
3901 atcttttaaa gttttttgc ataagttaaa ctgttcccag ctttaaattg tcctccctat
3961 agggcaagtt ggactaggtg tttctagtat ccgcattgag aagcccagtg ctgagccaca
4021 atactcacta aaaggctttc cccgtagagg tgtgactgcc cctaactgct aacacggatg
4081 gttcactgca gtgtaatgtc catccgctag aatacacctc aggtagtttt agaacttgca
4141 gcatttggtg tttgtaataa gccaaccagt tactttatgt tactcaattg ccacgaatgc
4201 agagtaaaac taatcaagct gacattcaag gtcaacactt agtaaggtca actcaggatc
4261 aagtcttagc ctagaaagcc gctttcttta cttcaccact ttctgaacat tctctttgta
4321 ccaatgggcc tataagaatc cgtatagtcc agagtgcatt ggccatcttt ccttaccaat
4381 ctagaacact gctgaattta aagttgtttc ttcttagaaa aatgcctacc ttactattga
4441 agatttttcc ccaagtcata tatttccctc ttagaaatca ggccagacgg cagttctagt
4501 ttggaagttg gttacagtcc tttggctgtt accatctcta gccattctgc tttcttctgg
4561 agaatgaaga ggagaaaagt gcattaaagt acaaaaggtg tcctctcacc ctcggaagat
4621 caactgacag gtgttggatg atctccaaca agtaaatttt gtgaccagta taaagttgaa
4681 tttgtaccaa tatcaaacaa agtctgacca atgtaaatta tgtgcacaat tagaatatct
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4921 ttgttacagt tttttatggg gtgtttggtg attagtttgc tggctgcctt gagggagtga
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5041 ctcagggtaa ctgtcatttg tttcaaagtt gatgtgattg tctgggaaat ggatggatgc
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5161 ctctcggtca caagcgttga agtcactcaa aaaggactaa gctagttatt tctctgtggg
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5341 ctgtcctgtg tctcctcagc ggagcccagc cctgcctgac acaggagaaa agggtggcct
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5461 agtgcaggct ccaggagagg gcagagaccc cattctggtc actgctgaac ccctgttttt
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5641 ttttttctgc ttttctcccg ggactttgct actgcagttc tcaaacatgg aagtgagttt
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5761 atgcttctat catgtaaatt ctaatttctc cttaaggctg ttcaatcctg aaataattaa
5821 acaacttgaa gttgtataaa attctccttg gaaacttgtg atattttatt gtaatttatc
5881 ttgtagcttc tgctttatgc caacttaaaa tttgtggaaa tgttgtgagg aactttactc
5941 ttatgtcttt gtctacagga gtatttttat aaaggattta tttgc
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SEQ ID NO: 57 Mouse TRAF6 Amino Acid Sequence (NP_033450.2)

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   1 msllncensc gssqsssdcc aamaascsaa vkddsvsgsa stgnlsssfm eeiqgydvef
  61 dppleskyec piclmalrea vqtpcghrfc kaciiksird aghkcpvdne illenqlfpd
 121 nfakreilsl tvkcpnkgcl qkmelrhled hqvhcefalv ncpqcqrpfq kcqvnthiie
 181 dcprrqvscv ncavsmayee keihdqscpl aniiceycgt ilireqmpnh ydldcptapi
 241 pctfsvfgch ekmqrnhlar hlqentqlhm rllaqavhnv nlalrpcdaa spsrgcrped
 301 pnyeetikql esrlvrqdhq ireltakmet qsmyvgelkr tirtledkva emeaqqcngi
 361 yiwkignfgm hlksqeeerp vvihspgfyt grpgyklcmr lhlqlptaqr canyislfvh
 421 tmqgeydshl pwpfqgtirl tildqseali rqnheevmda kpellafqrp tiprnpkgfg
 481 yvtfmhleal rqgtfikddt llvrcevstr fdmgglrkeg fqprstdagv
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SEQ ID NO: 58 Human TAB2 Transcript Variant 1 cDNA Sequence (NM_015093.5, CDS region from position 422-2503)

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   1 atcgagcgcc taggagagcc ccgggtgggg gagggcgcaa ggggctcggg agggcccctc
  61 cctgcgtcgg cgcccctgac ccgcccccag aggcagggtt ttgctgtatt gcacaggcta
 121 gtctcaaact ccttggctca agtgatcctc ttgctttggt ctcccaaagt gctgcgatta
```

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 181 caggctccag acccagttcc tggacctgcc ctggaaaaga agtatcccgt agagatgagc
 241 tcactgcagt tacttaatta acaatttgta agctgcaaaa atggcaatgg gccaaccaag
 301 aacagctaca atttgaattt ttctatttcc agaaaatgct tggacagaag agatgagtac
 361 tatttccact aaggcctaga attgcctact gtacaaatag tcctgatcag gcaatatacg
 421 aatggcccaa ggaagccacc aaattgattt tcaggtttta catgacctgc gacaaaaatt
 481 ccctgaagta cctgaagttg ttgtatccag gtgcatgtta cagaataata ataacctgga
 541 tgcctgctgt gctgttctct ctcaggagag tacaagatat ctttatggtg aaggagactt
 601 gaatttttca gatgattctg gaatttctgg tctacgcaat cacatgactt ctctcaactt
 661 ggacttgcaa tcacagaaca tttaccacca tggaagagaa ggaagtagga tgaatggaag
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 901 cagcaaagga acatctagcc tttctcaaca aactcccaga tttaatccca ttatggtaac
 961 tttagcccca aatatccaga ctggtcgtaa tactcctaca tctttgcaca tacatggtgt
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1081 aactcctggt ggtacaactc gacagacaca acagcattct ggctgggtat ctcagtttaa
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2101 attaaaatct gaggttaatg aaatggaaaa taatctaact cgaaggcgcc tgaaaagatc
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2281 accacatttt aaccccagcg ctattcataa cttttatgac aatattggat ttgtaggtcc
2341 tgtgccacca aaacccaaag atcaaaggtc catcatcaaa acaccaaaga ctcaagacac
2401 agaagatgat gagggagctc agtggaattg taccgcctgt actttttga accatccagc
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2521 cttctctaaa accacatcta aagttcaaga aactagtctg tcatcgggaa aaagtttcac
2581 tgctacatag gattttgtca aattgaaggt gtgacaagat ggtgttctgc taatgttaaa
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2881 tttgtaaagc tttcagtgaa acactacata cacgaagaaa aggaacaagg tttaactatt
2941 taagaaccat ttgctgccgc atagtgccat tggataggga agaacttcag aaatctgtgg
3001 tactcttggc cttgtctttg tcttccctga acgtgtctcc actctgtgaa gccagcatct
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3781 acaactgaag aaaagctgga aaaaaaaaca agcaaacttg aacactgaag caacctcaag
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3841 catctcttta ttttgatgat atatttttgt aaggaaaata ttcagatgat caggaatgta
3901 tataactgaa atcaagaaaa agaacagtat gcatttaaaa agacagaatt atgaaattat
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4261 atcagttttt acttgtttta ttaataaaac gtaatttgga tatcttgagt tgatggtttt
4321 gtgatttagc tgggtaaact atctttgtaa cagataagtt atttataaaa attaaaaaac
4381 ttatattcta atgtggaaaa aaaaaaaaaa aaaa
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SEQ ID NO: 59 Human TAB2 Transcript Variant 3 cDNA Sequence (NM_001292034.2, CDS region from position 179-2260)

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   1 ggaggcgacg gtggagacgg ctgccctagt gggagaggcg gcggcggcgg cggccgagga
  61 ggaggagggg gaagcggcgg cggcaaagga aaatgcttgg acagaagaga tgagtactat
 121 ttccactaag gcctagaatt gcctactgta caaatagtcc tgatcaggca atatacgaat
 181 ggcccaagga agccaccaaa ttgattttca ggttttacat gacctgcgac aaaaattccc
 241 tgaagtacct gaagttgttg tatccaggtg catgttacag aataataata acctggatgc
 301 ctgctgtgct gttctctctc aggagagtac aagatatctt tatggtgaag gagacttgaa
 361 tttttcagat gattctggaa tttctggtct acgcaatcac atgacttctc tcaacttgga
 421 cttgcaatca cagaacattt accaccatgg aagagaagga agtaggatga atggaagtag
 481 gactctaacg cacagcatta gtgatggaca acttcaaggt ggccagtcca atagtgaact
 541 atttcagcag gagccacaga cagcaccagc tcaagttcct caaggcttta atgtttttgg
 601 aatgtccagt tcctctggtg cttcaaattc agcaccacat cttggatttc acttaggcag
 661 caaaggaaca tctagccttt ctcaacaaac tcccagattt aatcccatta tggtaacttt
 721 agccccaaat atccagactg gtcgtaatac tcctacatct ttgcacatac atggtgtacc
 781 tccacctgta cttaacagtc cacagggaaa ttctatctat attaggcctt acattacaac
 841 tcctggtggt acaactcgac agacacaaca gcattctggc tgggtatctc agtttaatcc
 901 catgaaccct cagcaagttt atcagccttc acagcctggt ccctggacta cttgtcctgc
 961 atctaatcct ctgtcacata cctcatctca acagccaaat cagcaaggcc accagacctc
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1201 gcgttcttct ggacctcgaa cctccagcac ttcctcttca gtcaatagcc agaccttaaa
1261 cagaaatcag cccactgttt acatagctgc cagccccca aatacggatg agctgatgtc
1321 ccgtagtcaa cctaaggtct atatttcagc gaatgctgcc acaggagatg aacaggtcat
1381 gcggaatcag cccacactct tcatatccac aaactctgga gcatctgctg cctccaggaa
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1741 gaaactgagt atgggatctg atgatgctgc ctacacacaa gctcttttgg tacaccagaa
1801 ggccagaatg gaacgacttc aaagagaact tgagattcaa aagaaaaagc tggataaatt
1861 aaaatctgag gttaatgaaa tggaaaataa tctaactcga aggcgcctga aaagatcaaa
1921 ttctatatcc cagatacctt cccttgaaga aatgcagcag ctgagaagtt gtaatagaca
1981 actccagatt gacattgact gcttaaccaa agaaattgat ctttttcaag cccgaggacc
2041 acattttaac cccagcgcta ttcataactt ttatgacaat attggatttg taggtcctgt
2101 gccaccaaaa cccaaagatc aaaggtccat catcaaaaca ccaaagactc aagacacaga
2161 agatgatgag ggagctcagt ggaattgtac cgcctgtact tttttgaacc atccagcctt
2221 aattcgctgt gaacagtgtg agatgccaag gcatttctga gccaaatggc cctgtatctt
2281 ctctaaaacc acatctaaag ttcaagaaac tagtctgtca tcgggaaaaa gtttcactgc
2341 tacataggat tttgtcaaat tgaaggtgtg acaagatggt gttctgctaa tgttaaatgt
2401 cagcccacag agctaataat acctcagtat aatgtcatga gcagttgaaa ttcatcacat
2461 gaaaagtaat ctgctgaaag acttggttgc ccactgccta actgtgtaca gtgttaccag
2521 tgtcccatta tggataattc tcaatatgtt aacacctagg tgttcccaat acctttttcc
2581 cctcatgtca ctactgaatt ttgacaggag gaaggaatag aatgatagct tgttttattt
2641 gtaaagcttt cagtgaaaca ctacatacac gaagaaaagg aacaaggttt aactatttaa
2701 gaaccatttg ctgccgcata gtgccattgg atagggaaga acttcagaaa tctgtggtac
2761 tcttggcctt gtctttgtct tccctgaacg tgtctccact ctgtgaagcc agcatctagg
```

```
2821 ggctaaagat gcaaaggaaa gcagcatgca ttgtctgtac aaatgtgcag cgaaataccc
2881 caaagctttt cctactgtac agatctctcg agtctgcttt aagtgatttc ttttcttctt
2941 gattattttc ttatatttct atatgtatag tgtaatagcc ttttgttaac taatttttct
3001 ttttcctttt agtaattaag cacgatcatg tccctttttа agccttacct gagaggaaca
3061 atgccttaaa ataaaaaagc attaatgaga tgaaagtatg cacagaataa ctttcctcta
3121 cttattctgt actttgccct catgagttcc aatgttgtgt gaagacaggc agatgctgca
3181 cagtgaattg cagatgatat tacagaagtg atgtctgtag gtcacattaa atactgactt
3241 gagcagtggg tgacacaaca cagtgtttgt cttccacagg gaagcttaaa acaaaagata
3301 tttttaaccc actgacagaa caacaaggtt aagcttcatc tgcttggtgt cccacagaac
3361 ttgcacaagc agttgttatt gggaaagtac agtctcaaaa ccagcaacag cagcagtacc
3421 tacagccctt tttttggaga gaagtttaaa tgctttactg ttggggcagt ccatttctaa
3481 tcctgacttg gtgacagtat catgtgtatt tataaaacaa ggctagccat atttaggaca
3541 actgaagaaa agctggaaaa aaaaacaagc aaacttgaac actgaagcaa cctcaagcat
3601 ctctttattt tgatgatata tttttgtaag gaaaatattc agatgatcag gaatgtatat
3661 aactgaaatc aagaaaaaga acagtatgca tttaaaaaga cagaattatg aaattatatg
3721 agtgcttaga atggggctaa gggaagtgct gaaatagagc aaaggatgga agataatata
3781 gactaccacc cactgtaaat gtttgcaagt ggctgtgttt taaatgggat tattacagtt
3841 gatctctatg aatgtcagag ccctaacttt caggctttgc attttgtata tgggaagaaa
3901 tatgacaatc ctaggtaatt aaaccataga cccaaagccc ttacgtttga tgcaatttat
3961 ttttaaaata ggccttgttt ttcagcttca tctgcagttc tatgtgaaga ttgataaatc
4021 agtttttact tgttttatta ataaaacgta atttggatat cttgagttga tggttttgtg
4081 atttagctgg gtaaactatc tttgtaacag ataagttatt tataaaaatt aaaaaactta
4141 tattctaatg tggaaaaaaa aaaaaaaaaa a
```

SEQ ID NO: 60 Human TAB2 Isoform a Amino Acid Sequence (NP_055908.1)

```
   1 maqgshqidf qvlhdlrqkf pevpevvvsr cmlqnnnnld accavlsqes trylygegdl
  61 nfsddsgisg lrnhmtslnl dlqsqniyhh gregsrmngs rtlthsisdg qlqggqsnse
 121 lfqqepqtap aqvpqgfnvf gmssssgasn saphlgfhlg skgtsslsqq tprfnpimvt
 181 lapniqtgrn tptslhihgv pppvlnspqg nsiyirpyit tpggttrqtq qhsgwvsqfn
 241 pmnpqqvyqp sqpgpwttcp asnplshtss qqpnqqghqt shvympissp ttsqpptihs
 301 sgssqssahs qyniqnistg prknqieikl eppqrnnssk lrssgprtss tsssvnsqtl
 361 nrnqptvyia asppntdelm srsqpkvyis anaatgdeqv mrnqptlfis tnsgasaasr
 421 nmsgqvsmgp afihhhppks raignnsats prvvvtqpnt kytfkitvsp nkppavspgv
 481 vsptfeltnl lnhpdhyvet eniqhltdpt lahvdriset rklsmgsdda aytqallvhq
 541 karmerlqre leiqkkkldk lksevnemen nltrrrlkrs nsisqipsle emqqlrscnr
 601 qlqididclt keidlfqarg phfnpsaihn fydnigfvgp vppkpkdqrs iiktpktqdt
 661 eddegaqwnc tactflnhpa lirceqcemp rhf
```

SEQ ID NO: 61 Human TAB2 Transcript Variant 4 cDNA Sequence (NM_001292035.2, CDS region from position 310-2295)

```
   1 tcctttctgg agtctgaaat gttaagtatg gaggcaaaag ctgaagctgg agcaacagtc
  61 tcagaatatg acaacaacca tgagaatgga ggccattcat agcagaacta cgagacaaga
 121 ggagcctagg tccctgagct gcagaactgc atactaacct gatgagaaga ctaatgctca
 181 gtgaggtgaa ctccttcagg gccacaggct gtaaagatct aaacaccatc tgatccagtt
 241 tcaatatgac tgcctggatg aagaaggatt tgagactaag tttattcctc caaagcaaac
 301 caactgagaa tgcagaataa taataacctg gatgcctgct gtgctgttct ctctcaggag
 361 agtacaagat atctttatgg tgaaggagac ttgaattttt cagatgattc tggaatttct
 421 ggtctacgca atcacatgac ttctctcaac ttggacttgc aatcacagaa catttaccac
 481 catggaagag aaggaagtag gatgaatgga agtaggactc taacgcacag cattagtgat
 541 ggacaacttc aaggtggcca gtccaatagt gaactatttc agcaggagcc acagacagca
 601 ccagctcaag ttcctcaagg ctttaatgtt tttggaatgt ccagttcctc tggtgcttca
 661 aattcagcac cacatcttgg atttcactta ggcagcaaag gaacatctag cctttctcaa
 721 caaactccca gatttaatcc cattatggta actttagccc caaatatcca gactggtcgt
 781 aatactccta catctttgca catacatggt gtacctccac ctgtacttaa cagtccacag
 841 ggaaattcta tctatattag gccttacatt acaactcctg gtggtacaac tcgacagaca
 901 caacagcatt ctggctgggt atctcagttt aatcccatga accctcagca agtttatcag
 961 ccttcacagc ctggtccctg gactacttgt cctgcatcta atcctctgtc acatacctca
1021 tctcaacagc caaatcagca aggccaccag acctctcatg tctacatgcc aatcagttca
1081 cctactactt cacaaccacc aaccattcat tcatctggta gctcacagtc ttctgcccat
1141 agccaatata acattcagaa tatttcaaca ggacctcgaa aaaaccagat tgaaatcaaa
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1201 cttgaacccc cacaaagaaa taattcttca aaactgcgtt cttctggacc tcgaacctcc
1261 agcacttcct cttcagtcaa tagccagacc ttaaacagaa atcagcccac tgtttacata
1321 gctgccagcc ccccaaatac ggatgagctg atgtcccgta gtcaacctaa ggtctatatt
1381 tcagcgaatg ctgccacagg agatgaacag gtcatgcgga atcagcccac actcttcata
1441 tccacaaact ctggagcatc tgctgcctcc aggaacatgt ctgggcaagt gagcatgggt
1501 cctgccttta ttcatcacca tcctcccaaa agtcgagcaa taggcaataa ctctgcaacc
1561 tctcctcgag tggtagtcac tcagcccaat acgaaataca ctttcaaaat tacagtctct
1621 cccaataagc cccctgcagt ttcaccaggg gtggtgtccc ctacctttga acttacaaat
1681 cttcttaatc atcctgatca ttatgtagaa accgagaata ttcagcacct cacggaccct
1741 acattagcac atgtggatag aataagtgaa acacggaaac tgagtatggg atctgatgat
1801 gctgcctaca cacaagctct tttggtacac cagaaggcca gaatggaacg acttcaaaga
1861 gaacttgaga ttcaaaagaa aaagctggat aaattaaaat ctgaggttaa tgaaatggaa
1921 aataatctaa ctcgaaggcg cctgaaaaga tcaaattcta tatcccagat accttccctt
1981 gaagaaatgc agcagctgag aagttgtaat agacaactcc agattgacat tgactgctta
2041 accaaagaaa ttgatctttt tcaagcccga ggaccacatt ttaaccccag cgctattcat
2101 aacttttatg acaatattgg atttgtaggt cctgtgccac caaaacccaa agatcaaagg
2161 tccatcatca aaacaccaaa gactcaagac acagaagatg atgagggagc tcagtggaat
2221 tgtaccgcct gtacttttt gaaccatcca gccttaattc gctgtgaaca gtgtgagatg
2281 ccaaggcatt tctgagccaa atggccctgt atcttctcta aaaccacatc taaagttcaa
2341 gaaactagtc tgtcatcggg aaaaagtttc actgctacat aggattttgt caaattgaag
2401 gtgtgacaag atggtgttct gctaatgtta aatgtcagcc cacagagcta ataatacctc
2461 agtataatgt catgagcagt tgaaattcat cacatgaaaa gtaatctgct gaaagacttg
2521 gttgcccact gcctaactgt gtacagtgtt accagtgtcc cattatggat aattctcaat
2581 atgttaacac ctaggtgttc ccaatacctt tttcccctca tgtcactact gaattttgac
2641 aggaggaagg aatagaatga tagcttgttt tatttgtaaa gctttcagtg aaacactaca
2701 tacacgaaga aaaggaacaa ggtttaacta tttaagaacc atttgctgcc gcatagtgcc
2761 attggatagg gaagaacttc agaaatctgt ggtactcttg gccttgtctt tgtcttccct
2821 gaacgtgtct ccactctgtg aagccagcat ctaggggcta aagatgcaaa ggaaagcagc
2881 atgcattgtc tgtacaaatg tgcagcgaaa taccccaaag cttttcctac tgtacagatc
2941 tctcgagtct gctttaagtg atttcttttc ttcttgatta ttttcttata tttctatatg
3001 tatagtgtaa tagccttttg ttaactaatt ttctttttttc cttttagtaa ttaagcacga
3061 tcatgtccct ttttaagcct tacctgagag gaacaatgcc ttaaaataaa aaagcattaa
3121 tgagatgaaa gtatgcacag aataactttc ctctacttat tctgtacttt gccctcatga
3181 gttccaatgt tgtgtgaaga caggcagatg ctgcacagtg aattgcagat gatattacag
3241 aagtgatgtc tgtaggtcac attaaatact gacttgagca gtgggtgaca caacacagtg
3301 tttgtcttcc acagggaagc ttaaaacaaa agatattttt aacccactga cagaacaaca
3361 aggttaagct tcatctgctt ggtgtcccac agaacttgca caagcagttg ttattgggaa
3421 agtacagtct caaaaccagc aacagcagca gtacctacag ccctttttttt ggagagaagt
3481 ttaaatgctt tactgttggg gcagtccatt tctaatcctg acttggtgac agtatcatgt
3541 gtatttataa aacaaggcta gccatattta ggacaactga agaaaagctg gaaaaaaaaa
3601 caagcaaact tgaacactga agcaacctca agcatctctt tattttgatg atatattttt
3661 gtaaggaaaa tattcagatg atcaggaatg tatataactg aaatcaagaa aaagaacagt
3721 atgcatttaa aaagacagaa ttatgaaatt atatgagtgc ttagaatggg gctaagggaa
3781 gtgctgaaat agagcaaagg atggaagata atatagacta ccacccactg taaatgtttg
3841 caagtggctg tgttttaaat gggattatta cagttgatct ctatgaatgt cagagcccta
3901 actttcaggc tttgcatttt gtatatggga agaaatatga caatcctagg taattaaacc
3961 atagacccaa agcccttacg tttgatgcaa tttatttttа aaataggcct tgtttttcag
4021 cttcatctgc agttctatgt gaagattgat aaatcagttt ttacttgttt tattaataaa
4081 acgtaatttg gatatcttga gttgatggtt ttgtgattta gctgggtaaa ctatctttgt
4141 aacagataag ttatttataa aaattaaaaa acttatattc taatgtggaa aaaaaaaaaa
4201 aaaaaa
```

SEQ ID NO: 62 Human TAB2 Isoform b Amino Acid Sequence (NP_001278964.1)

```
   1 mqnnnnldac cavlsqestr ylygegdlnf sddsgisglr nhmtslnldl qsqniyhhgr
  61 egsrmngsrt lthsisdgql qgggqsnself qqepqtapaq vpqgfnvfgm ssssgasnsa
 121 phlgfhlgsk gtsslsqqtp rfnpimvtla pniqtgrntp tslhihgvpp pvlnspqgns
 181 iyirpyittp ggttrqtqqh sgwvsqfnpm npqqvyqpsq pgpwttcpas nplshtssqq
 241 pnqqghqtsh vympissptt sqpptihssg ssqssahsqy niqnistgpr knqieiklep
 301 pqrnnssklr ssgprtssts ssvnsqtlnr nqptvyiaas ppntdelmsr sqpkvyisan
```

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 361 aatgdeqvmr nqptlfistn sgasaasrnm sgqvsmgpaf ihhhppksra ignnsatspr
 421 vvvtqpntky tfkitvspnk ppavspgvvs ptfeltnlln hpdhyveten iqhltdptla
 481 hvdrisetrk lsmgsddaay tqallvhqka rmerlqrele iqkkkldklk sevnemennl
 541 trrrlkrsns isqipsleem qqlrscnrql qididcltke idlfqargph fnpsaihnfy
 601 dnigfvgpvp pkpkdqrsii ktpktqdted degaqwncta ctflnhpali rceqcemprh
 661 f
```

SEQ ID NO: 63 Mouse TAB2 cDNA Sequence (NM_138667.3, CDS region from position 410-2491)

```
   1 gtgcgagccg gagccgagca gcgggccgcg gcgacgccgc cccggccatc gctttgatcc
  61 gcggcaggcg ccggcggcgg ggcgccccag gccgaccccc tcccctcccc ctcagccgcg
 121 agcggcggcg gcggccggag ggagttggcg gcggccgggc ggaggggccc gagcggggag
 181 ggagggaggg ctgaggtgtc ccccctgccg ggtggaatcg gcgctggcgg cggccgcggt
 241 gacgacgggg gagacggctg cccaagtggg agaggcggcg gcggcggacg aggaggaggg
 301 ggaagccgcg gcggcggcga aggaaaatgc ttggccagaa gagatgagta ctaattccac
 361 tcaggcctag aattgcctat tgagatagac ctgagcaggc gacatacgaa tggcccaagg
 421 aagccaccaa attgattttc aggttttaca tgacctgcga caaaaattcc cagaagtgcc
 481 tgaagttgtt gtatccaggt gcatgttaca gaacaataat aacctggatg cctgctgcgc
 541 tgttctctct caggagagta caaggtatct ttatggtgaa ggagacctga atttttcaga
 601 tgagtctgga atttctggtc tacgcaatca catgacttct ctcaacttgg acttgcagtc
 661 acagaatgtt taccaccatg gaagagaagg aagtcgagtg aatggaagca ggactttaac
 721 acacagcgtt agtgatggac agcttcacgg tgggcagtcc aataacgaac tgtttcagca
 781 agagccacag acagcaccag ctcaagttcc tcaaggcttt aatgtttttg gaatgcccag
 841 tacatctggt gcttcaaact caacaccgca tcttggattt cacctaggca gcaaagggac
 901 atcaaacctt tctcaacaga ctcccaggtt caatcctatt atggtaactt tagccccaaa
 961 catacagact ggccgtagta ctcctacatc tttgcacata catggtgtac ctccgcctgt
1021 actgaacagt ccacagggaa attctatcta tattaggcct tatattacaa ctcctagtgg
1081 tacagctcgg cagacacaac aacattctgg ctgggtgtct cagtttaatc ccatgaaccc
1141 tcagcaagcc tatcaacctt cacagcctgg accttggact acttatcctg catccaatcc
1201 tttgccacat acctcaaccc agcagccgaa tcagcagggc catcagacct ctcatgtcta
1261 catgcctatc agttcgccaa ctactccaca accgccaacc attcactcat ctgggagctc
1321 tcagtcttct gcccatagcc aatataacat tcagaatatt tcaacaggac ctcgaaagaa
1381 ccagatagaa atcaaacttg aacccccaca aagaaacagt tcttcaaaat tacgttcttc
1441 tggacctcga actgccagca cttcctcatt ggtcaacagc cagaccttaa atagaaatca
1501 gcccactgtt tacatagctg ccagtcctcc aaatactgat gagatgatct cccgtagtca
1561 acccaaggtc tatatttcag ccaatgccac cgcaggagat gagcaaggca tgcggaatca
1621 accgacactc ttcatatcta caaactctgg gccatctgca gcctccagga acatgtctgg
1681 gcaagtgagc atgggtcctg cctttatcca tcaccatcca cccaaaagcc gagtgttagg
1741 tggtaactct gcaacgtctc ctcgagtggt ggtcactcaa cccaacacaa aatatacttt
1801 caaaattaca gtttccccca ataagccccc tgctgtttcc ccgggggtgg tctccccaac
1861 ctttgaactt acaaatcttc taaatcatcc tgaccattat gtagaaacag agaacattca
1921 gcatctcaca gacccggctc tagcacatgt ggatagaata agcgaagccc ggaaactgag
1981 tatgggatct gatgatgctg cctacacaca agctctgctg gtgcaccaga aggccaggat
2041 ggaacggctt caaagagagc tcgagatgca aaagaaaaag ctggataaac tcaaatctga
2101 ggtcaatgag atggaaaata atctaactcg aaggcgcctg aagagatcaa attccatttc
2161 ccagataccg tcactcgaag aaatgcagca gttgagaagt tgtaatagac aactccagat
2221 tgacattgac tgcttaacca aagaaattga tctttttcaa gcccgaggac cacattttaa
2281 ccccagcgct attcataact tttatgacaa tattggattt gtaggccctg tgccaccaaa
2341 acccaaagat caaaggtcca ccatcaaagc accaaagacc caagacgcag aggatgagga
2401 aggtgctcag tggaattgca ctgcctgtac gtttctgaat cacccggcct taatccgctg
2461 tgaacagtgt gagatgcctc ggcatttctg agccaaaggc cccgccttcc tctgaaacca
2521 catctaaagt tcaagaaggt cgcctgtcat cgggaaagtg tttcaccgcc acaggagtta
2581 gtccagttga aggtgtgacg agacagtggt gtgctgtgtt gagtgtcagc ccacagagct
2641 aatgatacct caatctaatg tcatgggcag tgagattcat cacctgaaag gcctgagaga
2701 ccgggctgcc tgctgcctga ccatgtccag tgtcacgtgt cagtgcctag gtgttcccag
2761 gactcctccc ctctttgtcac cactgatcct gacgggagga agggagagac aggatgattg
2821 ctttttgtgt tgttagagct tttggtgaaa cactacatac acaaagagaa ggaacaaggt
2881 ttgactgaga actctgctgc cacatagtgc cagtagatgt gggaaacgcc acaaatctgg
2941 ggtacttttg gccctgtcct ctgtcttctg aggacatctc tgtgaagcca gcatttaggt
3001 gtgagataaa aggaaagctg tgtctgtctg tacatccaca gtgcgctccc caagcctctc
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3061 ctactgtaca gatgtctcac gtctgctttg atttctccat tatcttctta tatttctata
3121 tgtatagtgt aatagacttg tgttaactag tgtccttttc ccttttagta agcacgatca
3181 tgctcctttt aagccttacc tgagaaaagc aatgccataa ggtagaaagc attaatgaaa
3241 tgaaactgtg cacaacataa ctccctctcc ccttcctcct cctgtccttt gtcccttggt
3301 gccaatgctc tgagaagaca tgcaggtgct gcatgtgagt cactgcaaga aacactgcag
3361 ccatcggacg gtcgaggtca cgctgtacac tgacttgagc agtgggtgac caaacatgtc
3421 gttttgtctt ctccagcagc ttaaagcaga aagtatttaa acccactgcc atcttgcccc
3481 atctgtgtgg tatcccatag aactccatgg gaggctcttg ttgggaaaat gcagttttaa
3541 gaagcagcct cagcagccgt tcccgcagcc tcttcttccc gggaagaagt gtggctgtcg
3601 gactgttggc acagtcatgt gtatttataa agcatagtca tctttaggac aactgaagaa
3661 aagctggaaa aaagaaagaa aacaagcaga cttgaacact gaagcaacct cagacatctc
3721 tttattttga tgatatattt ttgtaaggaa aataaatatt catatgatca ggaatgtata
3781 taactaaatg aaatcaagaa aaaataacca tatgcatttt aaaagaatta tgaaattatc
3841 agtgcttgga atggggctaa gggaagtgct gaaatataag aaaaaaatag gaaataatgt
3901 agattgtccc actgtaaatg ttcacaagtg gctatgtttt aaataaacag gattattatg
3961 aatgagcgtt aagaaggtca gagcctggcc ttccgggctt tgcactgtga gtatggaaag
4021 agatacggca accataggtc actaggcaac agaccccaaa actcgtacat ttgatgcatt
4081 gtgttttaaa agtaggcctt gtttttcagc ttcctctgca gttctatgtg aagattgata
4141 aatcagtttt tacttgtttt attaataaaa cgtaatttgg atatcttgag ttgatggttt
4201 tgtgatttag ctgggtaaac tatctttgta acagataagt tatttataaa aattaaaaaa
4261 acttatattc taatgtgg
```

SEQ ID NO: 64 Mouse TAB2 Amino Acid Sequence (NP_619608.1)

```
   1 maqgshqidf qvlhdlrqkf pevpevvvsr cmlqnnnnld accavlsqes trylygegdl
  61 nfsdesgisg lrnhmtslnl dlqsqnvyhh gregsrvngs rtlthsvsdg qlhggqsnne
 121 lfqqepqtap aqvpqgfnvf gmpstsgasn stphlgfhlg skgtsnlsqq tprfnpimvt
 181 lapniqtgrs tptslhihgv pppvlnspqg nsiyirpyit tpsgtarqtq qhsgwvsqfn
 241 pmnpqqayqp sqpgpwttyp asnplphtst qqpnqqghqt shvympissp ttpqpptihs
 301 sgssqssahs qyniqnistg prknqieikl eppqrnsssk lrssgprtas tsslvnsqtl
 361 nrnqptvyia asppntdemi srsqpkvyis anatagdeqg mrnqptlfis tnsgpsaasr
 421 nmsgqvsmgp afihhhppks rvlggnsats prvvvtqpnt kytfkitvsp nkppavspgv
 481 vsptfeltnl lnhpdhyvet eniqhltdpa lahvdrisea rklsmgsdda aytqallvhq
 541 karmerlqre lemqkkkldk lksevnemen nltrrrlkrs nsisqipsle emqqlrscnr
 601 qlqididclt keidlfqarg phfnpsaihn fydnigfvgp vppkpkdqrs tikapktqda
 661 edeegaqwnc tactflnhpa lirceqcemp rhf
```

SEQ ID NO: 65 Human TNIP1 Transcript Variant 1 cDNA Sequence (NM_001252385.1, CDS region from position 242-2101)

```
   1 agtctccggg gactttccca ggggtggggc ggcccggcca ggcccccggc acttcctcgt
  61 cctcggcccg ggtgccctgc ccccgtccag gagccctagg agtgctacgg ggggccggag
 121 ccttgcccgg gccgctgccc cgtccctgga ttcggggctg gacgcagcaa gcggggcgct
 181 gtgtccccaa gctccccgtc ctcggccagg cgggcaccac ggcaggggct gagctaccct
 241 catggaaggg agaggaccgt accggatcta cgaccctggg ggcagcgtgc cctcaggaga
 301 ggcatccgca gcttttgagc gcctagtgaa ggagaattcc cggctgaagg aaaaaatgca
 361 agggataaag atgttagggg agcttttgga agagtcccag atggaagcga ccaggctccg
 421 gcagaaggca gaggagctag tgaaggacaa cgagctgctc ccaccacctt ctccctcctt
 481 gggctccttc gacccctgg ctgagctcac aggaaaggac tcaaatgtca cagcatctcc
 541 cacagcccct gcatgcccca gtgacaagcc agcaccagtc cagaagcctc catccagtgg
 601 cacctcctct gaatttgaag tggtcactcc tgaggagcag aattcaccag agagcagcag
 661 ccatgccaat gcgatggcgc tgggccccct gccccgtgag gacggcaacc tgatgctgca
 721 cctgcagcgc ctggagacca cgctgagtgt gtgtgccgag gagccggacc acggccagct
 781 cttcacccac ctgggccgca tggccctgga gttcaaccga ctggcatcca aggtgcacaa
 841 gaatgagcag cgcacctcca ttctgcagac cctgtgtgag cagcttcgga aggagaacga
 901 ggctctgaag gccaagttgg ataagggcct ggaacagcgg gatcaggctg ccgagaggct
 961 gcgggaggaa aatttggagc tcaagaagtt gttgatgagc aatggcaaca aagagggtgc
1021 gtctgggcgg ccaggctcac cgaagatgga agggacaggc aagaaggcag tggctggaca
1081 gcagcaggct agtgtgacgg caggtaaggt cccagaggtg gtggccttgg gcgcagccga
1141 gaagaaggtg aagatgctgg agcagcagcg cagtgagctg ctggaagtga acaagcagtg
1201 ggaccagcat ttccggtcca tgaagcagca gtatgagcag aagatcactg agctgcgtca
1261 gaagctggct gatttgcaga agcaggtgac tgacctggag gccgagcggg agcagaagca
```

```
1321 gcgtgacttt gaccgcaagc tcctcctggc caagtccaag attgaaatgg aggagaccga
1381 caaggagcag ctgacagcag aggccaagga gctgcgccaa aaggtcaagt acctgcagga
1441 tcagctgagc ccactcaccc gacagcgtga gtaccaggaa aaggagatcc agcggctcaa
1501 caaggccctg gaggaagcac tgagcatcca aaccccgcca tcatctccac caacagcatt
1561 tgggagccca gaaggagcag gggccctcct aaggaaacag gagctggtca cgcagaatga
1621 gttgctgaaa cagcaggtga agatcttcga ggaggacttc cagagggagc gcagtgatcg
1681 tgagcgcatg aatgaggaga aggaagagct gaagaagcaa gtggagaagc tgcaggccca
1741 ggtcaccctg tcaaatgccc agctaaaagc attcaaagat gaggagaagg caagagaagc
1801 cctcagacag cagaagagga aagcaaaggc ctcaggagag cgttaccatg tggagcccca
1861 cccagaacat ctctgcgggg cctaccccta cgcctacccg cccatgccag ccatggtgcc
1921 acaccatggc ttcgaggact ggtcccagat ccgctacccc cctcccccca tggccatgga
1981 gcacccgccc ccactcccca actcgcgcct cttccatctg caccagtttt gtcgcagccg
2041 gaatacacct ggcgtctacc ctgtggaggg gttcgaaatc caaatcagag ctcccaagtg
2101 atggaccctc ccacagccag gcctacagaa ccagagtctc caaaaaatga ccgtgagggg
2161 cctcagtgag accagattgt gtcatttggc tccaccttca tcttgcagag ccagctgatc
2221 tcagattgcc aagaaactag aagccacttg cacggtgtgg ccagagcctc agctggatga
2281 gaggctgaga tgggtggcca gcttgtacac cagtccctga actgagctgt ttacaggact
2341 ggggaggctc cacccagaag gctttcattt gtactctgct gggagtgact gggaaaaact
2401 ccttccctgc tgctgagtgg agagaggcct catccggctt tgacccacca tccgttgcag
2461 aagcctccag gagcagcaat cctaagagtg ggaggcagcc aagacccccc tccttcaaaa
2521 cctcccggaa gtggtttcag gccctctagt tgccatgacc aatttgtgtg tgtgtttaat
2581 ttttgcttca agctctgtag caggacctgc cccacgcaca cccctacccc tctgtgagga
2641 gctgtgggaa gtgtgggttt gtctccagaa cagaagagaa tgatggatat tctggctctg
2701 gggccctctc caccaccact cacagtagcc ttgctgaagc catcacagat gggagaaggc
2761 catgccagcc acgtccgccg aggggcgcca gcctgaagct gccaggccct gaggttcaga
2821 ccctggaccc catagctgga ggcctgtggt gccagaagcc cagattaggg tggctgtcca
2881 tccctggata gctatttgca cgaatcatgg acataaatcc aagttgaaga agatcaacaa
2941 aaaaaaaaaa1
```

SEQ ID NO: 66 Human TNIP1 Isoform 1 Amino Acid Sequence (NP_001239314.1)

```
   1 megrgpyriy dpggsvpsge asaaferlvk ensrlkekmq gikmlgelle esqmeatrlr
  61 qkaeelvkdn ellpppspsl gsfdplaelt gkdsnvtasp tapacpsdkp apvqkppssg
 121 tssefevvtp eeqnspesss hanamalgpl predgnlmlh lqrlettlsv caeepdhgql
 181 fthlgrmale fnrlaskvhk neqrtsilqt lceqlrkene alkakldkgl eqrdqaaerl
 241 reenlelkkl lmsngnkega sgrpgspkme gtgkkavagq qqasvtagkv pevvalgaae
 301 kkvkmleqqr sellevnkqw dqhfrsmkqq yeqkitelrq kladlqkqvt dleaereqkq
 361 rdfdrkllla kskiemeetd keqltaeake lrqkvkylqd qlspltrqre yqekeiqrln
 421 kaleealsiq tppsspptaf gspegagall rkqelvtqne llkqqvkife edfqrersdr
 481 ermneekeel kkqveklqaq vtlsnaqlka fkdeekarea lrqqkrkaka sgeryhveph
 541 pehlcgaypy ayppmpamvp hhgfedwsqi ryppppmame hppplpnsrl fhlhqfcrsr
 601 ntpgvypveg feiqirapk
```

SEQ ID NO: 67 Human TNIP1 Transcript Variant 2 cDNA Sequence (NM_001252386.1, CDS region from position 229-1980)

```
   1 agtctccggg gactttccca ggggtggggc ggcccggcca ggcccccggc acttcctcgt
  61 cctcggcccg ggtgccctgc ccccgtccag gagccctagg agtgctacgg ggggccggag
 121 ccttgcccgg gccgctgccc cgtccctgga ttcggggctg gacgcagcaa gcggggcgct
 181 gtgtccccaa gctccccgtc ctcgggggag cttttggaag agtcccagat ggaagcgacc
 241 aggctccggc agaaggcaga ggagctagtg aaggacaacg agctgctccc accaccttct
 301 ccctccttgg gctccttcga ccccctggct gagctcacag gaaaggactc aaatgtcaca
 361 gcatctccca cagcccctgc atgccccagt gacaagccag caccagtcca gaagcctcca
 421 tccagtggca cctcctctga atttgaagtg gtcactcctg aggagcagaa ttcaccagag
 481 agcagcagcc atgccaatgc gatggcgctg ggccccctgc cccgtgagga cggcaacctg
 541 atgctgcacc tgcagcgcct ggagaccacg ctgagtgtgt gtgccgagga gccggaccac
 601 ggccagctct tcacccacct gggccgcatg gccctggagt tcaaccgact ggcatccaag
 661 gtgcacaaga atgagcagcg cacctccatt ctgcagaccc tgtgtgagca gcttcggaag
 721 gagaacgagg ctctgaaggc caagttggat aagggcctgg aacagcggga tcaggctgcc
 781 gagaggctgc gggaggaaaa tttggagctc aagaagttgt tgatgagcaa tggcaacaaa
 841 gagggtgcgt ctgggcggcc aggctcaccg aagatggaag ggacaggcaa gaaggcagtg
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```
 901 gctggacagc agcaggctag tgtgacggca ggtaaggtcc cagaggtggt ggccttgggc
 961 gcagccgaga agaaggtgaa gatgctggag cagcagcgca gtgagctgct ggaagtgaac
1021 aagcagtggg accagcattt ccggtccatg aagcagcagt atgagcagaa gatcactgag
1081 ctgcgtcaga agctggctga tttgcagaag caggtgactg acctggaggc cgagcgggag
1141 cagaagcagc gtgactttga ccgcaagctc ctcctggcca agtccaagat tgaaatggag
1201 gagaccgaca aggagcagct gacagcagag gccaaggagc tgcgccaaaa ggtcaagtac
1261 ctgcaggatc agctgagccc actcacccga cagcgtgagt accaggaaaa ggagatccag
1321 cggctcaaca aggccctgga ggaagcactg agcatccaaa ccccgccatc atctccacca
1381 acagcatttg ggagcccaga aggagcaggg gccctcctaa ggaaacagga gctggtcacg
1441 cagaatgagt tgctgaaaca gcaggtgaag atcttcgagg aggacttcca gagggagcgc
1501 agtgatcgtg agcgcatgaa tgaggagaag gaagagctga agaagcaagt ggagaagctg
1561 caggcccagg tcaccctgtc aaatgcccag ctaaaagcat tcaaagatga ggagaaggca
1621 agagaagccc tcagacagca gaagaggaaa gcaaaggcct caggagagcg ttaccatgtg
1681 gagccccacc cagaacatct ctgcggggcc tacccctacg cctacccgcc catgccagcc
1741 atggtgccac accatggctt cgaggactgg tcccagatcc gctacccccc tccccccatg
1801 gccatggagc acccgccccc actccccaac tcgcgcctct tccatctgcc ggaatacacc
1861 tggcgtctac cctgtggagg ggttcgaaat ccaaatcaga gctcccaagt gatggaccct
1921 cccacagcca ggcctacaga accagagtct ccaaaaaatg accgtgaggg gcctcagtga
1981 gaccagattg tgtcatttgg ctccaccttc atcttgcaga gccagctgat ctcagattgc
2041 caagaaacta gaagccactt gcacggtgtg gccagagcct cagctggatg agaggctgag
2101 atgggtggcc agcttgtaca ccagtccctg aactgagctg tttacaggac tggggaggct
2161 ccacccagaa ggctttcatt tgtactctgc tgggagtgac tgggaaaaac tccttccctg
2221 ctgctgagtg gagagaggcc tcatccggct ttgacccacc atccgttgca gaagcctcca
2281 ggagcagcaa tcctaagagt gggaggcagc caagaccccc ttccttcaaa acctcccgga
2341 agtggtttca ggccctctag ttgccatgac caatttgtgt gtgtgtttaa tttttgcttc
2401 aagctctgta gcaggacctg ccccacgcac acccctaccc ctctgtgagg agctgtggga
2461 agtgtgggtt tgtctccaga acagaagaga atgatggata ttctggctct ggggccctct
2521 ccaccaccac tcacagtagc cttgctgaag ccatcacaga tgggagaagg ccatgccagc
2581 cacgtccgcc gaggggcgcc agcctgaagc tgccaggccc tgaggttcag accctggacc
2641 ccatagctgg aggcctgtgg tgccagaagc ccagattagg gtggctgtcc atccctggat
2701 agctatttgc acgaatcatg gacataaatc caagttgaag aagatcaaca aaaaaaaaaa
2761 a
```

SEQ ID NO: 68 Human TNIP1 Isoform 2 Amino Acid Sequence (NP_001239315.1)

```
   1 meatrlrqka eelvkdnell pppspslgsf dplaeltgkd snvtasptap acpsdkpapv
  61 qkppssgtss efevvtpeeq nspessshan amalgplpre dgnlmlhlqr lettlsvcae
 121 epdhgqlfth lgrmalefnr laskvhkneq rtsilqtlce qlrkenealk akldkgleqr
 181 dqaaerlree nlelkkllms ngnkegasgr pgspkmegtg kkavagqqqa svtagkvpev
 241 valgaaekkv kmleqqrsel levnkqwdqh frsmkqqyeq kitelrqkla dlqkqvtdle
 301 aereqkqrdf drklllaksk iemeetdkeq ltaeakelrq kvkylqdqls pltrqreyqe
 361 keiqrlnkal eealsiqtpp sspptafgsp egagallrkq elvtqnellk qqvkifeedf
 421 qrersdrerm neekeelkkq veklqaqvtl snaqlkafkd eekarealrq qkrkakasge
 481 ryhvephpeh lcgaypyayp pmpamvphhg fedwsqiryp pppmamehpp plpnsrlfhl
 541 peytwrlpcg gvrnpnqssq vmdpptarpt epespkndre gpq
```

SEQ ID NO: 69 Human TNIP1 Transcript Variant 3 cDNA Sequence (NM_001252390.1, CDS region from position 107-2017)

```
   1 atgcaccaag gaaggagtgg gccccttctt cactatggat ggagaagcct cagagagtaa
  61 gtggcaacag ccaggcgggc accacggcag gggctgagct accctcatgg aagggagagg
 121 accgtaccgg atctacgacc ctgggggcag cgtgccctca ggagaggcat ccgcagcttt
 181 tgagcgccta gtgaaggaga attcccggct gaaggaaaaa atgcaaggga taaagatgtt
 241 aggggagctt ttggaagagt cccagatgga agcgaccagg ctccggcaga aggcagagga
 301 gctagtgaag gacaacgagc tgctcccacc accttctccc tccttgggct ccttcgaccc
 361 cctggctgag ctcacaggaa aggactcaaa tgtcacagca tctcccacag cccctgcatg
 421 ccccagtgac aagccagcac cagtccagaa gcctccatcc agtggcacct cctctgaatt
 481 tgaagtggtc actcctgagg agcagaattc accagagagc agcagccatg ccaatgcgat
 541 ggcgctgggc cccctgcccc gtgaggacgg caacctgatg ctgcacctgc agcgcctgga
 601 gaccacgctg agtgtgtgtg ccgaggagcc ggaccacggc cagctcttca cccacctggg
 661 ccgcatggcc ctggagttca accgactggc atccaaggtg cacaagaatg agcagcgcac
```

```
 721 ctccattctg cagaccctgt gtgagcagct tcggaaggag aacgaggctc tgaaggccaa
 781 gttggataag ggcctggaac agcgggatca ggctgccgag aggctgcggg aggaaaattt
 841 ggagctcaag aagttgttga tgagcaatgg caacaaagag ggtgcgtctg ggcggccagg
 901 ctcaccgaag atggaaggga caggcaagaa ggcagtggct ggacagcagc aggctagtgt
 961 gacggcaggt aaggtcccag aggtggtggc cttgggcgca gccgagaaga aggtgaagat
1021 gctggagcag cagcgcagtg agctgctgga agtgaacaag cagtgggacc agcatttccg
1081 gtccatgaag cagcagtatg agcagaagat cactgagctg cgtcagaagc tggctgattt
1141 gcagaagcag gtgactgacc tggaggccga gcgggagcag aagcagcgtg actttgaccg
1201 caagctcctc ctggccaagt ccaagattga aatggaggag accgacaagg agcagctgac
1261 agcagaggcc aaggagctgc gccaaaaggt caagtacctg caggatcagc tgagcccact
1321 cacccgacag cgtgagtacc aggaaaagga gatccagcgg ctcaacaagg ccctggagga
1381 agcactgagc atccaaaccc cgccatcatc tccaccaaca gcatttggga gcccagaagg
1441 agcaggggcc ctcctaagga aacaggagct ggtcacgcag aatgagttgc tgaaacagca
1501 ggtgaagatc ttcgaggagg acttccagag ggagcgcagt gatcgtgagc gcatgaatga
1561 ggagaaggaa gagctgaaga agcaagtgga gaagctgcag gcccaggtca ccctgtcaaa
1621 tgcccagcta aaagcattca aagatgagga gaaggcaaga gaagccctca gacagcagaa
1681 gaggaaagca aaggcctcag gagagcgtta ccatgtggag ccccacccag aacatctctg
1741 cggggcctac ccctacgcct acccgcccat gccagccatg gtgccacacc atggcttcga
1801 ggactggtcc cagatccgct acccccctcc ccccatggcc atggagcacc cgcccccact
1861 ccccaactcg cgcctcttcc atctgccgga atacacctgg cgtctaccct gtggaggggt
1921 tcgaaatcca aatcagagct cccaagtgat ggaccctccc acagccaggc ctacagaacc
1981 agagtctcca aaaaatgacc gtgaggggcc tcagtgagac cagattgtgt catttggctc
2041 caccttcatc ttgcagagcc agctgatctc agattgccaa gaaactagaa gccacttgca
2101 cggtgtggcc agagcctcag ctggatgaga ggctgagatg ggtggccagc ttgtacacca
2161 gtccctgaac tgagctgttt acaggactgg ggaggctcca cccagaaggc tttcatttgt
2221 actctgctgg gagtgactgg gaaaaactcc ttccctgctg ctgagtggag agaggcctca
2281 tccggctttg acccaccatc cgttgcagaa gcctccagga gcagcaatcc taagagtggg
2341 aggcagccaa gacccccttc cttcaaaacc tcccggaagt ggtttcaggc cctctagttg
2401 ccatgaccaa tttgtgtgtg tgtttaattt ttgcttcaag ctctgtagca ggacctgccc
2461 cacgcacacc cctacccctc tgtgaggagc tgtgggaagt gtgggtttgt ctccagaaca
2521 gaagagaatg atggatattc tggctctggg gccctctcca ccaccactca cagtagcctt
2581 gctgaagcca tcacagatgg gagaaggcca tgccagccac gtccgccgag gggcgccagc
2641 ctgaagctgc caggccctga ggttcagacc ctggacccca tagctggagg cctgtggtgc
2701 cagaagccca gattagggtg gctgtccatc cctggatagc tatttgcacg aatcatggac
2761 ataaatccaa gttgaagaag atcaacaaaa aaaaaaaa
```

SEQ ID NO: 70 Human TNIP1 Transcript Variant 4 cDNA Sequence (NM_001252391.1, CDS region from position 139-2049)

```
   1 agcccagccc tccttcctgc agaagcacag tgagccgagg agccttcata gggacagccg
  61 cccctggtgc acacaccctc gtattctcct gcccttcccc agccaggcgg gcaccacggc
 121 aggggctgag ctaccctcat ggaagggaga ggaccgtacc ggatctacga ccctgggggc
 181 agcgtgccct caggagaggc atccgcagct tttgagcgcc tagtgaagga gaattcccgg
 241 ctgaaggaaa aaatgcaagg gataaagatg ttaggggagc ttttggaaga gtcccagatg
 301 gaagcgacca ggctccggca gaaggcagag gagctagtga aggacaacga gctgctccca
 361 ccaccttctc cctccttggg ctccttcgac cccctggctg agctcacagg aaaggactca
 421 aatgtcacag catctcccac agcccctgca tgccccagtg acaagccagc accagtccag
 481 aagcctccat ccagtggcac ctcctctgaa tttgaagtgg tcactcctga ggagcagaat
 541 tcaccagaga gcagcagcca tgccaatgcg atggcgctgg gccccctgcc ccgtgaggac
 601 ggcaacctga tgctgcacct gcagcgcctg gagaccacgc tgagtgtgtg tgccgaggag
 661 ccggaccacg gccagctctt cacccacctg ggccgcatgg ccctggagtt caaccgactg
 721 gcatccaagg tgcacaagaa tgagcagcgc acctccattc tgcagaccct gtgtgagcag
 781 cttcggaagg agaacgaggc tctgaaggcc aagttggata agggcctgga acagcgggat
 841 caggctgccg agaggctgcg ggaggaaaat ttggagctca agaagttgtt gatgagcaat
 901 ggcaacaaag agggtgcgtc tgggcggcca ggctcaccga agatggaagg gacaggcaag
 961 aaggcagtgg ctggacagca gcaggctagt gtgacggcag gtaaggtccc agaggtggtg
1021 gccttgggcg cagccgagaa gaaggtgaag atgctggagc agcagcgcag tgagctgctg
1081 gaagtgaaca agcagtggga ccagcatttc cggtccatga agcagcagta tgagcagaag
1141 atcactgagc tgcgtcagaa gctggctgat ttgcagaagc aggtgactga cctggaggcc
1201 gagcgggagc agaagcagcg tgactttgac cgcaagctcc tcctggccaa gtccaagatt
1261 gaaatggagg agaccgacaa ggagcagctg acagcagagg ccaaggagct gcgccaaaag
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```
1321 gtcaagtacc tgcaggatca gctgagccca ctcacccgac agcgtgagta ccaggaaaag
1381 gagatccagc ggctcaacaa ggccctggag gaagcactga gcatccaaac cccgccatca
1441 tctccaccaa cagcatttgg gagcccagaa ggagcagggg ccctcctaag gaaacaggag
1501 ctggtcacgc agaatgagtt gctgaaacag caggtgaaga tcttcgagga ggacttccag
1561 agggagcgca gtgatcgtga gcgcatgaat gaggagaagg aagagctgaa gaagcaagtg
1621 gagaagctgc aggcccaggt caccctgtca aatgcccagc taaaagcatt caaagatgag
1681 gagaaggcaa gagaagccct cagacagcag aagaggaaag caaaggcctc aggagagcgt
1741 taccatgtgg agccccaccc agaacatctc tgcggggcct acccctacgc ctacccgccc
1801 atgccagcca tggtgccaca ccatggcttc gaggactggt cccagatccg ctacccccct
1861 ccccccatgg ccatggagca cccgccccca ctccccaact cgcgcctctt ccatctgccg
1921 gaatacacct ggcgtctacc ctgtggaggg gttcgaaatc caaatcagag ctcccaagtg
1981 atggaccctc ccacagccag gcctacagaa ccagagtctc caaaaaatga ccgtgagggg
2041 cctcagtgag accagattgt gtcatttggc tccaccttca tcttgcagag ccagctgatc
2101 tcagattgcc aagaaactag aagccacttg cacggtgtgg ccagagcctc agctggatga
2161 gaggctgaga tgggtggcca gcttgtacac cagtccctga actgagctgt ttacaggact
2221 ggggaggctc cacccagaag gctttcattt gtactctgct gggagtgact gggaaaaact
2281 ccttccctgc tgctgagtgg agagaggcct catccggctt tgacccacca tccgttgcag
2341 aagcctccag gagcagcaat cctaagagtg ggaggcagcc aagacccccc tccttcaaaa
2401 cctcccggaa gtggtttcag gccctctagt tgccatgacc aatttgtgtg tgtgtttaat
2461 ttttgcttca agctctgtag caggacctgc cccacgcaca cccctacccc tctgtgagga
2521 gctgtgggaa gtgtgggttt gtctccagaa cagaagagaa tgatggatat tctggctctg
2581 gggccctctc caccaccact cacagtagcc ttgctgaagc catcacagat gggagaaggc
2641 catgccagcc acgtccgccg aggggcgcca gcctgaagct gccaggccct gaggttcaga
2701 ccctggaccc catagctgga ggcctgtggt gccagaagcc cagattaggg tggctgtcca
2761 tccctggata gctatttgca cgaatcatgg acataaatcc aagttgaaga agatcaacaa
2821 aaaaaaaaaa
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SEQ ID NO: 71 Human TNIP1 Transcript Variant 5 cDNA Sequence (NM_006058.4, CDS region from position 242-2152)

```
   1 agtctccggg gactttccca ggggtggggc ggcccggcca ggcccccggc acttcctcgt
  61 cctcggcccg ggtgccctgc ccccgtccag gagccctagg agtgctacgg ggggccggag
 121 ccttgcccgg gccgctgccc cgtccctgga ttcggggctg gacgcagcaa gcggggcgct
 181 gtgtccccaa gctccccgtc ctcggccagg cgggcaccac ggcaggggct gagctaccct
 241 catggaaggg agaggaccgt accggatcta cgaccctggg ggcagcgtgc cctcaggaga
 301 ggcatccgca gcttttgagc gcctagtgaa ggagaattcc cggctgaagg aaaaaatgca
 361 agggataaag atgttagggg agcttttgga agagtcccag atggaagcga ccaggctccg
 421 gcagaaggca gaggagctag tgaaggacaa cgagctgctc ccaccacctt ctccctcctt
 481 gggctccttc gacccctgg ctgagctcac aggaaaggac tcaaatgtca cagcatctcc
 541 cacagcccct gcatgcccca gtgacaagcc agcaccagtc cagaagcctc catccagtgg
 601 cacctcctct gaatttgaag tggtcactcc tgaggagcag aattcaccag agagcagcag
 661 ccatgccaat gcgatggcgc tgggcccccc gccccgtgag gacggcaacc tgatgctgca
 721 cctgcagcgc ctggagacca cgctgagtgt gtgtgccgag gagccggacc acggccagct
 781 cttcacccac ctgggccgca tggccctgga gttcaaccga ctggcatcca aggtgcacaa
 841 gaatgagcag cgcacctcca ttctgcagac cctgtgtgag cagcttcgga aggagaacga
 901 ggctctgaag gccaagttgg ataagggcct ggaacagcgg gatcaggctg ccgagaggct
 961 gcgggaggaa aatttggagc tcaagaagtt gttgatgagc aatggcaaca aagagggtgc
1021 gtctgggcgg ccaggctcac cgaagatgga agggacaggc aagaaggcag tggctggaca
1081 gcagcaggct agtgtgacgg caggtaaggt cccagaggtg gtggccttgg gcgcagccga
1141 gaagaaggtg aagatgctgg agcagcagcg cagtgagctg ctggaagtga acaagcagtg
1201 ggaccagcat ttccggtcca tgaagcagca gtatgagcag aagatcactg agctgcgtca
1261 gaagctggct gatttgcaga agcaggtgac tgacctggag gccgagcggg agcagaagca
1321 gcgtgacttt gaccgcaagc tcctcctggc caagtccaag attgaaatgg aggagaccga
1381 caaggagcag ctgacagcag aggccaagga gctgcgccaa aaggtcaagt acctgcagga
1441 tcagctgagc ccactcaccc gacagcgtga gtaccaggaa aaggagatcc agcggctcaa
1501 caaggccctg gaggaagcac tgagcatcca aaccccgcca tcatctccac caacagcatt
1561 tgggagccca gaaggagcag gggccctcct aaggaaacag gagctggtca cgcagaatga
1621 gttgctgaaa cagcaggtga agatcttcga ggaggacttc cagagggagc gcagtgatcg
1681 tgagcgcatg aatgaggaga aggaagagct gaagaagcaa gtggagaagc tgcaggccca
1741 ggtcaccctg tcaaatgccc agctaaaagc attcaaagat gaggagaagg caagagaagc
1801 cctcagacag cagaagagga aagcaaaggc ctcaggagag cgttaccatg tggagcccca
```

```
1861 cccagaacat ctctgcgggg cctaccccta cgcctacccg cccatgccag ccatggtgcc
1921 acaccatggc ttcgaggact ggtcccagat ccgctacccc cctcccccca tggccatgga
1981 gcacccgccc ccactcccca actcgcgcct cttccatctg ccggaataca cctggcgtct
2041 accctgtgga ggggttcgaa atccaaatca gagctcccaa gtgatggacc ctcccacagc
2101 caggcctaca gaaccagagt ctccaaaaaa tgaccgtgag gggcctcagt gagaccagat
2161 tgtgtcattt ggctccacct tcatcttgca gagccagctg atctcagatt gccaagaaac
2221 tagaagccac ttgcacggtg tggccagagc ctcagctgga tgagaggctg agatgggtgg
2281 ccagcttgta caccagtccc tgaactgagc tgtttacagg actggggagg ctccacccag
2341 aaggctttca tttgtactct gctgggagtg actgggaaaa actccttccc tgctgctgag
2401 tggagagagg cctcatccgg ctttgaccca ccatccgttg cagaagcctc caggagcagc
2461 aatcctaaga gtgggaggca gccaagaccc ccttccttca aaacctcccg gaagtggttt
2521 caggccctct agttgccatg accaatttgt gtgtgtgttt aatttttgct tcaagctctg
2581 tagcaggacc tgccccacgc acacccctac ccctctgtga ggagctgtgg gaagtgtggg
2641 tttgtctcca gaacagaaga gaatgatgga tattctggct ctggggccct ctccaccacc
2701 actcacagta gccttgctga agccatcaca gatgggagaa ggccatgcca gccacgtccg
2761 ccgaggggcg ccagcctgaa gctgccaggc cctgaggttc agaccctgga ccccatagct
2821 ggaggcctgt ggtgccagaa gcccagatta gggtggctgt ccatccctgg atagctattt
2881 gcacgaatca tggacataaa tccaagttga agaagatcaa caaaaaaaaa aaa
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SEQ ID NO: 72 Human TNIP1 Transcript Variant 8 cDNA Sequence (NM_001258454.1, CDS region from position 242-2152)

```
   1 tgccagtctc cggggacttt cccaggggtg gggcggcccg gccaggcccc cggcacttcc
  61 tcgtcctcgg cccgggtgcc ctgcccccgt ccaggagccc taggagtgct acgggggccc
 121 ggagccttgc ccgggccgct gccccgtccc tggattcggg gctggacgca gcaagcgggg
 181 cgctgtgtcc ccaagctccc cgtcctcggg cgggcaccac ggcaggggct gagctaccct
 241 catggaaggg agaggaccgt accggatcta cgaccctggg ggcagcgtgc cctcaggaga
 301 ggcatccgca gcttttgagc gcctagtgaa ggagaattcc cggctgaagg aaaaaatgca
 361 agggataaag atgttagggg agcttttgga agagtcccag atggaagcga ccaggctccg
 421 gcagaaggca gaggagctag tgaaggacaa cgagctgctc ccaccacctt ctccctcctt
 481 gggctccttc gacccccctgg ctgagctcac aggaaaggac tcaaatgtca cagcatctcc
 541 cacagccccct gcatgcccca gtgacaagcc agcaccagtc cagaagcctc catccagtgg
 601 cacctcctct gaatttgaag tggtcactcc tgaggagcag aattcaccag agagcagcag
 661 ccatgccaat gcgatggcgc tgggcccccct gccccgtgag gacggcaacc tgatgctgca
 721 cctgcagcgc ctggagacca cgctgagtgt gtgtgccgag gagccggacc acggccagct
 781 cttcacccac ctgggccgca tggccctgga gttcaaccga ctggcatcca aggtgcacaa
 841 gaatgagcag cgcacctcca ttctgcagac cctgtgtgag cagcttcgga aggagaacga
 901 ggctctgaag gccaagttgg ataagggcct ggaacagcgg gatcaggctg ccgagaggct
 961 gcgggaggaa aatttggagc tcaagaagtt gttgatgagc aatggcaaca aagagggtgc
1021 gtctgggcgg ccaggctcac cgaagatgga agggacaggc aagaaggcag tggctggaca
1081 gcagcaggct agtgtgacgg caggtaaggt cccagaggtg gtggccttgg gcgcagccga
1141 gaagaaggtg aagatgctgg agcagcagcg cagtgagctg ctggaagtga acaagcagtg
1201 ggaccagcat ttccggtcca tgaagcagca gtatgagcag aagatcactg agctgcgtca
1261 gaagctggct gatttgcaga agcaggtgac tgacctggag gccgagcggg agcagaagca
1321 gcgtgacttt gaccgcaagc tcctcctggc caagtccaag attgaaatgg aggagaccga
1381 caaggagcag ctgacagcag aggccaagga gctgcgccaa aaggtcaagt acctgcagga
1441 tcagctgagc ccactcaccc gacagcgtga gtaccaggaa aaggagatcc agcggctcaa
1501 caaggccctg gaggaagcac tgagcatcca aaccccgcca tcatctccac caacagcatt
1561 tgggagccca gaaggagcag gggccctcct aaggaaacag gagctggtca cgcagaatga
1621 gttgctgaaa cagcaggtga agatcttcga ggaggacttc cagagggagc gcagtgatcg
1681 tgagcgcatg aatgaggaga aggaagagct gaagaagcaa gtggagaagc tgcaggccca
1741 ggtcaccctg tcaaatgccc agctaaaagc attcaaagat gaggagaagg caagagaagc
1801 cctcagacag cagaagagga aagcaaaggc ctcaggagag cgttaccatg tggagcccca
1861 cccagaacat ctctgcgggg cctaccccta cgcctacccg cccatgccag ccatggtgcc
1921 acaccatggc ttcgaggact ggtcccagat ccgctacccc cctcccccca tggccatgga
1981 gcacccgccc ccactcccca actcgcgcct cttccatctg ccggaataca cctggcgtct
2041 accctgtgga ggggttcgaa atccaaatca gagctcccaa gtgatggacc ctcccacagc
2101 caggcctaca gaaccagagt ctccaaaaaa tgaccgtgag gggcctcagt gagaccagat
2161 tgtgtcattt ggctccacct tcatcttgca gagccagctg atctcagatt gccaagaaac
2221 tagaagccac ttgcacggtg tggccagagc ctcagctgga tgagaggctg agatgggtgg
2281 ccagcttgta caccagtccc tgaactgagc tgtttacagg actggggagg ctccacccag
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2341 aaggctttca tttgtactct gctgggagtg actgggaaaa actccttccc tgctgctgag
2401 tggagagagg cctcatccgg ctttgaccca ccatccgttg cagaagcctc caggagcagc
2461 aatcctaaga gtgggaggca gccaagaccc ccttccttca aaacctcccg gaagtggttt
2521 caggccctct agttgccatg accaatttgt gtgtgtgttt aatttttgct tcaagctctg
2581 tagcaggacc tgccccacgc acacccctac ccctctgtga ggagctgtgg gaagtgtggg
2641 tttgtctcca gaacagaaga gaatgatgga tattctggct ctggggccct ctccaccacc
2701 actcacagta gccttgctga agccatcaca gatgggagaa ggccatgcca gccacgtccg
2761 ccgaggggcg ccagcctgaa gctgccaggc cctgaggttc agaccctgga ccccatagct
2821 ggaggcctgt ggtgccagaa gcccagatta gggtggctgt ccatccctgg atagctattt
2881 gcacgaatca tggacataaa tccaagttga agaagatcaa ca
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SEQ ID NO: 73 Human TNIP1 Isoform 3 Amino Acid Sequence (NP_001239319.1)

```
   1 megrgpyriy dpggsvpsge asaaferlvk ensrlkekmq gikmlgelle esqmeatrlr
  61 qkaeelvkdn ellpppspsl gsfdplaelt gkdsnvtasp tapacpsdkp apvqkppssg
 121 tssefevvtp eeqnspesss hanamalgpl predgnlmlh lqrlettlsv caeepdhgql
 181 fthlgrmale fnrlaskvhk neqrtsilqt lceqlrkene alkakldkgl eqrdqaaerl
 241 reenlelkkl lmsngnkega sgrpgspkme gtgkkavagq qqasvtagkv pevvalgaae
 301 kkvkmleqqr sellevnkqw dqhfrsmkqq yeqkitelrq kladlqkqvt dleaereqkq
 361 rdfdrkllla kskiemeetd keqltaeake lrqkvkylqd qlspltrqre yqekeiqrln
 421 kaleealsiq tppsspptaf gspegagall rkqelvtqne llkqqvkife edfqrersdr
 481 ermneekeel kkqveklqaq vtlsnaqlka fkdeekarea lrqqkrkaka sgeryhveph
 541 pehlcgaypy ayppmpamvp hhgfedwsqi ryppppmame hppplpnsrl fhlpeytwrl
 601 pcggvrnpnq ssqvmdppta rptepespkn dregpq
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SEQ ID NO: 74 Human TNIP1 Transcript Variant 6 cDNA Sequence (NM_001252392.1, CDS region from position 139-2046)

```
   1 agcccagccc tccttcctgc agaagcacag tgagccgagg agccttcata gggacagccg
  61 cccctggtgc acacaccctc gtattctcct gcccttcccc agccaggcgg gcaccacggc
 121 aggggctgag ctaccctcat ggaagggaga ggaccgtacc ggatctacga ccctgggggc
 181 agcgtgccct caggagaggc atccgcagct tttgagcgcc tagtgaagga gaattcccgg
 241 ctgaaggaaa aaatgcaagg gataaagatg ttaggggagc ttttggaaga gtcccagatg
 301 gaagcgacca ggctccggca gaaggcagag gagctagtga aggacaacga gctgctccca
 361 ccaccttctc cctccttggg ctccttcgac cccctggctg agctcacagg aaaggactca
 421 aatgtcacag catctcccac agcccctgca tgccccagtg acaagccagc accagtccag
 481 aagcctccat ccagtggcac ctcctctgaa tttgaagtgg tcactcctga ggagcagaat
 541 tcaccagaga gcagcagcca tgccaatgcg atggcgctgg gccccctgcc ccgtgaggac
 601 ggcaacctga tgctgcacct gcagcgcctg gagaccacgc tgagtgtgtg tgccgaggag
 661 ccggaccacg gccagctctt cacccacctg ggccgcatgg ccctggagtt caaccgactg
 721 gcatccaagg tgcacaagaa tgagcagcgc acctccattc tgcagaccct gtgtgagcag
 781 cttcggaagg agaacgaggc tctgaaggcc aagttggata agggcctgga acagcgggat
 841 caggctgccg agaggctgcg ggaggaaaat ttggagctca agaagttgtt gatgagcaat
 901 ggcaacaaag agggtgcgtc tgggcggcca ggctcaccga agatggaagg gacaggcaag
 961 aaggcagtgg ctggacagca gcaggctagt gtgacggcag gtaaggtccc agaggtggtg
1021 gccttgggcg cagccgagaa gaaggtgaag atgctggagc agcagcgcag tgagctgctg
1081 gaagtgaaca agcagtggga ccagcatttc cggtccatga agcagcagta tgagcagaag
1141 atcactgagc tgcgtcagaa gctggctgat ttgcagaagc aggtgactga cctggaggcc
1201 gagcgggagc agaagcagcg tgactttgac cgcaagctcc tcctggccaa gtccaagatt
1261 gaaatggagg agaccgacaa ggagcagctg acagcagagg ccaaggagct gcgccaaaag
1321 gtcaagtacc tgcaggatca gctgagccca ctcacccgac agcgtgagta ccaggaaaag
1381 gagatccagc ggctcaacaa ggccctggag gaagcactga gcatccaaac cccgccatca
1441 tctccaccaa cagcatttgg gagcccagaa ggagcagggg ccctcctaag gaaacaggag
1501 ctggtcacgc agaatgagtt gctgaaacag caggtgaaga tcttcgagga ggacttccag
1561 agggagcgca gtgatcgtga gcgcatgaat gaggagaagg aagagctgaa gaagcaagtg
1621 gagaagctgc aggcccaggt caccctgtca aatgcccagc taaaagcatt caaagatgag
1681 gagaaggcaa gagaagccct cagacagcag aagaggaaag caaaggcctc aggagagcgt
1741 taccatgtgg agccccaccc agaacatctc tgcggggcct acccctacgc ctacccgccc
1801 atgccagcca tggtgccaca ccatggcttc gaggactggt cccagatccg ctacccccct
1861 ccccccatgg ccatggagca cccgccccca ctccccaact cgcgcctctt ccatctgccg
1921 gaatacacct ggcgtctacc ctgtggaggg gttcgaaatc caaatcagag ctcccaagtg
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1981 atggaccctc ccacagccag gcctacagaa ccagagccag ctgatctcag attgccaaga
2041 aactagaagc cacttgcacg gtgtggccag agcctcagct ggatgagagg ctgagatggg
2101 tggccagctt gtacaccagt ccctgaactg agctgtttac aggactgggg aggctccacc
2161 cagaaggctt tcatttgtac tctgctggga gtgactggga aaaactcctt ccctgctgct
2221 gagtggagag aggcctcatc cggctttgac ccaccatccg ttgcagaagc ctccaggagc
2281 agcaatccta agagtgggag gcagccaaga cccccttcct tcaaaacctc ccggaagtgg
2341 tttcaggccc tctagttgcc atgaccaatt tgtgtgtgtg tttaattttt gcttcaagct
2401 ctgtagcagg acctgcccca cgcacacccc tacccctctg tgaggagctg tgggaagtgt
2461 gggtttgtct ccagaacaga agagaatgat ggatattctg gctctggggc cctctccacc
2521 accactcaca gtagccttgc tgaagccatc acagatggga gaaggccatg ccagccacgt
2581 ccgccgaggg gcgccagcct gaagctgcca ggccctgagg ttcagaccct ggaccccata
2641 gctggaggcc tgtggtgcca gaagcccaga ttagggtggc tgtccatccc tggatagcta
2701 tttgcacgaa tcatggacat aaatccaagt tgaagaagat caacaaaaaa aaaaaa
```

SEQ ID NO: 75 Human TNIP1 Transcript Variant 7 cDNA Sequence (NM_001252393.1, CDS region from position 242-2149)

```
   1 agtctccggg gactttccca ggggtggggc ggcccggcca ggcccccggc acttcctcgt
  61 cctcggcccg ggtgccctgc ccccgtccag gagccctagg agtgctacgg ggggccggag
 121 ccttgcccgg gccgctgccc cgtccctgga ttcggggctg gacgcagcaa gcggggcgct
 181 gtgtccccaa gctccccgtc ctcggccagg cgggcaccac ggcaggggct gagctaccct
 241 catggaaggg agaggaccgt accggatcta cgaccctggg ggcagcgtgc cctcaggaga
 301 ggcatccgca gcttttgagc gcctagtgaa ggagaattcc cggctgaagg aaaaaatgca
 361 agggataaag atgttagggg agcttttgga agagtcccag atggaagcga ccaggctccg
 421 gcagaaggca gaggagctag tgaaggacaa cgagctgctc ccaccacctt ctccctcctt
 481 gggctccttc gacccccctgg ctgagctcac aggaaaggac tcaaatgtca cagcatctcc
 541 cacagcccct gcatgcccca gtgacaagcc agcaccagtc cagaagcctc catccagtgg
 601 cacctcctct gaatttgaag tggtcactcc tgaggagcag aattcaccag agagcagcag
 661 ccatgccaat gcgatggcgc tgggcccccc gccccgtgag gacggcaacc tgatgctgca
 721 cctgcagcgc ctggagacca cgctgagtgt gtgtgccgag gagccggacc acggccagct
 781 cttcacccac ctgggccgca tggccctgga gttcaaccga ctggcatcca aggtgcacaa
 841 gaatgagcag cgcacctcca ttctgcagac cctgtgtgag cagcttcgga aggagaacga
 901 ggctctgaag gccaagttgg ataagggcct ggaacagcgg gatcaggctg ccgagaggct
 961 gcgggaggaa aatttggagc tcaagaagtt gttgatgagc aatggcaaca aagagggtgc
1021 gtctgggcgg ccaggctcac cgaagatgga agggacaggc aagaaggcag tggctggaca
1081 gcagcaggct agtgtgacgg caggtaaggt cccagaggtg gtggccttgg gcgcagccga
1141 gaagaaggtg aagatgctgg agcagcagcg cagtgagctg ctggaagtga acaagcagtg
1201 ggaccagcat ttccggtcca tgaagcagca gtatgagcag aagatcactg agctgcgtca
1261 gaagctggct gatttgcaga agcaggtgac tgacctggag gccgagcggg agcagaagca
1321 gcgtgacttt gaccgcaagc tcctcctggc caagtccaag attgaaatgg aggagaccga
1381 caaggagcag ctgacagcag aggccaagga gctgcgccaa aaggtcaagt acctgcagga
1441 tcagctgagc ccactcaccc gacagcgtga gtaccaggaa aaggagatcc agcggctcaa
1501 caaggccctg gaggaagcac tgagcatcca aaccccgcca tcatctccac caacagcatt
1561 tgggagccca gaaggagcag gggccctcct aaggaaacag gagctggtca cgcagaatga
1621 gttgctgaaa cagcaggtga agatcttcga ggaggacttc cagagggagc gcagtgatcg
1681 tgagcgcatg aatgaggaga aggaagagct gaagaagcaa gtggagaagc tgcaggccca
1741 ggtcaccctg tcaaatgccc agctaaaagc attcaaagat gaggagaagg caagagaagc
1801 cctcagacag cagaagagga aagcaaaggc ctcaggagag cgttaccatg tggagcccca
1861 cccagaacat ctctgcgggg cctaccccta cgcctacccg cccatgccag ccatggtgcc
1921 acaccatggc ttcgaggact ggtcccagat ccgctacccc cctcccccca tggccatgga
1981 gcacccgccc ccactcccca actcgcgcct cttccatctg ccggaataca cctggcgtct
2041 accctgtgga ggggttcgaa atccaaatca gagctcccaa gtgatggacc ctcccacagc
2101 caggcctaca gaaccagagc cagctgatct cagattgcca agaaactaga agccacttgc
2161 acggtgtggc cagagcctca gctggatgag aggctgagat gggtggccag cttgtacacc
2221 agtccctgaa ctgagctgtt tacaggactg gggaggctcc acccagaagg ctttcatttg
2281 tactctgctg ggagtgactg ggaaaaactc cttccctgct gctgagtgga gagaggcctc
2341 atccggcttt gacccaccat ccgttgcaga agcctccagg agcagcaatc ctaagagtgg
2401 gaggcagcca agacccccctt ccttcaaaac ctcccggaag tggtttcagg ccctctagtt
2461 gccatgacca atttgtgtgt gtgtttaatt tttgcttcaa gctctgtagc aggacctgcc
2521 ccacgcacac ccctacccct ctgtgaggag ctgtgggaag tgtgggtttg tctccagaac
2581 agaagagaat gatggatatt ctggctctgg ggccctctcc accaccactc acagtagcct
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2641 tgctgaagcc atcacagatg ggagaaggcc atgccagcca cgtccgccga ggggcgccag
2701 cctgaagctg ccaggccctg aggttcagac cctggacccc atagctggag gcctgtggtg
2761 ccagaagccc agattagggt ggctgtccat ccctggatag ctatttgcac gaatcatgga
2821 cataaatcca agttgaagaa gatcaacaaa aaaaaaaaa
```

SEQ ID NO: 76 Human TNIP1 Isoform 4 Amino Acid Sequence (NP_001239321.1)

```
   1 megrgpyriy dpggsvpsge asaaferlvk ensrlkekmq gikmlgelle esqmeatrlr
  61 qkaeelvkdn ellpppspsl gsfdplaelt gkdsnvtasp tapacpsdkp apvqkppssg
 121 tssefevvtp eeqnspesss hanamalgpl predgnlmlh lqrlettlsv caeepdhgql
 181 fthlgrmale fnrlaskvhk neqrtsilqt lceqlrkene alkakldkgl eqrdqaaerl
 241 reenlelkkl lmsngnkega sgrpgspkme gtgkkavagq qqasvtagkv pevvalgaae
 301 kkvkmleqqr sellevnkqw dqhfrsmkqq yeqkitelrq kladlqkqvt dleaereqkq
 361 rdfdrkllla kskiemeetd keqltaeake lrqkvkylqd qlspltrqre yqekeiqrln
 421 kaleealsiq tppsspptaf gspegagall rkqelvtqne llkqqvkife edfqrersdr
 481 ermneekeel kkqveklqaq vtlsnaqlka fkdeekarea lrqqkrkaka sgeryhveph
 541 pehlcgaypy ayppmpamvp hhgfedwsqi ryppppmame hppplpnsrl fhlpeytwrl
 601 pcggvrnpnq ssqvmdppta rptepepadl rlprn
```

SEQ ID NO: 77 Human TNIP1 Transcript Variant 9 cDNA Sequence (NM_001258455.1, CDS region from position 37-1755)

```
   1 ccaggcgggc accacggcag gggctgagct accctcatgg aagggagagg accgtaccgg
  61 atctacgacc ctgggggcag cgtgccctca ggagaggcat ccgcagcttt tgagcgccta
 121 gtgaaggaga attcccggct gaaggaaaaa atgcaaggga taaagatgtt aggggagctt
 181 ttggaagagt cccagatgga agcgaccagg ctccggcaga aggcagagga gctagtgaag
 241 gacaacgagc tgctcccacc accttctccc tccttgggct ccttcgaccc cctggctgag
 301 ctcacaggaa aggactcaaa tgtcacagca tctcccacag cccctgcatg ccccagtgac
 361 aagccagcac cagtccagaa gcctccatcc agtggcacct cctctgaatt tgaagtggtc
 421 actcctgagg agcagaattc accagagagc agcagccatg ccaatgcgat ggcgctgggc
 481 cccctgcccc gtgaggacgg caacctgatg ctgcacctgc agcgcctgga gaccacgctg
 541 agtgtgtgtg ccgaggagcc ggaccacggc cagctcttca cccacctggg ccgcatggcc
 601 ctggagttca accgactggc atccaaggtg cacaagaatg agcagcgcac ctccattctg
 661 cagaccctgt gtgagcagct tcggaaggag aacgaggctc tgaaggccaa gttggataag
 721 ggcctggaac agcgggatca ggctgccgag aggctgcggg aggaaaattt ggagctcaag
 781 aagttgttga tgagcaatgg caacaaagag ggtgcgtctg ggcggccagg ctcaccgaag
 841 atggaaggga caggcaagaa ggcagtggct ggacagcagc aggctagtgt gacggcaggt
 901 aaggtcccag aggtggtggc cttgggcgca gccgagaaga aggtgaagat gctggagcag
 961 cagcgcagtg agctgctgga agtgaacaag cagtgggacc agcatttccg gtccatgaag
1021 cagcagtatg agcagaagat cactgagctg cgtcagaagc tggctgattt gcagaagcag
1081 gtgactgacc tggaggccga gcgggagcag aagcagcgtg actttgaccg caagctcctc
1141 ctggccaagt ccaagattga aatggaggag accgacaagg agcagctgac agcagaggcc
1201 aaggagctgc gccaaaaggt caagtacctg caggatcagc tgagcccact cacccgacag
1261 cgtgagtacc aggaaaagga gatccagcgg ctcaacaagg ccctggagga agcactgagc
1321 atccaaaccc cgccatcatc tccaccaaca gcatttggga gcccagaagg agcaggggcc
1381 ctcctaagga aacaggagct ggtcacgcag aatgagttgc tgaaacagca ggtgaagatc
1441 ttcgaggagg acttccagag ggagcgcagt gatcgtgagc gcatgaatga ggagaaggaa
1501 gagctgaaga agcaagtgga gaagctgcag gcccaggtca ccctgtcaaa tgcccagcta
1561 aaagcattca aagatgagga gaaggcaaga gaagccctca gacagcagaa gaggaaagca
1621 aagccggaat acacctggcg tctaccctgt ggaggggttc gaaatccaaa tcagagctcc
1681 caagtgatgg accctcccac agccaggcct acagaaccag agtctccaaa aaatgaccgt
1741 gaggggcctc agtgagacca gattgtgtca tttggctcca ccttcatctt gcagagccag
1801 ctgatctcag attgccaaga aactagaagc cacttgcacg gtgtggccag agcctcagct
1861 ggatgagagg ctgagatggg tggccagctt gtacaccagt ccctgaactg agctgtttac
1921 aggactgggg aggctccacc cagaaggctt tcatttgtac tctgctggga gtgactggga
1981 aaaactcctt ccctgctgct gagtggagag aggcctcatc cggctttgac ccaccatccg
2041 ttgcagaagc ctccaggagc agcaatccta agagtgggag gcagccaaga ccccttcct
2101 tcaaaacctc ccggaagtgg tttcaggccc tctagttgcc atgaccaatt tgtgtgtgtg
2161 tttaattttt gcttcaagct ctgtagcagg acctgcccca cgcacacccc taccctctg
2221 tgaggagctg tgggaagtgt gggtttgtct ccagaacaga agagaatgat ggatattctg
2281 gctctggggc cctctccacc accactcaca gtagccttgc tgaagccatc acagatggga
```

```
2341 gaaggccatg ccagccacgt ccgccgaggg gcgccagcct gaagctgcca ggccctgagg
2401 ttcagaccct ggaccccata gctggaggcc tgtggtgcca gaagcccaga ttagggtggc
2461 tgtccatccc tggatagcta tttgcacgaa tcatggacat aaatccaagt tgaagaagat
2521 caaca
```

SEQ ID NO: 78 Human TNIP1 Isoform 5 Amino Acid Sequence (NP_001245384.1)

```
   1 megrgpyriy dpggsvpsge asaaferlvk ensrlkekmq gikmlgelle esqmeatrlr
  61 qkaeelvkdn ellpppspsl gsfdplaelt gkdsnvtasp tapacpsdkp apvqkppssg
 121 tssefevvtp eeqnspesss hanamalgpl predgnlmlh lqrlettlsv caeepdhgql
 181 fthlgrmale fnrlaskvhk neqrtsilqt lceqlrkene alkakldkgl eqrdqaaerl
 241 reenlelkkl lmsngnkega sgrpgspkme gtgkkavagq qqasvtagkv pevvalgaae
 301 kkvkmleqqr sellevnkqw dqhfrsmkqq yeqkitelrq kladlqkqvt dleaereqkq
 361 rdfdrkllla kskiemeetd keqltaeake lrqkvkylqd qlspltrqre yqekeiqrln
 421 kaleealsiq tppsspptaf gspegagall rkqelvtqne llkqqvkife edfqrersdr
 481 ermneekeel kkqveklqaq vtlsnaqlka fkdeekarea lrqqkrkakp eytwrlpcgg
 541 vrnpnqssqv mdpptarpte pespkndreg pq
```

SEQ ID NO: 79 Human TNIP1 Transcript Variant 10 cDNA Sequence (NM_001258456.1, CDS region from position 37-1707)

```
   1 ccaggcgggc accacggcag gggctgagct accctcatgg aagggagagg accgtaccgg
  61 atctacgacc ctgggggcag cgtgccctca ggagaggcat ccgcagcttt tgagcgccta
 121 gtgaaggaga attcccggct gaaggaaaaa atgcaaggga taaagatgtt aggggagctt
 181 ttggaagagt cccagatgga agcgaccagg ctccggcaga aggcagagga gctagtgaag
 241 gacaacgagc tgctcccacc accttctccc tccttgggct ccttcgaccc cctggctgag
 301 ctcacaggaa aggactcaaa tgtcacagca tctcccacag cccctgcatg ccccagtgac
 361 aagccagcac cagtccagaa gcctccatcc agtggcacct cctctgaatt tgaagtggtc
 421 actcctgagg agcagaattc accagagagc agcagccatg ccaatgcgat ggcgctgggc
 481 cccctgcccc gtgaggacgg caacctgatg ctgcacctgc agcgcctgga gaccacgctg
 541 agtgtgtgtg ccgaggagcc ggaccacggc cagctcttca cccacctggg ccgcatggcc
 601 ctggagttca accgactggc atccaaggtg cacaagaatg agcagcgcac ctccattctg
 661 cagaccctgt gtgagcagct tcggaaggag aacgaggctc tgaaggccaa gttggataag
 721 ggcctggaac agcgggatca ggctgccgag aggctgcggg aggaaaattt ggagctcaag
 781 aagttgttga tgagcaatgg caacaaagag ggtgcgtctg ggcggccagg ctcaccgaag
 841 atggaaggga caggcaagaa ggcagtggct ggacagcagc aggctagtgt gacggcaggt
 901 aaggtcccag aggtggtggc cttgggcgca gccgagaaga aggtgaagat gctggagcag
 961 cagcgcagtg agctgctgga agtgaacaag cagtgggacc agcatttccg gtccatgaag
1021 cagcagtatg agcagaagat cactgagctg cgtcagaagc tggctgattt gcagaagcag
1081 gtgactgacc tggaggccga gcgggagcag aagcagcgtg actttgaccg caagctcctc
1141 ctggccaagt ccaagattga aatggaggag accgacaagg agcagctgac agcagaggcc
1201 aaggagctgc gccaaaaggt caagtacctg caggatcagc tgagcccact cacccgacag
1261 cgtgagtacc aggaaaagga gatccagcgg ctcaacaagg ccctggagga agcactgagc
1321 atccaaaccc cgccatcatc tccaccaaca gcatttggga gcccagaagg agcaggggcc
1381 ctcctaagga aacaggagct ggtcacgcag aatgagttgc tgaaacagca ggtgaagatc
1441 ttcgaggagg acttccagag ggagcgcagt gatcgtgagc gcatgaatga ggagaaggaa
1501 gagctgaaga agcaagtgga gaagctgcag gcccaggtca ccctgtcaaa tgcccagcta
1561 aaagcattca aagatgagga gaaggcaaga gaagccctca gacagcagaa gaggaaagca
1621 aagagtctcc aaaaaatgac cgtgaggggc ctcagtgaga ccagattgtg tcatttggct
1681 ccaccttcat cttgcagagc cagctgatct cagattgcca agaaactaga agccacttgc
1741 acggtgtggc cagagcctca gctggatgag aggctgagat gggtggccag cttgtacacc
1801 agtccctgaa ctgagctgtt tacaggactg gggaggctcc acccagaagg ctttcatttg
1861 tactctgctg ggagtgactg ggaaaaactc cttccctgct gctgagtgga gagaggcctc
1921 atccggcttt gacccaccat ccgttgcaga agcctccagg agcagcaatc ctaagagtgg
1981 gaggcagcca agacccccct ccttcaaaac ctcccggaag tggtttcagg ccctctagtt
2041 gccatgacca atttgtgtgt gtgtttaatt tttgcttcaa gctctgtagc aggacctgcc
2101 ccacgcacac ccctacccct ctgtgaggag ctgtgggaag tgtgggtttg tctccagaac
2161 agaagagaat gatggatatt ctggctctgg ggccctctcc accaccactc acagtagcct
2221 tgctgaagcc atcacagatg ggagaaggcc atgccagcca cgtccgccga ggggcgccag
2281 cctgaagctg ccaggccctg aggttcagac cctggacccc atagctggag gcctgtggtg
2341 ccagaagccc agattagggt ggctgtccat ccctggatag ctatttgcac gaatcatgga
```

2401 cataaatcca agttgaagaa gatcaaca1

SEQ ID NO: 80 Human TNIP1 Isoform 6 Amino Acid Sequence (NP_001245385.1)

```
   1 megrgpyriy dpggsvpsge asaaferlvk ensrlkekmq gikmlgelle esqmeatrlr
  61 qkaeelvkdn ellpppspsl gsfdplaelt gkdsnvtasp tapacpsdkp apvqkppssg
 121 tssefevvtp eeqnspesss hanamalgpl predgnlmlh lqrlettlsv caeepdhgql
 181 fthlgrmale fnrlaskvhk neqrtsilqt lceqlrkene alkakldkgl eqrdqaaerl
 241 reenlelkkl lmsngnkega sgrpgspkme gtgkkavagq qqasvtagkv pevvalgaae
 301 kkvkmleqqr sellevnkqw dqhfrsmkqq yeqkitelrq kladlqkqvt dleaereqkq
 361 rdfdrkllla kskiemeetd keqltaeake lrqkvkylqd qlspltrqre yqekeiqrln
 421 kaleealsiq tppsspptaf gspegagall rkqelvtqne llkqqvkife edfqrersdr
 481 ermneekeel kkqveklqaq vtlsnaqlka fkdeekarea lrqqkrkaks lqkmtvrgls
 541 etrlchlapp sscras
```

SEQ ID NO: 81 Mouse TNIP1 Transcript Variant 1 cDNA Sequence (NM_021327.4, CDS region from position 145-2088)

```
   1 tcagaaagcc cagcaacctt cacagggaca cagggaggca tggccgcact cactgggcac
  61 atcttcagat cacctcgtgc attctcggat gagtgacctg ggctgaagct aggcggccgt
 121 cacggcaggg gttgagccac cctcatggaa gggagaggac cctacgggat ctacgaccca
 181 gggggcagca cgcctctggg agaggtgtcc gcagcttttg aacgtctagt ggaggagaat
 241 actcggctga agggaaaaat gcaagggata aagatgttag gggagcttct ggaggagtct
 301 cagatggaag cgtccagact ccggcagaag gcagaggagc tggtcaagga cagcgagctg
 361 tcaccaccga catctgcccc ctccttggtc tcctttgatg acctggctga gctcacagga
 421 caggatacaa aggtccaggt acatcctgct accagcactg ccgccaccac caccgccacc
 481 gccaccacgg gaaactccat ggagaagccc gagccagcct ccaaatctcc gtccaatggc
 541 gcctcctcgg actttgaagt ggtccctact gaggagcaga attcacccga aactggcagc
 601 caccctacga acatgatgga cctggggccc ccacccccag aggacagcaa cctgaagctc
 661 cacctgcagc gcctggagac cacccttagc gtgtgtgcag aggagccaga ccacagccag
 721 ctcttcaccc acctgggccg catggccctc gagttcaaca ggttggcctc caaagtgcat
 781 aaaaatgagc agcgcacctc catcctgcag accttatgtg agcagctgcg ccaggagaat
 841 gaagccctga aggccaagct ggacaagggc ctggaacagc gggatctggc tgctgagagg
 901 ctgcgggagg aaaacacgga gctcaagaaa ctgttgatga acagcagctg caaagaggga
 961 ctctgtgggc agcccagctc cccaaagcca gagggtgctg gcaagaaggg cgtggctgga
1021 cagcagcagg ccagtgtgat ggcgagtaaa gtccctgaag cgggggcctt tggagcagct
1081 gagaagaagg tgaagttgct agaacagcaa cgcatggagc tgctggaagt gaacaagcag
1141 tgggaccagc atttccggtc catgaagcag cagtatgagc agaagatcac agagcttcgc
1201 cagaagctgg tggacctgca gaaacaggta actgagctgg aggccgaacg ggagcagaag
1261 cagcgtgact ttgaccggaa actcctcctg gccaaatcga agatagagat ggaagagacc
1321 gacaaggagc agctgacagc agaggccaag gaactgcgcc agaaggtcag gtacctacag
1381 gatcagctga gcccgctcac aaggcaacga gaataccagg agaaggagat ccagcggctc
1441 aataaggccc tggaggaggc cctcagcatc caggcctctc catcatctcc gcctgcagct
1501 tttgggagtc cagaaggcgt tgggggccat ctgaggaagc aggaactagt gacacagaat
1561 gagttgctga aacagcaggt aaagatcttt gaagaggact tccagaggga acggagtgac
1621 cgtgaacgca tgaatgaaga gaaggaggag ctgaagaagc aagtagagaa gctgcaggcc
1681 caggtcaccc tgactaatgc ccagctcaaa actctcaaag aggaggagaa ggccaaggaa
1741 gccctcaaac agcagaagag gaaagcaaag gcttcgggag agcgctacca catggaaccc
1801 caccctgagc acgtctgcgg cgcctatccc tatgcctacc cacccatgcc agccatggta
1861 cctcaccatg cctacaagga ctggtcccag atccgatacc ctccaccccc tgtgcccatg
1921 gagcacccgc ccccacaccc caactctcgc ctcttccatc tgccggagta cacctggcgt
1981 ccaccctgtg cagggattcg gaatcagagc tctcaagtga tggacccgcc cccagacagg
2041 cctgcagagc cagagtctgc agacaatgac tgtgatgggc cccagtgagg ctgcagtggg
2101 tcatttggtt ccaccttcat ctttcagagc cagctgacct cagattgcca aaagtttgaa
2161 ggccatgtgc atgttctgtg tgacccaagc cttggcagag gagaggctgg gatgggtagc
2221 tggctcacat ccccagccaa gcctcgaact gttgacaaga ccagggagaa tccacccatg
2281 ggcgcccacc aggttcttat ggatgcaagc aggagaagct caacaccctg cctcttgcca
2341 agacaaggaa gcctcacctg gctttgacct gccatccgtt gctgaggcca ctggcttcca
2401 tcctaagaat gaggtgcaac aagaccccat tctcacagaa cctcaaagac ttggttccag
2461 gctctccaga gaccataccc aactcatgtg catgtgccgt ttttgcttca agctcagtag
2521 caggacctgc cccgagcccc ctgctccttg cccctctgtg aggagttacg gagagggctt
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```
2581 tgtctctaga gcagaagaga atgatgggac ggcctgatgc tgtcatgctc tccactgcac
2641 ctgtggcagc ctcctgagag ccaccaagat ctgggatgaa ggccacacca gccatgtctg
2701 ctgaagggcc ccagactgag atgactccgg cctccacagt tagatgttta tggtgccaga
2761 ggtctatatt aaggtagctg tctgttgcta ggcagccgtt tgcacaaatc ttggacataa
2821 atccaacttg aagatcaa
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SEQ ID NO: 82 Mouse TNIP1 Transcript Variant 2 cDNA Sequence (NM_001199275.2, CDS region from position 303-2246)

```
   1 tcagaaagcc cagcaacctt cacagggaca cagggaggca tggccgcact cactgggcac
  61 atcttcagat cacctcgtgc attctcggat gagtgacctg ggctgaagac cccgccccgg
 121 cgcccagcgc gcaacgccat tggacagacc gtacatcggt ctccggggac tttcccaggg
 181 gcgtggcgtg gccctggcat ttcctggtac cgaccggagc ttagattctg cgagtctggc
 241 cccggccccg aagctctcgt cctaggctag gcggccgtca cggcaggggt tgagccaccc
 301 tcatggaagg gagaggaccc tacgggatct acgacccagg gggcagcacg cctctgggag
 361 aggtgtccgc agcttttgaa cgtctagtgg aggagaatac tcggctgaag ggaaaaatgc
 421 aagggataaa gatgttaggg gagcttctgg aggagtctca gatggaagcg tccagactcc
 481 ggcagaaggc agaggagctg gtcaaggaca gcgagctgtc accaccgaca tctgcccccc
 541 ccttggtctc ctttgatgac ctggctgagc tcacaggaca ggatacaaag gtccaggtac
 601 atcctgctac cagcactgcc gccaccacca ccgccaccgc caccacggga aactccatgg
 661 agaagcccga gccagcctcc aaatctccgt ccaatggcgc ctcctcggac tttgaagtgg
 721 tccctactga ggagcagaat tcacccgaaa ctggcagcca ccctacgaac atgatggacc
 781 tggggccccc acccccagag gacagcaacc tgaagctcca cctgcagcgc ctggagacca
 841 cccttagcgt gtgtgcagag gagccagacc acagccagct cttcacccac ctgggccgca
 901 tggccctcga gttcaacagg ttggcctcca aagtgcataa aaatgagcag cgcacctcca
 961 tcctgcagac cttatgtgag cagctgcgcc aggagaatga agccctgaag gccaagctgg
1021 acaagggcct ggaacagcgg gatctggctg ctgagaggct gcgggaggaa aacacggagc
1081 tcaagaaact gttgatgaac agcagctgca aagagggact ctgtgggcag cccagctccc
1141 caaagccaga gggtgctggc aagaagggcg tggctggaca gcagcaggcc agtgtgatgg
1201 cgagtaaagt ccctgaagcg ggggcctttg gagcagctga gaagaaggtg aagttgctag
1261 aacagcaacg catggagctg ctggaagtga acaagcagtg ggaccagcat ttccggtcca
1321 tgaagcagca gtatgagcag aagatcacag agcttcgcca gaagctggtg gacctgcaga
1381 aacaggtaac tgagctggag gccgaacggg agcagaagca gcgtgacttt gaccggaaac
1441 tcctcctggc caaatcgaag atagagatgg aagagaccga caaggagcag ctgacagcag
1501 aggccaagga actgcgccag aaggtcaggt acctacagga tcagctgagc ccgctcacaa
1561 ggcaacgaga ataccaggag aaggagatcc agcggctcaa taaggccctg gaggaggccc
1621 tcagcatcca ggcctctcca tcatctccgc ctgcagcttt tgggagtcca gaaggcgttg
1681 ggggccatct gaggaagcag gaactagtga cacagaatga gttgctgaaa cagcaggtaa
1741 agatctttga agaggacttc cagagggaac ggagtgaccg tgaacgcatg aatgaagaga
1801 aggaggagct gaagaagcaa gtagagaagc tgcaggccca ggtcaccctg actaatgccc
1861 agctcaaaac tctcaaagag gaggagaagg ccaaggaagc cctcaaacag cagaagagga
1921 aagcaaaggc ttcgggagag cgctaccaca tggaacccca ccctgagcac gtctgcggcg
1981 cctatcccta tgcctaccca cccatgccag ccatggtacc tcaccatgcc tacaaggact
2041 ggtcccagat ccgataccct ccacccccctg tgcccatgga gcacccgccc ccacacccca
2101 actctcgcct cttccatctg ccggagtaca cctggcgtcc accctgtgca gggattcgga
2161 atcagagctc tcaagtgatg gacccgcccc cagacaggcc tgcagagcca gagtctgcag
2221 acaatgactg tgatgggccc cagtgaggct gcagtgggtc atttggttcc accttcatct
2281 ttcagagcca gctgacctca gattgccaaa agtttgaagg ccatgtgcat gttctgtgtg
2341 acccaagcct tggcagagga gaggctggga tgggtagctg gctcacatcc ccagccaagc
2401 ctcgaactgt tgacaagacc agggagaatc cacccatggg cgcccaccag gttcttatgg
2461 atgcaagcag gagaagctca acaccctgcc tcttgccaag acaaggaagc ctcacctggc
2521 tttgacctgc catccgttgc tgaggccact ggcttccatc ctaagaatga ggtgcaacaa
2581 gaccccattc tcacagaacc tcaaagactt ggttccaggc tctccagaga ccatacccaa
2641 ctcatgtgca tgtgccgttt ttgcttcaag ctcagtagca ggacctgccc cgagcccccct
2701 gctccttgcc cctctgtgag gagttacgga gagggctttg tctctagagc agaagagaat
2761 gatgggacgg cctgatgctg tcatgctctc cactgcacct gtggcagcct cctgagagcc
2821 accaagatct gggatgaagg ccacaccagc catgtctgct gaagggcccc agactgagat
2881 gactccggcc tccacagtta gatgtttatg gtgccagagg tctatattaa ggtagctgtc
2941 tgttgctagg cagccgtttg cacaaatctt ggacataaat ccaacttgaa gatcaa
```

SEQ ID NO: 83 Mouse TNIP1 Isoform 1 Amino Acid Sequence (NP_067302.2)

```
   1 megrgpygiy dpggstplge vsaaferlve entrlkgkmq gikmlgelle esqmeasrlr
  61 qkaeelvkds elspptsaps lvsfddlael tgqdtkvqvh patstaattt atattgnsme
 121 kpepasksps ngassdfevv pteeqnspet gshptnmmdl gppppedsnl klhlqrlett
 181 lsvcaeepdh sqlfthlgrm alefnrlask vhkneqrtsi lqtlceqlrq enealkakld
 241 kgleqrdlaa erlreentel kkllmnssck eglcgqpssp kpegagkkgv agqqqasvma
 301 skvpeagafg aaekkvklle qqrmellevn kqwdqhfrsm kqqyeqkite lrqklvdlqk
 361 qvteleaere qkqrdfdrkl llakskieme etdkeqltae akelrqkvry lqdqlspltr
 421 qreyqekeiq rlnkaleeal siqaspsspp aafgspegvg ghlrkqelvt qnellkqqvk
 481 ifeedfqrer sdrermneek eelkkqvekl qaqvtltnaq lktlkeeeka kealkqqkrk
 541 akasgeryhm ephpehvcga ypyayppmpa mvphhaykdw sqiryppppv pmehppphpn
 601 srlfhlpeyt wrppcagirn qssqvmdppp drpaepesad ndcdgpq
```

SEQ ID NO: 84 Mouse TNIP1 Transcript Variant 3 cDNA Sequence (NM_001199276.2, CDS region from position 132-1916)

```
   1 tcagaaagcc cagcaacctt cacagggaca cagggaggca tggccgcact cactgggcac
  61 atcttcagat cacctcgtgc attctcggat gagtgacctg ggctgaaggg gagcttctgg
 121 aggagtctca gatggaagcg tccagactcc ggcagaaggc agaggagctg gtcaaggaca
 181 gcgagctgtc accaccgaca tctgccccct ccttggtctc ctttgatgac ctggctgagc
 241 tcacaggaca ggatacaaag gtccaggtac atcctgctac cagcactgcc gccaccacca
 301 ccgccaccgc caccacggga aactccatgg agaagcccga gccagcctcc aaatctccgt
 361 ccaatggcgc ctcctcggac tttgaagtgg tccctactga ggagcagaat tcacccgaaa
 421 ctggcagcca ccctacgaac atgatggacc tggggccccc acccccagag gacagcaacc
 481 tgaagctcca cctgcagcgc ctggagacca cccttagcgt gtgtgcagag gagccagacc
 541 acagccagct cttcacccac ctgggccgca tggccctcga gttcaacagg ttggcctcca
 601 aagtgcataa aaatgagcag cgcacctcca tcctgcagac cttatgtgag cagctgcgcc
 661 aggagaatga agccctgaag gccaagctgg acaagggcct ggaacagcgg gatctggctg
 721 ctgagaggct gcgggaggaa aacacggagc tcaagaaact gttgatgaac agcagctgca
 781 aagagggact ctgtgggcag cccagctccc caaagccaga gggtgctggc aagaagggcg
 841 tggctggaca gcagcaggcc agtgtgatgg cgagtaaagt ccctgaagcg ggggcctttg
 901 gagcagctga gaagaaggtg aagttgctag aacagcaacg catggagctg ctggaagtga
 961 acaagcagtg ggaccagcat ttccggtcca tgaagcagca gtatgagcag aagatcacag
1021 agcttcgcca gaagctggtg gacctgcaga aacaggtaac tgagctggag gccgaacggg
1081 agcagaagca gcgtgacttt gaccggaaac tcctcctggc caaatcgaag atagagatgg
1141 aagagaccga caaggagcag ctgacagcag aggccaagga actgcgccag aaggtcaggt
1201 acctacagga tcagctgagc ccgctcacaa ggcaacgaga ataccaggag aaggagatcc
1261 agcggctcaa taaggccctg gaggaggccc tcagcatcca ggcctctcca tcatctccgc
1321 ctgcagcttt tgggagtcca gaaggcgttg ggggccatct gaggaagcag gaactagtga
1381 cacagaatga gttgctgaaa cagcaggtaa agatctttga agaggacttc cagagggaac
1441 ggagtgaccg tgaacgcatg aatgaagaga aggaggagct gaagaagcaa gtagagaagc
1501 tgcaggccca ggtcaccctg actaatgccc agctcaaaac tctcaaagag gaggagaagg
1561 ccaaggaagc cctcaaacag cagaagagga aagcaaaggc ttcgggagag cgctaccaca
1621 tggaacccca ccctgagcac gtctgcggcg cctatcccta tgcctaccca cccatgccag
1681 ccatggtacc tcaccatgcc tacaaggact ggtcccagat ccgataccct ccacccccctg
1741 tgcccatgga gcacccgccc ccacacccca actctcgcct cttccatctg ccggagtaca
1801 cctggcgtcc accctgtgca gggattcgga atcagagctc tcaagtgatg gacccgcccc
1861 cagacaggcc tgcagagcca gagtctgcag acaatgactg tgatgggccc cagtgaggct
1921 gcagtgggtc atttggttcc accttcatct ttcagagcca gctgacctca gattgccaaa
1981 agtttgaagg ccatgtgcat gttctgtgtg acccaagcct tggcagagga gaggctggga
2041 tgggtagctg gctcacatcc ccagccaagc ctcgaactgt tgacaagacc agggagaatc
2101 cacccatggg cgcccaccag gttcttatgg atgcaagcag gagaagctca acaccctgcc
2161 tcttgccaag acaaggaagc ctcacctggc tttgacctgc catccgttgc tgaggccact
2221 ggcttccatc ctaagaatga ggtgcaacaa gaccccattc tcacagaacc tcaaagactt
2281 ggttccaggc tctccagaga ccatacccaa ctcatgtgca tgtgccgttt ttgcttcaag
2341 ctcagtagca ggacctgccc cgagcccccct gctccttgcc cctctgtgag gagttacgga
2401 gagggctttg tctctagagc agaagagaat gatgggacgg cctgatgctg tcatgctctc
2461 cactgcacct gtggcagcct cctgagagcc accaagatct gggatgaagg ccacaccagc
2521 catgtctgct gaagggcccc agactgagat gactccggcc tccacagtta gatgtttatg
2581 gtgccagagg tctatattaa ggtagctgtc tgttgctagg cagccgtttg cacaaatctt
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2641 ggacataaat ccaacttgaa gatcaa

SEQ ID NO: 85 Mouse TNIP1 Transcript Variant 4 cDNA Sequence (NM_001271455.1, CDS region from position 185-1969)

```
   1 tcagaaagcc cagcaacctt cacagggaca cagggaggca tggccgcact cactgggcac
  61 atcttcagat cacctcgtgc attctcggat gagtgacctg ggctgaagga gacagaggaa
 121 ggcagatctc tctgacttca aggcccgcct gatctccaac cgggagcttc tggaggagtc
 181 tcagatggaa gcgtccagac tccggcagaa ggcagaggag ctggtcaagg acagcgagct
 241 gtcaccaccg acatctgccc cctccttggt ctcctttgat gacctggctg agctcacagg
 301 acaggataca aaggtccagg tacatcctgc taccagcact gccgccacca ccaccgccac
 361 cgccaccacg ggaaactcca tggagaagcc cgagccagcc tccaaatctc cgtccaatgg
 421 cgcctcctcg gactttgaag tggtccctac tgaggagcag aattcacccg aaactggcag
 481 ccaccctacg aacatgatgg acctggggcc cccaccccca gaggacagca acctgaagct
 541 ccacctgcag cgcctggaga ccacccttag cgtgtgtgca gaggagccag accacagcca
 601 gctcttcacc cacctgggcc gcatggccct cgagttcaac aggttggcct ccaaagtgca
 661 taaaaatgag cagcgcacct ccatcctgca gaccttatgt gagcagctgc gccaggagaa
 721 tgaagccctg aaggccaagc tggacaaggg cctggaacag cgggatctgg ctgctgagag
 781 gctgcgggag gaaaacacgg agctcaagaa actgttgatg aacagcagct gcaaagaggg
 841 actctgtggg cagcccagct ccccaaagcc agagggtgct ggcaagaagg gcgtggctgg
 901 acagcagcag gccagtgtga tggcgagtaa agtccctgaa gcgggggcct ttggagcagc
 961 tgagaagaag gtgaagttgc tagaacagca acgcatggag ctgctggaag tgaacaagca
1021 gtgggaccag catttccggt ccatgaagca gcagtatgag cagaagatca cagagcttcg
1081 ccagaagctg gtggacctgc agaaacaggt aactgagctg gaggccgaac gggagcagaa
1141 gcagcgtgac tttgaccgga aactcctcct ggccaaatcg aagatagaga tggaagagac
1201 cgacaaggag cagctgacag cagaggccaa ggaactgcgc cagaaggtca ggtacctaca
1261 ggatcagctg agcccgctca caaggcaacg agaataccag gagaaggaga tccagcggct
1321 caataaggcc ctggaggagg ccctcagcat ccaggcctct ccatcatctc cgcctgcagc
1381 ttttgggagt ccagaaggcg ttgggggcca tctgaggaag caggaactag tgacacagaa
1441 tgagttgctg aaacagcagg taaagatctt tgaagaggac ttccagaggg aacggagtga
1501 ccgtgaacgc atgaatgaag agaaggagga gctgaagaag caagtagaga agctgcaggc
1561 ccaggtcacc ctgactaatg cccagctcaa aactctcaaa gaggaggaga aggccaagga
1621 agccctcaaa cagcagaaga ggaaagcaaa ggcttcggga gagcgctacc acatggaacc
1681 ccaccctgag cacgtctgcg gcgcctatcc ctatgcctac ccacccatgc cagccatggt
1741 acctcaccat gcctacaagg actggtccca gatccgatac cctccacccc ctgtgcccat
1801 ggagcacccg cccccacacc ccaactctcg cctcttccat ctgccggagt acacctggcg
1861 tccaccctgt gcagggattc ggaatcagag ctctcaagtg atggacccgc ccccagacag
1921 gcctgcagag ccagagtctg cagacaatga ctgtgatggg ccccagtgag gctgcagtgg
1981 gtcatttggt tccaccttca tctttcagag ccagctgacc tcagattgcc aaaagtttga
2041 aggccatgtg catgttctgt gtgacccaag ccttggcaga ggagaggctg ggatgggtag
2101 ctggctcaca tccccagcca agcctcgaac tgttgacaag accagggaga atccacccat
2161 gggcgcccac caggttctta tggatgcaag caggagaagc tcaacaccct gcctcttgcc
2221 aagacaagga agcctcacct ggctttgacc tgccatccgt tgctgaggcc actggcttcc
2281 atcctaagaa tgaggtgcaa caagacccca ttctcacaga acctcaaaga cttggttcca
2341 ggctctccag agaccatacc caactcatgt gcatgtgccg tttttgcttc aagctcagta
2401 gcaggacctg ccccgagccc cctgctcctt gcccctctgt gaggagttac ggagagggct
2461 ttgtctctag agcagaagag aatgatggga cggcctgatg ctgtcatgct ctccactgca
2521 cctgtggcag cctcctgaga gccaccaaga tctgggatga aggccacacc agccatgtct
2581 gctgaagggc cccagactga gatgactccg gcctccacag ttagatgttt atggtgccag
2641 aggtctatat taaggtagct gtctgttgct aggcagccgt ttgcacaaat cttggacata
2701 aatccaactt gaagatcaa
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SEQ ID NO: 86 Mouse TNIP1 Isoform 2 Amino Acid Sequence (NP_001186205.1)

```
   1 measrlrqka eelvkdsels pptsapslvs fddlaeltgq dtkvqvhpat staatttata
  61 ttgnsmekpe paskspsnga ssdfevvpte eqnspetgsh ptnmmdlgpp ppedsnlklh
 121 lqrlettlsv caeepdhsql fthlgrmale fnrlaskvhk neqrtsilqt lceqlrqene
 181 alkakldkgl eqrdlaaerl reentelkkl lmnssckegl cgqpsspkpe gagkkgvagq
 241 qqasvmaskv peagafgaae kkvklleqqr mellevnkqw dqhfrsmkqq yeqkitelrq
 301 klvdlqkqvt eleaereqkq rdfdrkllla kskiemeetd keqltaeake lrqkvrylqd
 361 qlspltrqre yqekeiqrln kaleealsiq aspssppaaf gspegvgghl rkqelvtqne
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 421 llkqqvkife edfqrersdr ermneekeel kkqveklqaq vtltnaqlkt lkeeekakea
 481 lkqqkrkaka sgeryhmeph pehvcgaypy ayppmpamvp hhaykdwsqi ryppppvpme
 541 hppphpnsrl fhlpeytwrp pcagirnqss qvmdpppdrp aepesadndc dgpq
```

SEQ ID NO: 87 Mouse TNIP1 Transcript Variant 5 cDNA Sequence (NM_001271456.1, CDS region from position 198-2138)

```
   1 tcagaaagcc cagcaacctt cacagggaca cagggaggca tggccgcact cactgggcac
  61 atcttcagat cacctcgtgc attctcggat gagtgacctg ggctgaagga gacagaggaa
 121 ggcagatctc tctgacttca aggcccgcct gatctccaac cctaggcggc cgtcacggca
 181 ggggttgagc caccctcatg gaagggagag gaccctacgg gatctacgac ccaggggca
 241 gcacgcctct gggagaggtg tccgcagctt ttgaacgtct agtggaggag aatactcggc
 301 tgaagggaaa aatgcaaggg ataaagatgt taggggagct tctggaggag tctcagatgg
 361 aagcgtccag actccggcag aaggcagagg agctggtcaa ggacagcgag ctgtcaccac
 421 cgacatctgc cccctccttg gtctcctttg atgacctggc tgagctcaca ggacaggata
 481 caaaggtcca ggtacatcct gctaccagca ctgccgccac caccaccgcc accgccacca
 541 cgggaaactc catggagaag cccgagccag cctccaaatc tccgtccaat ggcgcctcct
 601 cggactttga agtggtccct actgaggagc agaattcacc cgaaactggc agccacccta
 661 cgaacatgat ggacctgggg cccccacccc cagaggacag caacctgaag ctccacctgc
 721 agcgcctgga gaccaccctt agcgtgtgtg cagaggagcc agaccacagc cagctcttca
 781 cccacctggg ccgcatggcc ctcgagttca acaggttggc ctccaaagtg cataaaaatg
 841 agcagcgcac ctccatcctg cagaccttat gtgagcagct gcgccaggag aatgaagccc
 901 tgaaggccaa gctggacaag ggcctggaac agcgggatct ggctgctgag aggctgcggg
 961 aggaaaacac ggagctcaag aaactgttga tgaacagcag ctgcaaagag ggactctgtg
1021 ggcagcccag ctccccaaag ccagagggtg ctggcaagaa gggcgtggct ggacagcagc
1081 aggccagtgt gatggcgagt aaagtccctg aagcgggggc ctttggagca gctgagaaga
1141 aggtgaagtt gctagaacag caacgcatgg agctgctgga agtgaacaag cagtgggacc
1201 agcatttccg gtccatgaag cagcagtatg agcagaagat cacagagctt cgccagaagc
1261 tggtggacct gcagaaacag gtaactgagc tggaggccga acgggagcag aagcagcgtg
1321 actttgaccg gaaactcctc ctggccaaat cgaagataga gatggaagag accgacaagg
1381 agcagctgac agcagaggcc aaggaactgc gccagaaggt caggtaccta caggatcagc
1441 tgagcccgct cacaaggcaa cgagaatacc aggagaagga gatccagcgg ctcaataagg
1501 ccctggagga ggccctcagc atccaggcct ctccatcatc tccgcctgca gcttttggga
1561 gtccagaagg cgttgggggc catctgagga agcaggaact agtgacacag aatgagttgc
1621 tgaaacagca ggtaaagatc tttgaagagg acttccagag ggaacggagt gaccgtgaac
1681 gcatgaatga agagaaggag gagctgaaga agcaagtaga gaagctgcag gcccaggtca
1741 ccctgactaa tgcccagctc aaaactctca aagaggagga gaaggccaag gaagccctca
1801 aacagcagaa gaggaaagca aaggcttcgg gagagcgcta ccacatggaa ccccaccctg
1861 agcacgtctg cggcgcctat ccctatgcct acccacccat gccagccatg gtacctcacc
1921 atgcctacaa ggactggtcc cagatccgat accctccacc ccctgtgccc atggagcacc
1981 cgcccccaca ccccaactct cgcctcttcc atctgccgga gtacacctgg cgtccaccct
2041 gtgcagggat tcggaatcag agctctcaag tgatggaccc gcccccagac aggcctgcag
2101 agccagagcc agctgacctc agattgccaa aagtttgaag gccatgtgca tgttctgtgt
2161 gacccaagcc ttggcagagg agaggctggg atgggtagct ggctcacatc cccagccaag
2221 cctcgaactg ttgacaagac cagggagaat ccacccatgg gcgcccacca ggttcttatg
2281 gatgcaagca ggagaagctc aacaccctgc ctcttgccaa gacaaggaag cctcacctgg
2341 ctttgacctg ccatccgttg ctgaggccac tggcttccat cctaagaatg aggtgcaaca
2401 agaccccatt ctcacagaac ctcaaagact tggttccagg ctctccagag accataccca
2461 actcatgtgc atgtgccgtt tttgcttcaa gctcagtagc aggacctgcc ccgagccccc
2521 tgctccttgc ccctctgtga ggagttacgg agagggcttt gtctctagag cagaagagaa
2581 tgatgggacg gcctgatgct gtcatgctct ccactgcacc tgtggcagcc tcctgagagc
2641 caccaagatc tgggatgaag gccacaccag ccatgtctgc tgaagggccc cagactgaga
2701 tgactccggc ctccacagtt agatgtttat ggtgccagag gtctatatta aggtagctgt
2761 ctgttgctag gcagccgttt gcacaaatct tggacataaa tccaacttga agatcaa
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SEQ ID NO: 88 Mouse TNIP1 Isoform 3 Amino Acid Sequence (NP_001258385.1)

```
   1 megrgpygiy dpggstplge vsaaferlve entrlkgkmq gikmlgelle esqmeasrlr
  61 qkaeelvkds elspptsaps lvsfddlael tgqdtkvqvh patstaattt atattgnsme
 121 kpepasksps ngassdfevv pteeqnspet gshptnmmdl gppppedsnl klhlqrlett
 181 lsvcaeepdh sqlfthlgrm alefnrlask vhkneqrtsi lqtlceqlrq enealkakld
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241 kgleqrdlaa erlreentel kkllmnssck eglcgqpssp kpegagkkgv agqqqasvma
301 skvpeagafg aaekkvklle qqrmellevn kqwdqhfrsm kqqyeqkite lrqklvdlqk
361 qvteleaere qkqrdfdrkl llakskieme etdkeqltae akelrqkvry lqdqlspltr
421 qreyqekeiq rlnkaleeal siqaspsspp aafgspegvg ghlrkqelvt qnellkqqvk
481 ifeedfqrer sdrermneek eelkkqvekl qaqvtltnaq lktlkeeeka kealkqqkrk
541 akasgeryhm ephpehvcga ypyayppmpa mvphhaykdw sqiryppppv pmehppphpn
601 srlfhlpeyt wrppcagirn qssqvmdppp drpaepepad lrlpkv
```

* Included in Table 1 are RNA nucleic acid molecules (*e.g.*, thymines replaced with uredines), nucleic acid molecules encoding orthologs of the encoded proteins, as well as DNA or RNA nucleic acid sequences comprising a nucleic acid sequence having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or more identity across their full length with the nucleic acid sequence of any SEQ ID NO listed in Table 1, or a portion thereof. Such nucleic acid molecules can have a function of the full-length nucleic acid as described further herein.

* Included in Table 1 are orthologs of the proteins, as well as polypeptide molecules comprising an amino acid sequence having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or more identity across their full length with an amino acid sequence of any SEQ ID NO listed in Table 1, or a portion thereof. Such polypeptides can have a function of the full-length polypeptide as described further herein.

II. Subjects

In one embodiment, the subject for whom predicted likelihood of efficacy of an inhibitor of one or more biomarkers listed in Table 1 and an immunotherapy combination treatment is determined, is a mammal (*e.g.*, mouse, rat, primate, non-human mammal, domestic animal, such as a dog, cat, cow, horse, and the like), and is preferably a human. In another embodiment, the subject is an animal model of cancer. For example, the animal model can be an orthotopic xenograft animal model of a human-derived cancer.

In another embodiment of the methods of the present invention, the subject has not undergone treatment, such as chemotherapy, radiation therapy, targeted therapy, and/or immunotherapies. In still another embodiment, the subject has undergone treatment, such as chemotherapy, radiation therapy, targeted therapy, and/or immunotherapies.

In certain embodiments, the subject has had surgery to remove cancerous or precancerous tissue. In other embodiments, the cancerous tissue has not been removed, *e.g.*, the cancerous tissue may be located in an inoperable region of the body, such as in a

tissue that is essential for life, or in a region where a surgical procedure would cause considerable risk of harm to the patient.

The methods of the present invention can be used to determine the responsiveness to inhibitors of one or more biomarkers listed in Table 1 and immunotherapy combination treatment of many different cancers in subjects such as those described herein.

III. Sample Collection, Preparation and Separation

In some embodiments, biomarker amount and/or activity measurement(s) in a sample from a subject is compared to a predetermined control (standard) sample. The sample from the subject is typically from a diseased tissue, such as cancer cells or tissues. The control sample can be from the same subject or from a different subject. The control sample is typically a normal, non-diseased sample. However, in some embodiments, such as for staging of disease or for evaluating the efficacy of treatment, the control sample can be from a diseased tissue. The control sample can be a combination of samples from several different subjects. In some embodiments, the biomarker amount and/or activity measurement(s) from a subject is compared to a pre-determined level. This pre-determined level is typically obtained from normal samples. As described herein, a "pre-determined" biomarker amount and/or activity measurement(s) may be a biomarker amount and/or activity measurement(s) used to, by way of example only, evaluate a subject that may be selected for treatment (*e.g.*, based on the number of genomic mutations and/or the number of genomic mutations causing non-functional proteins for DNA repair genes), evaluate a response to an inhibitor of one or more biomarkers listed in Table 1 and an immunotherapy combination treatment, and/or evaluate a response to inhibitors of one or more biomarkers listed in Table 1, in combination with an immunotherapy with or without additional anti-cancer therapies. A pre-determined biomarker amount and/or activity measurement(s) may be determined in populations of patients with or without cancer. The pre-determined biomarker amount and/or activity measurement(s) can be a single number, equally applicable to every patient, or the pre-determined biomarker amount and/or activity measurement(s) can vary according to specific subpopulations of patients. Age, weight, height, and other factors of a subject may affect the pre-determined biomarker amount and/or activity measurement(s) of the individual. Furthermore, the pre-determined biomarker amount and/or activity can be determined for each subject individually. In one

embodiment, the amounts determined and/or compared in a method described herein are based on absolute measurements.

In another embodiment, the amounts determined and/or compared in a method described herein are based on relative measurements, such as ratios (*e.g.*, biomarker copy numbers, level, and/or activity before a treatment vs. after a treatment, such biomarker measurements relative to a spiked or man-made control, such biomarker measurements relative to the expression of a housekeeping gene, and the like). For example, the relative analysis can be based on the ratio of pre-treatment biomarker measurement as compared to post-treatment biomarker measurement. Pre-treatment biomarker measurement can be made at any time prior to initiation of anti-cancer therapy. Post-treatment biomarker measurement can be made at any time after initiation of anti-cancer therapy. In some embodiments, post-treatment biomarker measurements are made 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 weeks or more after initiation of anti-cancer therapy, and even longer toward indefinitely for continued monitoring. Treatment can comprise anti-cancer therapy, such as a therapeutic regimen comprising one or more inhibitors of one or more biomarkers listed in Table 1 and immunotherapy combination treatment alone or in combination with other anti-cancer agents, such as with immune checkpoint inhibitors.

The pre-determined biomarker amount and/or activity measurement(s) can be any suitable standard. For example, the pre-determined biomarker amount and/or activity measurement(s) can be obtained from the same or a different human for whom a patient selection is being assessed. In one embodiment, the pre-determined biomarker amount and/or activity measurement(s) can be obtained from a previous assessment of the same patient. In such a manner, the progress of the selection of the patient can be monitored over time. In addition, the control can be obtained from an assessment of another human or multiple humans, *e.g.*, selected groups of humans, if the subject is a human. In such a manner, the extent of the selection of the human for whom selection is being assessed can be compared to suitable other humans, *e.g.*, other humans who are in a similar situation to the human of interest, such as those suffering from similar or the same condition(s) and/or of the same ethnic group.

In some embodiments of the present invention the change of biomarker amount and/or activity measurement(s) from the pre-determined level is about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, or 5.0 fold or greater, or any range in

between, inclusive. Such cutoff values apply equally when the measurement is based on relative changes, such as based on the ratio of pre-treatment biomarker measurement as compared to post-treatment biomarker measurement.

Biological samples can be collected from a variety of sources from a patient including a body fluid sample, cell sample, or a tissue sample comprising nucleic acids and/or proteins. "Body fluids" refer to fluids that are excreted or secreted from the body as well as fluids that are normally not (*e.g.*, amniotic fluid, aqueous humor, bile, blood and blood plasma, cerebrospinal fluid, cerumen and earwax, cowper's fluid or pre-ejaculatory fluid, chyle, chyme, stool, female ejaculate, interstitial fluid, intracellular fluid, lymph, menses, breast milk, mucus, pleural fluid, pus, saliva, sebum, semen, serum, sweat, synovial fluid, tears, urine, vaginal lubrication, vitreous humor, vomit). In a preferred embodiment, the subject and/or control sample is selected from the group consisting of cells, cell lines, histological slides, paraffin embedded tissues, biopsies, whole blood, nipple aspirate, serum, plasma, buccal scrape, saliva, cerebrospinal fluid, urine, stool, and bone marrow. In one embodiment, the sample is serum, plasma, or urine. In another embodiment, the sample is serum.

The samples can be collected from individuals repeatedly over a longitudinal period of time (*e.g.*, once or more on the order of days, weeks, months, annually, biannually, etc.). Obtaining numerous samples from an individual over a period of time can be used to verify results from earlier detections and/or to identify an alteration in biological pattern as a result of, for example, disease progression, drug treatment, *etc.* For example, subject samples can be taken and monitored every month, every two months, or combinations of one, two, or three month intervals according to the present invention. In addition, the biomarker amount and/or activity measurements of the subject obtained over time can be conveniently compared with each other, as well as with those of normal controls during the monitoring period, thereby providing the subject's own values, as an internal, or personal, control for long-term monitoring.

Sample preparation and separation can involve any of the procedures, depending on the type of sample collected and/or analysis of biomarker measurement(s). Such procedures include, by way of example only, concentration, dilution, adjustment of pH, removal of high abundance polypeptides (*e.g.*, albumin, gamma globulin, and transferrin, etc.), addition of preservatives and calibrants, addition of protease inhibitors, addition of denaturants, desalting of samples, concentration of sample proteins, extraction and

purification of lipids.

The sample preparation can also isolate molecules that are bound in non-covalent complexes to other protein (*e.g.*, carrier proteins). This process may isolate those molecules bound to a specific carrier protein (*e.g.*, albumin), or use a more general process, such as the release of bound molecules from all carrier proteins via protein denaturation, for example using an acid, followed by removal of the carrier proteins.

Removal of undesired proteins (*e.g.*, high abundance, uninformative, or undetectable proteins) from a sample can be achieved using high affinity reagents, high molecular weight filters, ultracentrifugation and/or electrodialysis. High affinity reagents include antibodies or other reagents (*e.g.*, aptamers) that selectively bind to high abundance proteins. Sample preparation could also include ion exchange chromatography, metal ion affinity chromatography, gel filtration, hydrophobic chromatography, chromatofocusing, adsorption chromatography, isoelectric focusing and related techniques. Molecular weight filters include membranes that separate molecules on the basis of size and molecular weight. Such filters may further employ reverse osmosis, nanofiltration, ultrafiltration and microfiltration.

Ultracentrifugation is a method for removing undesired polypeptides from a sample. Ultracentrifugation is the centrifugation of a sample at about 15,000-60,000 rpm while monitoring with an optical system the sedimentation (or lack thereof) of particles. Electrodialysis is a procedure which uses an electromembrane or semipermable membrane in a process in which ions are transported through semi-permeable membranes from one solution to another under the influence of a potential gradient. Since the membranes used in electrodialysis may have the ability to selectively transport ions having positive or negative charge, reject ions of the opposite charge, or to allow species to migrate through a semipermable membrane based on size and charge, it renders electrodialysis useful for concentration, removal, or separation of electrolytes.

Separation and purification in the present invention may include any procedure known in the art, such as capillary electrophoresis (*e.g.*, in capillary or on-chip) or chromatography (*e.g.*, in capillary, column or on a chip). Electrophoresis is a method which can be used to separate ionic molecules under the influence of an electric field. Electrophoresis can be conducted in a gel, capillary, or in a microchannel on a chip. Examples of gels used for electrophoresis include starch, acrylamide, polyethylene oxides, agarose, or combinations thereof. A gel can be modified by its cross-linking, addition of

detergents, or denaturants, immobilization of enzymes or antibodies (affinity electrophoresis) or substrates (zymography) and incorporation of a pH gradient. Examples of capillaries used for electrophoresis include capillaries that interface with an electrospray.

Capillary electrophoresis (CE) is preferred for separating complex hydrophilic molecules and highly charged solutes. CE technology can also be implemented on microfluidic chips. Depending on the types of capillary and buffers used, CE can be further segmented into separation techniques such as capillary zone electrophoresis (CZE), capillary isoelectric focusing (CIEF), capillary isotachophoresis (cITP) and capillary electrochromatography (CEC). An embodiment to couple CE techniques to electrospray ionization involves the use of volatile solutions, for example, aqueous mixtures containing a volatile acid and/or base and an organic such as an alcohol or acetonitrile.

Capillary isotachophoresis (cITP) is a technique in which the analytes move through the capillary at a constant speed but are nevertheless separated by their respective mobilities. Capillary zone electrophoresis (CZE), also known as free-solution CE (FSCE), is based on differences in the electrophoretic mobility of the species, determined by the charge on the molecule, and the frictional resistance the molecule encounters during migration which is often directly proportional to the size of the molecule. Capillary isoelectric focusing (CIEF) allows weakly-ionizable amphoteric molecules, to be separated by electrophoresis in a pH gradient. CEC is a hybrid technique between traditional high performance liquid chromatography (HPLC) and CE.

Separation and purification techniques used in the present invention include any chromatography procedures known in the art. Chromatography can be based on the differential adsorption and elution of certain analytes or partitioning of analytes between mobile and stationary phases. Different examples of chromatography include, but not limited to, liquid chromatography (LC), gas chromatography (GC), high performance liquid chromatography (HPLC), etc.

IV. Biomarker Nucleic Acids and Polypeptides

One aspect of the present invention pertains to the use of isolated nucleic acid molecules that correspond to biomarker nucleic acids that encode a biomarker polypeptide or a portion of such a polypeptide. As used herein, the term “nucleic acid molecule” is intended to include DNA molecules (*e.g.*, cDNA or genomic DNA) and RNA molecules (*e.g.*, mRNA) and analogs of the DNA or RNA generated using nucleotide analogs. The

nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA.

An "isolated" nucleic acid molecule is one which is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid molecule. Preferably, an "isolated" nucleic acid molecule is free of sequences (preferably protein-encoding sequences) which naturally flank the nucleic acid (*i.e.*, sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated nucleic acid molecule can contain less than about 5 kB, 4 kB, 3 kB, 2 kB, 1 kB, 0.5 kB or 0.1 kB of nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized.

A biomarker nucleic acid molecule of the present invention can be isolated using standard molecular biology techniques and the sequence information in the database records described herein. Using all or a portion of such nucleic acid sequences, nucleic acid molecules of the present invention can be isolated using standard hybridization and cloning techniques (*e.g.*, as described in Sambrook *et al.*, ed., *Molecular Cloning: A Laboratory Manual, 2nd ed.*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989).

A nucleic acid molecule of the present invention can be amplified using cDNA, mRNA, or genomic DNA as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. The nucleic acid molecules so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. Furthermore, oligonucleotides corresponding to all or a portion of a nucleic acid molecule of the present invention can be prepared by standard synthetic techniques, *e.g.*, using an automated DNA synthesizer.

Moreover, a nucleic acid molecule of the present invention can comprise only a portion of a nucleic acid sequence, wherein the full length nucleic acid sequence comprises a marker of the present invention or which encodes a polypeptide corresponding to a marker of the present invention. Such nucleic acid molecules can be used, for example, as a probe or primer. The probe/primer typically is used as one or more substantially purified

oligonucleotides. The oligonucleotide typically comprises a region of nucleotide sequence that hybridizes under stringent conditions to at least about 7, preferably about 15, more preferably about 25, 50, 75, 100, 125, 150, 175, 200, 250, 300, 350, or 400 or more consecutive nucleotides of a biomarker nucleic acid sequence. Probes based on the sequence of a biomarker nucleic acid molecule can be used to detect transcripts or genomic sequences corresponding to one or more markers of the present invention. The probe comprises a label group attached thereto, *e.g.*, a radioisotope, a fluorescent compound, an enzyme, or an enzyme co-factor.

A biomarker nucleic acid molecules that differ, due to degeneracy of the genetic code, from the nucleotide sequence of nucleic acid molecules encoding a protein which corresponds to the biomarker, and thus encode the same protein, are also contemplated.

In addition, it will be appreciated by those skilled in the art that DNA sequence polymorphisms that lead to changes in the amino acid sequence can exist within a population (*e.g.*, the human population). Such genetic polymorphisms can exist among individuals within a population due to natural allelic variation. An allele is one of a group of genes which occur alternatively at a given genetic locus. In addition, it will be appreciated that DNA polymorphisms that affect RNA expression levels can also exist that may affect the overall expression level of that gene (*e.g.*, by affecting regulation or degradation).

The term "allele," which is used interchangeably herein with "allelic variant," refers to alternative forms of a gene or portions thereof. Alleles occupy the same locus or position on homologous chromosomes. When a subject has two identical alleles of a gene, the subject is said to be homozygous for the gene or allele. When a subject has two different alleles of a gene, the subject is said to be heterozygous for the gene or allele. For example, biomarker alleles can differ from each other in a single nucleotide, or several nucleotides, and can include substitutions, deletions, and insertions of nucleotides. An allele of a gene can also be a form of a gene containing one or more mutations.

The term "allelic variant of a polymorphic region of gene" or "allelic variant", used interchangeably herein, refers to an alternative form of a gene having one of several possible nucleotide sequences found in that region of the gene in the population. As used herein, allelic variant is meant to encompass functional allelic variants, non-functional allelic variants, SNPs, mutations and polymorphisms.

The term "single nucleotide polymorphism" (SNP) refers to a polymorphic site occupied by a single nucleotide, which is the site of variation between allelic sequences. The site is usually preceded by and followed by highly conserved sequences of the allele (*e.g.*, sequences that vary in less than 1/100 or 1/1000 members of a population). A SNP usually arises due to substitution of one nucleotide for another at the polymorphic site. SNPs can also arise from a deletion of a nucleotide or an insertion of a nucleotide relative to a reference allele. Typically the polymorphic site is occupied by a base other than the reference base. For example, where the reference allele contains the base "T" (thymidine) at the polymorphic site, the altered allele can contain a "C" (cytidine), "G" (guanine), or "A" (adenine) at the polymorphic site. SNP's may occur in protein-coding nucleic acid sequences, in which case they may give rise to a defective or otherwise variant protein, or genetic disease. Such a SNP may alter the coding sequence of the gene and therefore specify another amino acid (a "missense" SNP) or a SNP may introduce a stop codon (a "nonsense" SNP). When a SNP does not alter the amino acid sequence of a protein, the SNP is called "silent." SNP's may also occur in noncoding regions of the nucleotide sequence. This may result in defective protein expression, *e.g.*, as a result of alternative spicing, or it may have no effect on the function of the protein.

As used herein, the terms "gene" and "recombinant gene" refer to nucleic acid molecules comprising an open reading frame encoding a polypeptide corresponding to a marker of the present invention. Such natural allelic variations can typically result in 1-5% variance in the nucleotide sequence of a given gene. Alternative alleles can be identified by sequencing the gene of interest in a number of different individuals. This can be readily carried out by using hybridization probes to identify the same genetic locus in a variety of individuals. Any and all such nucleotide variations and resulting amino acid polymorphisms or variations that are the result of natural allelic variation and that do not alter the functional activity are intended to be within the scope of the present invention.

In another embodiment, a biomarker nucleic acid molecule is at least 7, 15, 20, 25, 30, 40, 60, 80, 100, 150, 200, 250, 300, 350, 400, 450, 550, 650, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000, 2200, 2400, 2600, 2800, 3000, 3500, 4000, 4500, or more nucleotides in length and hybridizes under stringent conditions to a nucleic acid molecule corresponding to a marker of the present invention or to a nucleic acid molecule encoding a protein corresponding to a marker of the present invention. As used herein, the term "hybridizes under stringent conditions" is intended to describe

conditions for hybridization and washing under which nucleotide sequences at least 60% (65%, 70%, 75%, 80%, preferably 85%) identical to each other typically remain hybridized to each other. Such stringent conditions are known to those skilled in the art and can be found in sections 6.3.1-6.3.6 of *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989). A preferred, non-limiting example of stringent hybridization conditions are hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by one or more washes in 0.2X SSC, 0.1% SDS at 50-65°C.

In addition to naturally-occurring allelic variants of a nucleic acid molecule of the present invention that can exist in the population, the skilled artisan will further appreciate that sequence changes can be introduced by mutation thereby leading to changes in the amino acid sequence of the encoded protein, without altering the biological activity of the protein encoded thereby. For example, one can make nucleotide substitutions leading to amino acid substitutions at "non-essential" amino acid residues. A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequence without altering the biological activity, whereas an "essential" amino acid residue is required for biological activity. For example, amino acid residues that are not conserved or only semi-conserved among homologs of various species may be non-essential for activity and thus would be likely targets for alteration. Alternatively, amino acid residues that are conserved among the homologs of various species (*e.g.*, murine and human) may be essential for activity and thus would not be likely targets for alteration.

Accordingly, another aspect of the present invention pertains to nucleic acid molecules encoding a polypeptide of the present invention that contain changes in amino acid residues that are not essential for activity. Such polypeptides differ in amino acid sequence from the naturally-occurring proteins which correspond to the markers of the present invention, yet retain biological activity. In one embodiment, a biomarker protein has an amino acid sequence that is at least about 40% identical, 50%, 60%, 70%, 75%, 80%, 83%, 85%, 87.5%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or identical to the amino acid sequence of a biomarker protein described herein.

An isolated nucleic acid molecule encoding a variant protein can be created by introducing one or more nucleotide substitutions, additions or deletions into the nucleotide sequence of nucleic acids of the present invention, such that one or more amino acid residue substitutions, additions, or deletions are introduced into the encoded protein. Mutations can be introduced by standard techniques, such as site-directed mutagenesis and PCR-mediated

mutagenesis. Preferably, conservative amino acid substitutions are made at one or more predicted non-essential amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), non-polar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Alternatively, mutations can be introduced randomly along all or part of the coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for biological activity to identify mutants that retain activity. Following mutagenesis, the encoded protein can be expressed recombinantly and the activity of the protein can be determined.

In some embodiments, the present invention further contemplates the use of anti-biomarker antisense nucleic acid molecules, *i.e.*, molecules which are complementary to a sense nucleic acid of the present invention, *e.g.*, complementary to the coding strand of a double-stranded cDNA molecule corresponding to a marker of the present invention or complementary to an mRNA sequence corresponding to a marker of the present invention. Accordingly, an antisense nucleic acid molecule of the present invention can hydrogen bond to (*i.e.* anneal with) a sense nucleic acid of the present invention. The antisense nucleic acid can be complementary to an entire coding strand, or to only a portion thereof, *e.g.*, all or part of the protein coding region (or open reading frame). An antisense nucleic acid molecule can also be antisense to all or part of a non-coding region of the coding strand of a nucleotide sequence encoding a polypeptide of the present invention. The non-coding regions ("5' and 3' untranslated regions") are the 5' and 3' sequences which flank the coding region and are not translated into amino acids.

An antisense oligonucleotide can be, for example, about 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 or more nucleotides in length. An antisense nucleic acid can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (*e.g.*, an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the

physical stability of the duplex formed between the antisense and sense nucleic acids, *e.g.*, phosphorothioate derivatives and acridine substituted nucleotides can be used. Examples of modified nucleotides which can be used to generate the antisense nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2- methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. Alternatively, the antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been sub-cloned in an antisense orientation (*i.e.*, RNA transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest, described further in the following subsection).

The antisense nucleic acid molecules of the present invention are typically administered to a subject or generated *in situ* such that they hybridize with or bind to cellular mRNA and/or genomic DNA encoding a polypeptide corresponding to a selected marker of the present invention to thereby inhibit expression of the marker, *e.g.*, by inhibiting transcription and/or translation. The hybridization can be by conventional nucleotide complementarity to form a stable duplex, or, for example, in the case of an antisense nucleic acid molecule which binds to DNA duplexes, through specific interactions in the major groove of the double helix. Examples of a route of administration of antisense nucleic acid molecules of the present invention includes direct injection at a tissue site or infusion of the antisense nucleic acid into a blood- or bone marrow-associated body fluid. Alternatively, antisense nucleic acid molecules can be modified to target selected cells and then administered systemically. For example, for systemic administration, antisense molecules can be modified such that they specifically bind to receptors or antigens expressed on a selected cell surface, *e.g.*, by linking the antisense nucleic acid molecules to peptides or antibodies which bind to cell surface receptors or antigens. The antisense

nucleic acid molecules can also be delivered to cells using the vectors described herein. To achieve sufficient intracellular concentrations of the antisense molecules, vector constructs in which the antisense nucleic acid molecule is placed under the control of a strong pol II or pol III promoter are preferred.

An antisense nucleic acid molecule of the present invention can be an α-anomeric nucleic acid molecule. An α-anomeric nucleic acid molecule forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual α-units, the strands run parallel to each other (Gaultier *et al.,* 1987, *Nucleic Acids Res.* 15:6625-6641). The antisense nucleic acid molecule can also comprise a 2'-o-methylribonucleotide (Inoue *et al.,* 1987, *Nucleic Acids Res.* 15:6131-6148) or a chimeric RNA-DNA analogue (Inoue *et al.,* 1987, *FEBS Lett.* 215:327-330).

The present invention also encompasses ribozymes. Ribozymes are catalytic RNA molecules with ribonuclease activity which are capable of cleaving a single-stranded nucleic acid, such as an mRNA, to which they have a complementary region. Thus, ribozymes (*e.g.*, hammerhead ribozymes as described in Haselhoff and Gerlach, 1988, *Nature* 334:585-591) can be used to catalytically cleave mRNA transcripts to thereby inhibit translation of the protein encoded by the mRNA. A ribozyme having specificity for a nucleic acid molecule encoding a polypeptide corresponding to a marker of the present invention can be designed based upon the nucleotide sequence of a cDNA corresponding to the marker. For example, a derivative of a *Tetrahymena* L-19 IVS RNA can be constructed in which the nucleotide sequence of the active site is complementary to the nucleotide sequence to be cleaved (see Cech *et al.* U.S. Patent No. 4,987,071; and Cech *et al.* U.S. Patent No. 5,116,742). Alternatively, an mRNA encoding a polypeptide of the present invention can be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules (see, *e.g.*, Bartel and Szostak, 1993, *Science* 261:1411-1418).

The present invention also encompasses nucleic acid molecules which form triple helical structures. For example, expression of a biomarker protein can be inhibited by targeting nucleotide sequences complementary to the regulatory region of the gene encoding the polypeptide (*e.g.*, the promoter and/or enhancer) to form triple helical structures that prevent transcription of the gene in target cells. See generally Helene (1991) *Anticancer Drug Des.* 6(6):569-84; Helene (1992) *Ann. N.Y. Acad. Sci.* 660:27-36; and Maher (1992) *Bioassays* 14(12):807-15.

In various embodiments, the nucleic acid molecules of the present invention can be modified at the base moiety, sugar moiety or phosphate backbone to improve, *e.g.*, the stability, hybridization, or solubility of the molecule. For example, the deoxyribose phosphate backbone of the nucleic acid molecules can be modified to generate peptide nucleic acid molecules (see Hyrup *et al.,* 1996, *Bioorganic & Medicinal Chemistry* 4(1): 5-23). As used herein, the terms "peptide nucleic acids" or "PNAs" refer to nucleic acid mimics, *e.g.*, DNA mimics, in which the deoxyribose phosphate backbone is replaced by a pseudopeptide backbone and only the four natural nucleobases are retained. The neutral backbone of PNAs has been shown to allow for specific hybridization to DNA and RNA under conditions of low ionic strength. The synthesis of PNA oligomers can be performed using standard solid phase peptide synthesis protocols as described in Hyrup *et al.* (1996), *supra*; Perry-O'Keefe *et al.* (1996) *Proc. Natl. Acad. Sci. USA* 93:14670-675.

PNAs can be used in therapeutic and diagnostic applications. For example, PNAs can be used as antisense or antigene agents for sequence-specific modulation of gene expression by, *e.g.*, inducing transcription or translation arrest or inhibiting replication. PNAs can also be used, *e.g.*, in the analysis of single base pair mutations in a gene by, *e.g.*, PNA directed PCR clamping; as artificial restriction enzymes when used in combination with other enzymes, *e.g.*, S1 nucleases (Hyrup (1996), *supra*; or as probes or primers for DNA sequence and hybridization (Hyrup, 1996, *supra*; Perry-O'Keefe *et al.,* 1996, *Proc. Natl. Acad. Sci. USA* 93:14670-675).

In another embodiment, PNAs can be modified, *e.g.*, to enhance their stability or cellular uptake, by attaching lipophilic or other helper groups to PNA, by the formation of PNA-DNA chimeras, or by the use of liposomes or other techniques of drug delivery known in the art. For example, PNA-DNA chimeras can be generated which can combine the advantageous properties of PNA and DNA. Such chimeras allow DNA recognition enzymes, *e.g.*, RNASE H and DNA polymerases, to interact with the DNA portion while the PNA portion would provide high binding affinity and specificity. PNA-DNA chimeras can be linked using linkers of appropriate lengths selected in terms of base stacking, number of bonds between the nucleobases, and orientation (Hyrup, 1996, *supra*). The synthesis of PNA-DNA chimeras can be performed as described in Hyrup (1996), *supra,* and Finn *et al.* (1996) *Nucleic Acids Res.* 24(17):3357-63. For example, a DNA chain can be synthesized on a solid support using standard phosphoramidite coupling chemistry and modified nucleoside analogs. Compounds such as 5'-(4-methoxytrityl)amino-5'-deoxy-

thymidine phosphoramidite can be used as a link between the PNA and the 5' end of DNA (Mag *et al.,* 1989, *Nucleic Acids Res.* 17:5973-88). PNA monomers are then coupled in a step-wise manner to produce a chimeric molecule with a 5' PNA segment and a 3' DNA segment (Finn *et al.,* 1996, *Nucleic Acids Res.* 24(17):3357-63). Alternatively, chimeric molecules can be synthesized with a 5' DNA segment and a 3' PNA segment (Peterser *et al.,* 1975, *Bioorganic Med. Chem. Lett.* 5:1119-11124).

In other embodiments, the oligonucleotide can include other appended groups such as peptides (*e.g.*, for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane (see, *e.g.*, Letsinger *et al.,* 1989, *Proc. Natl. Acad. Sci. USA* 86:6553-6556; Lemaitre *et al.,* 1987, *Proc. Natl. Acad. Sci. USA* 84:648-652; PCT Publication No. WO 88/09810) or the blood-brain barrier (see, *e.g.*, PCT Publication No. WO 89/10134). In addition, oligonucleotides can be modified with hybridization-triggered cleavage agents (see, *e.g.,* Krol *et al.,* 1988, *Bio/Techniques* 6:958-976) or intercalating agents (see, *e.g.,* Zon, 1988, *Pharm. Res.* 5:539-549). To this end, the oligonucleotide can be conjugated to another molecule, *e.g.*, a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, *etc.*

Another aspect of the present invention pertains to the use of biomarker proteins and biologically active portions thereof. In one embodiment, the native polypeptide corresponding to a marker can be isolated from cells or tissue sources by an appropriate purification scheme using standard protein purification techniques. In another embodiment, polypeptides corresponding to a marker of the present invention are produced by recombinant DNA techniques. Alternative to recombinant expression, a polypeptide corresponding to a marker of the present invention can be synthesized chemically using standard peptide synthesis techniques.

An "isolated" or "purified" protein or biologically active portion thereof is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the protein is derived, or substantially free of chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of protein in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly produced. Thus, protein that is substantially free of cellular material includes preparations of protein having less than about 30%, 20%, 10%, or 5% (by dry weight) of heterologous protein (also referred to herein as a "contaminating protein"). When the protein or biologically active portion

thereof is recombinantly produced, it is also preferably substantially free of culture medium, *i.e.*, culture medium represents less than about 20%, 10%, or 5% of the volume of the protein preparation. When the protein is produced by chemical synthesis, it is preferably substantially free of chemical precursors or other chemicals, *i.e.*, it is separated from chemical precursors or other chemicals which are involved in the synthesis of the protein. Accordingly such preparations of the protein have less than about 30%, 20%, 10%, 5% (by dry weight) of chemical precursors or compounds other than the polypeptide of interest.

Biologically active portions of a biomarker polypeptide include polypeptides comprising amino acid sequences sufficiently identical to or derived from a biomarker protein amino acid sequence described herein, but which includes fewer amino acids than the full length protein, and exhibit at least one activity of the corresponding full-length protein. Typically, biologically active portions comprise a domain or motif with at least one activity of the corresponding protein. A biologically active portion of a protein of the present invention can be a polypeptide which is, for example, 10, 25, 50, 100 or more amino acids in length. Moreover, other biologically active portions, in which other regions of the protein are deleted, can be prepared by recombinant techniques and evaluated for one or more of the functional activities of the native form of a polypeptide of the present invention.

Preferred polypeptides have an amino acid sequence of a biomarker protein encoded by a nucleic acid molecule described herein. Other useful proteins are substantially identical (*e.g.*, at least about 40%, preferably 50%, 60%, 70%, 75%, 80%, 83%, 85%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%) to one of these sequences and retain the functional activity of the protein of the corresponding naturally-occurring protein yet differ in amino acid sequence due to natural allelic variation or mutagenesis.

To determine the percent identity of two amino acid sequences or of two nucleic acids, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is

a function of the number of identical positions shared by the sequences (*i.e.*, % identity = # of identical positions/total # of positions (*e.g.*, overlapping positions) x100). In one embodiment the two sequences are the same length.

The determination of percent identity between two sequences can be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA* 87:2264-2268, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul, *et al.* (1990) *J. Mol. Biol.* 215:403-410. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to a nucleic acid molecules of the present invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to a protein molecules of the present invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res.* 25:3389-3402. Alternatively, PSI-Blast can be used to perform an iterated search which detects distant relationships between molecules. When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov. Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, (1988) *Comput Appl Biosci,* 4:11-7. Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used. Yet another useful algorithm for identifying regions of local sequence similarity and alignment is the FASTA algorithm as described in Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. USA* 85:2444-2448. When using the FASTA algorithm for comparing nucleotide or amino acid sequences, a PAM120 weight residue table can, for example, be used with a *k*-tuple value of 2.

The percent identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, only exact matches are counted.

The present invention also provides chimeric or fusion proteins corresponding to a biomarker protein. As used herein, a "chimeric protein" or "fusion protein" comprises all or part (preferably a biologically active part) of a polypeptide corresponding to a marker of the present invention operably linked to a heterologous polypeptide (*i.e.*, a polypeptide other than the polypeptide corresponding to the marker). Within the fusion protein, the term "operably linked" is intended to indicate that the polypeptide of the present invention and the heterologous polypeptide are fused in-frame to each other. The heterologous polypeptide can be fused to the amino-terminus or the carboxyl-terminus of the polypeptide of the present invention.

One useful fusion protein is a GST fusion protein in which a polypeptide corresponding to a marker of the present invention is fused to the carboxyl terminus of GST sequences. Such fusion proteins can facilitate the purification of a recombinant polypeptide of the present invention.

In another embodiment, the fusion protein contains a heterologous signal sequence, immunoglobulin fusion protein, toxin, or other useful protein sequence. Chimeric and fusion proteins of the present invention can be produced by standard recombinant DNA techniques. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed and re-amplified to generate a chimeric gene sequence (see, *e.g.*, Ausubel *et al.*, *supra*). Moreover, many expression vectors are commercially available that already encode a fusion moiety (*e.g.*, a GST polypeptide). A nucleic acid encoding a polypeptide of the present invention can be cloned into such an expression vector such that the fusion moiety is linked in-frame to the polypeptide of the present invention.

A signal sequence can be used to facilitate secretion and isolation of the secreted protein or other proteins of interest. Signal sequences are typically characterized by a core of hydrophobic amino acids which are generally cleaved from the mature protein during secretion in one or more cleavage events. Such signal peptides contain processing sites that allow cleavage of the signal sequence from the mature proteins as they pass through the secretory pathway. Thus, the present invention pertains to the described polypeptides having a signal sequence, as well as to polypeptides from which the signal sequence has been proteolytically cleaved (*i.e.*, the cleavage products). In one embodiment, a nucleic

acid sequence encoding a signal sequence can be operably linked in an expression vector to a protein of interest, such as a protein which is ordinarily not secreted or is otherwise difficult to isolate. The signal sequence directs secretion of the protein, such as from a eukaryotic host into which the expression vector is transformed, and the signal sequence is subsequently or concurrently cleaved. The protein can then be readily purified from the extracellular medium by art recognized methods. Alternatively, the signal sequence can be linked to the protein of interest using a sequence which facilitates purification, such as with a GST domain.

The present invention also pertains to variants of the biomarker polypeptides described herein. Such variants have an altered amino acid sequence which can function as either agonists (mimetics) or as antagonists. Variants can be generated by mutagenesis, *e.g.*, discrete point mutation or truncation. An agonist can retain substantially the same, or a subset, of the biological activities of the naturally occurring form of the protein. An antagonist of a protein can inhibit one or more of the activities of the naturally occurring form of the protein by, for example, competitively binding to a downstream or upstream member of a cellular signaling cascade which includes the protein of interest. Thus, specific biological effects can be elicited by treatment with a variant of limited function. Treatment of a subject with a variant having a subset of the biological activities of the naturally occurring form of the protein can have fewer side effects in a subject relative to treatment with the naturally occurring form of the protein.

Variants of a biomarker protein which function as either agonists (mimetics) or as antagonists can be identified by screening combinatorial libraries of mutants, *e.g.*, truncation mutants, of the protein of the present invention for agonist or antagonist activity. In one embodiment, a variegated library of variants is generated by combinatorial mutagenesis at the nucleic acid level and is encoded by a variegated gene library. A variegated library of variants can be produced by, for example, enzymatically ligating a mixture of synthetic oligonucleotides into gene sequences such that a degenerate set of potential protein sequences is expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (*e.g.*, for phage display). There are a variety of methods which can be used to produce libraries of potential variants of the polypeptides of the present invention from a degenerate oligonucleotide sequence. Methods for synthesizing degenerate oligonucleotides are known in the art (see, *e.g.*, Narang, 1983, *Tetrahedron*

39:3; Itakura *et al.,* 1984, *Annu. Rev. Biochem.* 53:323; Itakura *et al.,* 1984, *Science* 198:1056; Ike *et al.,* 1983 *Nucleic Acid Res.* 11:477).

In addition, libraries of fragments of the coding sequence of a polypeptide corresponding to a marker of the present invention can be used to generate a variegated population of polypeptides for screening and subsequent selection of variants. For example, a library of coding sequence fragments can be generated by treating a double stranded PCR fragment of the coding sequence of interest with a nuclease under conditions wherein nicking occurs only about once per molecule, denaturing the double stranded DNA, renaturing the DNA to form double stranded DNA which can include sense/antisense pairs from different nicked products, removing single stranded portions from reformed duplexes by treatment with S1 nuclease, and ligating the resulting fragment library into an expression vector. By this method, an expression library can be derived which encodes amino terminal and internal fragments of various sizes of the protein of interest.

Several techniques are known in the art for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening cDNA libraries for gene products having a selected property. The most widely used techniques, which are amenable to high throughput analysis, for screening large gene libraries typically include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates isolation of the vector encoding the gene whose product was detected. Recursive ensemble mutagenesis (REM), a technique which enhances the frequency of functional mutants in the libraries, can be used in combination with the screening assays to identify variants of a protein of the present invention (Arkin and Yourvan, 1992, *Proc. Natl. Acad. Sci. USA 8*9:7811-7815; Delgrave *et al.,* 1993, *Protein Engineering* 6(3):327- 331).

An isolated polypeptide or a fragment thereof (or a nucleic acid encoding such a polypeptide) corresponding to one or more biomarkers of the invention, including the biomarkers listed in Table 1 or fragments thereof, can be used as an immunogen to generate antibodies that bind to said immunogen, using standard techniques for polyclonal and monoclonal antibody preparation according to well-known methods in the art. An antigenic peptide comprises at least 8 amino acid residues and encompasses an epitope present in the respective full length molecule such that an antibody raised against the peptide forms a specific immune complex with the respective full length molecule. Preferably, the

antigenic peptide comprises at least 10 amino acid residues. In one embodiment such epitopes can be specific for a given polypeptide molecule from one species, such as mouse or human (*i.e.*, an antigenic peptide that spans a region of the polypeptide molecule that is not conserved across species is used as immunogen; such non conserved residues can be determined using an alignment such as that provided herein).

In some embodiments, the immunotherapy utilizes an inhibitor of at least one immune checkpoint, such as an antibody binds substantially specifically to an immune checkpoint, such as PD-1, and inhibits or blocks its immunoinhibitory function, such as by interrupting its interaction with a binding partner of the immune checkpoint, such as PD-L1 and/or PD-L2 binding partners of PD-1. In one embodiment, an antibody, especially an intrbody, binds substantially specifically to one or more biomarkers listed in Table 1 and inhibits or blocks its biological function, such as by interrupting its interaction with a substrate like STAT or JAK proteins. In another embodiment, an antibody, especially an intrbody, binds substantially specifically to a biomarker binding partner, such as biomarker substrates descrbied herein, and inhibits or blocks its biological function, such as by interrupting its interaction to one or more biomarkers listed in Table 1.

For example, a polypeptide immunogen typically is used to prepare antibodies by immunizing a suitable subject (*e.g.*, rabbit, goat, mouse or other mammal) with the immunogen. A preferred animal is a mouse deficeint in the desired target antigen. For example, a PD-1 knockout mouse if the desired antibody is an anti-PD-1 antibody, may be used. This results in a wider spectrum of antibody recognition possibilities as antibodies reactive to common mouse and human epitopes are not removed by tolerance mechanisms. An appropriate immunogenic preparation can contain, for example, a recombinantly expressed or chemically synthesized molecule or fragment thereof to which the immune response is to be generated. The preparation can further include an adjuvant, such as Freund's complete or incomplete adjuvant, or similar immunostimulatory agent. Immunization of a suitable subject with an immunogenic preparation induces a polyclonal antibody response to the antigenic peptide contained therein.

Polyclonal antibodies can be prepared as described above by immunizing a suitable subject with a polypeptide immunogen. The polypeptide antibody titer in the immunized subject can be monitored over time by standard techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized polypeptide. If desired, the antibody directed against the antigen can be isolated from the mammal (*e.g.*, from the blood) and

further purified by well-known techniques, such as protein A chromatography, to obtain the IgG fraction. At an appropriate time after immunization, *e.g.*, when the antibody titers are highest, antibody-producing cells can be obtained from the subject and used to prepare monoclonal antibodies by standard techniques, such as the hybridoma technique (originally described by Kohler and Milstein (1975) *Nature* 256:495-497) (see also Brown *et al.* (1981) *J. Immunol.* 127:539-46; Brown *et al.* (1980) *J. Biol. Chem.* 255:4980-83; Yeh *et al.* (1976) *Proc. Natl. Acad. Sci.* 76:2927-31; Yeh *et al.* (1982) *Int. J. Cancer* 29:269-75), the more recent human B cell hybridoma technique (Kozbor *et al.* (1983) *Immunol. Today* 4:72), the EBV-hybridoma technique (Cole *et al.* (1985) *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96) or trioma techniques. The technology for producing monoclonal antibody hybridomas is well-known (see generally Kenneth, R. H. in *Monoclonal Antibodies: A New Dimension In Biological Analyses*, Plenum Publishing Corp., New York, New York (1980); Lerner, E. A. (1981) *Yale J. Biol. Med.* 54:387-402; Gefter, M. L. *et al.* (1977) *Somatic Cell Genet.* 3:231-36). Briefly, an immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal immunized with an immunogen as described above, and the culture supernatants of the resulting hybridoma cells are screened to identify a hybridoma producing a monoclonal antibody that binds to the polypeptide antigen, preferably specifically. In some embodiments, the immunization is performed in a cell or animal host that has a knockout of a target antigen of interest (*e.g.*, does not produce the antigen prior to immunization).

Any of the many well-known protocols used for fusing lymphocytes and immortalized cell lines can be applied for the purpose of generating a monoclonal antibody against one or more biomarkers of the invention, including the biomarkers listed in Table 1, or a fragment thereof (see, *e.g.*, Galfre, G. *et al.* (1977) *Nature* 266:55052; Gefter *et al.* (1977) *supra*; Lerner (1981) *supra*; Kenneth (1980) *supra*). Moreover, the ordinary skilled worker will appreciate that there are many variations of such methods which also would be useful. Typically, the immortal cell line (*e.g.*, a myeloma cell line) is derived from the same mammalian species as the lymphocytes. For example, murine hybridomas can be made by fusing lymphocytes from a mouse immunized with an immunogenic preparation of the present invention with an immortalized mouse cell line. Preferred immortal cell lines are mouse myeloma cell lines that are sensitive to culture medium containing hypoxanthine, aminopterin and thymidine ("HAT medium"). Any of a number of myeloma cell lines can be used as a fusion partner according to standard techniques, *e.g.*, the P3-NS1/1-Ag4-1, P3-

x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. These myeloma lines are available from the American Type Culture Collection (ATCC), Rockville, MD. Typically, HAT-sensitive mouse myeloma cells are fused to mouse splenocytes using polyethylene glycol ("PEG"). Hybridoma cells resulting from the fusion are then selected using HAT medium, which kills unfused and unproductively fused myeloma cells (unfused splenocytes die after several days because they are not transformed). Hybridoma cells producing a monoclonal antibody of the invention are detected by screening the hybridoma culture supernatants for antibodies that bind a given polypeptide, *e.g.*, using a standard ELISA assay.

As an alternative to preparing monoclonal antibody-secreting hybridomas, a monoclonal specific for one of the above described polypeptides can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (*e.g.*, an antibody phage display library) with the appropriate polypeptide to thereby isolate immunoglobulin library members that bind the polypeptide. Kits for generating and screening phage display libraries are commercially available (*e.g.*, the Pharmacia *Recombinant Phage Antibody System*, Catalog No. 27-9400-01; and the Stratagene *SurfZAP*^{TM} *Phage Display Kit*, Catalog No. 240612). Additionally, examples of methods and reagents particularly amenable for use in generating and screening an antibody display library can be found in, for example, Ladner *et al.* U.S. Patent No. 5,223,409; Kang *et al.* International Publication No. WO 92/18619; Dower *et al.* International Publication No. WO 91/17271; Winter *et al.* International Publication WO 92/20791; Markland *et al.* International Publication No. WO 92/15679; Breitling *et al.* International Publication WO 93/01288; McCafferty *et al.* International Publication No. WO 92/01047; Garrard *et al.* International Publication No. WO 92/09690; Ladner *et al.* International Publication No. WO 90/02809; Fuchs *et al.* (1991) *Biotechnology (NY)* 9:1369-1372; Hay *et al.* (1992) *Hum. Antibod. Hybridomas* 3:81-85; Huse *et al.* (1989) *Science* 246:1275-1281; Griffiths *et al.* (1993) *EMBO J.* 12:725-734; Hawkins *et al.* (1992) *J. Mol. Biol.* 226:889-896; Clarkson *et al.* (1991) *Nature* 352:624-628; Gram *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:3576-3580; Garrard *et al.* (1991) *Biotechnology (NY)* 9:1373-1377; Hoogenboom *et al.* (1991) *Nucleic Acids Res.* 19:4133-4137; Barbas *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:7978-7982; and McCafferty *et al.* (1990) *Nature* 348:552-554.

Since it is well-known in the art that antibody heavy and light chain CDR3 domains play a particularly important role in the binding specificity/affinity of an antibody for an antigen, the recombinant monoclonal antibodies of the present invention prepared as set

forth above preferably comprise the heavy and light chain CDR3s of variable regions of the antibodies described herein and well-known in the art. Similarly, the antibodies can further comprise the CDR2s of variable regions of said antibodies. The antibodies can further comprise the CDR1s of variable regions of said antibodies. In other embodiments, the antibodies can comprise any combinations of the CDRs.

The CDR1, 2, and/or 3 regions of the engineered antibodies described above can comprise the exact amino acid sequence(s) as those of variable regions of the present invention described herein. However, the ordinarily skilled artisan will appreciate that some deviation from the exact CDR sequences may be possible while still retaining the ability of the antibody, especially an introbody, to bind a desired target, such as one or more biomarkers listed in Table 1 and/or a binding partner thereof, either alone or in combination with an immunotherapy, such as one or more biomarkers listed in Table 1, biomarker binding partners/substrates, or an immunotherapy effectively (*e.g.*, conservative sequence modifications). Accordingly, in another embodiment, the engineered antibody may be composed of one or more CDRs that are, for example, 50%, 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 99.5% identical to one or more CDRs of the present invention described herein or otherwise publicly available.

For example, the structural features of non-human or human antibodies (*e.g.*, a rat anti-mouse/anti-human antibody) can be used to create structurally related human antibodies, especially introbodies, that retain at least one functional property of the antibodies of the present invention, such as binding to one or more biomarkers listed in Table 1, biomarker binding partners/substrates, and/or an immune checkpoint. Another functional property includes inhibiting binding of the original known, non-human or human antibodies in a competition ELISA assay.

Antibodies, immunoglobulins, and polypeptides of the invention can be used in an isolated (*e.g.*, purified) form or contained in a vector, such as a membrane or lipid vesicle (*e.g.* a liposome). Moreover, amino acid sequence modification(s) of the antibodies described herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody. It is known that when a humanized antibody is produced by simply grafting only CDRs in VH and VL of an antibody derived from a non-human animal in FRs of the VH and VL of a human antibody, the antigen binding activity is reduced in comparison with that of the original antibody derived from a non-human animal. It is considered that several amino acid residues of the

VH and VL of the non-human antibody, not only in CDRs but also in FRs, are directly or indirectly associated with the antigen binding activity. Hence, substitution of these amino acid residues with different amino acid residues derived from FRs of the VH and VL of the human antibody would reduce binding activity and can be corrected by replacing the amino acids with amino acid residues of the original antibody derived from a non-human animal.

Similarly, modifications and changes may be made in the structure of the antibodies described herein, and in the DNA sequences encoding them, and still obtain a functional molecule that encodes an antibody and polypeptide with desirable characteristics. For example, antibody glycosylation patterns can be modulated to, for example, increase stability. By "altering" is meant deleting one or more carbohydrate moieties found in the antibody, and/or adding one or more glycosylation sites that are not present in the antibody. Glycosylation of antibodies is typically N-linked. "N-linked" refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagines-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. Addition of glycosylation sites to the antibody is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). Another type of covalent modification involves chemically or enzymatically coupling glycosides to the antibody. These procedures are advantageous in that they do not require production of the antibody in a host cell that has glycosylation capabilities for N- or O-linked glycosylation. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, orhydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. For example, such methods are described in WO87/05330.

Similarly, removal of any carbohydrate moieties present on the antibody may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the antibody to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the antibody intact. Chemical

deglycosylation is described by *Sojahr et al.* (1987) and by Edge *et al.* (1981). Enzymatic cleavage of carbohydrate moieties on antibodies can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura *et al.* (1987).

Other modifications can involve the formation of immunoconjugates. For example, in one type of covalent modification, antibodies or proteins are covalently linked to one of a variety of non proteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Pat. No. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

Conjugation of antibodies or other proteins of the present invention with heterologous agents can be made using a variety of bifunctional protein coupling agents including but not limited to N-succinimidyl (2-pyridyldithio) propionate (SPDP), succinimidyl (N-maleimidomethyl)cyclohexane-1-carboxylate, iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, carbon labeled 1-isothiocyanatobenzyl methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody (WO 94/11026).

In another aspect, the present invention features antibodies conjugated to a therapeutic moiety, such as a cytotoxin, a drug, and/or a radioisotope. When conjugated to a cytotoxin, these antibody conjugates are referred to as "immunotoxins." A cytotoxin or cytotoxic agent includes any agent that is detrimental to (*e.g.*, kills) cells. Examples include taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Therapeutic agents include, but are not limited to, antimetabolites (*e.g.*, methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (*e.g.*, mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclothosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin),

anthracyclines (*e.g.*, daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (*e.g.*, dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (*e.g.*, vincristine and vinblastine). An antibody of the present invention can be conjugated to a radioisotope, *e.g.*, radioactive iodine, to generate cytotoxic radiopharmaceuticals for treating a related disorder, such as a cancer.

Conjugated antibodies, in addition to therapeutic utility, can be useful for diagnostically or prognostically to monitor polypeptide levels in tissue as part of a clinical testing procedure, *e.g.*, to determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling (*i e.*, physically linking) the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate (FITC), rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin (PE); an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include ^{125}I, ^{131}I, ^{35}S, or ^{3}H. [0134] As used herein, the term "labeled", with regard to the antibody, is intended to encompass direct labeling of the antibody by coupling (*i.e.*, physically linking) a detectable substance, such as a radioactive agent or a fluorophore (*e.g.* fluorescein isothiocyanate (FITC) or phycoerythrin (PE) or Indocyanine (Cy5)) to the antibody, as well as indirect labeling of the antibody by reactivity with a detectable substance.

The antibody conjugates of the present invention can be used to modify a given biological response. The therapeutic moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, an enzymatically active toxin, or active fragment thereof, such as abrin, ricin A, *Pseudomonas* exotoxin, or diphtheria toxin; a protein such as tumor necrosis factor or interferon-.gamma.; or, biological response modifiers such as, for example, lymphokines, interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"), granulocyte macrophage colony stimulating

factor ("GM-CSF"), granulocyte colony stimulating factor ("G-CSF"), or other cytokines or growth factors.

In one embodiment, an antibody for use in the instant invention is a bispecific or multispecific antibody. A bispecific antibody has binding sites for two different antigens within a single antibody polypeptide. Antigen binding may be simultaneous or sequential. Triomas and hybrid hybridomas are two examples of cell lines that can secrete bispecific antibodies. Examples of bispecific antibodies produced by a hybrid hybridoma or a trioma are disclosed in U.S. Patent 4,474,893. Bispecific antibodies have been constructed by chemical means (Staerz *et al.* (1985) *Nature* 314:628, and Perez *et al.* (1985) *Nature* 316:354) and hybridoma technology (Staerz and Bevan (1986) *Proc. Natl. Acad. Sci. USA*, 83:1453, and Staerz and Bevan (1986) *Immunol. Today* 7:241). Bispecific antibodies are also described in U.S. Patent 5,959,084. Fragments of bispecific antibodies are described in U.S. Patent 5,798,229.

Bispecific agents can also be generated by making heterohybridomas by fusing hybridomas or other cells making different antibodies, followed by identification of clones producing and co-assembling both antibodies. They can also be generated by chemical or genetic conjugation of complete immunoglobulin chains or portions thereof such as Fab and Fv sequences. The antibody component can bind to a polypeptide or a fragment thereof of one or more biomarkers of the invention, including one or more biomarkers listed in Table 1, or a fragment thereof. In one embodiment, the bispecific antibody could specifically bind to both a polypeptide or a fragment thereof and its natural binding partner(s) or a fragment(s) thereof.

Techniques for modulating antibodies, such as humanization, conjugation, recombinant techniques, and the like are well-known in the art.

In another aspect of this invention, peptides or peptide mimetics can be used to antagonize the activity of one or more biomarkers of the invention, including one or more biomarkers listed in Table 1, or a fragment(s) thereof. In one embodiment, variants of one or more biomarkers listed in Table 1 which function as a modulating agent for the respective full length protein, can be identified by screening combinatorial libraries of mutants, *e.g.*, truncation mutants, for antagonist activity. In one embodiment, a variegated library of variants is generated by combinatorial mutagenesis at the nucleic acid level and is encoded by a variegated gene library. A variegated library of variants can be produced, for instance, by enzymatically ligating a mixture of synthetic oligonucleotides into gene

sequences such that a degenerate set of potential polypeptide sequences is expressible as individual polypeptides containing the set of polypeptide sequences therein. There are a variety of methods which can be used to produce libraries of polypeptide variants from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be performed in an automatic DNA synthesizer, and the synthetic gene then ligated into an appropriate expression vector. Use of a degenerate set of genes allows for the provision, in one mixture, of all of the sequences encoding the desired set of potential polypeptide sequences. Methods for synthesizing degenerate oligonucleotides are known in the art (see, *e.g.*, Narang, S. A. (1983) *Tetrahedron* 39:3; Itakura *et al.* (1984) *Annu. Rev. Biochem.* 53:323; Itakura *et al.* (1984) *Science* 198:1056; Ike *et al.* (1983) *Nucleic Acid Res.* 11:477.

In addition, libraries of fragments of a polypeptide coding sequence can be used to generate a variegated population of polypeptide fragments for screening and subsequent selection of variants of a given polypeptide. In one embodiment, a library of coding sequence fragments can be generated by treating a double stranded PCR fragment of a polypeptide coding sequence with a nuclease under conditions wherein nicking occurs only about once per polypeptide, denaturing the double stranded DNA, renaturing the DNA to form double stranded DNA which can include sense/antisense pairs from different nicked products, removing single stranded portions from reformed duplexes by treatment with S1 nuclease, and ligating the resulting fragment library into an expression vector. By this method, an expression library can be derived which encodes N-terminal, C-terminal and internal fragments of various sizes of the polypeptide.

Several techniques are known in the art for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening cDNA libraries for gene products having a selected property. Such techniques are adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of polypeptides. The most widely used techniques, which are amenable to high through-put analysis, for screening large gene libraries typically include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates isolation of the vector encoding the gene whose product was detected. Recursive ensemble mutagenesis (REM), a technique which enhances the frequency of functional mutants in the libraries, can be used in combination with the screening assays to identify variants of interest (Arkin and Youvan (1992) *Proc. Natl. Acad.*

Sci. USA 89:7811-7815; Delagrave *et al.* (1993) *Protein Eng.* 6(3):327-331). In one embodiment, cell based assays can be exploited to analyze a variegated polypeptide library. For example, a library of expression vectors can be transfected into a cell line which ordinarily synthesizes one or more biomarkers of the invention, including one or more biomarkers listed in Table 1, or a fragment thereof. The transfected cells are then cultured such that the full length polypeptide and a particular mutant polypeptide are produced and the effect of expression of the mutant on the full length polypeptide activity in cell supernatants can be detected, *e.g.*, by any of a number of functional assays. Plasmid DNA can then be recovered from the cells which score for inhibition, or alternatively, potentiation of full length polypeptide activity, and the individual clones further characterized.

Systematic substitution of one or more amino acids of a polypeptide amino acid sequence with a D-amino acid of the same type (*e.g.*, D-lysine in place of L-lysine) can be used to generate more stable peptides. In addition, constrained peptides comprising a polypeptide amino acid sequence of interest or a substantially identical sequence variation can be generated by methods known in the art (Rizo and Gierasch (1992) *Annu. Rev. Biochem.* 61:387, incorporated herein by reference); for example, by adding internal cysteine residues capable of forming intramolecular disulfide bridges which cyclize the peptide.

The amino acid sequences described herein will enable those of skill in the art to produce polypeptides corresponding peptide sequences and sequence variants thereof. Such polypeptides can be produced in prokaryotic or eukaryotic host cells by expression of polynucleotides encoding the peptide sequence, frequently as part of a larger polypeptide. Alternatively, such peptides can be synthesized by chemical methods. Methods for expression of heterologous proteins in recombinant hosts, chemical synthesis of polypeptides, and *in vitro* translation are well-known in the art and are described further in Maniatis *et al. Molecular Cloning: A Laboratory Manual* (1989), 2nd Ed., Cold Spring Harbor, N.Y.; Berger and Kimmel, Methods in Enzymology, Volume 152, Guide to Molecular Cloning Techniques (1987), Academic Press, Inc., San Diego, Calif.; Merrifield, J. (1969) *J. Am. Chem. Soc.* 91:501; Chaiken I. M. (1981) *CRC Crit. Rev. Biochem.* 11: 255; Kaiser *et al.* (1989) *Science* 243:187; Merrifield, B. (1986) *Science* 232:342; Kent, S. B. H. (1988) *Annu. Rev. Biochem.* 57:957; and Offord, R. E. (1980) *Semisynthetic Proteins*, Wiley Publishing, which are incorporated herein by reference).

Peptides can be produced, typically by direct chemical synthesis. Peptides can be produced as modified peptides, with nonpeptide moieties attached by covalent linkage to the N-terminus and/or C-terminus. In certain preferred embodiments, either the carboxy-terminus or the amino-terminus, or both, are chemically modified. The most common modifications of the terminal amino and carboxyl groups are acetylation and amidation, respectively. Amino-terminal modifications such as acylation (*e.g.*, acetylation) or alkylation (*e.g.*, methylation) and carboxy-terminal-modifications such as amidation, as well as other terminal modifications, including cyclization, can be incorporated into various embodiments of the invention. Certain amino-terminal and/or carboxy-terminal modifications and/or peptide extensions to the core sequence can provide advantageous physical, chemical, biochemical, and pharmacological properties, such as: enhanced stability, increased potency and/or efficacy, resistance to serum proteases, desirable pharmacokinetic properties, and others. Peptides described herein can be used therapeutically to treat disease, *e.g.*, by altering costimulation in a patient.

Peptidomimetics (Fauchere (1986) *Adv. Drug Res.* 15:29; Veber and Freidinger (1985) TINS p.392; and Evans *et al.* (1987) *J. Med. Chem.* 30:1229, which are incorporated herein by reference) are usually developed with the aid of computerized molecular modeling. Peptide mimetics that are structurally similar to therapeutically useful peptides can be used to produce an equivalent therapeutic or prophylactic effect. Generally, peptidomimetics are structurally similar to a paradigm polypeptide (*i.e.*, a polypeptide that has a biological or pharmacological activity), but have one or more peptide linkages optionally replaced by a linkage selected from the group consisting of: -CH2NH-, -CH2S-, -CH2-CH2-, -CH=CH- (cis and trans), -COCH2-, -CH(OH)CH2-, and -CH2SO-, by methods known in the art and further described in the following references: Spatola, A. F. in "*Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins*" Weinstein, B., ed., Marcel Dekker, New York, p. 267 (1983); Spatola, A. F., Vega Data (March 1983), Vol. 1, Issue 3, "Peptide Backbone Modifications" (general review); Morley, J. S. (1980) *Trends Pharm. Sci.* pp. 463-468 (general review); Hudson, D. *et al.* (1979) *Int. J. Pept. Prot. Res.* 14:177-185 (-CH2NH-, CH2CH2-); Spatola, A. F. *et al.* (1986) *Life Sci.* 38:1243-1249 (-CH2-S); Hann, M. M. (1982) *J. Chem. Soc. Perkin Trans. I.* 307-314 (-CH-CH-, cis and trans); Almquist, R. G. *et al.* (190) *J. Med. Chem.* 23:1392-1398 (-COCH2-); Jennings-White, C. *et al.* (1982) *Tetrahedron Lett.* 23:2533 (-COCH2-); Szelke, M. *et al.* European Appln. EP 45665 (1982) CA: 97:39405 (1982)(-CH(OH)CH2-

); Holladay, M. W. *et al.* (1983) *Tetrahedron Lett.* (1983) 24:4401-4404 (-C(OH)CH2-); and Hruby, V. J. (1982) *Life Sci.* (1982) 31:189-199 (-CH2-S-); each of which is incorporated herein by reference. A particularly preferred non-peptide linkage is -CH2NH-. Such peptide mimetics may have significant advantages over polypeptide embodiments, including, for example: more economical production, greater chemical stability, enhanced pharmacological properties (half-life, absorption, potency, efficacy, etc.), altered specificity (*e.g.*, a broad-spectrum of biological activities), reduced antigenicity, and others. Labeling of peptidomimetics usually involves covalent attachment of one or more labels, directly or through a spacer (*e.g.*, an amide group), to non-interfering position(s) on the peptidomimetic that are predicted by quantitative structure-activity data and/or molecular modeling. Such non-interfering positions generally are positions that do not form direct contacts with the macropolypeptides(s) to which the peptidomimetic binds to produce the therapeutic effect. Derivatization (*e.g.*, labeling) of peptidomimetics should not substantially interfere with the desired biological or pharmacological activity of the peptidomimetic.

Also encompassed by the present invention are small molecules which can modulate (either enhance or inhibit) interactions, *e.g.*, between biomarkers described herein or listed in Table 1 and their natural binding partners. The small molecules of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. (Lam, K. S. (1997) *Anticancer Drug Des.* 12:145).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:6909; Erb *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann *et al.* (1994) *J. Med. Chem.* 37:2678; Cho *et al.* (1993) *Science* 261:1303; Carrell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and in Gallop *et al.* (1994) *J. Med. Chem.* 37:1233.

Libraries of compounds can be presented in solution (*e.g.*, Houghten (1992) *Biotechniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364:555-556), bacteria (Ladner USP 5,223,409), spores (Ladner USP '409), plasmids (Cull *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:1865-1869) or on phage (Scott

and Smith (1990) *Science* 249:386-390); (Devlin (1990) *Science* 249:404-406); (Cwirla *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:6378-6382); (Felici (1991) *J. Mol. Biol.* 222:301-310); (Ladner *supra.*). Compounds can be screened in cell based or non-cell based assays. Compounds can be screened in pools (*e.g.* multiple compounds in each testing sample) or as individual compounds.

Chimeric or fusion proteins can be prepared for the biomarker inhibitors and/or agents for the immunotherapies described herein, such as inhibitors to the biomarkers of the invention, including the biomarkers listed in Table 1, or fragments thereof. As used herein, a "chimeric protein" or "fusion protein" comprises one or more biomarkers of the invention, including one or more biomarkers listed in Table 1, or a fragment thereof, operatively linked to another polypeptide having an amino acid sequence corresponding to a protein which is not substantially homologous to the respective biomarker. In a preferred embodiment, the fusion protein comprises at least one biologically active portion of one or more biomarkers of the invention, including one or more biomarkers listed in Table 1, or fragments thereof. Within the fusion protein, the term "operatively linked" is intended to indicate that the biomarker sequences and the non-biomarker sequences are fused in-frame to each other in such a way as to preserve functions exhibited when expressed independently of the fusion. The "another" sequences can be fused to the N-terminus or C-terminus of the biomarker sequences, respectively.

Such a fusion protein can be produced by recombinant expression of a nucleotide sequence encoding the first peptide and a nucleotide sequence encoding the second peptide. The second peptide may optionally correspond to a moiety that alters the solubility, affinity, stability or valency of the first peptide, for example, an immunoglobulin constant region. In another preferred embodiment, the first peptide consists of a portion of a biologically active molecule (*e.g.* the extracellular portion of the polypeptide or the ligand binding portion). The second peptide can include an immunoglobulin constant region, for example, a human Cγ1 domain or Cγ4 domain (*e.g.*, the hinge, CH2 and CH3 regions of human IgCγ 1, or human IgCγ4, see *e.g.*, Capon *et al.* U.S. Patents 5,116,964; 5,580,756; 5,844,095 and the like, incorporated herein by reference). Such constant regions may retain regions which mediate effector function (*e.g.* Fc receptor binding) or may be altered to reduce effector function. A resulting fusion protein may have altered solubility, binding affinity, stability and/or valency (*i.e.*, the number of binding sites available per polypeptide) as compared to the independently expressed first peptide, and may increase the efficiency of protein

purification. Fusion proteins and peptides produced by recombinant techniques can be secreted and isolated from a mixture of cells and medium containing the protein or peptide. Alternatively, the protein or peptide can be retained cytoplasmically and the cells harvested, lysed and the protein isolated. A cell culture typically includes host cells, media and other byproducts. Suitable media for cell culture are well-known in the art. Protein and peptides can be isolated from cell culture media, host cells, or both using techniques known in the art for purifying proteins and peptides. Techniques for transfecting host cells and purifying proteins and peptides are known in the art.

Preferably, a fusion protein of the invention is produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different polypeptide sequences are ligated together in-frame in accordance with conventional techniques, for example employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed and reamplified to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel *et al.* John Wiley & Sons: 1992).

The fusion proteins of the invention can be used as immunogens to produce antibodies in a subject. Such antibodies may be used to purify the respective natural polypeptides from which the fusion proteins were generated, or in screening assays to identify polypeptides which inhibit the interactions between one or more biomarkers polypeptide or a fragment thereof and its natural binding partner(s) or a fragment(s) thereof.

Also provided herein are compositions comprising one or more nucleic acids comprising or capable of expressing at least 1, 2, 3, 4, 5, 10, 20 or more small nucleic acids or antisense oligonucleotides or derivatives thereof, wherein said small nucleic acids or antisense oligonucleotides or derivatives thereof in a cell specifically hybridize (*e.g.*, bind) under cellular conditions, with cellular nucleic acids (*e.g.*, small non-coding RNAS such as miRNAs, pre-miRNAs, pri-miRNAs, miRNA*, anti-miRNA, a miRNA binding site, a variant and/or functional variant thereof, cellular mRNAs or a fragments thereof). In one embodiment, expression of the small nucleic acids or antisense oligonucleotides or

derivatives thereof in a cell can inhibit expression or biological activity of cellular nucleic acids and/or proteins, *e.g.*, by inhibiting transcription, translation and/or small nucleic acid processing of, for example, one or more biomarkers of the invention, including one or more biomarkers listed in Table 1, or fragment(s) thereof. In one embodiment, the small nucleic acids or antisense oligonucleotides or derivatives thereof are small RNAs (*e.g.*, microRNAs) or complements of small RNAs. In another embodiment, the small nucleic acids or antisense oligonucleotides or derivatives thereof can be single or double stranded and are at least six nucleotides in length and are less than about 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100, 50, 40, 30, 25, 24, 23, 22, 21,20, 19, 18, 17, 16, 15, or 10 nucleotides in length. In another embodiment, a composition may comprise a library of nucleic acids comprising or capable of expressing small nucleic acids or antisense oligonucleotides or derivatives thereof, or pools of said small nucleic acids or antisense oligonucleotides or derivatives thereof. A pool of nucleic acids may comprise about 2-5, 5-10, 10-20, 10-30 or more nucleic acids comprising or capable of expressing small nucleic acids or antisense oligonucleotides or derivatives thereof.

In one embodiment, binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix. In general, "antisense" refers to the range of techniques generally employed in the art, and includes any process that relies on specific binding to oligonucleotide sequences.

It is well-known in the art that modifications can be made to the sequence of a miRNA or a pre-miRNA without disrupting miRNA activity. As used herein, the term "functional variant" of a miRNA sequence refers to an oligonucleotide sequence that varies from the natural miRNA sequence, but retains one or more functional characteristics of the miRNA (*e.g.* cancer cell proliferation inhibition, induction of cancer cell apoptosis, enhancement of cancer cell susceptibility to chemotherapeutic agents, specific miRNA target inhibition). In some embodiments, a functional variant of a miRNA sequence retains all of the functional characteristics of the miRNA. In certain embodiments, a functional variant of a miRNA has a nucleobase sequence that is a least about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to the miRNA or precursor thereof over a region of about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more nucleobases, or that the functional variant hybridizes to the complement of the

miRNA or precursor thereof under stringent hybridization conditions. Accordingly, in certain embodiments the nucleobase sequence of a functional variant is capable of hybridizing to one or more target sequences of the miRNA.

miRNAs and their corresponding stem-loop sequences described herein may be found in miRBase, an online searchable database of miRNA sequences and annotation, found on the world wide web at microrna.sanger.ac.uk. Entries in the miRBase Sequence database represent a predicted hairpin portion of a miRNA transcript (the stem-loop), with information on the location and sequence of the mature miRNA sequence. The miRNA stem-loop sequences in the database are not strictly precursor miRNAs (pre-miRNAs), and may in some instances include the pre-miRNA and some flanking sequence from the presumed primary transcript. The miRNA nucleobase sequences described herein encompass any version of the miRNA, including the sequences described in Release 10.0 of the miRBase sequence database and sequences described in any earlier Release of the miRBase sequence database. A sequence database release may result in the re-naming of certain miRNAs. A sequence database release may result in a variation of a mature miRNA sequence.

In some embodiments, miRNA sequences of the invention may be associated with a second RNA sequence that may be located on the same RNA molecule or on a separate RNA molecule as the miRNA sequence. In such cases, the miRNA sequence may be referred to as the active strand, while the second RNA sequence, which is at least partially complementary to the miRNA sequence, may be referred to as the complementary strand. The active and complementary strands are hybridized to create a double-stranded RNA that is similar to a naturally occurring miRNA precursor. The activity of a miRNA may be optimized by maximizing uptake of the active strand and minimizing uptake of the complementary strand by the miRNA protein complex that regulates gene translation. This can be done through modification and/or design of the complementary strand.

In some embodiments, the complementary strand is modified so that a chemical group other than a phosphate or hydroxyl at its 5' terminus. The presence of the 5' modification apparently eliminates uptake of the complementary strand and subsequently favors uptake of the active strand by the miRNA protein complex. The 5' modification can be any of a variety of molecules known in the art, including NH_2, $NHCOCH_3$, and biotin.

In another embodiment, the uptake of the complementary strand by the miRNA pathway is reduced by incorporating nucleotides with sugar modifications in the first 2-6

nucleotides of the complementary strand. It should be noted that such sugar modifications can be combined with the 5' terminal modifications described above to further enhance miRNA activities.

In some embodiments, the complementary strand is designed so that nucleotides in the 3' end of the complementary strand are not complementary to the active strand. This results in double-strand hybrid RNAs that are stable at the 3' end of the active strand but relatively unstable at the 5' end of the active strand. This difference in stability enhances the uptake of the active strand by the miRNA pathway, while reducing uptake of the complementary strand, thereby enhancing miRNA activity.

Small nucleic acid and/or antisense constructs of the methods and compositions presented herein can be delivered, for example, as an expression plasmid which, when transcribed in the cell, produces RNA which is complementary to at least a unique portion of cellular nucleic acids (*e.g.*, small RNAs, mRNA, and/or genomic DNA). Alternatively, the small nucleic acid molecules can produce RNA which encodes mRNA, miRNA, pre-miRNA, pri-miRNA, miRNA*, anti-miRNA, or a miRNA binding site, or a variant thereof. For example, selection of plasmids suitable for expressing the miRNAs, methods for inserting nucleic acid sequences into the plasmid, and methods of delivering the recombinant plasmid to the cells of interest are within the skill in the art. See, for example, Zeng *et al.* (2002) *Mol. Cell* 9:1327-1333; Tuschl (2002), *Nat. Biotechnol.* 20:446-448; Brummelkamp *et al.* (2002) *Science* 296:550-553; Miyagishi *et al.* (2002) *Nat. Biotechnol.* 20:497-500; Paddison *et al.* (2002) *Genes Dev.* 16:948-958; Lee *et al.* (2002) *Nat. Biotechnol.* 20:500-505; and Paul *et al.* (2002) *Nat. Biotechnol.* 20:505-508, the entire disclosures of which are herein incorporated by reference.

Alternatively, small nucleic acids and/or antisense constructs are oligonucleotide probes that are generated *ex vivo* and which, when introduced into the cell, results in hybridization with cellular nucleic acids. Such oligonucleotide probes are preferably modified oligonucleotides that are resistant to endogenous nucleases, *e.g.*, exonucleases and/or endonucleases, and are therefore stable *in vivo*. Exemplary nucleic acid molecules for use as small nucleic acids and/or antisense oligonucleotides are phosphoramidate, phosphothioate and methylphosphonate analogs of DNA (see also U.S. Patents 5,176,996; 5,264,564; and 5,256,775). Additionally, general approaches to constructing oligomers useful in antisense therapy have been reviewed, for example, by Van der Krol et al. (1988) BioTechniques 6:958-976; and Stein et al. (1988) Cancer Res 48:2659-2668.

Antisense approaches may involve the design of oligonucleotides (either DNA or RNA) that are complementary to cellular nucleic acids (*e.g.*, complementary to biomarkers listed in Table 1). Absolute complementarity is not required. In the case of double-stranded antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with a nucleic acid (*e.g.*, RNA) it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

Oligonucleotides that are complementary to the 5' end of the mRNA, *e.g.*, the 5' untranslated sequence up to and including the AUG initiation codon, should work most efficiently at inhibiting translation. However, sequences complementary to the 3' untranslated sequences of mRNAs have recently been shown to be effective at inhibiting translation of mRNAs as well (Wagner (1994) *Nature* 372:333). Therefore, oligonucleotides complementary to either the 5' or 3' untranslated, non-coding regions of genes could be used in an antisense approach to inhibit translation of endogenous mRNAs. Oligonucleotides complementary to the 5' untranslated region of the mRNA may include the complement of the AUG start codon. Antisense oligonucleotides complementary to mRNA coding regions are less efficient inhibitors of translation but could also be used in accordance with the methods and compositions presented herein. Whether designed to hybridize to the 5', 3' or coding region of cellular mRNAs, small nucleic acids and/or antisense nucleic acids should be at least six nucleotides in length, and can be less than about 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100, 50, 40, 30, 25, 24, 23, 22, 21,20, 19, 18, 17, 16, 15, or 10 nucleotides in length.

Regardless of the choice of target sequence, it is preferred that *in vitro* studies are first performed to quantitate the ability of the antisense oligonucleotide to inhibit gene expression. In one embodiment these studies utilize controls that distinguish between antisense gene inhibition and nonspecific biological effects of oligonucleotides. In another embodiment these studies compare levels of the target nucleic acid or protein with that of an internal control nucleic acid or protein. Additionally, it is envisioned that results obtained using the antisense oligonucleotide are compared with those obtained using a control oligonucleotide. It is preferred that the control oligonucleotide is of approximately

the same length as the test oligonucleotide and that the nucleotide sequence of the oligonucleotide differs from the antisense sequence no more than is necessary to prevent specific hybridization to the target sequence.

Small nucleic acids and/or antisense oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. Small nucleic acids and/or antisense oligonucleotides can be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule, hybridization, etc., and may include other appended groups such as peptides (*e.g.*, for targeting host cell receptors), or agents facilitating transport across the cell membrane (see, *e.g.*, Letsinger *et al.* (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86:6553-6556; Lemaitre *et al.* (1987) *Proc. Natl. Acad. Sci. U.S.A.* 84:648-652; PCT Publication No. W088/09810) or the blood-brain barrier (see, *e.g.*, PCT Publication No. W089/10134), hybridization-triggered cleavage agents. (See, *e.g.*, Krol *et al.* (1988) *BioTech.* 6:958-976) or intercalating agents. (See, *e.g.*, Zon (1988) *Pharm. Res.* 5:539-549). To this end, small nucleic acids and/or antisense oligonucleotides may be conjugated to another molecule, *e.g.*, a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

Small nucleic acids and/or antisense oligonucleotides may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxytiethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. Small nucleic acids and/or antisense oligonucleotides may also comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

In certain embodiments, a compound comprises an oligonucleotide (*e.g.*, a miRNA or miRNA encoding oligonucleotide) conjugated to one or more moieties which enhance the activity, cellular distribution or cellular uptake of the resulting oligonucleotide. In certain such embodiments, the moiety is a cholesterol moiety (*e.g.*, antagomirs) or a lipid moiety or liposome conjugate. Additional moieties for conjugation include carbohydrates, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes. In certain embodiments, a conjugate group is attached directly to the oligonucleotide. In certain embodiments, a conjugate group is attached to the oligonucleotide by a linking moiety selected from amino, hydroxyl, carboxylic acid, thiol, unsaturations (*e.g.*, double or triple bonds), 8-amino-3,6-dioxaoctanoic acid (ADO), succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), 6-aminohexanoic acid (AHEX or AHA), substituted C1-C10 alkyl, substituted or unsubstituted C2-C10 alkenyl, and substituted or unsubstituted C2-C10 alkynyl. In certain such embodiments, a substituent group is selected from hydroxyl, amino, alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl and alkynyl.

In certain such embodiments, the compound comprises the oligonucleotide having one or more stabilizing groups that are attached to one or both termini of the oligonucleotide to enhance properties such as, for example, nuclease stability. Included in stabilizing groups are cap structures. These terminal modifications protect the oligonucleotide from exonuclease degradation, and can help in delivery and/or localization within a cell. The cap can be present at the 5'-terminus (5'-cap), or at the 3'-terminus (3'-cap), or can be present on both termini. Cap structures include, for example, inverted deoxy abasic caps.

Suitable cap structures include a 4',5'-methylene nucleotide, a 1-(beta-D-erythrofuranosyl) nucleotide, a 4'-thio nucleotide, a carbocyclic nucleotide, a 1,5-anhydrohexitol nucleotide, an L-nucleotide, an alpha-nucleotide, a modified base nucleotide, a phosphorodithioate linkage, a threo-pentofuranosyl nucleotide, an acyclic 3',4'-seco nucleotide, an acyclic 3,4-dihydroxybutyl nucleotide, an acyclic 3,5-dihydroxypentyl nucleotide, a 3'-3'-inverted nucleotide moiety, a 3'-3'-inverted abasic moiety, a 3'-2'-inverted nucleotide moiety, a 3'-2'-inverted abasic moiety, a 1,4-butanediol phosphate, a 3'-phosphoramidate, a hexylphosphate, an aminohexyl phosphate, a 3'-phosphate, a 3'-phosphorothioate, a phosphorodithioate, a bridging methylphosphonate

moiety, and a non-bridging methylphosphonate moiety 5'-amino-alkyl phosphate, a 1,3-diamino-2-propyl phosphate, 3-aminopropyl phosphate, a 6-aminohexyl phosphate, a 1,2-aminododecyl phosphate, a hydroxypropyl phosphate, a 5'-5'-inverted nucleotide moiety, a 5'-5'-inverted abasic moiety, a 5'-phosphoramidate, a 5'-phosphorothioate, a 5'-amino, a bridging and/or non-bridging 5'-phosphoramidate, a phosphorothioate, and a 5'-mercapto moiety.

Small nucleic acids and/or antisense oligonucleotides can also contain a neutral peptide-like backbone. Such molecules are termed peptide nucleic acid (PNA)-oligomers and are described, *e.g.*, in Perry-O'Keefe et al. (1996) Proc. Natl. Acad. Sci. U.S.A. 93:14670 and in Eglom et al. (1993) Nature 365:566. One advantage of PNA oligomers is their capability to bind to complementary DNA essentially independently from the ionic strength of the medium due to the neutral backbone of the DNA. In yet another embodiment, small nucleic acids and/or antisense oligonucleotides comprises at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In a further embodiment, small nucleic acids and/or antisense oligonucleotides are α-anomeric oligonucleotides. An α-anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual b-units, the strands run parallel to each other (Gautier et al. (1987) Nucl. Acids Res. 15:6625-6641). The oligonucleotide is a 2'-0-methylribonucleotide (Inoue et al. (1987) Nucl. Acids Res. 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue et al. (1987) FEBS Lett. 215:327-330).

Small nucleic acids and/or antisense oligonucleotides of the methods and compositions presented herein may be synthesized by standard methods known in the art, *e.g.*, by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein et al. (1988) Nucl. Acids Res. 16:3209, methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin et al. (1988) Proc. Natl. Acad. Sci. U.S.A. 85:7448-7451), etc. For example, an isolated miRNA can be chemically synthesized or recombinantly produced using methods known in the art. In some instances, miRNA are chemically synthesized using appropriately protected ribonucleoside phosphoramidites and a conventional

DNA/RNA synthesizer. Commercial suppliers of synthetic RNA molecules or synthesis reagents include, *e.g.*, Proligo (Hamburg, Germany), Dharmacon Research (Lafayette, Colo., USA), Pierce Chemical (part of Perbio Science, Rockford, Ill., USA), Glen Research (Sterling, Va., USA), ChemGenes (Ashland, Mass., USA), Cruachem (Glasgow, UK), and Exiqon (Vedbaek, Denmark).

Small nucleic acids and/or antisense oligonucleotides can be delivered to cells *in vivo*. A number of methods have been developed for delivering small nucleic acids and/or antisense oligonucleotides DNA or RNA to cells; *e.g.*, antisense molecules can be injected directly into the tissue site, or modified antisense molecules, designed to target the desired cells (*e.g.*, antisense linked to peptides or antibodies that specifically bind receptors or antigens expressed on the target cell surface) can be administered systematically.

In one embodiment, small nucleic acids and/or antisense oligonucleotides may comprise or be generated from double stranded small interfering RNAs (siRNAs), in which sequences fully complementary to cellular nucleic acids (*e.g.* mRNAs) sequences mediate degradation or in which sequences incompletely complementary to cellular nucleic acids (*e.g.*, mRNAs) mediate translational repression when expressed within cells, or piwiRNAs. In another embodiment, double stranded siRNAs can be processed into single stranded antisense RNAs that bind single stranded cellular RNAs (*e.g.*, microRNAs) and inhibit their expression. RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing in animals and plants, initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene. *in vivo*, long dsRNA is cleaved by ribonuclease III to generate 21- and 22-nucleotide siRNAs. It has been shown that 21-nucleotide siRNA duplexes specifically suppress expression of endogenous and heterologous genes in different mammalian cell lines, including human embryonic kidney (293) and HeLa cells (Elbashir et al. (2001) *Nature* 411:494-498). Accordingly, translation of a gene in a cell can be inhibited by contacting the cell with short double stranded RNAs having a length of about 15 to 30 nucleotides or of about 18 to 21 nucleotides or of about 19 to 21 nucleotides. Alternatively, a vector encoding for such siRNAs or short hairpin RNAs (shRNAs) that are metabolized into siRNAs can be introduced into a target cell (see, *e.g.*, McManus *et al.* (2002) *RNA* 8:842; Xia *et al.* (2002) *Nat. Biotechnol.* 20:1006; and Brummelkamp *et al.* (2002) *Science* 296:550). Vectors that can be used are commercially available, *e.g.*, from OligoEngine under the name pSuper RNAi System™.

Ribozyme molecules designed to catalytically cleave cellular mRNA transcripts can also be used to prevent translation of cellular mRNAs and expression of cellular polypeptides, or both (See, *e.g.*, PCT International Publication WO90/11364, published October 4, 1990; Sarver et al. (1990) Science 247:1222-1225 and U.S. Patent No. 5,093,246). While ribozymes that cleave mRNA at site specific recognition sequences can be used to destroy cellular mRNAs, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well-known in the art and is described more fully in Haseloff and Gerlach (1988) Nature 334:585-591. The ribozyme may be engineered so that the cleavage recognition site is located near the 5' end of cellular mRNAs; *i.e.*, to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

The ribozymes of the methods presented herein also include RNA endoribonucleases (hereinafter "Cech-type ribozymes") such as the one which occurs naturally in *Tetrahymena thermophila* (known as the IVS, or L-19 IVS RNA) and which has been extensively described by Thomas Cech and collaborators (Zaug *et al.* (1984) *Science* 224:574-578; Zaug *et al.* (1986) *Science* 231:470-475; Zaug *et al.* (1986) *Nature* 324:429-433; WO 88/04300; and Been *et al.* (1986) *Cell* 47:207-216). The Cech-type ribozymes have an eight base pair active site which hybridizes to a target RNA sequence whereafter cleavage of the target RNA takes place. The methods and compositions presented herein encompasses those Cech-type ribozymes which target eight base-pair active site sequences that are present in cellular genes.

As in the antisense approach, the ribozymes can be composed of modified oligonucleotides (*e.g.*, for improved stability, targeting, etc.). A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy endogenous cellular messages and inhibit translation. Because ribozymes unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficiency.

Nucleic acid molecules to be used in triple helix formation for the inhibition of transcription of cellular genes are preferably single stranded and composed of

deoxyribonucleotides. The base composition of these oligonucleotides should promote triple helix formation via Hoogsteen base pairing rules, which generally require sizable stretches of either purines or pyrimidines to be present on one strand of a duplex. Nucleotide sequences may be pyrimidine-based, which will result in TAT and CGC triplets across the three associated strands of the resulting triple helix. The pyrimidine-rich molecules provide base complementarity to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. In addition, nucleic acid molecules may be chosen that are purine-rich, for example, containing a stretch of G residues. These molecules will form a triple helix with a DNA duplex that is rich in GC pairs, in which the majority of the purine residues are located on a single strand of the targeted duplex, resulting in CGC triplets across the three strands in the triplex.

Alternatively, the potential sequences that can be targeted for triple helix formation may be increased by creating a so called "switchback" nucleic acid molecule. Switchback molecules are synthesized in an alternating 5'-3', 3'-5' manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a sizable stretch of either purines or pyrimidines to be present on one strand of a duplex.

Small nucleic acids (*e.g.*, miRNAs, pre-miRNAs, pri-miRNAs, miRNA*, anti-miRNA, or a miRNA binding site, or a variant thereof), antisense oligonucleotides, ribozymes, and triple helix molecules of the methods and compositions presented herein may be prepared by any method known in the art for the synthesis of DNA and RNA molecules. These include techniques for chemically synthesizing oligodeoxyribonucleotides and oligoribonucleotides well-known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by in vitro and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

Moreover, various well-known modifications to nucleic acid molecules may be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribonucleotides or deoxyribonucleotides to the 5' and/or 3' ends of the molecule or the use

of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the oligodeoxyribonucleotide backbone. One of skill in the art will readily understand that polypeptides, small nucleic acids, and antisense oligonucleotides can be further linked to another peptide or polypeptide (*e.g.*, a heterologous peptide), *e.g.*, that serves as a means of protein detection. Non-limiting examples of label peptide or polypeptide moieties useful for detection in the invention include, without limitation, suitable enzymes such as horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase; epitope tags, such as FLAG, MYC, HA, or HIS tags; fluorophores such as green fluorescent protein; dyes; radioisotopes; digoxygenin; biotin; antibodies; polymers; as well as others known in the art, for example, in Principles of Fluorescence Spectroscopy, Joseph R. Lakowicz (Editor), Plenum Pub Corp, 2nd edition (July 1999).

The modulatory agents described herein (*e.g.,* antibodies, small molecules, peptides, fusion proteins, or small nucleic acids) can be incorporated into pharmaceutical compositions and administered to a subject *in vivo*. The compositions may contain a single such molecule or agent or any combination of agents described herein. "Single active agents" described herein can be combined with other pharmacologically active compounds ("second active agents") known in the art according to the methods and compositions provided herein.

The production and use of biomarker nucleic acid and/or biomarker polypeptide molecules described herein can be facilitated by using standard recombinant techniques. In some embodiments, such techniques use vectors, preferably expression vectors, containing a nucleic acid encoding a biomarker polypeptide or a portion of such a polypeptide. As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors, namely expression vectors, are capable of directing the expression of genes to which they are operably linked. In general, expression vectors of utility in recombinant DNA

techniques are often in the form of plasmids (vectors). However, the present invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The recombinant expression vectors of the present invention comprise a nucleic acid of the present invention in a form suitable for expression of the nucleic acid in a host cell. This means that the recombinant expression vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression, which is operably linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner which allows for expression of the nucleotide sequence (*e.g.*, in an *in vitro* transcription/translation system or in a host cell when the vector is introduced into the host cell). The term "regulatory sequence" is intended to include promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel, *Methods in Enzymology: Gene Expression Technology* vol.185, Academic Press, San Diego, CA (1991). Regulatory sequences include those which direct constitutive expression of a nucleotide sequence in many types of host cell and those which direct expression of the nucleotide sequence only in certain host cells (*e.g.*, tissue-specific regulatory sequences). It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, and the like. The expression vectors of the present invention can be introduced into host cells to thereby produce proteins or peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein.

The recombinant expression vectors for use in the present invention can be designed for expression of a polypeptide corresponding to a marker of the present invention in prokaryotic (*e.g.*, *E. coli*) or eukaryotic cells (*e.g.*, insect cells {using baculovirus expression vectors}, yeast cells or mammalian cells). Suitable host cells are discussed further in Goeddel, *supra*. Alternatively, the recombinant expression vector can be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

Expression of proteins in prokaryotes is most often carried out in *E. coli* with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, usually to the amino terminus of the recombinant protein. Such fusion vectors typically serve three purposes: 1) to increase expression of recombinant protein; 2) to increase the solubility of the recombinant protein; and 3) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith and Johnson, 1988, *Gene* 67:31-40), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein.

Examples of suitable inducible non-fusion *E. coli* expression vectors include pTrc (Amann *et al.*, 1988, *Gene* 69:301-315) and pET 11d (Studier *et al.*, p. 60-89, In *Gene Expression Technology: Methods in Enzymology* vol.185, Academic Press, San Diego, CA, 1991). Target biomarker nucleic acid expression from the pTrc vector relies on host RNA polymerase transcription from a hybrid trp-lac fusion promoter. Target biomarker nucleic acid expression from the pET 11d vector relies on transcription from a T7 gn10-lac fusion promoter mediated by a co-expressed viral RNA polymerase (T7 gn1). This viral polymerase is supplied by host strains BL21 (DE3) or HMS174(DE3) from a resident prophage harboring a T7 gn1 gene under the transcriptional control of the lacUV 5 promoter.

One strategy to maximize recombinant protein expression in *E. coli* is to express the protein in a host bacterium with an impaired capacity to proteolytically cleave the recombinant protein (Gottesman, p. 119-128, In *Gene Expression Technology: Methods in Enzymology* vol. 185, Academic Press, San Diego, CA, 1990. Another strategy is to alter the nucleic acid sequence of the nucleic acid to be inserted into an expression vector so that the individual codons for each amino acid are those preferentially utilized in *E. coli* (Wada *et al.*, 1992, *Nucleic Acids Res.* 20:2111-2118). Such alteration of nucleic acid sequences of the present invention can be carried out by standard DNA synthesis techniques.

In another embodiment, the expression vector is a yeast expression vector. Examples of vectors for expression in yeast *S. cerevisiae* include pYepSec1 (Baldari *et al.*, 1987, *EMBO J.* 6:229-234), pMFa (Kurjan and Herskowitz, 1982, *Cell* 30:933-943), pJRY88 (Schultz *et al.*, 1987, *Gene* 54:113-123), pYES2 (Invitrogen Corporation, San Diego, CA), and pPicZ (Invitrogen Corp, San Diego, CA).

Alternatively, the expression vector is a baculovirus expression vector. Baculovirus vectors available for expression of proteins in cultured insect cells (*e.g.*, Sf 9 cells) include the pAc series (Smith *et al.*, 1983, *Mol. Cell Biol.* 3:2156-2165) and the pVL series (Lucklow and Summers, 1989, *Virology* 170:31-39).

In yet another embodiment, a nucleic acid of the present invention is expressed in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8 (Seed, 1987, *Nature* 329:840) and pMT2PC (Kaufman *et al.*, 1987, *EMBO J.* 6:187-195). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40. For other suitable expression systems for both prokaryotic and eukaryotic cells see chapters 16 and 17 of Sambrook *et al.*, *supra*.

In another embodiment, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (*e.g.*, tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert *et al.*, 1987, *Genes Dev.* 1:268-277), lymphoid-specific promoters (Calame and Eaton, 1988, *Adv. Immunol.* 43:235-275), in particular promoters of T cell receptors (Winoto and Baltimore, 1989, *EMBO J.* 8:729-733) and immunoglobulins (Banerji *et al.*, 1983, *Cell* 33:729-740; Queen and Baltimore, 1983, *Cell* 33:741-748), neuron-specific promoters (*e.g.*, the neurofilament promoter; Byrne and Ruddle, 1989, *Proc. Natl. Acad. Sci. USA* 86:5473-5477), pancreas-specific promoters (Edlund *et al.*, 1985, *Science* 230:912-916), and mammary gland-specific promoters (*e.g.*, milk whey promoter; U.S. Patent No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, for example the murine hox promoters (Kessel and Gruss, 1990, *Science* 249:374-379) and the α-fetoprotein promoter (Camper and Tilghman, 1989, *Genes Dev.* 3:537-546).

The present invention further provides a recombinant expression vector comprising a DNA molecule cloned into the expression vector in an antisense orientation. That is, the DNA molecule is operably linked to a regulatory sequence in a manner which allows for expression (by transcription of the DNA molecule) of an RNA molecule which is antisense to the mRNA encoding a polypeptide of the present invention. Regulatory sequences operably linked to a nucleic acid cloned in the antisense orientation can be chosen which direct the continuous expression of the antisense RNA molecule in a variety of cell types, for instance viral promoters and/or enhancers, or regulatory sequences can be chosen which direct constitutive, tissue-specific or cell type specific expression of antisense RNA. The antisense expression vector can be in the form of a recombinant plasmid, phagemid, or attenuated virus in which antisense nucleic acids are produced under the control of a high efficiency regulatory region, the activity of which can be determined by the cell type into which the vector is introduced. For a discussion of the regulation of gene expression using antisense genes (see Weintraub *et al.*, 1986, *Trends in Genetics*, Vol. 1(1)).

Another aspect of the present invention pertains to host cells into which a recombinant expression vector of the present invention has been introduced. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

A host cell can be any prokaryotic (*e.g.*, *E. coli*) or eukaryotic cell (*e.g.*, insect cells, yeast or mammalian cells).

Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook, *et al.* (*supra*), and other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these

integrants, a gene that encodes a selectable marker (*e.g.*, for resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (*e.g.*, cells that have incorporated the selectable marker gene will survive, while the other cells die).

V. Analyzing Biomarker Nucleic Acids and Polypeptides

Biomarker nucleic acids and/or biomarker polypeptides can be analyzed according to the methods described herein and techniques known to the skilled artisan to identify such genetic or expression alterations useful for the present invention including, but not limited to, 1) an alteration in the level of a biomarker transcript or polypeptide, 2) a deletion or addition of one or more nucleotides from a biomarker gene, 4) a substitution of one or more nucleotides of a biomarker gene, 5) aberrant modification of a biomarker gene, such as an expression regulatory region, and the like.

a. Methods for Detection of Copy Number

Methods of evaluating the copy number of a biomarker nucleic acid are well-known to those of skill in the art. The presence or absence of chromosomal gain or loss can be evaluated simply by a determination of copy number of the regions or markers identified herein.

In one embodiment, a biological sample is tested for the presence of copy number changes in genomic loci containing the genomic marker. A copy number of at least 3, 4, 5, 6, 7, 8, 9, or 10 is predictive of poorer outcome of biomarker inhibitor and immunotherapy combination treatments.

Methods of evaluating the copy number of a biomarker locus include, but are not limited to, hybridization-based assays. Hybridization-based assays include, but are not limited to, traditional "direct probe" methods, such as Southern blots, *in situ* hybridization (*e.g.,* FISH and FISH plus SKY) methods, and "comparative probe" methods, such as comparative genomic hybridization (CGH), *e.g.,* cDNA-based or oligonucleotide-based CGH. The methods can be used in a wide variety of formats including, but not limited to, substrate (*e.g.* membrane or glass) bound methods or array-based approaches.

In one embodiment, evaluating the biomarker gene copy number in a sample involves a Southern Blot. In a Southern Blot, the genomic DNA (typically fragmented and

separated on an electrophoretic gel) is hybridized to a probe specific for the target region. Comparison of the intensity of the hybridization signal from the probe for the target region with control probe signal from analysis of normal genomic DNA (*e.g.,* a non-amplified portion of the same or related cell, tissue, organ, *etc.*) provides an estimate of the relative copy number of the target nucleic acid. Alternatively, a Northern blot may be utilized for evaluating the copy number of encoding nucleic acid in a sample. In a Northern blot, mRNA is hybridized to a probe specific for the target region. Comparison of the intensity of the hybridization signal from the probe for the target region with control probe signal from analysis of normal RNA (*e.g.,* a non-amplified portion of the same or related cell, tissue, organ, *etc.*) provides an estimate of the relative copy number of the target nucleic acid. Alternatively, other methods well-known in the art to detect RNA can be used, such that higher or lower expression relative to an appropriate control (*e.g.*, a non-amplified portion of the same or related cell tissue, organ, etc.) provides an estimate of the relative copy number of the target nucleic acid.

An alternative means for determining genomic copy number is *in situ* hybridization (*e.g.,* Angerer (1987) *Meth. Enzymol* 152: 649). Generally, *in situ* hybridization comprises the following steps: (1) fixation of tissue or biological structure to be analyzed; (2) prehybridization treatment of the biological structure to increase accessibility of target DNA, and to reduce nonspecific binding; (3) hybridization of the mixture of nucleic acids to the nucleic acid in the biological structure or tissue; (4) post-hybridization washes to remove nucleic acid fragments not bound in the hybridization and (5) detection of the hybridized nucleic acid fragments. The reagent used in each of these steps and the conditions for use vary depending on the particular application. In a typical *in situ* hybridization assay, cells are fixed to a solid support, typically a glass slide. If a nucleic acid is to be probed, the cells are typically denatured with heat or alkali. The cells are then contacted with a hybridization solution at a moderate temperature to permit annealing of labeled probes specific to the nucleic acid sequence encoding the protein. The targets (*e.g.,* cells) are then typically washed at a predetermined stringency or at an increasing stringency until an appropriate signal to noise ratio is obtained. The probes are typically labeled, *e.g.,* with radioisotopes or fluorescent reporters. In one embodiment, probes are sufficiently long so as to specifically hybridize with the target nucleic acid(s) under stringent conditions. Probes generally range in length from about 200 bases to about 1000 bases. In some applications it is necessary to block the hybridization capacity of repetitive sequences.

Thus, in some embodiments, tRNA, human genomic DNA, or Cot-I DNA is used to block non-specific hybridization.

An alternative means for determining genomic copy number is comparative genomic hybridization. In general, genomic DNA is isolated from normal reference cells, as well as from test cells (*e.g.*, tumor cells) and amplified, if necessary. The two nucleic acids are differentially labeled and then hybridized *in situ* to metaphase chromosomes of a reference cell. The repetitive sequences in both the reference and test DNAs are either removed or their hybridization capacity is reduced by some means, for example by prehybridization with appropriate blocking nucleic acids and/or including such blocking nucleic acid sequences for said repetitive sequences during said hybridization. The bound, labeled DNA sequences are then rendered in a visualizable form, if necessary. Chromosomal regions in the test cells which are at increased or decreased copy number can be identified by detecting regions where the ratio of signal from the two DNAs is altered. For example, those regions that have decreased in copy number in the test cells will show relatively lower signal from the test DNA than the reference compared to other regions of the genome. Regions that have been increased in copy number in the test cells will show relatively higher signal from the test DNA. Where there are chromosomal deletions or multiplications, differences in the ratio of the signals from the two labels will be detected and the ratio will provide a measure of the copy number. In another embodiment of CGH, array CGH (aCGH), the immobilized chromosome element is replaced with a collection of solid support bound target nucleic acids on an array, allowing for a large or complete percentage of the genome to be represented in the collection of solid support bound targets. Target nucleic acids may comprise cDNAs, genomic DNAs, oligonucleotides (*e.g.*, to detect single nucleotide polymorphisms) and the like. Array-based CGH may also be performed with single-color labeling (as opposed to labeling the control and the possible tumor sample with two different dyes and mixing them prior to hybridization, which will yield a ratio due to competitive hybridization of probes on the arrays). In single color CGH, the control is labeled and hybridized to one array and absolute signals are read, and the possible tumor sample is labeled and hybridized to a second array (with identical content) and absolute signals are read. Copy number difference is calculated based on absolute signals from the two arrays. Methods of preparing immobilized chromosomes or arrays and performing comparative genomic hybridization are well-known in the art (see, *e.g.*, U.S. Pat. Nos: 6,335,167; 6,197,501; 5,830,645; and 5,665,549 and Albertson (1984)

EMBO J. 3: 1227-1234; Pinkel (1988) *Proc. Natl. Acad. Sci. USA* 85: 9138-9142; EPO Pub. No. 430,402; *Methods in Molecular Biology*, Vol. 33: *In situ* Hybridization Protocols, Choo, ed., Humana Press, Totowa, N.J. (1994), *etc.*) In another embodiment, the hybridization protocol of Pinkel, *et al.* (1998) *Nature Genetics* 20: 207-211, or of Kallioniemi (1992) *Proc. Natl Acad Sci USA* 89:5321-5325 (1992) is used.

In still another embodiment, amplification-based assays can be used to measure copy number. In such amplification-based assays, the nucleic acid sequences act as a template in an amplification reaction (*e.g.,* Polymerase Chain Reaction (PCR). In a quantitative amplification, the amount of amplification product will be proportional to the amount of template in the original sample. Comparison to appropriate controls, *e.g.* healthy tissue, provides a measure of the copy number.

Methods of "quantitative" amplification are well-known to those of skill in the art. For example, quantitative PCR involves simultaneously co-amplifying a known quantity of a control sequence using the same primers. This provides an internal standard that may be used to calibrate the PCR reaction. Detailed protocols for quantitative PCR are provided in Innis, *et al.* (1990) PCR Protocols, *A Guide to Methods and Applications*, Academic Press, Inc. N.Y.). Measurement of DNA copy number at microsatellite loci using quantitative PCR analysis is described in Ginzonger, *et al.* (2000) *Cancer Research* 60:5405-5409. The known nucleic acid sequence for the genes is sufficient to enable one of skill in the art to routinely select primers to amplify any portion of the gene. Fluorogenic quantitative PCR may also be used in the methods of the present invention. In fluorogenic quantitative PCR, quantitation is based on amount of fluorescence signals, *e.g.,* TaqMan and SYBR green.

Other suitable amplification methods include, but are not limited to, ligase chain reaction (LCR) (see Wu and Wallace (1989) *Genomics* 4: 560, Landegren, *et al.* (1988) *Science* 241:1077, and Barringer *et al.* (1990) *Gene* 89: 117), transcription amplification (Kwoh, *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86: 1173), self-sustained sequence replication (Guatelli, *et al.* (1990) *Proc. Nat. Acad. Sci. USA* 87: 1874), dot PCR, and linker adapter PCR, *etc.*

Loss of heterozygosity (LOH) and major copy proportion (MCP) mapping (Wang, Z.C., *et al.* (2004) *Cancer Res* 64(1):64-71; Seymour, A. B., *et al.* (1994) *Cancer Res* 54, 2761-4; Hahn, S. A., *et al.* (1995) *Cancer Res* 55, 4670-5; Kimura, M., *et al.* (1996) *Genes Chromosomes Cancer* 17, 88-93; Li *et al.*, (2008) *MBC Bioinform.* 9, 204-219) may also be used to identify regions of amplification or deletion.

b. Methods for Detection of Biomarker Nucleic Acid Expression

Biomarker expression may be assessed by any of a wide variety of well-known methods for detecting expression of a transcribed molecule or protein. Non-limiting examples of such methods include immunological methods for detection of secreted, cell-surface, cytoplasmic, or nuclear proteins, protein purification methods, protein function or activity assays, nucleic acid hybridization methods, nucleic acid reverse transcription methods, and nucleic acid amplification methods.

In preferred embodiments, activity of a particular gene is characterized by a measure of gene transcript (*e.g.* mRNA), by a measure of the quantity of translated protein, or by a measure of gene product activity. Marker expression can be monitored in a variety of ways, including by detecting mRNA levels, protein levels, or protein activity, any of which can be measured using standard techniques. Detection can involve quantification of the level of gene expression (*e.g.,* genomic DNA, cDNA, mRNA, protein, or enzyme activity), or, alternatively, can be a qualitative assessment of the level of gene expression, in particular in comparison with a control level. The type of level being detected will be clear from the context.

In another embodiment, detecting or determining expression levels of a biomarker and functionally similar homologs thereof, including a fragment or genetic alteration thereof (*e.g.*, in regulatory or promoter regions thereof) comprises detecting or determining RNA levels for the marker of interest. In one embodiment, one or more cells from the subject to be tested are obtained and RNA is isolated from the cells. In a preferred embodiment, a sample of breast tissue cells is obtained from the subject.

In one embodiment, RNA is obtained from a single cell. For example, a cell can be isolated from a tissue sample by laser capture microdissection (LCM). Using this technique, a cell can be isolated from a tissue section, including a stained tissue section, thereby assuring that the desired cell is isolated (*see, e.g.*, Bonner *et al.* (1997) Science 278: 1481; Emmert-Buck *et al.* (1996) Science 274:998; Fend *et al.* (1999) Am. J. Path. 154: 61 and Murakami *et al.* (2000) Kidney Int. 58:1346). For example, Murakami *et al.*, *supra*, describe isolation of a cell from a previously immunostained tissue section.

It is also be possible to obtain cells from a subject and culture the cells *in vitro*, such as to obtain a larger population of cells from which RNA can be extracted. Methods for establishing cultures of non-transformed cells, *i.e.*, primary cell cultures, are known in the art.

When isolating RNA from tissue samples or cells from individuals, it may be important to prevent any further changes in gene expression after the tissue or cells has been removed from the subject. Changes in expression levels are known to change rapidly following perturbations, *e.g.*, heat shock or activation with lipopolysaccharide (LPS) or other reagents. In addition, the RNA in the tissue and cells may quickly become degraded. Accordingly, in a preferred embodiment, the tissue or cells obtained from a subject is snap frozen as soon as possible.

RNA can be extracted from the tissue sample by a variety of methods, *e.g.*, the guanidium thiocyanate lysis followed by CsCl centrifugation (Chirgwin *et al.*, 1979, Biochemistry 18:5294-5299). RNA from single cells can be obtained as described in methods for preparing cDNA libraries from single cells, such as those described in Dulac, C. (1998) Curr. Top. Dev. Biol. 36, 245 and Jena *et al.* (1996) J. Immunol. Methods 190:199. Care to avoid RNA degradation must be taken, *e.g.*, by inclusion of RNAsin.

The RNA sample can then be enriched in particular species. In one embodiment, poly(A)+ RNA is isolated from the RNA sample. In general, such purification takes advantage of the poly-A tails on mRNA. In particular and as noted above, poly-T oligonucleotides may be immobilized within on a solid support to serve as affinity ligands for mRNA. Kits for this purpose are commercially available, *e.g.*, the MessageMaker kit (Life Technologies, Grand Island, NY).

In a preferred embodiment, the RNA population is enriched in marker sequences. Enrichment can be undertaken, *e.g.*, by primer-specific cDNA synthesis, or multiple rounds of linear amplification based on cDNA synthesis and template-directed *in vitro* transcription (*see, e.g.*, Wang *et al.* (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86: 9717; Dulac *et al.*, *supra*, and Jena *et al.*, *supra*).

The population of RNA, enriched or not in particular species or sequences, can further be amplified. As defined herein, an "amplification process" is designed to strengthen, increase, or augment a molecule within the RNA. For example, where RNA is mRNA, an amplification process such as RT-PCR can be utilized to amplify the mRNA, such that a signal is detectable or detection is enhanced. Such an amplification process is beneficial particularly when the biological, tissue, or tumor sample is of a small size or volume.

Various amplification and detection methods can be used. For example, it is within the scope of the present invention to reverse transcribe mRNA into cDNA followed by

polymerase chain reaction (RT-PCR); or, to use a single enzyme for both steps as described in U.S. Pat. No. 5,322,770, or reverse transcribe mRNA into cDNA followed by symmetric gap ligase chain reaction (RT-AGLCR) as described by R. L. Marshall, *et al.*, PCR Methods and Applications 4: 80-84 (1994). Real time PCR may also be used.

Other known amplification methods which can be utilized herein include but are not limited to the so-called "NASBA" or "3SR" technique described in PNAS USA 87: 1874-1878 (1990) and also described in Nature 350 (No. 6313): 91-92 (1991); Q-beta amplification as described in published European Patent Application (EPA) No. 4544610; strand displacement amplification (as described in G. T. Walker *et al.*, Clin. Chem. 42: 9-13 (1996) and European Patent Application No. 684315; target mediated amplification, as described by PCT Publication WO9322461; PCR; ligase chain reaction (LCR) (*see, e.g.*, Wu and Wallace, Genomics 4, 560 (1989), Landegren *et al.*, Science 241, 1077 (1988)); self-sustained sequence replication (SSR) (*see, e.g.*, Guatelli *et al.*, Proc. Nat. Acad. Sci. USA, 87, 1874 (1990)); and transcription amplification (*see, e.g.*, Kwoh *et al.*, Proc. Natl. Acad. Sci. USA 86, 1173 (1989)).

Many techniques are known in the state of the art for determining absolute and relative levels of gene expression, commonly used techniques suitable for use in the present invention include Northern analysis, RNase protection assays (RPA), microarrays and PCR-based techniques, such as quantitative PCR and differential display PCR. For example, Northern blotting involves running a preparation of RNA on a denaturing agarose gel, and transferring it to a suitable support, such as activated cellulose, nitrocellulose or glass or nylon membranes. Radiolabeled cDNA or RNA is then hybridized to the preparation, washed and analyzed by autoradiography.

In situ hybridization visualization may also be employed, wherein a radioactively labeled antisense RNA probe is hybridized with a thin section of a biopsy sample, washed, cleaved with RNase and exposed to a sensitive emulsion for autoradiography. The samples may be stained with hematoxylin to demonstrate the histological composition of the sample, and dark field imaging with a suitable light filter shows the developed emulsion. Non-radioactive labels such as digoxigenin may also be used.

Alternatively, mRNA expression can be detected on a DNA array, chip or a microarray. Labeled nucleic acids of a test sample obtained from a subject may be hybridized to a solid surface comprising biomarker DNA. Positive hybridization signal is obtained with the sample containing biomarker transcripts. Methods of preparing DNA

arrays and their use are well-known in the art (see, *e.g.*, U.S. Pat. Nos: 6,618,6796; 6,379,897; 6,664,377; 6,451,536; 548,257; U.S. 20030157485 and Schena *et al.* (1995) *Science* 20, 467-470; Gerhold *et al.* (1999) *Trends In Biochem. Sci.* 24, 168-173; and Lennon *et al.* (2000) *Drug Discovery Today* 5, 59-65, which are herein incorporated by reference in their entirety). Serial Analysis of Gene Expression (SAGE) can also be performed (See for example U.S. Patent Application 20030215858).

To monitor mRNA levels, for example, mRNA is extracted from the biological sample to be tested, reverse transcribed, and fluorescently-labeled cDNA probes are generated. The microarrays capable of hybridizing to marker cDNA are then probed with the labeled cDNA probes, the slides scanned and fluorescence intensity measured. This intensity correlates with the hybridization intensity and expression levels.

Types of probes that can be used in the methods described herein include cDNA, riboprobes, synthetic oligonucleotides and genomic probes. The type of probe used will generally be dictated by the particular situation, such as riboprobes for *in situ* hybridization, and cDNA for Northern blotting, for example. In one embodiment, the probe is directed to nucleotide regions unique to the RNA. The probes may be as short as is required to differentially recognize marker mRNA transcripts, and may be as short as, for example, 15 bases; however, probes of at least 17, 18, 19 or 20 or more bases can be used. In one embodiment, the primers and probes hybridize specifically under stringent conditions to a DNA fragment having the nucleotide sequence corresponding to the marker. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% identity in nucleotide sequences. In another embodiment, hybridization under "stringent conditions" occurs when there is at least 97% identity between the sequences.

The form of labeling of the probes may be any that is appropriate, such as the use of radioisotopes, for example, ^{32}P and ^{35}S. Labeling with radioisotopes may be achieved, whether the probe is synthesized chemically or biologically, by the use of suitably labeled bases.

In one embodiment, the biological sample contains polypeptide molecules from the test subject. Alternatively, the biological sample can contain mRNA molecules from the test subject or genomic DNA molecules from the test subject.

In another embodiment, the methods further involve obtaining a control biological sample from a control subject, contacting the control sample with a compound or agent capable of detecting marker polypeptide, mRNA, genomic DNA, or fragments thereof, such

that the presence of the marker polypeptide, mRNA, genomic DNA, or fragments thereof, is detected in the biological sample, and comparing the presence of the marker polypeptide, mRNA, genomic DNA, or fragments thereof, in the control sample with the presence of the marker polypeptide, mRNA, genomic DNA, or fragments thereof in the test sample.

c. Methods for Detection of Biomarker Protein Expression

The activity or level of a biomarker protein can be detected and/or quantified by detecting or quantifying the expressed polypeptide. The polypeptide can be detected and quantified by any of a number of means well-known to those of skill in the art. Aberrant levels of polypeptide expression of the polypeptides encoded by a biomarker nucleic acid and functionally similar homologs thereof, including a fragment or genetic alteration thereof (*e.g.*, in regulatory or promoter regions thereof) are associated with the likelihood of response of a cancer to biomarker inhibitor and immunotherapy combination treatments. Any method known in the art for detecting polypeptides can be used. Such methods include, but are not limited to, immunodiffusion, immunoelectrophoresis, radioimmunoassay (RIA), enzyme-linked immunosorbent assays (ELISAs), immunofluorescent assays, Western blotting, binder-ligand assays, immunohistochemical techniques, agglutination, complement assays, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), hyperdiffusion chromatography, and the like (*e.g.*, Basic and Clinical Immunology, Sites and Terr, eds., Appleton and Lange, Norwalk, Conn. pp 217-262, 1991 which is incorporated by reference). Preferred are binder-ligand immunoassay methods including reacting antibodies with an epitope or epitopes and competitively displacing a labeled polypeptide or derivative thereof.

For example, ELISA and RIA procedures may be conducted such that a desired biomarker protein standard is labeled (with a radioisotope such as ^{125}I or ^{35}S, or an assayable enzyme, such as horseradish peroxidase or alkaline phosphatase), and, together with the unlabeled sample, brought into contact with the corresponding antibody, whereon a second antibody is used to bind the first, and radioactivity or the immobilized enzyme assayed (competitive assay). Alternatively, the biomarker protein in the sample is allowed to react with the corresponding immobilized antibody, radioisotope- or enzyme-labeled anti-biomarker protein antibody is allowed to react with the system, and radioactivity or the enzyme assayed (ELISA-sandwich assay). Other conventional methods may also be employed as suitable.

The above techniques may be conducted essentially as a “one-step” or “two-step”

assay. A "one-step" assay involves contacting antigen with immobilized antibody and, without washing, contacting the mixture with labeled antibody. A "two-step" assay involves washing before contacting, the mixture with labeled antibody. Other conventional methods may also be employed as suitable.

In one embodiment, a method for measuring biomarker protein levels comprises the steps of: contacting a biological specimen with an antibody or variant (*e.g.*, fragment) thereof which selectively binds the biomarker protein, and detecting whether said antibody or variant thereof is bound to said sample and thereby measuring the levels of the biomarker protein.

Enzymatic and radiolabeling of biomarker protein and/or the antibodies may be effected by conventional means. Such means will generally include covalent linking of the enzyme to the antigen or the antibody in question, such as by glutaraldehyde, specifically so as not to adversely affect the activity of the enzyme, by which is meant that the enzyme must still be capable of interacting with its substrate, although it is not necessary for all of the enzyme to be active, provided that enough remains active to permit the assay to be effected. Indeed, some techniques for binding enzyme are non-specific (such as using formaldehyde), and will only yield a proportion of active enzyme.

It is usually desirable to immobilize one component of the assay system on a support, thereby allowing other components of the system to be brought into contact with the component and readily removed without laborious and time-consuming labor. It is possible for a second phase to be immobilized away from the first, but one phase is usually sufficient.

It is possible to immobilize the enzyme itself on a support, but if solid-phase enzyme is required, then this is generally best achieved by binding to antibody and affixing the antibody to a support, models and systems for which are well-known in the art. Simple polyethylene may provide a suitable support.

Enzymes employable for labeling are not particularly limited, but may be selected from the members of the oxidase group, for example. These catalyze production of hydrogen peroxide by reaction with their substrates, and glucose oxidase is often used for its good stability, ease of availability and cheapness, as well as the ready availability of its substrate (glucose). Activity of the oxidase may be assayed by measuring the concentration of hydrogen peroxide formed after reaction of the enzyme-labeled antibody with the substrate under controlled conditions well-known in the art.

Other techniques may be used to detect biomarker protein according to a practitioner's preference based upon the present disclosure. One such technique is Western blotting (Towbin et at., Proc. Nat. Acad. Sci. 76:4350 (1979)), wherein a suitably treated sample is run on an SDS-PAGE gel before being transferred to a solid support, such as a nitrocellulose filter. Anti-biomarker protein antibodies (unlabeled) are then brought into contact with the support and assayed by a secondary immunological reagent, such as labeled protein A or anti-immunoglobulin (suitable labels including ^{125}I, horseradish peroxidase and alkaline phosphatase). Chromatographic detection may also be used.

Immunohistochemistry may be used to detect expression of biomarker protein, *e.g.*, in a biopsy sample. A suitable antibody is brought into contact with, for example, a thin layer of cells, washed, and then contacted with a second, labeled antibody. Labeling may be by fluorescent markers, enzymes, such as peroxidase, avidin, or radiolabeling. The assay is scored visually, using microscopy.

Anti-biomarker protein antibodies, such as intrabodies, may also be used for imaging purposes, for example, to detect the presence of biomarker protein in cells and tissues of a subject. Suitable labels include radioisotopes, iodine (^{125}I, ^{121}I), carbon (^{14}C), sulphur (^{35}S), tritium (^{3}H), indium (^{112}In), and technetium (^{99}mTc), fluorescent labels, such as fluorescein and rhodamine, and biotin.

For *in vivo* imaging purposes, antibodies are not detectable, as such, from outside the body, and so must be labeled, or otherwise modified, to permit detection. Markers for this purpose may be any that do not substantially interfere with the antibody binding, but which allow external detection. Suitable markers may include those that may be detected by X-radiography, NMR or MRI. For X-radiographic techniques, suitable markers include any radioisotope that emits detectable radiation but that is not overtly harmful to the subject, such as barium or cesium, for example. Suitable markers for NMR and MRI generally include those with a detectable characteristic spin, such as deuterium, which may be incorporated into the antibody by suitable labeling of nutrients for the relevant hybridoma, for example.

The size of the subject, and the imaging system used, will determine the quantity of imaging moiety needed to produce diagnostic images. In the case of a radioisotope moiety, for a human subject, the quantity of radioactivity injected will normally range from about 5 to 20 millicuries of technetium-99. The labeled antibody or antibody fragment will then

preferentially accumulate at the location of cells which contain biomarker protein. The labeled antibody or antibody fragment can then be detected using known techniques.

Antibodies that may be used to detect biomarker protein include any antibody, whether natural or synthetic, full length or a fragment thereof, monoclonal or polyclonal, that binds sufficiently strongly and specifically to the biomarker protein to be detected. An antibody may have a K_d of at most about 10^{-6}M, 10^{-7}M, 10^{-8}M, 10^{-9}M, 10^{-10}M, 10^{-11}M, 10^{-12}M. The phrase "specifically binds" refers to binding of, for example, an antibody to an epitope or antigen or antigenic determinant in such a manner that binding can be displaced or competed with a second preparation of identical or similar epitope, antigen or antigenic determinant. An antibody may bind preferentially to the biomarker protein relative to other proteins, such as related proteins.

Antibodies are commercially available or may be prepared according to methods known in the art.

Antibodies and derivatives thereof that may be used encompass polyclonal or monoclonal antibodies, chimeric, human, humanized, primatized (CDR-grafted), veneered or single-chain antibodies as well as functional fragments, *i.e.*, biomarker protein binding fragments, of antibodies. For example, antibody fragments capable of binding to a biomarker protein or portions thereof, including, but not limited to, Fv, Fab, Fab' and F(ab') 2 fragments can be used. Such fragments can be produced by enzymatic cleavage or by recombinant techniques. For example, papain or pepsin cleavage can generate Fab or F(ab') 2 fragments, respectively. Other proteases with the requisite substrate specificity can also be used to generate Fab or F(ab') 2 fragments. Antibodies can also be produced in a variety of truncated forms using antibody genes in which one or more stop codons have been introduced upstream of the natural stop site. For example, a chimeric gene encoding a F(ab') 2 heavy chain portion can be designed to include DNA sequences encoding the CH, domain and hinge region of the heavy chain.

Synthetic and engineered antibodies are described in, *e.g.*, Cabilly *et al.*, U.S. Pat. No. 4,816,567 Cabilly *et al.*, European Patent No. 0,125,023 B1; Boss *et al.*, U.S. Pat. No. 4,816,397; Boss *et al.*, European Patent No. 0,120,694 B1; Neuberger, M. S. *et al.*, WO 86/01533; Neuberger, M. S. *et al.*, European Patent No. 0,194,276 B1; Winter, U.S. Pat. No. 5,225,539; Winter, European Patent No. 0,239,400 B1; Queen *et al.*, European Patent No. 0451216 B1; and Padlan, E. A. *et al.*, EP 0519596 A1. See also, Newman, R. *et al.*, BioTechnology, 10: 1455-1460 (1992), regarding primatized antibody, and Ladner *et al.*,

U.S. Pat. No. 4,946,778 and Bird, R. E. *et al.*, Science, 242: 423-426 (1988)) regarding single-chain antibodies. Antibodies produced from a library, *e.g.*, phage display library, may also be used.

In some embodiments, agents that specifically bind to a biomarker protein other than antibodies are used, such as peptides. Peptides that specifically bind to a biomarker protein can be identified by any means known in the art. For example, specific peptide binders of a biomarker protein can be screened for using peptide phage display libraries.

d. Methods for Detection of Biomarker Structural Alterations

The following illustrative methods can be used to identify the presence of a structural alteration in a biomarker nucleic acid and/or biomarker polypeptide molecule in order to, for example, identify one or more biomarkers listed in Table 1 or other biomarkers used in the immunotherapies described herein that are overexpressed, overfunctional, and the like.

In certain embodiments, detection of the alteration involves the use of a probe/primer in a polymerase chain reaction (PCR) (see, *e.g.*, U.S. Pat. Nos. 4,683,195 and 4,683,202), such as anchor PCR or RACE PCR, or, alternatively, in a ligation chain reaction (LCR) (see, *e.g.*, Landegran *et al.* (1988) Science 241:1077-1080; and Nakazawa *et al.* (1994) Proc. Natl. Acad. Sci. USA 91:360-364), the latter of which can be particularly useful for detecting point mutations in a biomarker nucleic acid such as a biomarker gene (see Abravaya *et al.* (1995) Nucleic Acids Res. 23:675-682). This method can include the steps of collecting a sample of cells from a subject, isolating nucleic acid (*e.g.*, genomic, mRNA or both) from the cells of the sample, contacting the nucleic acid sample with one or more primers which specifically hybridize to a biomarker gene under conditions such that hybridization and amplification of the biomarker gene (if present) occurs, and detecting the presence or absence of an amplification product, or detecting the size of the amplification product and comparing the length to a control sample. It is anticipated that PCR and/or LCR may be desirable to use as a preliminary amplification step in conjunction with any of the techniques used for detecting mutations described herein.

Alternative amplification methods include: self-sustained sequence replication (Guatelli, J. C. *et al.* (1990) Proc. Natl. Acad. Sci. USA 87:1874-1878), transcriptional amplification system (Kwoh, D. Y. *et al.* (1989) Proc. Natl. Acad. Sci. USA 86:1173-1177), Q-Beta Replicase (Lizardi, P. M. *et al.* (1988) Bio-Technology 6:1197), or any other nucleic acid amplification method, followed by the detection of the amplified molecules

using techniques well-known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers.

In an alternative embodiment, mutations in a biomarker nucleic acid from a sample cell can be identified by alterations in restriction enzyme cleavage patterns. For example, sample and control DNA is isolated, amplified (optionally), digested with one or more restriction endonucleases, and fragment length sizes are determined by gel electrophoresis and compared. Differences in fragment length sizes between sample and control DNA indicates mutations in the sample DNA. Moreover, the use of sequence specific ribozymes (see, for example, U.S. Pat. No. 5,498,531) can be used to score for the presence of specific mutations by development or loss of a ribozyme cleavage site.

In other embodiments, genetic mutations in biomarker nucleic acid can be identified by hybridizing a sample and control nucleic acids, *e.g.*, DNA or RNA, to high density arrays containing hundreds or thousands of oligonucleotide probes (Cronin, M. T. *et al.* (1996) Hum. Mutat. 7:244-255; Kozal, M. J. *et al.* (1996) Nat. Med. 2:753-759). For example, biomarker genetic mutations can be identified in two dimensional arrays containing light-generated DNA probes as described in Cronin *et al.* (1996) supra. Briefly, a first hybridization array of probes can be used to scan through long stretches of DNA in a sample and control to identify base changes between the sequences by making linear arrays of sequential, overlapping probes. This step allows the identification of point mutations. This step is followed by a second hybridization array that allows the characterization of specific mutations by using smaller, specialized probe arrays complementary to all variants or mutations detected. Each mutation array is composed of parallel probe sets, one complementary to the wild-type gene and the other complementary to the mutant gene. Such biomarker genetic mutations can be identified in a variety of contexts, including, for example, germline and somatic mutations.

In yet another embodiment, any of a variety of sequencing reactions known in the art can be used to directly sequence a biomarker gene and detect mutations by comparing the sequence of the sample biomarker with the corresponding wild-type (control) sequence. Examples of sequencing reactions include those based on techniques developed by Maxam and Gilbert (1977) *Proc. Natl. Acad. Sci. USA* 74:560 or Sanger (1977) *Proc. Natl. Acad Sci. USA* 74:5463. It is also contemplated that any of a variety of automated sequencing procedures can be utilized when performing the diagnostic assays (Naeve (1995)

Biotechniques 19:448-53), including sequencing by mass spectrometry (see, *e.g.*, PCT International Publication No. WO 94/16101; Cohen *et al.* (1996) *Adv. Chromatogr.* 36:127-162; and Griffin *et al.* (1993) *Appl. Biochem. Biotechnol.* 38:147-159).

Other methods for detecting mutations in a biomarker gene include methods in which protection from cleavage agents is used to detect mismatched bases in RNA/RNA or RNA/DNA heteroduplexes (Myers *et al.* (1985) Science 230:1242). In general, the art technique of "mismatch cleavage" starts by providing heteroduplexes formed by hybridizing (labeled) RNA or DNA containing the wild-type biomarker sequence with potentially mutant RNA or DNA obtained from a tissue sample. The double-stranded duplexes are treated with an agent which cleaves single-stranded regions of the duplex such as which will exist due to base pair mismatches between the control and sample strands. For instance, RNA/DNA duplexes can be treated with RNase and DNA/DNA hybrids treated with SI nuclease to enzymatically digest the mismatched regions. In other embodiments, either DNA/DNA or RNA/DNA duplexes can be treated with hydroxylamine or osmium tetroxide and with piperidine in order to digest mismatched regions. After digestion of the mismatched regions, the resulting material is then separated by size on denaturing polyacrylamide gels to determine the site of mutation. See, for example, Cotton *et al.* (1988) Proc. Natl. Acad. Sci. USA 85:4397 and Saleeba *et al.* (1992) Methods Enzymol. 217:286-295. In a preferred embodiment, the control DNA or RNA can be labeled for detection.

In still another embodiment, the mismatch cleavage reaction employs one or more proteins that recognize mismatched base pairs in double-stranded DNA (so called "DNA mismatch repair" enzymes) in defined systems for detecting and mapping point mutations in biomarker cDNAs obtained from samples of cells. For example, the mutY enzyme of *E. coli* cleaves A at G/A mismatches and the thymidine DNA glycosylase from HeLa cells cleaves T at G/T mismatches (Hsu *et al.* (1994) *Carcinogenesis* 15:1657-1662). According to an exemplary embodiment, a probe based on a biomarker sequence, *e.g.*, a wild-type biomarker treated with a DNA mismatch repair enzyme, and the cleavage products, if any, can be detected from electrophoresis protocols or the like (*e.g.*, U.S. Pat. No. 5,459,039.)

In other embodiments, alterations in electrophoretic mobility can be used to identify mutations in biomarker genes. For example, single strand conformation polymorphism (SSCP) may be used to detect differences in electrophoretic mobility between mutant and wild type nucleic acids (Orita *et al.* (1989) *Proc Natl. Acad. Sci USA* 86:2766; see also

Cotton (1993) *Mutat. Res.* 285:125-144 and Hayashi (1992) *Genet. Anal. Tech. Appl.* 9:73-79). Single-stranded DNA fragments of sample and control biomarker nucleic acids will be denatured and allowed to renature. The secondary structure of single-stranded nucleic acids varies according to sequence, the resulting alteration in electrophoretic mobility enables the detection of even a single base change. The DNA fragments may be labeled or detected with labeled probes. The sensitivity of the assay may be enhanced by using RNA (rather than DNA), in which the secondary structure is more sensitive to a change in sequence. In a preferred embodiment, the subject method utilizes heteroduplex analysis to separate double stranded heteroduplex molecules on the basis of changes in electrophoretic mobility (Keen *et al.* (1991) *Trends Genet.* 7:5).

In yet another embodiment the movement of mutant or wild-type fragments in polyacrylamide gels containing a gradient of denaturant is assayed using denaturing gradient gel electrophoresis (DGGE) (Myers *et al.* (1985) *Nature* 313:495). When DGGE is used as the method of analysis, DNA will be modified to ensure that it does not completely denature, for example by adding a GC clamp of approximately 40 bp of high-melting GC-rich DNA by PCR. In a further embodiment, a temperature gradient is used in place of a denaturing gradient to identify differences in the mobility of control and sample DNA (Rosenbaum and Reissner (1987) *Biophys. Chem.* 265:12753).

Examples of other techniques for detecting point mutations include, but are not limited to, selective oligonucleotide hybridization, selective amplification, or selective primer extension. For example, oligonucleotide primers may be prepared in which the known mutation is placed centrally and then hybridized to target DNA under conditions which permit hybridization only if a perfect match is found (Saiki *et al.* (1986) *Nature* 324:163; Saiki *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:6230). Such allele specific oligonucleotides are hybridized to PCR amplified target DNA or a number of different mutations when the oligonucleotides are attached to the hybridizing membrane and hybridized with labeled target DNA.

Alternatively, allele specific amplification technology which depends on selective PCR amplification may be used in conjunction with the instant invention. Oligonucleotides used as primers for specific amplification may carry the mutation of interest in the center of the molecule (so that amplification depends on differential hybridization) (Gibbs *et al.* (1989) *Nucleic Acids Res.* 17:2437-2448) or at the extreme 3' end of one primer where, under appropriate conditions, mismatch can prevent, or reduce polymerase extension

(Prossner (1993) *Tibtech* 11:238). In addition it may be desirable to introduce a novel restriction site in the region of the mutation to create cleavage-based detection (Gasparini *et al.* (1992) *Mol. Cell Probes* 6:1). It is anticipated that in certain embodiments amplification may also be performed using Taq ligase for amplification (Barany (1991) *Proc. Natl. Acad. Sci USA* 88:189). In such cases, ligation will occur only if there is a perfect match at the 3' end of the 5' sequence making it possible to detect the presence of a known mutation at a specific site by looking for the presence or absence of amplification.

3. Anti-Cancer Therapies

The efficacy of inhibitors of one or more biomarkers listed in Table 1, in combination with an immunotherapy is predicted according to biomarker amount and/or activity associated with a cancer in a subject according to the methods described herein. In one embodiment, such biomarker inhibitor and immunotherapy combination treatments (*e.g.*, one or more biomarker inhibitor and immunotherapy combination treatment in combination with one or more additional anti-cancer therapies, such as another immune checkpoint inhibitor) can be administered, particularly if a subject has first been indicated as being a likely responder to biomarker inhibitor and immunotherapy combination treatment. In another embodiment, such biomarker inhibitor and immunotherapy combination treatment can be avoided once a subject is indicated as not being a likely responder to biomarker inhibitor and immunotherapy combination treatment and an alternative treatment regimen, such as targeted and/or untargeted anti-cancer therapies can be administered. Combination therapies are also contemplated and can comprise, for example, one or more chemotherapeutic agents and radiation, one or more chemotherapeutic agents and immunotherapy, or one or more chemotherapeutic agents, radiation and chemotherapy, each combination of which can be with anti-immune checkpoint therapy. In addition, any representative embodiment of an agent to modulate a particular target can be adapted to any other target described herein by the ordinarily skilled artisn (*e.g.*, direct and indirect PD-1 inhibitors described herein can be applied to other immune checkpoint inhibitors and/or one or more biomarkers listed in Table 1, such as monospecific antibodies, bispecific antibodies, non-activiting forms, small molecules, peptides, interfering nucleic acids, and the like).

The term "targeted therapy" refers to administration of agents that selectively interact with a chosen biomolecule to thereby treat cancer. One example includes

immunotherapies such as immune checkpoint inhibitors, which are well-known in the art. For example, anti-PD-1 pathway agents, such as therapeutic monoclonal blocking antibodies, which are well-known in the art and described above, can be used to target tumor microenvironments and cells expressing unwanted components of the PD-1 pathway, such as PD-1, PD-L1, and/or PD-L2.

For example, the term "PD-1 pathway" refers to the PD-1 receptor and its ligands, PD-L1 and PD-L2. "PD-1 pathway inhibitors" block or otherwise reduce the interaction between PD-1 and one or both of its ligands such that the immunoinhibitory signaling otherwise generated by the interaction is blocked or otherwise reduced. Anti-immune checkpoint inhibitors can be direct or indirect. Direct anti-immune checkpoint inhibitors block or otherwise reduce the interaction between an immune checkpoint and at least one of its ligands. For example, PD-1 inhibitors can block PD-1 binding with one or both of its ligands. Direct PD-1 combination inhibitors are well-known in the art, especially since the natural binding partners of PD-1 (*e.g.*, PD-L1 and PD-L2), PD-L1 (*e.g.*, PD-1 and B7-1), and PD-L2 (*e.g.*, PD-1 and RGMb) are known.

For example, agents which directly block the interaction between PD-1 and PD-L1, PD-1 and PD-L2, PD-1 and both PD-L1 and PD-L2, such as a bispecific antibody, can prevent inhibitory signaling and upregulate an immune response (*i.e.*, as a PD-1 pathway inhibitor). Alternatively, agents that indirectly block the interaction between PD-1 and one or both of its ligands can prevent inhibitory signaling and upregulate an immune response. For example, B7-1 or a soluble form thereof, by binding to a PD-L1 polypeptide indirectly reduces the effective concentration of PD-L1 polypeptide available to bind to PD-1. Exemplary agents include monospecific or bispecific blocking antibodies against PD-1, PD-L1, and/or PD-L2 that block the interaction between the receptor and ligand(s); a non-activating form of PD-1, PD-L1, and/or PD-L2 (*e.g.*, a dominant negative or soluble polypeptide), small molecules or peptides that block the interaction between PD-1, PD-L1, and/or PD-L2; fusion proteins (*e.g.* the extracellular portion of PD-1, PD-L1, and/or PD-L2, fused to the Fc portion of an antibody or immunoglobulin) that bind to PD-1, PD-L1, and/or PD-L2 and inhibit the interaction between the receptor and ligand(s); a non-activating form of a natural PD-1, PD-L2, and/or PD-L2 ligand, and a soluble form of a natural PD-1, PD-L2, and/or PD-L2 ligand.

Indirect anti-immune checkpoint inhibitors block or otherwise reduce the immunoinhibitory signaling generated by the interaction between the immune checkpoint

and at least one of its ligands. For example, an inhibitor can block the interaction between PD-1 and one or both of its ligands without necessarily directly blocking the interaction between PD-1 and one or both of its ligands. For example, indirect inhibitors include intrabodies that bind the intracellular portion of PD-1 and/or PD-L1 required to signal to block or otherwise reduce the immunoinhibitory signaling. Similarly, nucleic acids that reduce the expression of PD-1, PD-L1, and/or PD-L2 can indirectly inhibit the interaction between PD-1 and one or both of its ligands by removing the availability of components for interaction. Such nucleic acid molecules can block PD-L1, PD-L2, and/or PD-L2 transcription or translation.

Similary, agents which directly block the interaction between one or more biomarkers listed in Table 1 and their binding partners/substrates, and the like, can remove the inhibition to biomarker-regulated signaling and its downstream immune responses, such as increasing sensitivity to interferon signaling. Alternatively, agents that indirectly block the interaction between one or more biomarkers listed in Table 1 and their binding partners/substrates can remove the inhibition to biomarker-regulated signaling and its downstream immune responses. For example, a truncated or dominant negative form of one or more biomarkers listed in Table 1, such as biomarker fragments without phosphatase activity, by binding to a biomarker substrate indirectly reduces the effective concentration of such substrate available to bind to one or more biomarkers listed in Table 1 in cell. Exemplary agents include monospecific or bispecific blocking antibodies, especially intrbodies, against one or more biomarkers listed in Table 1 and/or biomarker substrate(s) that block the interaction between the biomarker and its substrate(s); a non-active form of one or more biomarkers listed in Table 1 and/or biomarker substrate(s) (*e.g.*, a dominant negative polypeptide), small molecules or peptides that block the interaction between one or more biomarkers listed in Table 1 and their substrate(s) or the catalytic activity of one or more biomarkers listed in Table 1; and a non-activating form of a natural biomarker and/or its substrate(s).

Immunotherapies that are designed to elicit or amplify an immune response are referred to as "activation immunotherapies." Immunotherapies that are designed to reduce or suppress an immune response are referred to as "suppression immunotherapies." Any agent believed to have an immune system effect on the genetically modified transplanted cancer cells can be assayed to determine whether the agent is an immunotherapy and the effect that a given genetic modification has on the modulation of immune response. In

some embodiments, the immunotherapy is cancer cell-specific. In some embodiments, immunotherapy can be "untargeted," which refers to administration of agents that do not selectively interact with immune system cells, yet modulates immune system function. Representative examples of untargeted therapies include, without limitation, chemotherapy, gene therapy, and radiation therapy.

Immunotherapy can involve passive immunity for short-term protection of a host, achieved by the administration of pre-formed antibody directed against a cancer antigen or disease antigen (*e.g.*, administration of a monoclonal antibody, optionally linked to a chemotherapeutic agent or toxin, to a tumor antigen). Immunotherapy can also focus on using the cytotoxic lymphocyte-recognized epitopes of cancer cell lines. Alternatively, antisense polynucleotides, ribozymes, RNA interference molecules, triple helix polynucleotides and the like, can be used to selectively modulate biomolecules that are linked to the initiation, progression, and/or pathology of a tumor or cancer.

In one embodiment, immunotherapy comprises adoptive cell-based immunotherapies. Well-known adoptive cell-based immunotherapeutic modalities, including, without limitation, Irradiated autologous or allogeneic tumor cells, tumor lysates or apoptotic tumor cells, antigen-presenting cell-based immunotherapy, dendritic cell-based immunotherapy, adoptive T cell transfer, adoptive CAR T cell therapy, autologous immune enhancement therapy (AIET), cancer vaccines, and/or antigen presenting cells. Such cell-based immunotherapies can be further modified to express one or more gene products to further modulate immune responses, such as expressing cytokines like GM-CSF, and/or to express tumor-associated antigen (TAA) antigens, such as Mage-1, gp-100, patient-specific neoantigen vaccines, and the like.

In another embodiment, immunotherapy comprises non-cell-based immunotherapies. In one embodiment, compositions comprising antigens with or without vaccine-enhancing adjuvants are used. Such compositions exist in many well-known forms, such as peptide compositions, oncolytic viruses, recombinant antigen comprising fusion proteins, and the like. In still another embodiment, immunomodulatory interleukins, such as IL-2, IL-6, IL-7, IL-12, IL-17, IL-23, and the like, as well as modulators thereof (*e.g.*, blocking antibodies or more potent or longer lasting forms) are used. In yet another embodiment, immunomodulatory cytokines, such as interferons, G-CSF, imiquimod, TNFalpha, and the like, as well as modulators thereof (*e.g.*, blocking antibodies or more potent or longer lasting forms) are used. In another embodiment, immunomodulatory

chemokines, such as CCL3, CCL26, and CXCL7, and the like, as well as modulators thereof (*e.g.*, blocking antibodies or more potent or longer lasting forms) are used. In another embodiment, immunomodulatory molecules targeting immunosuppression, such as STAT3 signaling modulators, NFkappaB signaling modulators, and immune checkpoint modulators, are used. The terms "immune checkpoint" and "anti-immune checkpoint therapy" are described above.

In still another embodiment, immunomodulatory drugs, such as immunocytostatic drugs, glucocorticoids, cytostatics, immunophilins and modulators thereof (*e.g.*, rapamycin, a calcineurin inhibitor, tacrolimus, ciclosporin (cyclosporin), pimecrolimus, abetimus, gusperimus, ridaforolimus, everolimus, temsirolimus, zotarolimus, etc.), hydrocortisone (cortisol), cortisone acetate, prednisone, prednisolone, methylprednisolone, dexamethasone, betamethasone, triamcinolone, beclometasone, fludrocortisone acetate, deoxycorticosterone acetate (doca) aldosterone, a non-glucocorticoid steroid, a pyrimidine synthesis inhibitor, leflunomide, teriflunomide, a folic acid analog, methotrexate, anti-thymocyte globulin, anti-lymphocyte globulin, thalidomide, lenalidomide, pentoxifylline, bupropion, curcumin, catechin, an opioid, an IMPDH inhibitor, mycophenolic acid, myriocin, fingolimod, an NF-xB inhibitor, raloxifene, drotrecogin alfa, denosumab, an NF-xB signaling cascade inhibitor, disulfiram, olmesartan, dithiocarbamate, a proteasome inhibitor, bortezomib, MG132, Prol, NPI-0052, curcumin, genistein, resveratrol, parthenolide, thalidomide, lenalidomide, flavopiridol, non-steroidal anti-inflammatory drugs (NSAIDs), arsenic trioxide, dehydroxymethylepoxyquinomycin (DHMEQ), I3C(indole-3-carbinol)/DIM(di-indolmethane) (13C/DIM), Bay 11-7082, luteolin, cell permeable peptide SN-50, IKBa.-super repressor overexpression, NFKB decoy oligodeoxynucleotide (ODN), or a derivative or analog of any thereo, are used. In yet another embodiment, immunomodulatory antibodies or protein are used. For example, antibodies that bind to CD40, Toll-like receptor (TLR), OX40, GITR, CD27, or to 4-1BB, T-cell bispecific antibodies, an anti-IL-2 receptor antibody, an anti-CD3 antibody, OKT3 (muromonab), otelixizumab, teplizumab, visilizumab, an anti-CD4 antibody, clenoliximab, keliximab, zanolimumab, an anti-CD11 a antibody, efalizumab, an anti-CD18 antibody, erlizumab, rovelizumab, an anti-CD20 antibody, afutuzumab, ocrelizumab, ofatumumab, pascolizumab, rituximab, an anti-CD23 antibody, lumiliximab, an anti-CD40 antibody, teneliximab, toralizumab, an anti-CD40L antibody, ruplizumab, an anti-CD62L antibody, aselizumab, an anti-CD80 antibody, galiximab, an anti-CD147 antibody, gavilimomab, a B-Lymphocyte stimulator (BLyS)

inhibiting antibody, belimumab, an CTLA4-Ig fusion protein, abatacept, belatacept, an anti-CTLA4 antibody, ipilimumab, tremelimumab, an anti-eotaxin 1 antibody, bertilimumab, an anti-a4-integrin antibody, natalizumab, an anti-IL-6R antibody, tocilizumab, an anti-LFA-1 antibody, odulimomab, an anti-CD25 antibody, basiliximab, daclizumab, inolimomab, an anti-CD5 antibody, zolimomab, an anti-CD2 antibody, siplizumab, nerelimomab, faralimomab, atlizumab, atorolimumab, cedelizumab, dorlimomab aritox, dorlixizumab, fontolizumab, gantenerumab, gomiliximab, lebrilizumab, maslimomab, morolimumab, pexelizumab, reslizumab, rovelizumab, talizumab, telimomab aritox, vapaliximab, vepalimomab, aflibercept, alefacept, rilonacept, an IL-1 receptor antagonist, anakinra, an anti-IL-5 antibody, mepolizumab, an IgE inhibitor, omalizumab, talizumab, an IL12 inhibitor, an IL23 inhibitor, ustekinumab, and the like.

Nutritional supplements that enhance immune responses, such as vitamin A, vitamin E, vitamin C, and the like, are well-known in the art (see, for example, U.S. Pat. Nos. 4,981,844 and 5,230,902 and PCT Publ. No. WO 2004/004483) can be used in the methods described herein.

Similarly, agents and therapies other than immunotherapy or in combination thereof can be used with in combination with inhibitors of one or more biomarkers listed in Table 1 and immunotherapies to stimulate an immune response to thereby treat a condition that would benefit therefrom. For example, chemotherapy, radiation, epigenetic modifiers (*e.g.*, histone deacetylase (HDAC) modifiers, methylation modifiers, phosphorylation modifiers, and the like), targeted therapy, and the like are well-known in the art.

The term "untargeted therapy" refers to administration of agents that do not selectively interact with a chosen biomolecule yet treat cancer. Representative examples of untargeted therapies include, without limitation, chemotherapy, gene therapy, and radiation therapy.

In one embodiment, chemotherapy is used. Chemotherapy includes the administration of a chemotherapeutic agent. Such a chemotherapeutic agent may be, but is not limited to, those selected from among the following groups of compounds: platinum compounds, cytotoxic antibiotics, antimetabolites, anti-mitotic agents, alkylating agents, arsenic compounds, DNA topoisomerase inhibitors, taxanes, nucleoside analogues, plant alkaloids, and toxins; and synthetic derivatives thereof. Exemplary compounds include, but are not limited to, alkylating agents: cisplatin, treosulfan, and trofosfamide; plant alkaloids: vinblastine, paclitaxel, docetaxol; DNA topoisomerase inhibitors: teniposide, crisnatol, and

mitomycin; anti-folates: methotrexate, mycophenolic acid, and hydroxyurea; pyrimidine analogs: 5-fluorouracil, doxifluridine, and cytosine arabinoside; purine analogs: mercaptopurine and thioguanine; DNA antimetabolites: 2'-deoxy-5-fluorouridine, aphidicolin glycinate, and pyrazoloimidazole; and antimitotic agents: halichondrin, colchicine, and rhizoxin. Compositions comprising one or more chemotherapeutic agents (*e.g.*, FLAG, CHOP) may also be used. FLAG comprises fludarabine, cytosine arabinoside (Ara-C) and G-CSF. CHOP comprises cyclophosphamide, vincristine, doxorubicin, and prednisone. In another embodiments, PARP (*e.g.*, PARP-1 and/or PARP-2) inhibitors are used and such inhibitors are well-known in the art (*e.g.*, Olaparib, ABT-888, BSI-201, BGP-15 (N-Gene Research Laboratories, Inc.); INO-1001 (Inotek Pharmaceuticals Inc.); PJ34 (Soriano *et al.*, 2001; Pacher *et al.*, 2002b); 3-aminobenzamide (Trevigen); 4-amino-1,8-naphthalimide; (Trevigen); 6(5H)-phenanthridinone (Trevigen); benzamide (U.S. Pat. Re. 36,397); and NU1025 (Bowman *et al.*). The mechanism of action is generally related to the ability of PARP inhibitors to bind PARP and decrease its activity. PARP catalyzes the conversion of .beta.-nicotinamide adenine dinucleotide (NAD+) into nicotinamide and poly-ADP-ribose (PAR). Both poly (ADP-ribose) and PARP have been linked to regulation of transcription, cell proliferation, genomic stability, and carcinogenesis (Bouchard V. J. et.al. Experimental Hematology, Volume 31, Number 6, June 2003, pp. 446-454(9); Herceg Z.; Wang Z.-Q. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, Volume 477, Number 1, 2 Jun. 2001, pp. 97-110(14)). Poly(ADP-ribose) polymerase 1 (PARP1) is a key molecule in the repair of DNA single-strand breaks (SSBs) (de Murcia J. *et al.* 1997. Proc Natl Acad Sci USA 94:7303-7307; Schreiber V, Dantzer F, Ame J C, de Murcia G (2006) Nat Rev Mol Cell Biol 7:517-528; Wang Z Q, *et al.* (1997) Genes Dev 11:2347-2358). Knockout of SSB repair by inhibition of PARP1 function induces DNA double-strand breaks (DSBs) that can trigger synthetic lethality in cancer cells with defective homology-directed DSB repair (Bryant H E, *et al.* (2005) Nature 434:913-917; Farmer H, *et al.* (2005) Nature 434:917-921). The foregoing examples of chemotherapeutic agents are illustrative, and are not intended to be limiting.

In another embodiment, radiation therapy is used. The radiation used in radiation therapy can be ionizing radiation. Radiation therapy can also be gamma rays, X-rays, or proton beams. Examples of radiation therapy include, but are not limited to, external-beam radiation therapy, interstitial implantation of radioisotopes (I-125, palladium, iridium), radioisotopes such as strontium-89, thoracic radiation therapy, intraperitoneal P-32

radiation therapy, and/or total abdominal and pelvic radiation therapy. For a general overview of radiation therapy, see Hellman, Chapter 16: Principles of Cancer Management: Radiation Therapy, 6th edition, 2001, DeVita *et al.*, eds., J. B. Lippencott Company, Philadelphia. The radiation therapy can be administered as external beam radiation or teletherapy wherein the radiation is directed from a remote source. The radiation treatment can also be administered as internal therapy or brachytherapy wherein a radioactive source is placed inside the body close to cancer cells or a tumor mass. Also encompassed is the use of photodynamic therapy comprising the administration of photosensitizers, such as hematoporphyrin and its derivatives, Vertoporfin (BPD-MA), phthalocyanine, photosensitizer Pc4, demethoxy-hypocrellin A; and 2BA-2-DMHA.

In another embodiment, surgical intervention can occur to physically remove cancerous cells and/or tissues.

In still another embodiment, hormone therapy is used. Hormonal therapeutic treatments can comprise, for example, hormonal agonists, hormonal antagonists (*e.g.*, flutamide, bicalutamide, tamoxifen, raloxifene, leuprolide acetate (LUPRON), LH-RH antagonists), inhibitors of hormone biosynthesis and processing, and steroids (*e.g.*, dexamethasone, retinoids, deltoids, betamethasone, cortisol, cortisone, prednisone, dehydrotestosterone, glucocorticoids, mineralocorticoids, estrogen, testosterone, progestins), vitamin A derivatives (*e.g.*, all-trans retinoic acid (ATRA)); vitamin D3 analogs; antigestagens (*e.g.*, mifepristone, onapristone), or antiandrogens (*e.g.*, cyproterone acetate).

In yet another embodiment, hyperthermia, a procedure in which body tissue is exposed to high temperatures (up to 106°F.) is used. Heat may help shrink tumors by damaging cells or depriving them of substances they need to live. Hyperthermia therapy can be local, regional, and whole-body hyperthermia, using external and internal heating devices. Hyperthermia is almost always used with other forms of therapy (*e.g.*, radiation therapy, chemotherapy, and biological therapy) to try to increase their effectiveness. Local hyperthermia refers to heat that is applied to a very small area, such as a tumor. The area may be heated externally with high-frequency waves aimed at a tumor from a device outside the body. To achieve internal heating, one of several types of sterile probes may be used, including thin, heated wires or hollow tubes filled with warm water; implanted microwave antennae; and radiofrequency electrodes. In regional hyperthermia, an organ or a limb is heated. Magnets and devices that produce high energy are placed over the region

to be heated. In another approach, called perfusion, some of the patient's blood is removed, heated, and then pumped (perfused) into the region that is to be heated internally. Whole-body heating is used to treat metastatic cancer that has spread throughout the body. It can be accomplished using warm-water blankets, hot wax, inductive coils (like those in electric blankets), or thermal chambers (similar to large incubators). Hyperthermia does not cause any marked increase in radiation side effects or complications. Heat applied directly to the skin, however, can cause discomfort or even significant local pain in about half the patients treated. It can also cause blisters, which generally heal rapidly.

In still another embodiment, photodynamic therapy (also called PDT, photoradiation therapy, phototherapy, or photochemotherapy) is used for the treatment of some types of cancer. It is based on the discovery that certain chemicals known as photosensitizing agents can kill one-celled organisms when the organisms are exposed to a particular type of light. PDT destroys cancer cells through the use of a fixed-frequency laser light in combination with a photosensitizing agent. In PDT, the photosensitizing agent is injected into the bloodstream and absorbed by cells all over the body. The agent remains in cancer cells for a longer time than it does in normal cells. When the treated cancer cells are exposed to laser light, the photosensitizing agent absorbs the light and produces an active form of oxygen that destroys the treated cancer cells. Light exposure must be timed carefully so that it occurs when most of the photosensitizing agent has left healthy cells but is still present in the cancer cells. The laser light used in PDT can be directed through a fiber-optic (a very thin glass strand). The fiber-optic is placed close to the cancer to deliver the proper amount of light. The fiber-optic can be directed through a bronchoscope into the lungs for the treatment of lung cancer or through an endoscope into the esophagus for the treatment of esophageal cancer. An advantage of PDT is that it causes minimal damage to healthy tissue. However, because the laser light currently in use cannot pass through more than about 3 centimeters of tissue (a little more than one and an eighth inch), PDT is mainly used to treat tumors on or just under the skin or on the lining of internal organs. Photodynamic therapy makes the skin and eyes sensitive to light for 6 weeks or more after treatment. Patients are advised to avoid direct sunlight and bright indoor light for at least 6 weeks. If patients must go outdoors, they need to wear protective clothing, including sunglasses. Other temporary side effects of PDT are related to the treatment of specific areas and can include coughing, trouble swallowing, abdominal pain, and painful breathing or shortness of breath. In December 1995, the U.S. Food and Drug Administration (FDA)

approved a photosensitizing agent called porfimer sodium, or Photofrin®, to relieve symptoms of esophageal cancer that is causing an obstruction and for esophageal cancer that cannot be satisfactorily treated with lasers alone. In January 1998, the FDA approved porfimer sodium for the treatment of early non-small cell lung cancer in patients for whom the usual treatments for lung cancer are not appropriate. The National Cancer Institute and other institutions are supporting clinical trials (research studies) to evaluate the use of photodynamic therapy for several types of cancer, including cancers of the bladder, brain, larynx, and oral cavity.

In yet another embodiment, laser therapy is used to harness high-intensity light to destroy cancer cells. This technique is often used to relieve symptoms of cancer such as bleeding or obstruction, especially when the cancer cannot be cured by other treatments. It may also be used to treat cancer by shrinking or destroying tumors. The term "laser" stands for light amplification by stimulated emission of radiation. Ordinary light, such as that from a light bulb, has many wavelengths and spreads in all directions. Laser light, on the other hand, has a specific wavelength and is focused in a narrow beam. This type of high-intensity light contains a lot of energy. Lasers are very powerful and may be used to cut through steel or to shape diamonds. Lasers also can be used for very precise surgical work, such as repairing a damaged retina in the eye or cutting through tissue (in place of a scalpel). Although there are several different kinds of lasers, only three kinds have gained wide use in medicine: Carbon dioxide (CO_2) laser--This type of laser can remove thin layers from the skin's surface without penetrating the deeper layers. This technique is particularly useful in treating tumors that have not spread deep into the skin and certain precancerous conditions. As an alternative to traditional scalpel surgery, the CO_2 laser is also able to cut the skin. The laser is used in this way to remove skin cancers. Neodymium:yttrium-aluminum-garnet (Nd:YAG) laser-- Light from this laser can penetrate deeper into tissue than light from the other types of lasers, and it can cause blood to clot quickly. It can be carried through optical fibers to less accessible parts of the body. This type of laser is sometimes used to treat throat cancers. Argon laser--This laser can pass through only superficial layers of tissue and is therefore useful in dermatology and in eye surgery. It also is used with light-sensitive dyes to treat tumors in a procedure known as photodynamic therapy (PDT). Lasers have several advantages over standard surgical tools, including: Lasers are more precise than scalpels. Tissue near an incision is protected, since there is little contact with surrounding skin or other tissue. The heat produced by lasers

sterilizes the surgery site, thus reducing the risk of infection. Less operating time may be needed because the precision of the laser allows for a smaller incision. Healing time is often shortened; since laser heat seals blood vessels, there is less bleeding, swelling, or scarring. Laser surgery may be less complicated. For example, with fiber optics, laser light can be directed to parts of the body without making a large incision. More procedures may be done on an outpatient basis. Lasers can be used in two ways to treat cancer: by shrinking or destroying a tumor with heat, or by activating a chemical--known as a photosensitizing agent--that destroys cancer cells. In PDT, a photosensitizing agent is retained in cancer cells and can be stimulated by light to cause a reaction that kills cancer cells. CO_2 and Nd:YAG lasers are used to shrink or destroy tumors. They may be used with endoscopes, tubes that allow physicians to see into certain areas of the body, such as the bladder. The light from some lasers can be transmitted through a flexible endoscope fitted with fiber optics. This allows physicians to see and work in parts of the body that could not otherwise be reached except by surgery and therefore allows very precise aiming of the laser beam. Lasers also may be used with low-power microscopes, giving the doctor a clear view of the site being treated. Used with other instruments, laser systems can produce a cutting area as small as 200 microns in diameter--less than the width of a very fine thread. Lasers are used to treat many types of cancer. Laser surgery is a standard treatment for certain stages of glottis (vocal cord), cervical, skin, lung, vaginal, vulvar, and penile cancers. In addition to its use to destroy the cancer, laser surgery is also used to help relieve symptoms caused by cancer (palliative care). For example, lasers may be used to shrink or destroy a tumor that is blocking a patient's trachea (windpipe), making it easier to breathe. It is also sometimes used for palliation in colorectal and anal cancer. Laser-induced interstitial thermotherapy (LITT) is one of the most recent developments in laser therapy. LITT uses the same idea as a cancer treatment called hyperthermia; that heat may help shrink tumors by damaging cells or depriving them of substances they need to live. In this treatment, lasers are directed to interstitial areas (areas between organs) in the body. The laser light then raises the temperature of the tumor, which damages or destroys cancer cells.

The duration and/or dose of treatment with therapies may vary according to the particular therapeutic agent or combination thereof. An appropriate treatment time for a particular cancer therapeutic agent will be appreciated by the skilled artisan. The present invention contemplates the continued assessment of optimal treatment schedules for each

cancer therapeutic agent, where the phenotype of the cancer of the subject as determined by the methods of the present invention is a factor in determining optimal treatment doses and schedules.

Any means for the introduction of a polynucleotide into mammals, human or non-human, or cells thereof may be adapted to the practice of this invention for the delivery of the various constructs of the present invention into the intended recipient. In one embodiment of the present invention, the DNA constructs are delivered to cells by transfection, *i.e.*, by delivery of "naked" DNA or in a complex with a colloidal dispersion system. A colloidal system includes macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. The preferred colloidal system of this invention is a lipid-complexed or liposome-formulated DNA. In the former approach, prior to formulation of DNA, *e.g.*, with lipid, a plasmid containing a transgene bearing the desired DNA constructs may first be experimentally optimized for expression (*e.g.*, inclusion of an intron in the 5' untranslated region and elimination of unnecessary sequences (Felgner, *et al.*, Ann NY Acad Sci 126-139, 1995). Formulation of DNA, *e.g.* with various lipid or liposome materials, may then be effected using known methods and materials and delivered to the recipient mammal. See, *e.g.*, Canonico et al, Am J Respir Cell Mol Biol 10:24-29, 1994; Tsan et al, Am J Physiol 268; Alton *et al.*, Nat Genet. 5:135-142, 1993 and U.S. patent No. 5,679,647 by Carson *et al.*

The targeting of liposomes can be classified based on anatomical and mechanistic factors. Anatomical classification is based on the level of selectivity, for example, organ-specific, cell-specific, and organelle-specific. Mechanistic targeting can be distinguished based upon whether it is passive or active. Passive targeting utilizes the natural tendency of liposomes to distribute to cells of the reticulo-endothelial system (RES) in organs, which contain sinusoidal capillaries. Active targeting, on the other hand, involves alteration of the liposome by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein, or by changing the composition or size of the liposome in order to achieve targeting to organs and cell types other than the naturally occurring sites of localization.

The surface of the targeted delivery system may be modified in a variety of ways. In the case of a liposomal targeted delivery system, lipid groups can be incorporated into the lipid bilayer of the liposome in order to maintain the targeting ligand in stable

association with the liposomal bilayer. Various linking groups can be used for joining the lipid chains to the targeting ligand. Naked DNA or DNA associated with a delivery vehicle, *e.g.*, liposomes, can be administered to several sites in a subject (see below).

Nucleic acids can be delivered in any desired vector. These include viral or non-viral vectors, including adenovirus vectors, adeno-associated virus vectors, retrovirus vectors, lentivirus vectors, and plasmid vectors. Exemplary types of viruses include HSV (herpes simplex virus), AAV (adeno associated virus), HIV (human immunodeficiency virus), BIV (bovine immunodeficiency virus), and MLV (murine leukemia virus). Nucleic acids can be administered in any desired format that provides sufficiently efficient delivery levels, including in virus particles, in liposomes, in nanoparticles, and complexed to polymers.

The nucleic acids encoding a protein or nucleic acid of interest may be in a plasmid or viral vector, or other vector as is known in the art. Such vectors are well-known and any can be selected for a particular application. In one embodiment of the present invention, the gene delivery vehicle comprises a promoter and a demethylase coding sequence. Preferred promoters are tissue-specific promoters and promoters which are activated by cellular proliferation, such as the thymidine kinase and thymidylate synthase promoters. Other preferred promoters include promoters which are activatable by infection with a virus, such as the α- and β-interferon promoters, and promoters which are activatable by a hormone, such as estrogen. Other promoters which can be used include the Moloney virus LTR, the CMV promoter, and the mouse albumin promoter. A promoter may be constitutive or inducible.

In another embodiment, naked polynucleotide molecules are used as gene delivery vehicles, as described in WO 90/11092 and U.S. Patent 5,580,859. Such gene delivery vehicles can be either growth factor DNA or RNA and, in certain embodiments, are linked to killed adenovirus. Curiel *et al.*, Hum. Gene. Ther. 3:147-154, 1992. Other vehicles which can optionally be used include DNA-ligand (Wu *et al.*, J. Biol. Chem. 264:16985-16987, 1989), lipid-DNA combinations (Felgner *et al.*, Proc. Natl. Acad. Sci. USA 84:7413 7417, 1989), liposomes (Wang *et al.*, Proc. Natl. Acad. Sci. 84:7851-7855, 1987) and microprojectiles (Williams *et al.*, Proc. Natl. Acad. Sci. 88:2726-2730, 1991).

A gene delivery vehicle can optionally comprise viral sequences such as a viral origin of replication or packaging signal. These viral sequences can be selected from viruses such as astrovirus, coronavirus, orthomyxovirus, papovavirus, paramyxovirus,

parvovirus, picornavirus, poxvirus, retrovirus, togavirus or adenovirus. In a preferred embodiment, the growth factor gene delivery vehicle is a recombinant retroviral vector. Recombinant retroviruses and various uses thereof have been described in numerous references including, for example, Mann *et al.*, Cell 33:153, 1983, Cane and Mulligan, Proc. Nat'l. Acad. Sci. USA 81:6349, 1984, Miller *et al.*, Human Gene Therapy 1:5-14, 1990, U.S. Patent Nos. 4,405,712, 4,861,719, and 4,980,289, and PCT Application Nos. WO 89/02,468, WO 89/05,349, and WO 90/02,806. Numerous retroviral gene delivery vehicles can be utilized in the present invention, including for example those described in EP 0,415,731; WO 90/07936; WO 94/03622; WO 93/25698; WO 93/25234; U.S. Patent No. 5,219,740; WO 9311230; WO 9310218; Vile and Hart, Cancer Res. 53:3860-3864, 1993; Vile and Hart, Cancer Res. 53:962-967, 1993; Ram *et al.*, Cancer Res. 53:83-88, 1993; Takamiya *et al.*, J. Neurosci. Res. 33:493-503, 1992; Baba *et al.*, J. Neurosurg. 79:729-735, 1993 (U.S. Patent No. 4,777,127, GB 2,200,651, EP 0,345,242 and WO91/02805).

Other viral vector systems that can be used to deliver a polynucleotide of the present invention have been derived from herpes virus, *e.g.*, Herpes Simplex Virus (U.S. Patent No. 5,631,236 by Woo *et al.*, issued May 20, 1997 and WO 00/08191 by Neurovex), vaccinia virus (Ridgeway (1988) Ridgeway, "Mammalian expression vectors," In: Rodriguez R L, Denhardt D T, ed. Vectors: A survey of molecular cloning vectors and their uses. Stoneham: Butterworth,; Baichwal and Sugden (1986) "Vectors for gene transfer derived from animal DNA viruses: Transient and stable expression of transferred genes," In: Kucherlapati R, ed. Gene transfer. New York: Plenum Press; Coupar *et al.* (1988) Gene, 68:1-10), and several RNA viruses. Preferred viruses include an alphavirus, a poxivirus, an arena virus, a vaccinia virus, a polio virus, and the like. They offer several attractive features for various mammalian cells (Friedmann (1989) Science, 244:1275-1281; Ridgeway, 1988, supra; Baichwal and Sugden, 1986, supra; Coupar *et al.*, 1988; Horwich *et al.*(1990) J.Virol., 64:642-650).

In other embodiments, target DNA in the genome can be manipulated using well-known methods in the art. For example, the target DNA in the genome can be manipulated by deletion, insertion, and/or mutation are retroviral insertion, artificial chromosome techniques, gene insertion, random insertion with tissue specific promoters, gene targeting, transposable elements and/or any other method for introducing foreign DNA or producing modified DNA/modified nuclear DNA. Other modification techniques include deleting

DNA sequences from a genome and/or altering nuclear DNA sequences. Nuclear DNA sequences, for example, may be altered by site-directed mutagenesis.

In other embodiments, recombinant biomarker polypeptides, and fragments thereof, can be administered to subjects. In some embodiments, fusion proteins can be constructed and administered which have enhanced biological properties. In addition, the biomarker polypeptides, and fragment thereof, can be modified according to well-known pharmacological methods in the art (*e.g.*, pegylation, glycosylation, oligomerization, *etc.*) in order to further enhance desirable biological activities, such as increased bioavailability and decreased proteolytic degradation.

4. Clincal Efficacy

Clinical efficacy can be measured by any method known in the art. For example, the response to a therapy, such as inhibitors of one or more biomarkers listed in Table 1 and immunotherapy combination treatment, relates to any response of the cancer, *e.g.*, a tumor, to the therapy, preferably to a change in tumor mass and/or volume after initiation of neoadjuvant or adjuvant chemotherapy. Tumor response may be assessed in a neoadjuvant or adjuvant situation where the size of a tumor after systemic intervention can be compared to the initial size and dimensions as measured by CT, PET, mammogram, ultrasound or palpation and the cellularity of a tumor can be estimated histologically and compared to the cellularity of a tumor biopsy taken before initiation of treatment. Response may also be assessed by caliper measurement or pathological examination of the tumor after biopsy or surgical resection. Response may be recorded in a quantitative fashion like percentage change in tumor volume or cellularity or using a semi-quantitative scoring system such as residual cancer burden (Symmans *et al.*, *J. Clin. Oncol.* (2007) 25:4414-4422) or Miller-Payne score (Ogston *et al.*, (2003) *Breast* (Edinburgh, Scotland) 12:320-327) in a qualitative fashion like "pathological complete response" (pCR), "clinical complete remission" (cCR), "clinical partial remission" (cPR), "clinical stable disease" (cSD), "clinical progressive disease" (cPD) or other qualitative criteria. Assessment of tumor response may be performed early after the onset of neoadjuvant or adjuvant therapy, *e.g.*, after a few hours, days, weeks or preferably after a few months. A typical endpoint for response assessment is upon termination of neoadjuvant chemotherapy or upon surgical removal of residual tumor cells and/or the tumor bed.

In some embodiments, clinical efficacy of the therapeutic treatments described herein may be determined by measuring the clinical benefit rate (CBR). The clinical benefit rate is measured by determining the sum of the percentage of patients who are in complete remission (CR), the number of patients who are in partial remission (PR) and the number of patients having stable disease (SD) at a time point at least 6 months out from the end of therapy. The shorthand for this formula is CBR=CR+PR+SD over 6 months. In some embodiments, the CBR for a particular anti-immune checkpoint therapeutic regimen is at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, or more.

Additional criteria for evaluating the response to immunotherapies, such as anti-immune checkpoint therapies, are related to "survival," which includes all of the following: survival until mortality, also known as overall survival (wherein said mortality may be either irrespective of cause or tumor related); "recurrence-free survival" (wherein the term recurrence shall include both localized and distant recurrence); metastasis free survival; disease free survival (wherein the term disease shall include cancer and diseases associated therewith). The length of said survival may be calculated by reference to a defined start point (*e.g.*, time of diagnosis or start of treatment) and end point (*e.g.*, death, recurrence or metastasis). In addition, criteria for efficacy of treatment can be expanded to include response to chemotherapy, probability of survival, probability of metastasis within a given time period, and probability of tumor recurrence.

For example, in order to determine appropriate threshold values, a particular anti-cancer therapeutic regimen can be administered to a population of subjects and the outcome can be correlated to biomarker measurements that were determined prior to administration of any immunotherapy, such as anti-immune checkpoint therapy. The outcome measurement may be pathologic response to therapy given in the neoadjuvant setting. Alternatively, outcome measures, such as overall survival and disease-free survival can be monitored over a period of time for subjects following immunotherapies for whom biomarker measurement values are known. In certain embodiments, the same doses of immunotherapy agents, if any, are administered to each subject. In related embodiments, the doses administered are standard doses known in the art for those agents used in immunotherapies. The period of time for which subjects are monitored can vary. For example, subjects may be monitored for at least 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 55, or 60 months. Biomarker measurement threshold values that correlate to

outcome of an immunotherapy can be determined using methods such as those described in the Examples section.

5. Further Uses and Methods of the Present Invention

The compositions described herein can be used in a variety of diagnostic, prognostic, and therapeutic applications. In any method described herein, such as a diagnostic method, prognostic method, therapeutic method, or combination thereof, all steps of the method can be performed by a single actor or, alternatively, by more than one actor. For example, diagnosis can be performed directly by the actor providing therapeutic treatment. Alternatively, a person providing a therapeutic agent can request that a diagnostic assay be performed. The diagnostician and/or the therapeutic interventionist can interpret the diagnostic assay results to determine a therapeutic strategy. Similarly, such alternative processes can apply to other assays, such as prognostic assays.

a. Screening Methods

One aspect of the present invention relates to screening assays, including non-cell based assays and xenograft animal model assays. In one embodiment, the assays provide a method for identifying whether a cancer is likely to respond to inhibitors of one or more biomarkers listed in Table 1, in combination with an immunotherapy, such as in a human by using a xenograft animal model assay, and/or whether an agent can inhibit the growth of or kill a cancer cell that is unlikely to respond to biomarker inhibitor and immunotherapy combination treatments.

In one embodiment, the present invention relates to assays for screening test agents which bind to, or modulate the biological activity of, at least one biomarker described herein (*e.g.*, in the tables, figures, examples, or otherwise in the specification). In one embodiment, a method for identifying such an agent entails determining the ability of the agent to modulate, *e.g.* inhibit, the at least one biomarker described herein.

In one embodiment, an assay is a cell-free or cell-based assay, comprising contacting at least one biomarker described herein, with a test agent, and determining the ability of the test agent to modulate (*e.g.*, inhibit) the enzymatic activity of the biomarker, such as by measuring direct binding of substrates or by measuring indirect parameters as described below.

For example, in a direct binding assay, biomarker protein (or their respective target polypeptides or molecules) can be coupled with a radioisotope or enzymatic label such that

binding can be determined by detecting the labeled protein or molecule in a complex. For example, the targets can be labeled with ^{125}I, ^{35}S, ^{14}C, or ^{3}H, either directly or indirectly, and the radioisotope detected by direct counting of radioemmission or by scintillation counting. Alternatively, the targets can be enzymatically labeled with, for example, horseradish peroxidase, alkaline phosphatase, or luciferase, and the enzymatic label detected by determination of conversion of an appropriate substrate to product. Determining the interaction between biomarker and substrate can also be accomplished using standard binding or enzymatic analysis assays. In one or more embodiments of the above described assay methods, it may be desirable to immobilize polypeptides or molecules to facilitate separation of complexed from uncomplexed forms of one or both of the proteins or molecules, as well as to accommodate automation of the assay.

Binding of a test agent to a target can be accomplished in any vessel suitable for containing the reactants. Non-limiting examples of such vessels include microtiter plates, test tubes, and micro-centrifuge tubes. Immobilized forms of the antibodies described herein can also include antibodies bound to a solid phase like a porous, microporous (with an average pore diameter less than about one micron) or macroporous (with an average pore diameter of more than about 10 microns) material, such as a membrane, cellulose, nitrocellulose, or glass fibers; a bead, such as that made of agarose or polyacrylamide or latex; or a surface of a dish, plate, or well, such as one made of polystyrene.

In an alternative embodiment, determining the ability of the agent to modulate the interaction between the biomarker and a substrate or a biomarker and its natural binding partner can be accomplished by determining the ability of the test agent to modulate the activity of a polypeptide or other product that functions downstream or upstream of its position within the signaling pathway (*e.g.*, feedback loops). Such feedback loops are well-known in the art (see, for example, Chen and Guillemin (2009) *Int. J. Tryptophan Res.* 2:1-19).

The present invention further pertains to novel agents identified by the above-described screening assays. Accordingly, it is within the scope of this invention to further use an agent identified as described herein, such as in an appropriate animal model. For example, an agent identified as described herein can be used in an animal model to determine the efficacy, toxicity, or side effects of treatment with such an agent. Alternatively, an antibody identified as described herein can be used in an animal model to determine the mechanism of action of such an agent.

b. Predictive Medicine

The present invention also pertains to the field of predictive medicine in which diagnostic assays, prognostic assays, and monitoring clinical trials are used for prognostic (predictive) purposes to thereby treat an individual prophylactically. Accordingly, one aspect of the present invention relates to diagnostic assays for determining the amount and/or activity level of a biomarker described herein in the context of a biological sample (*e.g.*, blood, serum, cells, or tissue) to thereby determine whether an individual afflicted with a cancer is likely to respond to inhibitors of one or more biomarkers listed in Table 1, in combination with an immunotherapy , such as in a cancer. Such assays can be used for prognostic or predictive purpose alone, or can be coupled with a therapeutic intervention to thereby prophylactically treat an individual prior to the onset or after recurrence of a disorder characterized by or associated with biomarker polypeptide, nucleic acid expression or activity. The skilled artisan will appreciate that any method can use one or more (*e.g.*, combinations) of biomarkers described herein, such as those in the tables, figures, examples, and otherwise described in the specification.

Another aspect of the present invention pertains to monitoring the influence of agents (*e.g.*, drugs, compounds, and small nucleic acid-based molecules) on the expression or activity of a biomarker described herein. These and other agents are described in further detail in the following sections.

The skilled artisan will also appreciated that, in certain embodiments, the methods of the present invention implement a computer program and computer system. For example, a computer program can be used to perform the algorithms described herein. A computer system can also store and manipulate data generated by the methods of the present invention which comprises a plurality of biomarker signal changes/profiles which can be used by a computer system in implementing the methods of this invention. In certain embodiments, a computer system receives biomarker expression data; (ii) stores the data; and (iii) compares the data in any number of ways described herein (*e.g.*, analysis relative to appropriate controls) to determine the state of informative biomarkers from cancerous or pre-cancerous tissue. In other embodiments, a computer system (i) compares the determined expression biomarker level to a threshold value; and (ii) outputs an indication of whether said biomarker level is significantly modulated (*e.g.*, above or below) the threshold value, or a phenotype based on said indication.

In certain embodiments, such computer systems are also considered part of the present invention. Numerous types of computer systems can be used to implement the analytic methods of this invention according to knowledge possessed by a skilled artisan in the bioinformatics and/or computer arts. Several software components can be loaded into memory during operation of such a computer system. The software components can comprise both software components that are standard in the art and components that are special to the present invention (*e.g.*, dCHIP software described in Lin *et al.* (2004) *Bioinformatics* 20, 1233-1240; radial basis machine learning algorithms (RBM) known in the art).

The methods of the present invention can also be programmed or modeled in mathematical software packages that allow symbolic entry of equations and high-level specification of processing, including specific algorithms to be used, thereby freeing a user of the need to procedurally program individual equations and algorithms. Such packages include, *e.g.*, Matlab from Mathworks (Natick, Mass.), Mathematica from Wolfram Research (Champaign, Ill.) or S-Plus from MathSoft (Seattle, Wash.).

In certain embodiments, the computer comprises a database for storage of biomarker data. Such stored profiles can be accessed and used to perform comparisons of interest at a later point in time. For example, biomarker expression profiles of a sample derived from the non-cancerous tissue of a subject and/or profiles generated from population-based distributions of informative loci of interest in relevant populations of the same species can be stored and later compared to that of a sample derived from the cancerous tissue of the subject or tissue suspected of being cancerous of the subject.

In addition to the exemplary program structures and computer systems described herein, other, alternative program structures and computer systems will be readily apparent to the skilled artisan. Such alternative systems, which do not depart from the above described computer system and programs structures either in spirit or in scope, are therefore intended to be comprehended within the accompanying claims.

c. Diagnostic Assays

The present invention provides, in part, methods, systems, and code for accurately classifying whether a biological sample is associated with a cancer that is likely to respond to inhibitors of one or more biomarkers listed in Table 1, in combination with an immunotherapy. In some embodiments, the present invention is useful for classifying a sample (*e.g.*, from a subject) as associated with or at risk for responding to or not

responding to such biomarker inhibitor and immunotherapy combination treatments using a statistical algorithm and/or empirical data (*e.g.*, the amount or activity of a biomarker described herein, such as in the tables, figures, examples, and otherwise described in the specification).

An exemplary method for detecting the amount or activity of a biomarker described herein, and thus useful for classifying whether a sample is likely or unlikely to respond to such biomarker inhibitor and immunotherapy combination treatments involves obtaining a biological sample from a test subject and contacting the biological sample with an agent, such as a protein-binding agent like an antibody or antigen-binding fragment thereof, or a nucleic acid-binding agent like an oligonucleotide, capable of detecting the amount or activity of the biomarker in the biological sample. In some embodiments, at least one antibody or antigen-binding fragment thereof is used, wherein two, three, four, five, six, seven, eight, nine, ten, or more such antibodies or antibody fragments can be used in combination (*e.g.*, in sandwich ELISAs) or in serial. In certain instances, the statistical algorithm is a single learning statistical classifier system. For example, a single learning statistical classifier system can be used to classify a sample as a based upon a prediction or probability value and the presence or level of the biomarker. The use of a single learning statistical classifier system typically classifies the sample as, for example, a likely immunotherapy responder or progressor sample with a sensitivity, specificity, positive predictive value, negative predictive value, and/or overall accuracy of at least about 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%.

Other suitable statistical algorithms are well-known to those of skill in the art. For example, learning statistical classifier systems include a machine learning algorithmic technique capable of adapting to complex data sets (*e.g.*, panel of markers of interest) and making decisions based upon such data sets. In some embodiments, a single learning statistical classifier system such as a classification tree (*e.g.*, random forest) is used. In other embodiments, a combination of 2, 3, 4, 5, 6, 7, 8, 9, 10, or more learning statistical classifier systems are used, preferably in tandem. Examples of learning statistical classifier systems include, but are not limited to, those using inductive learning (*e.g.*, decision/classification trees such as random forests, classification and regression trees (C&RT), boosted trees, etc.), Probably Approximately Correct (PAC) learning, connectionist learning (*e.g.*, neural networks (NN), artificial neural networks (ANN), neuro

fuzzy networks (NFN), network structures, perceptrons such as multi-layer perceptrons, multi-layer feed-forward networks, applications of neural networks, Bayesian learning in belief networks, etc.), reinforcement learning (*e.g.*, passive learning in a known environment such as naive learning, adaptive dynamic learning, and temporal difference learning, passive learning in an unknown environment, active learning in an unknown environment, learning action-value functions, applications of reinforcement learning, etc.), and genetic algorithms and evolutionary programming. Other learning statistical classifier systems include support vector machines (*e.g.*, Kernel methods), multivariate adaptive regression splines (MARS), Levenberg-Marquardt algorithms, Gauss-Newton algorithms, mixtures of Gaussians, gradient descent algorithms, and learning vector quantization (LVQ). In certain embodiments, the method of the present invention further comprises sending the sample classification results to a clinician, *e.g.*, an oncologist.

In another embodiment, the diagnosis of a subject is followed by administering to the individual a therapeutically effective amount of a defined treatment based upon the diagnosis.

In one embodiment, the methods further involve obtaining a control biological sample (*e.g.*, biological sample from a subject who does not have a cancer or whose cancer is susceptible to biomarker inhibitor and immunotherapy combination treatments), a biological sample from the subject during remission, or a biological sample from the subject during treatment for developing a cancer progressing despite biomarker inhibitor and immunotherapy combination treatments.

d. Prognostic Assays

The diagnostic methods described herein can furthermore be utilized to identify subjects having or at risk of developing a cancer that is likely or unlikely to be responsive to biomarker inhibitor and immunotherapy combination treatments. The assays described herein, such as the preceding diagnostic assays or the following assays, can be utilized to identify a subject having or at risk of developing a disorder associated with a misregulation of the amount or activity of at least one biomarker described herein, such as in cancer. Alternatively, the prognostic assays can be utilized to identify a subject having or at risk for developing a disorder associated with a misregulation of the at least one biomarker described herein, such as in cancer. Furthermore, the prognostic assays described herein can be used to determine whether a subject can be administered an agent (*e.g.*, an agonist, antagonist, peptidomimetic, polypeptide, peptide, nucleic acid, small molecule, or other

drug candidate) to treat a disease or disorder associated with the aberrant biomarker expression or activity.

e. Treatment Methods

The therapeutic compositions described herein, such as the combination of inhibitors of one or more biomarkers listed in Table 1 and immunotherapy, can be used in a variety of *in vitro* and *in vivo* therapeutic applications using the formulations and/or combinations described herein. In one embodiment, the therapeutic agents can be used to treat cancers determined to be responsive thereto. For example, single or multiple agents that inhibit or block both a biomarker inhibitor and an immunotherapy can be used to treat cancers in subjects identified as likely responders thereto.

Modulatory methods of the present invention involve contacting a cell, such as an immune cell with an agent that inhibits or blocks the expression and/or activity of one or more biomarkers listed in Table 1 and an immunotherapy, such as an immune checkpoint inhibitor (*e.g.*, PD-1). Exemplary agents useful in such methods are described above. Such agents can be administered *in vitro* or *ex vivo* (*e.g.*, by contacting the cell with the agent) or, alternatively, *in vivo* (*e.g.*, by administering the agent to a subject). As such, the present invention provides methods useful for treating an individual afflicted with a condition that would benefit from an increased immune response, such as an infection or a cancer like colorectal cancer.

Agents that upregulate immune responses can be in the form of enhancing an existing immune response or eliciting an initial immune response. Thus, enhancing an immune response using the subject compositions and methods is useful for treating cancer, but can also be useful for treating an infectious disease (*e.g.*, bacteria, viruses, or parasites), a parasitic infection, and an immunosuppressive disease.

Exemplary infectious disorders include viral skin diseases, such as Herpes or shingles, in which case such an agent can be delivered topically to the skin. In addition, systemic viral diseases, such as influenza, the common cold, and encephalitis might be alleviated by systemic administration of such agents. In one preferred embodiment, agents that upregulate the immune response described herein are useful for modulating the arginase/iNOS balance during *Trypanosoma cruzi* infection in order to facilitate a protective immune response against the parasite.

Immune responses can also be enhanced in an infected patient through an *ex vivo* approach, for instance, by removing immune cells from the patient, contacting immune

cells *in vitro* with an agent described herein and reintroducing the *in vitro* stimulated immune cells into the patient.

In certain instances, it may be desirable to further administer other agents that upregulate immune responses, for example, forms of other B7 family members that transduce signals via costimulatory receptors, in order to further augment the immune response. Such additional agents and therapies are described further below.

Agents that upregulate an immune response can be used prophylactically in vaccines against various polypeptides (*e.g.*, polypeptides derived from pathogens). Immunity against a pathogen (*e.g.*, a virus) can be induced by vaccinating with a viral protein along with an agent that upregulates an immune response, in an appropriate adjuvant.

In another embodiment, upregulation or enhancement of an immune response function, as described herein, is useful in the induction of tumor immunity.

In another embodiment, the immune response can be stimulated by the methods described herein, such that preexisting tolerance, clonal deletion, and/or exhaustion (*e.g.*, T cell exhaustion) is overcome. For example, immune responses against antigens to which a subject cannot mount a significant immune response, *e.g.*, to an autologous antigen, such as a tumor specific antigens can be induced by administering appropriate agents described herein that upregulate the immune response. In one embodiment, an autologous antigen, such as a tumor-specific antigen, can be coadministered. In another embodiment, the subject agents can be used as adjuvants to boost responses to foreign antigens in the process of active immunization.

In one embodiment, immune cells are obtained from a subject and cultured *ex vivo* in the presence of an agent as described herein, to expand the population of immune cells and/or to enhance immune cell activation. In a further embodiment the immune cells are then administered to a subject. Immune cells can be stimulated *in vitro* by, for example, providing to the immune cells a primary activation signal and a costimulatory signal, as is known in the art. Various agents can also be used to costimulate proliferation of immune cells. In one embodiment immune cells are cultured *ex vivo* according to the method described in PCT Application No. WO 94/29436. The costimulatory polypeptide can be soluble, attached to a cell membrane, or attached to a solid surface, such as a bead.

6. Administration of Agents

The immune modulating agents of the invention are administered to subjects in a biologically compatible form suitable for pharmaceutical administration *in vivo,* to enhance immune cell mediated immune responses. By "biologically compatible form suitable for administration *in vivo*" is meant a form to be administered in which any toxic effects are outweighed by the therapeutic effects. The term "subject" is intended to include living organisms in which an immune response can be elicited, *e.g.*, mammals. Examples of subjects include humans, dogs, cats, mice, rats, and transgenic species thereof. Administration of an agent as described herein can be in any pharmacological form including a therapeutically active amount of an agent alone or in combination with a pharmaceutically acceptable carrier.

Administration of a therapeutically active amount of the therapeutic composition of the present invention is defined as an amount effective, at dosages and for periods of time necessary, to achieve the desired result. For example, a therapeutically active amount of an agent may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of peptide to elicit a desired response in the individual. Dosage regimens can be adjusted to provide the optimum therapeutic response. For example, several divided doses can be administered daily or the dose can be proportionally reduced as indicated by the exigencies of the therapeutic situation.

Inhibiting or blocking biomarker expression and/or activity, alone or in combination with an immunotherapy, can be accomplished by combination therapy with the modulatory agents described herein. Combination therapy describes a therapy in which one or more biomarkers listed in Table 1 is inhibited or blocked with an immunotherapy simultaneously. This may be achieved by administration of the modulatory agent described herein swith the immunotherapy imultaneously (*e.g.*, in a combination dosage form or by simultaneous administration of single agents) or by administration of single inhibitory agent for one or more biomarkers listed in Table 1 and the immunotherapy, according to a schedule that results in effective amounts of each modulatory agent present in the patient at the same time.

The therapeutic agents described herein can be administered in a convenient manner such as by injection (subcutaneous, intravenous, etc.), oral administration, inhalation, transdermal application, or rectal administration. Depending on the route of administration, the active compound can be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound. For

example, for administration of agents, by other than parenteral administration, it may be desirable to coat the agent with, or co-administer the agent with, a material to prevent its inactivation.

An agent can be administered to an individual in an appropriate carrier, diluent or adjuvant, co-administered with enzyme inhibitors or in an appropriate carrier such as liposomes. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Adjuvant is used in its broadest sense and includes any immune stimulating compound such as interferon. Adjuvants contemplated herein include resorcinols, non-ionic surfactants such as polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether. Enzyme inhibitors include pancreatic trypsin inhibitor, diisopropylfluorophosphate (DEEP) and trasylol. Liposomes include water-in-oil-in-water emulsions as well as conventional liposomes (Sterna *et al.* (1984) *J. Neuroimmunol.* 7:27).

As described in detail below, the pharmaceutical compositions of the present invention may be specially formulated for administration in solid or liquid form, including those adapted for the following: (1) oral administration, for example, drenches (aqueous or non-aqueous solutions or suspensions), tablets, boluses, powders, granules, pastes; (2) parenteral administration, for example, by subcutaneous, intramuscular or intravenous injection as, for example, a sterile solution or suspension; (3) topical application, for example, as a cream, ointment or spray applied to the skin; (4) intravaginally or intrarectally, for example, as a pessary, cream or foam; or (5) aerosol, for example, as an aqueous aerosol, liposomal preparation or solid particles containing the compound.

The phrase "therapeutically-effective amount" as used herein means that amount of an agent that modulates (*e.g.*, inhibits) biomarker expression and/or activity, or expression and/or activity of the complex, or composition comprising an agent that modulates (*e.g.*, inhibits) biomarker expression and/or activity, or expression and/or activity of the complex, which is effective for producing some desired therapeutic effect, *e.g.*, cancer treatment, at a reasonable benefit/risk ratio.

The phrase "pharmaceutically acceptable" is employed herein to refer to those agents, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

The phrase "pharmaceutically-acceptable carrier" as used herein means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject chemical from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the subject. Some examples of materials which can serve as pharmaceutically-acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) phosphate buffer solutions; and (21) other non-toxic compatible substances employed in pharmaceutical formulations.

The term "pharmaceutically-acceptable salts" refers to the relatively non-toxic, inorganic and organic acid addition salts of the agents that modulates (*e.g.*, inhibits) biomarker expression and/or activity, or expression and/or activity of the complex encompassed by the present invention. These salts can be prepared *in situ* during the final isolation and purification of the therapeutic agents, or by separately reacting a purified therapeutic agent in its free base form with a suitable organic or inorganic acid, and isolating the salt thus formed. Representative salts include the hydrobromide, hydrochloride, sulfate, bisulfate, phosphate, nitrate, acetate, valerate, oleate, palmitate, stearate, laurate, benzoate, lactate, phosphate, tosylate, citrate, maleate, fumarate, succinate, tartrate, napthylate, mesylate, glucoheptonate, lactobionate, and laurylsulphonate salts and the like (See, for example, Berge *et al.* (1977) "Pharmaceutical Salts", *J. Pharm. Sci.* 66:1-19).

In other cases, the agents useful in the methods of the present invention may contain one or more acidic functional groups and, thus, are capable of forming pharmaceutically-acceptable salts with pharmaceutically-acceptable bases. The term "pharmaceutically-

acceptable salts" in these instances refers to the relatively non-toxic, inorganic and organic base addition salts of agents that modulates (*e.g.*, inhibits) biomarker expression and/or activity, or expression and/or activity of the complex. These salts can likewise be prepared in situ during the final isolation and purification of the therapeutic agents, or by separately reacting the purified therapeutic agent in its free acid form with a suitable base, such as the hydroxide, carbonate or bicarbonate of a pharmaceutically-acceptable metal cation, with ammonia, or with a pharmaceutically-acceptable organic primary, secondary or tertiary amine. Representative alkali or alkaline earth salts include the lithium, sodium, potassium, calcium, magnesium, and aluminum salts and the like. Representative organic amines useful for the formation of base addition salts include ethylamine, diethylamine, ethylenediamine, ethanolamine, diethanolamine, piperazine and the like (see, for example, Berge *et al.*, *supra*).

Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the compositions.

Examples of pharmaceutically-acceptable antioxidants include: (1) water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

Formulations useful in the methods of the present invention include those suitable for oral, nasal, topical (including buccal and sublingual), rectal, vaginal, aerosol and/or parenteral administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well-known in the art of pharmacy. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the host being treated, the particular mode of administration. The amount of active ingredient, which can be combined with a carrier material to produce a single dosage form will generally be that amount of the compound which produces a therapeutic effect. Generally, out of one hundred per cent, this amount will range from about 1 per cent to about ninety-nine percent of active ingredient,

preferably from about 5 per cent to about 70 per cent, most preferably from about 10 per cent to about 30 per cent.

Methods of preparing these formulations or compositions include the step of bringing into association an agent that modulates (*e.g.*, inhibits) biomarker expression and/or activity, with the carrier and, optionally, one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association a therapeutic agent with liquid carriers, or finely divided solid carriers, or both, and then, if necessary, shaping the product.

Formulations suitable for oral administration may be in the form of capsules, cachets, pills, tablets, lozenges (using a flavored basis, usually sucrose and acacia or tragacanth), powders, granules, or as a solution or a suspension in an aqueous or non-aqueous liquid, or as an oil-in-water or water-in-oil liquid emulsion, or as an elixir or syrup, or as pastilles (using an inert base, such as gelatin and glycerin, or sucrose and acacia) and/or as mouth washes and the like, each containing a predetermined amount of a therapeutic agent as an active ingredient. A compound may also be administered as a bolus, electuary or paste.

In solid dosage forms for oral administration (capsules, tablets, pills, dragees, powders, granules and the like), the active ingredient is mixed with one or more pharmaceutically-acceptable carriers, such as sodium citrate or dicalcium phosphate, and/or any of the following: (1) fillers or extenders, such as starches, lactose, sucrose, glucose, mannitol, and/or silicic acid; (2) binders, such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinyl pyrrolidone, sucrose and/or acacia; (3) humectants, such as glycerol; (4) disintegrating agents, such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; (5) solution retarding agents, such as paraffin; (6) absorption accelerators, such as quaternary ammonium compounds; (7) wetting agents, such as, for example, acetyl alcohol and glycerol monostearate; (8) absorbents, such as kaolin and bentonite clay; (9) lubricants, such a talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof; and (10) coloring agents. In the case of capsules, tablets and pills, the pharmaceutical compositions may also comprise buffering agents. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugars, as well as high molecular weight polyethylene glycols and the like.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared using binder (for example, gelatin or hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (for example, sodium starch glycolate or cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered peptide or peptidomimetic moistened with an inert liquid diluent.

Tablets, and other solid dosage forms, such as dragees, capsules, pills and granules, may optionally be scored or prepared with coatings and shells, such as enteric coatings and other coatings well-known in the pharmaceutical-formulating art. They may also be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile, other polymer matrices, liposomes and/or microspheres. They may be sterilized by, for example, filtration through a bacteria-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions, which can be dissolved in sterile water, or some other sterile injectable medium immediately before use. These compositions may also optionally contain opacifying agents and may be of a composition that they release the active ingredient(s) only, or preferentially, in a certain portion of the gastrointestinal tract, optionally, in a delayed manner. Examples of embedding compositions, which can be used include polymeric substances and waxes. The active ingredient can also be in micro-encapsulated form, if appropriate, with one or more of the above-described excipients.

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups and elixirs. In addition to the active ingredient, the liquid dosage forms may contain inert diluents commonly used in the art, such as, for example, water or other solvents, solubilizing agents and emulsifiers, such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor and sesame oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof.

Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, coloring, perfuming and preservative agents.

Suspensions, in addition to the active agent may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

Formulations for rectal or vaginal administration may be presented as a suppository, which may be prepared by mixing one or more therapeutic agents with one or more suitable nonirritating excipients or carriers comprising, for example, cocoa butter, polyethylene glycol, a suppository wax or a salicylate, and which is solid at room temperature, but liquid at body temperature and, therefore, will melt in the rectum or vaginal cavity and release the active agent.

Formulations which are suitable for vaginal administration also include pessaries, tampons, creams, gels, pastes, foams or spray formulations containing such carriers as are known in the art to be appropriate.

Dosage forms for the topical or transdermal administration of an agent that modulates (*e.g.*, inhibits) biomarker expression and/or activity include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. The active component may be mixed under sterile conditions with a pharmaceutically-acceptable carrier, and with any preservatives, buffers, or propellants which may be required.

The ointments, pastes, creams and gels may contain, in addition to a therapeutic agent, excipients, such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof.

Powders and sprays can contain, in addition to an agent that modulates (*e.g.*, inhibits) biomarker expression and/or activity, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants, such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

The agent that modulates (*e.g.*, inhibits) biomarker expression and/or activity, can be alternatively administered by aerosol. This is accomplished by preparing an aqueous aerosol, liposomal preparation or solid particles containing the compound. A nonaqueous (*e.g.*, fluorocarbon propellant) suspension could be used. Sonic nebulizers are preferred

because they minimize exposing the agent to shear, which can result in degradation of the compound.

Ordinarily, an aqueous aerosol is made by formulating an aqueous solution or suspension of the agent together with conventional pharmaceutically acceptable carriers and stabilizers. The carriers and stabilizers vary with the requirements of the particular compound, but typically include nonionic surfactants (Tweens, Pluronics, or polyethylene glycol), innocuous proteins like serum albumin, sorbitan esters, oleic acid, lecithin, amino acids such as glycine, buffers, salts, sugars or sugar alcohols. Aerosols generally are prepared from isotonic solutions.

Transdermal patches have the added advantage of providing controlled delivery of a therapeutic agent to the body. Such dosage forms can be made by dissolving or dispersing the agent in the proper medium. Absorption enhancers can also be used to increase the flux of the peptidomimetic across the skin. The rate of such flux can be controlled by either providing a rate controlling membrane or dispersing the peptidomimetic in a polymer matrix or gel.

Ophthalmic formulations, eye ointments, powders, solutions and the like, are also contemplated as being within the scope of this invention.

Pharmaceutical compositions of this invention suitable for parenteral administration comprise one or more therapeutic agents in combination with one or more pharmaceutically-acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents.

Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the present invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of

microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

In some cases, in order to prolong the effect of a drug, it is desirable to slow the absorption of the drug from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material having poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution, which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally-administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle.

Injectable depot forms are made by forming microencapsule matrices of an agent that modulates (*e.g.*, inhibits) biomarker expression and/or activity, in biodegradable polymers such as polylactide-polyglycolide. Depending on the ratio of drug to polymer, and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions, which are compatible with body tissue.

When the therapeutic agents of the present invention are administered as pharmaceuticals, to humans and animals, they can be given per se or as a pharmaceutical composition containing, for example, 0.1 to 99.5% (more preferably, 0.5 to 90%) of active ingredient in combination with a pharmaceutically acceptable carrier.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of this invention may be determined by the methods of the present invention so as to obtain an amount of the active ingredient, which is effective to achieve the desired therapeutic response for a particular subject, composition, and mode of administration, without being toxic to the subject.

The nucleic acid molecules of the present invention can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Pat. No. 5,328,470) or by stereotactic injection (see *e.g.*, Chen *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:3054-

3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, *e.g.*, retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

In one embodiment, an agent of the invention is an antibody. As defined herein, a therapeutically effective amount of antibody (*i.e.*, an effective dosage) ranges from about 0.001 to 30 mg/kg body weight, preferably about 0.01 to 25 mg/kg body weight, more preferably about 0.1 to 20 mg/kg body weight, and even more preferably about 1 to 10 mg/kg, 2 to 9 mg/kg, 3 to 8 mg/kg, 4 to 7 mg/kg, or 5 to 6 mg/kg body weight. The skilled artisan will appreciate that certain factors may influence the dosage required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of an antibody can include a single treatment or, preferably, can include a series of treatments. In a preferred example, a subject is treated with antibody in the range of between about 0.1 to 20 mg/kg body weight, one time per week for between about 1 to 10 weeks, preferably between 2 to 8 weeks, more preferably between about 3 to 7 weeks, and even more preferably for about 4, 5, or 6 weeks. It will also be appreciated that the effective dosage of antibody used for treatment may increase or decrease over the course of a particular treatment. Changes in dosage may result from the results of diagnostic assays.

7. Kits

The present invention also encompasses kits for detecting and/or modulating biomarkers described herein. A kit of the present invention may also include instructional materials disclosing or describing the use of the kit or an antibody of the disclosed invention in a method of the disclosed invention as provided herein. A kit may also include additional components to facilitate the particular application for which the kit is designed. For example, a kit may additionally contain means of detecting the label (*e.g.*, enzyme substrates for enzymatic labels, filter sets to detect fluorescent labels, appropriate secondary labels such as a sheep anti-mouse-HRP, *etc.*) and reagents necessary for controls (*e.g.*, control biological samples or standards). A kit may additionally include buffers and other

reagents recognized for use in a method of the disclosed invention. Non-limiting examples include agents to reduce non-specific binding, such as a carrier protein or a detergent.

EXAMPLES

Example 1: Materials and methods for Examples 2-4

a. *In vivo* CRISPR screening in B16 tumor cells

A Cas9-expressing version of the B16 melanoma cell line was created and confirmed that it could edit DNA efficiently with CRISPR using sgRNAs targeting the PD-L1 gene. For screening the B16-Cas9 cell line, a library of 9,992 optimized sgRNAs was created to target 2,398 genes, selected from the GO term categories: kinase, phosphatase, cell surface, plasma membrane, antigen processing and presentation, immune system process, and chromatin remodeling. The transcript abundance of the genes in these categories were then filtered to include only those that were expressed > RPKM (log_2) = 0.9. These genes were then ranked for expression in the B16 cell line using RNAseq to select for the top 2,398 expressed genes. The library was divided into 4 sub-pools, each containing one sgRNA per gene and 100 non-targeting control sgRNAs. The 4 sub-pools were screened individually and sgRNAs were delivered to B16-Cas9 cells via lentiviral infection at an infection rate of 30%. Transduced B16 cells were purified using a hCD19 reporter by positive magnetic selection (Miltenyi Biotech, Cambridge, MA) and then expanded *in vitro* before being implanted into animals. For each sub-pool, B16 cells were implanted into 10 TCR$\alpha^{-/-}$ mice, 10 WT mice treated with GVAX, and 10 WT mice treated with GVAX and PD-1 blockade (see below for treatment protocols). B16 cells transduced with libraries were also grown *in vitro* at approximately 2000 x library coverage for the same time period as the animal experiment. Mice were sacrificed 12-14 days after tumor implantation tumor genomic DNA was prepared from whole tumor tissue using the Qiagen DNA Blood Midi kit (Qiagen, Hilden, Germany). PCR was used to amplify the sgRNA region and sequencing to determine sgRNA abundance was performed on an Illumina HiSeq system (Illumina, San Diego, CA). Significantly enriched or depleted sgRNAs from any comparison of conditions were identified using the STARS algorithm (Doench *et al.* (2014) *Nat. Biotechnol.* 32:1262-1267).

b. Animal treatment and tumor challenges

The designs of these animal studies and procedures were approved by the Dana Farber Cancer Institute IACUC committee. Dana Farber's specific-pathogen free facility

was used for the storage and care of all mice. Seven-week old wild-type female C57BL/6J mice were obtained from Jackson laboratories (Bar Harbor, ME). A colony of B6.129S2-Tcra^{tm1Mom}/J (Tcra) T cell-deficient mice were bred on site. Mice were aged matched to be 7-12 weeks old at the time of tumor inoculation. For screening, 2.0 x 10^6 library-transduced B16-Cas9 cells resuspended in Hanks Balanced Salt Solution (Gibco, Thermo Fisher Scientific, Waltham, MA) were mixed 1:1 by volume with Matrigel® (Corning, Corning, NY) and subcutaneously injected into the right flank on day 0. Mice were vaccinated with 1.0 x 10^6 GM-CSF-secreting B16 (GVAX) cells that had been irradiated with 3500 Gy on days 1 and 4 to elicit an anti-tumor immune response. Subsequently, mice were treated with 100 μg of rat monoclonal anti-PD1 antibody (clone: 29F.1A12) on days 9 and 12 via intraperitoneal injection. For validation assays, 1.0 x 10^6 tumor cells were subcutaneously injected into the right flank without matrigel. Tumors were measured every 3 days beginning on day 6 after challenge until time of death. Measurements were taken manually by collecting the longest dimension (length) and the longest perpendicular dimension (width). Tumor volume was estimated with the formula: $(L \times W^2)/2$. CO_2 inhalation was used to euthanize mice on the day of sacrifice.

c. Creation of CRISPR edited tumor cell lines

Transient transfection of Cas9-sgRNA plasmid (pX459, Addgene, Cambridge, MA) was used to edit B16 and *Braf/Pten* melanoma cell lines. pX459 was digested with the enzyme Bpil (Thermo Fisher Scientific) as per the manufacturer's instructions and sgRNA oligos were cloned in using standard molecular cloning. For B16 cells, 5 x 10^5 cells were plated in a well of a 6-well plate and were transfected the following day using 2 μg of pX459 plasmid DNA and Turbofect™ (3:1 ratio, Thermo Fisher Scientific). Twenty-four hours after transfection, transfectants were selected in puromycin (6 μg/mL, Thermo Fisher Scientific). For *Braf/Pten* melanoma cells, 5 x 10^5 cells were plated in a well of a 6-well plate and were transfected the following day using 4 μg of pX459 plasmid DNA and Turbofect™ (3:1 ratio). After selection, cells were grown for 14 days *in vitro* before being implanted into mice.

d. *In vivo* competition assays

B16 cells were engineered to express GFP or TdTomato by lentiviral transduction to differentiate populations. Cas9-target sgRNA-transfected cells and Cas9-control sgRNA-transfected cells were mixed and then grown for at least two passages *in vitro* before implantation into mice. Mixes were analyzed by flow cytometry on the day of tumor

inoculation. Mice were euthanized 15-21 days after tumor inoculation for tumor harvest. Tumors were macerated on ice and incubated in collagenase P (2 mg/mL, Sigma, St. Louis, MO) and DNase I (50 µg/mL, Sigma) supplemented DMEM for 10 minutes at 37˚C. After incubation, tumor cells were passed through 70µm filters to remove undigested tumor. Tumor cells were washed with ice-cold MACS media and stained with Near-IR LIVE/DEAD® (1:1000, BD Biosciences, Franklin Lakes, NJ) for 10 minutes on ice. Tumor cells were then washed and resuspended in ice-cold PBS with 2% FBS. An Accuri™ C6 flow cytometry system (BD Biosiences) was used to analyze final GFP/TdTomato tumor cell ratios.

e. Analysis tumor-infiltrating lymphocytes by flow cytometry

Mice were injected subcutaneously (s.c.) with 1.0 x 10^6 CRISPR/Cas9 modified B16 cells and treated with GVAX + anti-PD-1 mAb as described above. Tumors were harvested on day 12-13, weighed, mechanically diced, incubated with collagenase P (2 mg/mL, Sigma Aldrich) and DNAse I (50 µg/mL, Sigma Aldrich) for 10 minutes, and pipetted into a single-cell suspension. After filtering through a 70 µm filter, cells were blocked with anti-mouse CD16/32 antibody (BioLegend, San Diego, CA) and stained with indicated antibodies for 30 minutes on ice. Dead cells were excluded using Aqua Live/Dead® (1:1000, ThermoFisher Scientific) added concurrently with surface antibodies. After washing, cells were fixed with Foxp3/transcription factor staining buffer set (eBiosciences, San Diego, CA) as per manufacturer's instructions, blocked with mouse and rat serum, then stained with intracellular antibodies. AccuCount Fluorescent particles (Spherotech, Lake Forest, IL) were added for cell quantification prior to analysis on an LSR Fortessa™ cell analyzer (BD Biosciences) using single-color compensation controls and fluorescence-minus-one thresholds to set gate margins. Comparisons between groups performed using Student's t test.

f. Flow cytometry analysis of B16 tumor cells

B16 cells were trypsinized and washed in PBS + 2% FBS, stained with antibodies for cell surface proteins as per the manufacturer's instructions and then analyzed on an Accuri™ C6 flow cytometry system (BD Biosciences).

g. RNAseq analysis of tumor cells

Null or control sgRNA-transfected B16 cells were stimulated with IFNγ (100 ng/mL, Cell Signaling Technology), TNFα (10 ng/mL, Peprotech) or both for 48 hours. RNA was extracted from cell pellets using the Qiagen RNeasy Mini kit according to

manufacturer's instructions. First-strand Illumina-barcoded libraries were generated using the NEB RNA Ultra™ Directional kit according to manufacturer's instructions, including a 12-cycle PCR enrichment. Libraries were sequenced on an Illumina NextSeq™ 500 instrument using paired-end 37bp reads. Data were trimmed for quality using the Trimmomatic pipeline with the following parameters: LEADING:15 TRAILING:15 SLIDINGWINDOW:4:15 MINLEN:16. Data were aligned to mouse reference genome mm10 using the Bowtie 2 aligning sequencing tool (available at the World Wide Web website of Johns Hopkins University). HTSeq was used to map aligned reads to genes and to generate a gene count matrix and it is available at the World Wide Web address of www-huber.embl.de/users/anders/HTSeq/doc/overview.html. Normalized counts and differential expression analysis was performed using the DESeq2 R package. The gene set enrichment analysis was performed as described previously in Subramanian *et al.* (2005) *Proc Natl Acad Sci USA* 102:15545-15550. Principle Components Analysis (PCA) was performed on the normalized gene counts including all genes that passed a minimal expression filter. Signature scores for the individual samples were generated using FastProject (available at the World Wide Web address of bmcbioinformatics.biomedcentral.com/articles/10.1186/s12859-016-1176-5) and the Hallmark gene signature collection (Liberzon *et al.* (2015) *Cell Sys.* 1:417-425). Pearson correlation coefficients were calculated between the Hallmark gene signatures and PC1 and PC2. Selected signatures were plotted on a normalized PCA projection of the dataset.

h. Western blotting

Whole cell lysates were prepared in lysis buffer (60 mM Tris HCl, 2% SDS, 10% glycerol, complete EDTA-free protease-inhibitor (Roche, Basel, Switzerland), and 500 U/mL benzonase nuclease (Novagen, Merck, Darmstadt, Germany)). Samples were boiled at 100°C and clarified by centrifugation. Protein concentration was measured with a BCA protein assay kit (Pierce, Dallas, Texas). Fifty to one hundred and fifty micrograms of protein was loaded on 4-12% Bolt® Bis-Tris Plus gels (Life Technologies, Carlsbad, CA) in MES buffer (Life Technologies). Protein was transferred to 0.45 μm nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were blocked in Tris-buffered saline plus 0.1% Tween 20 (TBS-T) containing 5% non-fat dry milk for 1 hour at room temperature followed by overnight incubation with primary antibody at 4°C. Membranes were washed with TBS-T and incubated with HRP-conjugated secondary antibodies for 1 hour at room temperature. HRP was activated with Supersignal® West Dura Extended

Duration Substrate (Pierce) and visualized with a chemiluminscent detection system using Fuji ImageQuant™ LAS4000 (GE Healthcare Life Sciences, Pittsburgh, PA). Blots were then analyzed using ImageJ and Adobe® Photoshop® software.

i. Antibodies

For Western blotting, primary antibodies against β-ACTIN (Abcam, Cambridge, UK, Cat. #8227) and FLAG (clone M2, Sigma Aldrich) were used. Peroxidase-conjugated secondaries against Rabbit-IgG (Cat. #111-035-046) and Mouse-IgG (Cat. #115-035-174) were purchased from Jackson Laboratories (Bar Harbor, ME).

For flow cytometry, the following anti-mouse (m) fluorochrome-conjugated antibodies were used: CD47 (clone miap301, BioLegend), Granzyme B (clone GB11, BioLegend), and PD-1 (clone RPMI-30, BioLegend).

j. CRISPR sgRNA sequences.

Gene Name/sg#	**sgRNA Sequence**	
Cd274 sgRNA 1	GCCTGCTGTCACTTGCTACG	(SEQ ID NO: 89)
Cd274 sgRNA 2	AATCAACCAGAGAATTTCCG	(SEQ ID NO: 90)
Cd274 sgRNA 3	GGTCCAGCTCCCGTTCTACA	(SEQ ID NO: 91)
Cd274 sgRNA 4	GTATGGCAGCAACGTCACGA	(SEQ ID NO: 92)
Cd47 sgRNA 1	TATAGAGCTGAAAAACCGCA	(SEQ ID NO: 93)
Cd47 sgRNA 2	CCACATTACGGACGATGCAA	(SEQ ID NO: 94)
Cd47 sgRNA 3	TCTTACGAGGAGGAGAAAGG	(SEQ ID NO: 95)
Cd47 sgRNA 4	GCAAGTGTAGTTTCCCACCA	(SEQ ID NO: 96)
control sgRNA 1	GCGAGGTATTCGGCTCCGCG	(SEQ ID NO: 97)
control sgRNA 2	GCTTTCACGGAGGTTCGACG	(SEQ ID NO: 98)
control sgRNA 3	ATGTTGCAGTTCGGCTCGAT	(SEQ ID NO: 99)
control sgRNA 4	ACGTGTAAGGCGAACGCCTT	(SEQ ID NO: 100)
control sgRNA 5	ATTGTTCGACCGTCTACGGG	(SEQ ID NO: 101)
Ripk1 sgRNA 1	CACCGTACACGTCCGACTTCTCCG	(SEQ ID NO: 102)

Examples 2-4 disclose the development of a pooled loss-of-function *in vivo* genetic screening approach that uses CRISPR-Cas9 genome editing to discover genes that increase sensitivity or cause resistance to immunotherapy in a mouse transplantable tumor model. About 2,400 genes expressed by tumor cells were screened in the B16 murine melanoma model to identify those that increase or decrease sensitivity to immunotherapy with tumor vaccination and PD-1 checkpoint blockade. The screen identified known immune evasion

molecules PD-L1 and CD47, as tumor cells bearing sgRNAs for these targets were significantly depleted in animals treated with immunotherapy. In contrast, loss of function of any of the genes that sense or signal in response to interferon-γ (IFNγ) rendered cells resistant to immunotherapy with PD-1 checkpoint blockade and vaccination recapitulating resistance mutations identified in melanoma patients (Zaretsky *et al.* (2016) *N. Engl. J. Med.* 375:819-829; Gao *et al.* (2016) *Cell* 167:397-404.e9). It was discovered that deletion of one or more regulators of TNF signaling and/or NF-κB activation, preferably RIPK1, BIRC2, TBK1, TRAF3, RNF31, RBCK1, OTULIN, TRAF6, TAB2, and TNIP1significantly increased sensitivity of tumor cells to immunotherapy by increasing IFNγ-mediated effects on antigen presentation and cell growth. These findings reveal that therapeutic strategies, such as *Ripk1* inhibition, that sensitize tumor cells to the effects of IFNγ are capable of increasing the efficacy of cancer immunotherapy. Moreover, this screening approach can discover new immunotherapy targets and prioritize their combination with existing immunotherapies.

Example 2: A pooled loss-of-function *in vivo* genetic screen recovers known immune evasion molecules expressed by tumors

In order to systematically identify new cancer immunotherapy targets and resistance mechanisms, a pooled genetic screening approach was developed to identify genes that increase or decrease the fitness of tumor cells growing *in vivo* in animals treated with immunotherapy (Figure 1A). First, a B16 melanoma cell line was engineered to express Cas9 (Figure 2A), confirmed of efficient DNA editing using sgRNAs targeting PD-L1 (Figure 1D). Next, a library of lentiviral vectors was created to encode 9,992 sgRNAs targeting 2,398 genes from relevant functional classes that were expressed at detectable levels in the tumor cell line (Figure 2B). After transduction and *in vitro* passage to allow gene editing to take place, the tumor cells were transplanted into animals that were then treated with either a GM-CSF-secreting, irradiated tumor cell vaccine (GVAX) or GVAX plus PD-1 blockade using a monoclonal antibody for PD-1, in order to apply immune selective pressure on the tumor cells (Figure 1B) (see Dranoff (2003) *Oncogene* 22:3188-3192; Dranoff *et al.* (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:3539-3543; and Duraiswamy *et al.* (2013) *Cancer Res.* 73:3591-3603). In parallel, the library-transduced tumor cells were transplanted into TCRα$^{-/-}$ mice, which lack CD4^{+} and CD8^{+} T cells and were therefore unable to apply adaptive immune selective pressure on the tumors. This allowed to

distinguish the effect of immune selective pressure on library representation from nonspecific effects on tumor cell viability. After 12 – 14 days, tumors were harvested (Figure 1B), with all sgRNAs recovered from each animal with good inter-animal reproducibility (Figures 2C-2E).

The library representation in tumors from immunotherapy-treated wild-type (WT) animals were compared with that found in tumors growing in TCR$\alpha^{-/-}$ mice, in which deletion of genes that result in resistance to immunotherapy would be expected to increase tumor sgRNA representation in WT animals, while deletion of genes that result in increased sensitivity of tumors to immunotherapy would decrease sgRNA representation. Analysis of sgRNAs enriched by immune selective pressure revealed those targeting genes involved in cytokine-mediating signaling and immune-system processes (Figure 1C). sgRNAs depleted by immunotherapy included those targeting genes involved in antigen processing, necroptosis, and regulation of immune responses (Figure 1C). These results indicate that the genetic screening approach used here identified genes expressed by tumors cells that play a role in interaction with the immune system.

Inspection of the list of genes targeted by sgRNAs depleted from tumors treated with immunotherapy revealed the known immune evasion molecule PD-L1, indicating that loss of PD-L1 increased the sensitivity of tumor cells to immune attack. sgRNAs targeting PD-L1 were not depleted from tumors in TCR$\alpha^{-/-}$ mice relative to cells growing *in vitro*, presumably due to the absence of T cell-mediated selective pressure (Figure 1D), but were significantly depleted in WT mice treated with GVAX relative to TCR$\alpha^{-/-}$ mice (FDR = 0.004). However, the depletion of PD-L1-targeting sgRNAs seen in GVAX-treated tumors was not observed in tumors treated with GVAX and anti-PD-1, indicating that loss of PD-L1 does not confer a selective disadvantage to tumors when PD-L1:PD-1 interactions are blocked (Figure 1D).

It was also found that sgRNAs targeting CD47, which enables immune evasion by impairing engulfment of tumors cells by phagocytes (as in Liu *et al.* (2015) *Nat. Med.* 21:1209-1215; Weiskopf *et al.* (2016) *J. Clin. Invest.* 126:2610-2620; and Tseng *et al.* (2013) *Proc. Natl. Acad. Sci. U.S.A.* 110: 11103-11108), were markedly depleted in tumors treated with either GVAX or with GVAX plus PD-1 blockade (FDR = 0.005, 0.002 respectively) (Figure 1E). To confirm that CD47 null tumors were more susceptible to GVAX and PD-1 blockade, CD47 null B16 melanoma cells were generated using transient transfection of a Cas9-sgRNA plasmid (as in Ran *et al.* (2013) *Nat. Protoc.* 8:2281-2308)

(Figure 2F). It was found that loss of CD47 significantly improved control of tumor growth mediated by GVAX plus anti-PD-1 immunotherapy (Figure 1F, $p< 0.01$).

Using the pooled loss-of-function in vivo genetic screen for identifying immune evasion molecules expressed by tumors described above, genes that increase or decrease the fitness of MC38 colon cancer cells growing *in vivo* in animal treated with immunotherapy.

Thus, *in vivo* genetic screening recovered genes known to confer tumor evasion properties on cancer cells.

Example 3: Discovery of novel gene targets to increase the efficacy of immunotherapy

Deletion of novel candidate immunotherapy targets was found to increase sensitivity of tumor cells to immunotherapy. sgRNAs targeting genes involved in TNF signaling and/or NF-κB activation (*e.g.*, RIPK1, BIRC2, TBK1, TRAF3, RNF31, RBCK1, OTULIN, TRAF6, TAB2, and TNIP1) were markedly depleted in mice treated with GVAX and PD-1 blockade (Figure 3A) relative to growth in $TCR\alpha^{-/-}$ mice. In many cases, multiple members of the same pathway (*e.g.*, *Ripk1*) or even the same multi-protein complex were depleted under immune selective pressure, underscoring the importance of these diverse biological pathways.

Example 4: *In vivo* validation of RIPK1 as a target for combination with immunotherapy

Representative genes as regulators of TNF signaling and/or NF-κB activation, *e.g.*, *Ripk1*, were selected to validate based on their highest cumulative score as ranked by the STARS algorithm (Doench *et al.* (2014) *Nat. Biotechnol.* 32:1262-1267). *In vivo* competition assays showed that tumor cells deleted for *Stub1* were strongly selected against in WT animals treated with immunotherapy but grew at equivalent rates to control tumor cells *in vitro* and in $TCR\alpha^{-/-}$ mice (Figure 3B and 3C; $p<0.0001$, Student's t test). This suggests that regulators of TNF signaling and/or NF-κB pathways, *e.g.*, *Ripk1*, are synthetically lethal with an anti-tumor immune response, rendering tumor cells more sensitive to immunotherapy but not altering their cell growth or survival in the absence of T cells.

One of the central challenges in cancer biology is to identify the genes that underlie the hallmarks of cancer. To date, functional genomic approaches using genome editing

have largely focused on identifying genes required by tumor cells for the cancer hallmarks of growth, metastasis and drug resistance (Ebert *et al.* (2008) *Nature* 451:335-339; Hart *et al.* (2015) *Cell* 163:1515-1526; Yu *et al.* (2016) *Nat. Biotechnol.* 34:419-423; Chen *et al.* (2015) *Cell* 160:1246-1260). This study extends this approach to the interrogation of the interaction of the tumor cell with the immune system, which can be broadly applied to multiple tumor models and immunotherapy modalities to systematically define genes that govern interactions between cancer cells and the immune system.

As described above, Ripk1 (Receptor-Interacting Protein Kinase 1) is a regulatory protein important for: i) mediating inflammation and ii) regulating apoptotic and necroptotic cell death (Ofengeim and Yaun (2013) *Nat. Rev.* 14:727). Using murine *in vivo* CRISPR/Cas9 genetic screening, it has been determined herein that Ripk1 is an immunotherapy target that sensitizes tumors cells to the immune effects of anti-PD-1 checkpoint blockade. *Ripk1*-deficient B16 melanoma cancer cells were significantly more sensitive to anti-PD-1 checkpoint blockade as demonstrated by dramatic overall decrease in tumor burden and increase in animal survival. Thus, inhibition of RIPK1 and other associated proteins is believed to provide a potent strategy for increasing clinical efficacy of anti-PD-1 checkpoint blockade in patients.

Following the discovery of the key role Ripk1 plays in mediating NF-κB-driven inflammation and necroptosis upon TNF or TLR activation, Ripk1 has been speculated as a promising therapeutic target for chronic inflammatory pathology (Fauster *et al.* (2015) *Cell Death and Disease* 6:e1767; Danneppal *et al.* (2014) *Nature* 513:90-94). However, RIPK1 inhibition has been reported to have both pro- and anti-inflammatory effects. In some cases, targeted inhibition of Ripk1 reduces TNF-driven inflammation in mice (Bullock and Degtreve (2015) *Oncotarget* 6:34057-34058; Fauster *et al.* (2015), *supra*). However, loss of Ripk1 function *in vivo* is highly correlated with chronic gastrointestinal and other systemic inflammatory and/or cell death-associated diseases in both mice and humans (Danneppal *et al.* (2014), *supra*). This may be due to the fact Ripk1 enzymatic function is sufficient for activating NF-κB transcription, but is not necessary for inducing necroptosis or apoptosis (Danneppal *et al.* (2014), *supra*). Furthermore, Ripk1 can be ubiquitylated at multiple residues, most notably on Lys-337 through Lys-63-linked ubiquitylation (*a.k.a.*, ubiquitination) during NF-κB induction (Ofengeim and Yuan (2013), *supra*), suggesting complex regulations of Ripk1 functions. Thus, it was unexpected that inhibition of RIPK1 function would be beneficial for the immune response to cancer.

Ripk1 was the top depleted screening hit when comparing guide abundance between TCRα and anti-PD1 checkpoint blockade treated mice (FDR = <0.0001). In addition, a number of other genes were identified that form a multi-protien complex with Ripk1 or are in the same pathway, such as Sharpin, Tab2, A20, and Caspase 8. Preliminary pre-clinical validation efforts have shown that ablation of Ripk1 in murine B16 cancer cells dramatically improves *in vivo* efficacy of anti-PD-1 monoclonal antibody therapy in WT C57bl/6j mice. Mice injected with Ripk1-ablated tumor cells show decreased tumor burden when treated with anti-PD-1 checkpoint blockade. These result are further validated by competitive assay data in which 50% of cancer cells transplanted are *Ripk1* deficient and are dramatically out-competed by wild-type cells 14 days post injection, indicating a severe competitive disadvantage for *Ripk1*-ablated cells in the presence of effective immunity. By contrast, *Ripk1*-ablated cells show no competitive disadvantage in TCRα mice which lack functional CD8 T cells, suggesting that the phenotype is T cell-dependent.

Incorporation by Reference

All publications, patents, and patent applications mentioned herein are hereby incorporated by reference in their entirety as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference. In case of conflict, the present application, including any definitions herein, will control.

Also incorporated by reference in their entirety are any polynucleotide and polypeptide sequences which reference an accession number correlating to an entry in a public database, such as those maintained by The Institute for Genomic Research (TIGR) on the world wide web at tigr.org and/or the National Center for Biotechnology Information (NCBI) on the World Wide Web at ncbi.nlm.nih.gov.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

What is claimed is:

1. A method of treating a subject afflicted with a cancer comprising administering to the subject a therapeutically effective amount of an agent that inhibits the copy number, the expression level, and/or the activity of one or more biomarkers listed in Table 1 or a fragment thereof, in combination with an immunotherapy.

2. The method of claim 1, wherein the agent decreases the copy number, the expression level, and/or the activity of one or more regulators of TNF signaling and/or NF-κB activation, preferably RIPK1, BIRC2, TBK1, TRAF3, RNF31, RBCK1, OTULIN, TRAF6, TAB2, and TNIP1.

3. The method of claim 1 or 2, wherein the agent selectively decreases the biological activity and/or the substrate binding activity of RIPK1, preferably the serine/threonine-protein kinase activity and/or the receptor binding activity of RIPK1.

4. The method of any one of claims 1-3, wherein the agent is a small molecule inhibitor, CRISPR single-guide RNA (sgRNA), RNA interfering agent, antisense oligonucleotide, peptide or peptidomimetic inhibitor, aptamer, or intrabody.

5. The method of claim 4, wherein the RNA interfering agent is a small interfering RNA (siRNA), CRISPR RNA (crRNA), a small hairpin RNA (shRNA), a microRNA (miRNA), or a piwi-interacting RNA (piRNA).

6. The method of claim 5, wherein the RNA interfering agent is a CRISPR single-guide RNA (sgRNA).

7. The method of claim 6, wherein the sgRNA comprises a nucleic acid sequence selected from the group consisting of nucleic acid sequence listed in Table 2.

8. The method of claim 4, wherein the agent comprises an intrabody, or an antigen binding fragment thereof, which specifically binds to the one or more biomarkers and/or a substrate of the one or more biomarkers.

9. The method of claim 8, wherein the intrabody, or antigen binding fragment thereof, is murine, chimeric, humanized, composite, or human.

10. The method of claim 8 or 9, wherein the intrabody, or antigen binding fragment thereof, is detectably labeled, comprises an effector domain, comprises an Fc domain, and/or is selected from the group consisting of Fv, Fav, F(ab')2, Fab', dsFv, scFv, sc(Fv)2, and diabodies fragments.

11. The method of any one of claims 8-10, wherein the intrabody, or antigen binding fragment thereof, is conjugated to a cytotoxic agent.

12. The method of claim 11, wherein the cytotoxic agent is selected from the group consisting of a chemotherapeutic agent, a biologic agent, a toxin, and a radioactive isotope.

13. The method of any one of claims 1-12, wherein the agent increases the sensitivity of the cancer cells to an immunotherapy.

14. The method of any one of claims 1-13, wherein the immunotherapy and/or a cancer therapy is administered before, after, or concurrently with the agent.

15. The method of claim 13 or 14, wherein the immunotherapy comprises an anti-cancer vaccine and/or virus.

16. The method of any one of claims 13-15, wherein the immunotherapy is cell-based.

17. The method of any one of claims 13-16, wherein the immunotherapy inhibits an immune checkpoint.

18. The method of claim 17, wherein the immune checkpoint is selected from the group consisting of CTLA-4, PD-1, VISTA, B7-H2, B7-H3, PD-L1, B7-H4, B7-H6, ICOS, HVEM, PD-L2, CD160, gp49B, PIR-B, KIR family receptors, TIM-1, TIM-3, TIM-4, LAG-3, GITR, 4-IBB, OX-40, BTLA, SIRP, CD47, CD48, 2B4 (CD244), B7.1, B7.2, ILT-2, ILT-4, TIGIT, HHLA2, butyrophilins, IDO, CD39, CD73 and A2aR.

19. The method of claim 18, wherein the immune checkpoint is selected from the group consisting of PD-1, PD-L1, and PD-L2, optionally wherein the immune checkpoint is PD-1.

20. The method of any one of claims 1-19, wherein the one or more biomarkers comprise a nucleic acid sequence having at least 95% identity to a nucleic acid sequence

listed in Table 1 and/or an amino acid sequence having at least 95% identity to an amino acid sequence listed in Table 1.

21. The method of any one of claims 1-20, wherein the one or more biomarkers are human, mouse, chimeric, or a fusion.

22. The method of any one of claims 1-21, wherein the agent reduces the number of proliferating cells in the cancer and/or reduces the volume or size of a tumor comprising the cancer cells.

23. The method of any one of claims 1-22, wherein the one or more biomarkers comprise an amino acid sequence listed in Table 1, optionally wherein the amino acid sequence is selected from the group consisting of SEQ ID Nos: 2, 4, 6, 9, 11, 14, 16, 18, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 42, 44, 47, 49, 51, 54, 57, 60, 62, 64, 66, 68, 73, 76, 78, 80, 83, 86, and 88.

24. The method of any one of claims 1-23, wherein the one or more biomarkers are encoded by a nucleic acid sequence listed in Table 1, optionally wherein the nucleic acid sequence is selected from the group consisting of SEQ ID Nos: 1, 3, 5, 7, 8, 10, 12, 13, 15, 17, 19, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 41, 43, 45, 46, 48, 50, 52, 53, 55, 56, 58, 59, 61, 63, 65, 67, 69-72, 74, 75, 77, 79, 81, 82, 84, 85, and 87.

25. The method of any one of claims 1-24, wherein the cancer is melanoma.

26. The method of any one of claims 1-25, wherein the subject is an animal model of the cancer, preferably a mouse model, or a human.

27. The method of any one of claims 1-26, further comprising administering to the subject at least one additional cancer therapy or regimen, optionally wherein the at least one additional cancer therapy or regimen is administered before, after, or concurrently with the agent and/or the immunotherapy.

28. The method of any one of claims 1-27, wherein the agent is administered in a pharmaceutically acceptable formulation.

29. A method of killing cancer cells comprising contacting the cancer cells with an agent that inhibits the copy number, the expression level, and/or the activity of one or more biomarkers listed in Table 1 or a fragment thereof, in combination with an immunotherapy.

30. The method of claim 29, wherein the agent decreases the copy number, the expression level, and/or the activity of one or more regulators of TNF signaling and/or NF-κB activation, preferably RIPK1, BIRC2, TBK1, TRAF3, RNF31, RBCK1, OTULIN, TRAF6, TAB2, and TNIP1.

31. The method of claim 29 or 30, wherein the agent selectively decreases the biological activity and/or the substrate binding activity of RIPK1, preferably the serine/threonine-protein kinase activity and/or the receptor binding activity of RIPK1.

32. The method of any one of claims 29-31, wherein the agent is a small molecule inhibitor, CRISPR single-guide RNA (sgRNA), RNA interfering agent, antisense oligonucleotide, peptide or peptidomimetic inhibitor, aptamer, or intrabody.

33. The method of claim 32, wherein the RNA interfering agent is a small interfering RNA (siRNA), CRISPR RNA (crRNA), a small hairpin RNA (shRNA), a microRNA (miRNA), or a piwi-interacting RNA (piRNA).

34. The method of claim 33, wherein the RNA interfering agent is a CRISPR single-guide RNA (sgRNA).

35. The method of claim 34, wherein the sgRNA comprises a nucleic acid sequence selected from the group consisting of nucleic acid sequence listed in Table 2.

36. The method of claim 32, wherein the agent comprises an intrabody, or an antigen binding fragment thereof, which specifically binds to the one or more biomarkers and/or a substrate of the one or more biomarkers.

37. The method of claim 36, wherein the intrabody, or antigen binding fragment thereof,
is murine, chimeric, humanized, composite, or human.

38. The method of claim 36 or 37, wherein the intrabody, or antigen binding fragment thereof, is detectably labeled, comprises an effector domain, comprises an Fc domain, and/or is selected from the group consisting of Fv, Fav, F(ab')2, Fab', dsFv, scFv, sc(Fv)2, and diabodies fragments.

39. The method of any one of claims 36-38, wherein the intrabody, or antigen binding fragment thereof, is conjugated to a cytotoxic agent.

40. The method of claim 39, wherein the cytotoxic agent is selected from the group consisting of a chemotherapeutic agent, a biologic agent, a toxin, and a radioactive isotope.

41. The method of any one of claims 29-40, wherein the agent increases the sensitivity of the cancer cells to an immunotherapy.

42. The method of any one of claims 29-41, wherein the cancer cells are contacted with an immunotherapy and/or a cancer therapy before, after, or concurrently with the agent.

43. The method of claim 41 or 42, wherein the immunotherapy comprises an anti-cancer vaccine and/or virus.

44. The method of any one of claims 41-43, wherein the immunotherapy is cell-based.

45. The method of any one of claims 41-44, wherein the immunotherapy inhibits an immune checkpoint.

46. The method of claim 45, wherein the immune checkpoint is selected from the group consisting of CTLA-4, PD-1, VISTA, B7-H2, B7-H3, PD-L1, B7-H4, B7-H6, ICOS, HVEM, PD-L2, CD160, gp49B, PIR-B, KIR family receptors, TIM-1, TIM-3, TIM-4, LAG-3, GITR, 4-IBB, OX-40, BTLA, SIRP, CD47, CD48, 2B4 (CD244), B7.1, B7.2, ILT-2, ILT-4, TIGIT, HHLA2, butyrophilins, IDO, CD39, CD73 and A2aR.

47. The method of claim 46, wherein the immune checkpoint is selected from the group consisting of PD-1, PD-L1, and PD-L2, optionally wherein the immune checkpoint is PD-1.

48. The method of any one of claims 29-47, wherein the one or more biomarker comprise a nucleic acid sequence having at least 95% identity to a nucleic acid sequence

listed in Table 1 and/or an amino acid sequence having at least 95% identity to an amino acid sequence listed in Table 1.

49. The method of any one of claims 29-48, wherein the one or more biomarkers are human, mouse, chimeric, or a fusion.

50. The method of any one of claims 29-49, wherein the agent reduces the number of proliferating cells in the cancer and/or reduces the volume or size of a tumor comprising the cancer cells.

51. The method of any one of claims 29-50, wherein the one or more biomarkers comprise an amino acid sequence listed in Table 1, optionally wherein the amino acid sequence is selected from the group consisting of SEQ ID Nos: 2, 4, 6, 9, 11, 14, 16, 18, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 42, 44, 47, 49, 51, 54, 57, 60, 62, 64, 66, 68, 73, 76, 78, 80, 83, 86, and 88.

52. The method of any one of claims 29-51, wherein the one or more biomarkers are encoded by a nucleic acid sequence listed in Table 1, optionally wherein the nucleic acid sequence is selected from the group consisting of SEQ ID Nos: 1, 3, 5, 7, 8, 10, 12, 13, 15, 17, 19, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 41, 43, 45, 46, 48, 50, 52, 53, 55, 56, 58, 59, 61, 63, 65, 67, 69-72, 74, 75, 77, 79, 81, 82, 84, 85, and 87.

53. The method of any one of claims 29-52, wherein the cancer is melanoma.

54. The method of any one of claims 29-53, wherein the subject is an animal model of the cancer, preferably a mouse model, or a human.

55. The method of any one of claims 29-54, further comprising administering to the subject at least one additional cancer therapy or regimen, optionally wherein the at least one additional cancer therapy or regimen is administered before, after, or concurrently with the agent and/or the immunotherapy.

56. The method of any one of claims 29-55, wherein the agent is administered in a pharmaceutically acceptable formulation.

57. A method of determining whether a subject afflicted with a cancer or at risk for developing a cancer would benefit from inhibiting the copy number, amount, and/or activity of at least one biomarker listed in Table 1, the method comprising:

a) obtaining a biological sample from the subject;

b) determining the copy number, amount, and/or activity of at least one biomarker listed in Table 1;

c) determining the copy number, amount, and/or activity of the at least one biomarker in a control; and

d) comparing the copy number, amount, and/or activity of the at least one biomarker detected in steps b) and c);

wherein the presence of, or a significant increase in, the copy number, amount, and/or activity of, the at least one biomarker listed in Table 1 in the subject sample relative to the control copy number, amount, and/or activity of the at least one biomarker indicates that the subject afflicted with the cancer or at risk for developing the cancer would benefit from inhibiting the copy number, amount, and/or activity of the at least one biomarker listed in Table 1.

58. The method of claim 57, further comprising recommending, prescribing, or administering an agent that inhibits the at least one biomarker listed in Table 1 if the cancer is determined to benefit from the agent, optionally further administering at least one additional cancer therapy that is administered before, after, or concurrently with the agent.

59. The method of claim 57, further comprising recommending, prescribing, or administering cancer therapy other than an agent that inhibits the at least one biomarker listed in Table 1 if the cancer is determined to not benefit from the agent.

60. The method of claim 58 or 59, wherein the cancer therapy is selected from the group consisting of immunotherapy, targeted therapy, chemotherapy, radiation therapy, hormonal therapy, an anti-cancer vaccine, an anti-cancer virus, and a checkpoint inhibitor.

61. The method of any one of claims 57-60, wherein the control sample is determined from a cancerous or non-cancerous sample from either the patient or a member of the same species to which the patient belongs.

62. The method of any one of claims 57-61, wherein the control sample comprises cells.

63. A method for predicting the clinical outcome of a subject afflicted with a cancer expressing one or more biomarkers listed in Table 1 or a fragment thereof to treatment with an immunotherapy, the method comprising:

a) determining the copy number, amount, and/or activity of at least one biomarker listed in Table 1 in a subject sample;

b) determining the copy number, amount, and/or activity of the at least one biomarker in a control having a good clinical outcome; and

c) comparing the copy number, amount, and/or activity of the at least one biomarker in the subject sample and in the control;

wherein the presence of, or a significant increase in, the copy number, amount, and/or activity of, the at least one biomarker listed in Table 1 in the subject sample as compared to the copy number, amount and/or activity in the control, is an indication that the subject has a poor clinical outcome.

64. A method for monitoring the progression of a cancer in a subject, wherein the subject is administered a therapeutically effective amount of an agent that inhibits the copy number, amount, and/or activity of at least one biomarker listed in Table 1 and an immunotherapy, the method comprising:

a) detecting in a subject sample at a first point in time the copy number, amount, and/or activity of at least one biomarker listed in Table 1;

b) repeating step a) at a subsequent point in time; and

c) comparing the amount or activity of at least one biomarker listed in Table 1 detected in steps a) and b) to monitor the progression of the cancer in the subject.

65. A method of assessing the efficacy of an agent that inhibits the copy number, amount, and/or activity of at least one biomarker listed in Table 1 and an immunotherapy for treating a cancer in a subject, comprising:

a) detecting in a subject sample at a first point in time the copy number, amount, and/or or activity of at least one biomarker listed in Table 1;

b) repeating step a) during at least one subsequent point in time after administration of the agent and the immunotherapy; and

c) comparing the copy number, amount, and/or activity detected in steps a) and b), wherein the absence of, or a significant decrease in, the copy number, amount, and/or activity of, the at least one biomarker listed in Table 1, in the subsequent sample as

compared to the copy number, amount, and/or activity in the sample at the first point in time, indicates that the agent and immunotherapy treats the cancer in the subject.

66. The method of claim 64 or 65, wherein between the first point in time and the subsequent point in time, the subject has undergone treatment, completed treatment, and/or is in remission for the cancer.

67. The method of claim 66, wherein the cancer treatment is selected from the group consisting of immunotherapy, targeted therapy, chemotherapy, radiation therapy, hormonal therapy, an anti-cancer vaccine, an anti-cancer virus, and a checkpoint inhibitor.

68. The method of any one of claims 64-67, wherein the first and/or at least one subsequent sample is selected from the group consisting of *ex vivo* and *in vivo* samples.

69. The method of any one of claims 64-68, wherein the first and/or at least one subsequent sample is a portion of a single sample or pooled samples obtained from the subject.

70. The method of any one of claims 57-69, wherein the sample comprises cells, serum, peritumoral tissue, and/or intratumoral tissue obtained from the subject.

71. The method of any one of claims 57-70, wherein the one or more biomarkers listed in Table 1 comprise RIPK1, BIRC2, TBK1, TRAF3, RNF31, RBCK1, OTULIN, TRAF6, TAB2, and/or TNIP1.

72. The method of any one of claims 57-71, wherein the cancer is melanoma.

73. The method of any one of claims 57-72, wherein the cancer is in a subject and the subject is a mammal.

74. The method of claim 73, wherein the mammal is a mouse or a human.

75. The method of claim 74, wherein the mammal is a human.

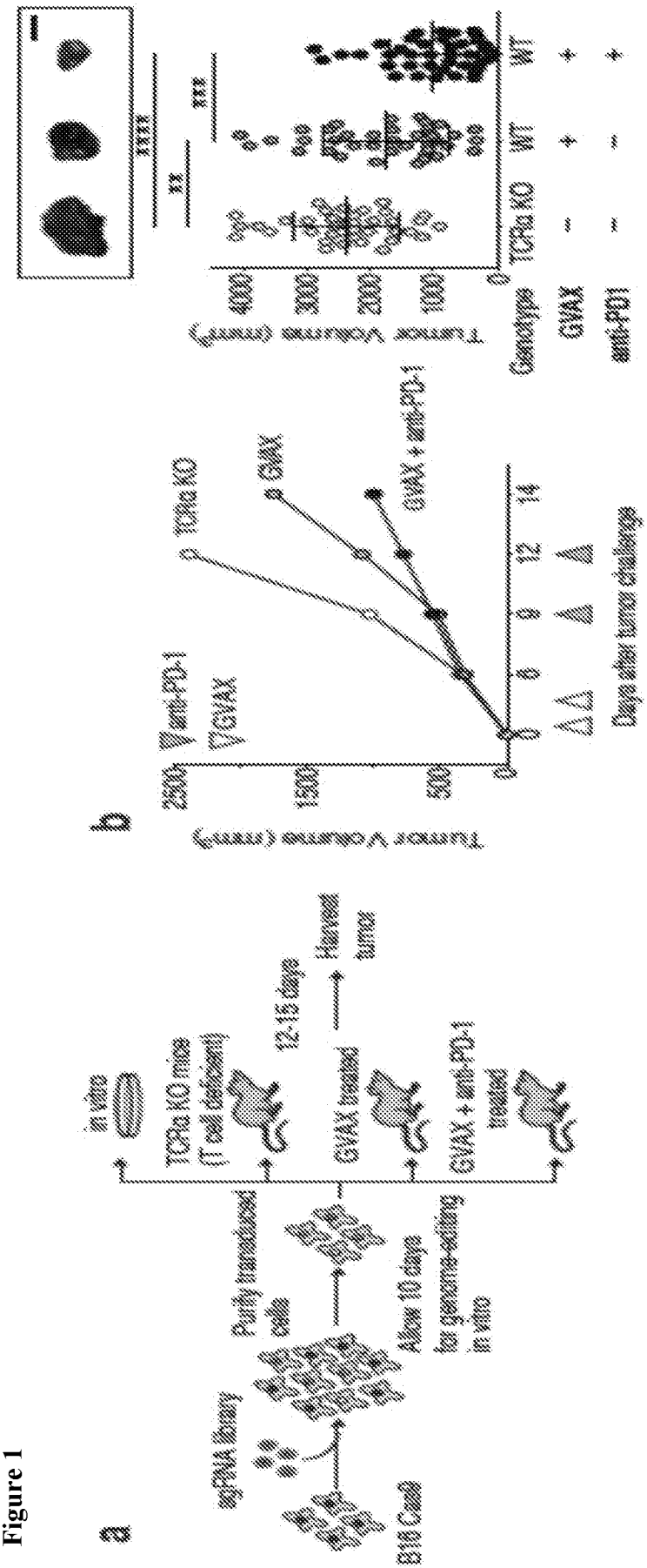

a
B16 Cas9
sgRNA library
Purify transduced cells
Allow 10 days for genome-editing in vitro
in vitro
TCRa KO mice (T cell deficient)
12-15 days
Harvest tumor
GVAX treated
GVAX + anti-PD-1 treated
b
Tumor volume (mm3)
2500
1500
500
0
anti-PD-1
GVAX
TCRa KO
GVAX
GVAX + anti-PD-1
0
6
9
12
14
Days after tumor challenge
Tumor weight (g)
400
300
200
100
0
Genotype
GVAX
anti-PD1
TCRa KO
WT
WT
+
+
-
+
-
-
+
+

Figure 1

Figure 1 (cont.)

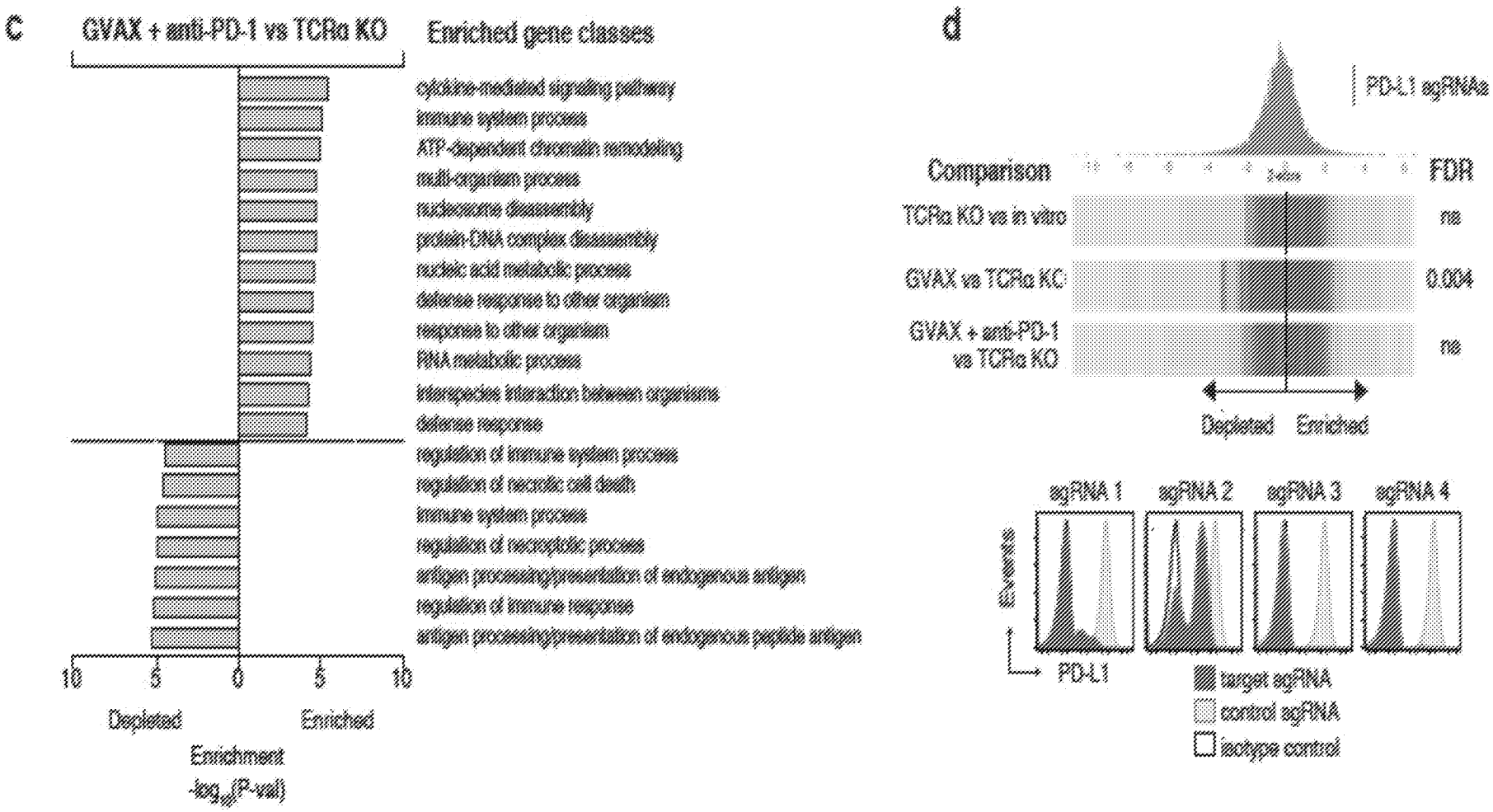
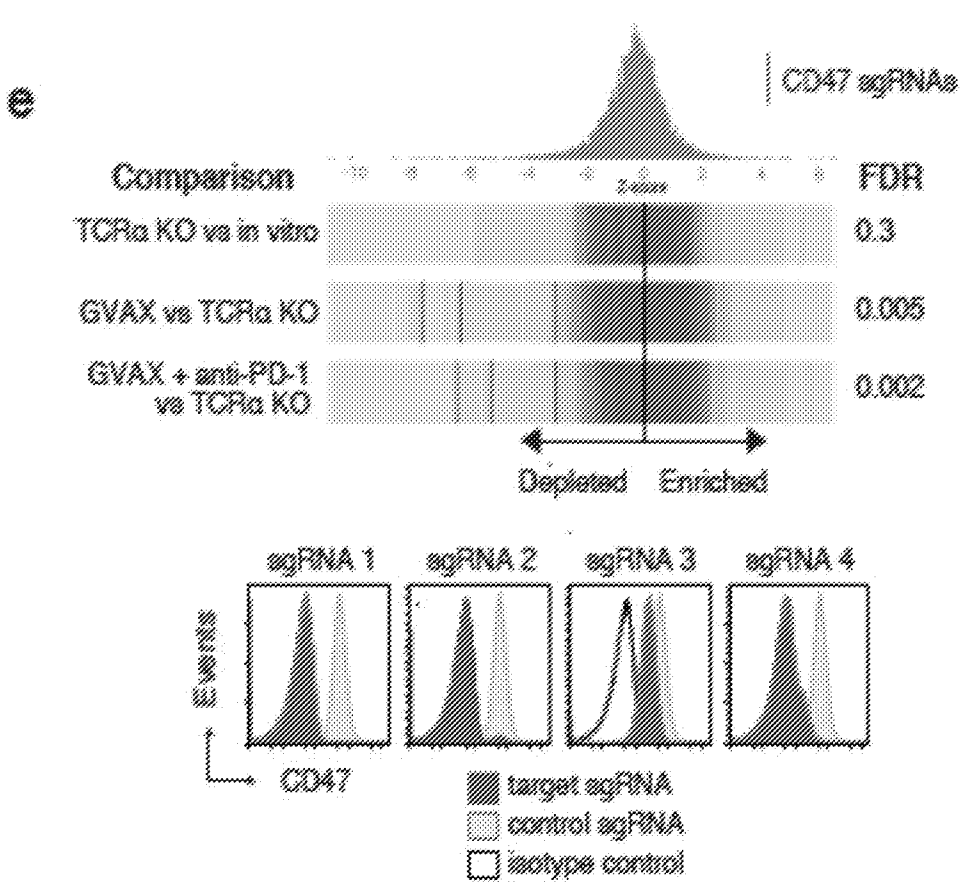


Figure 1 (cont.)



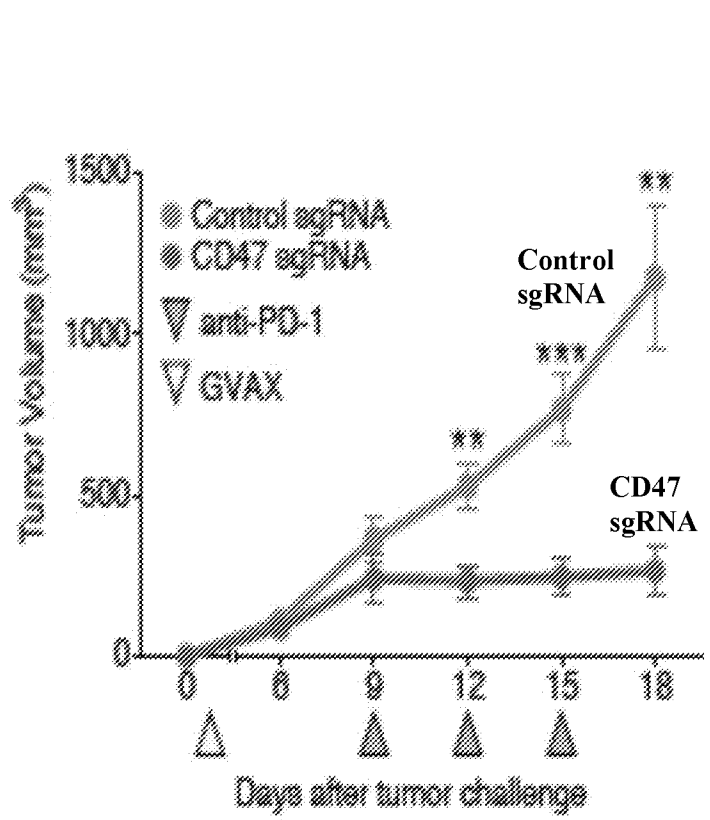


Figure 2

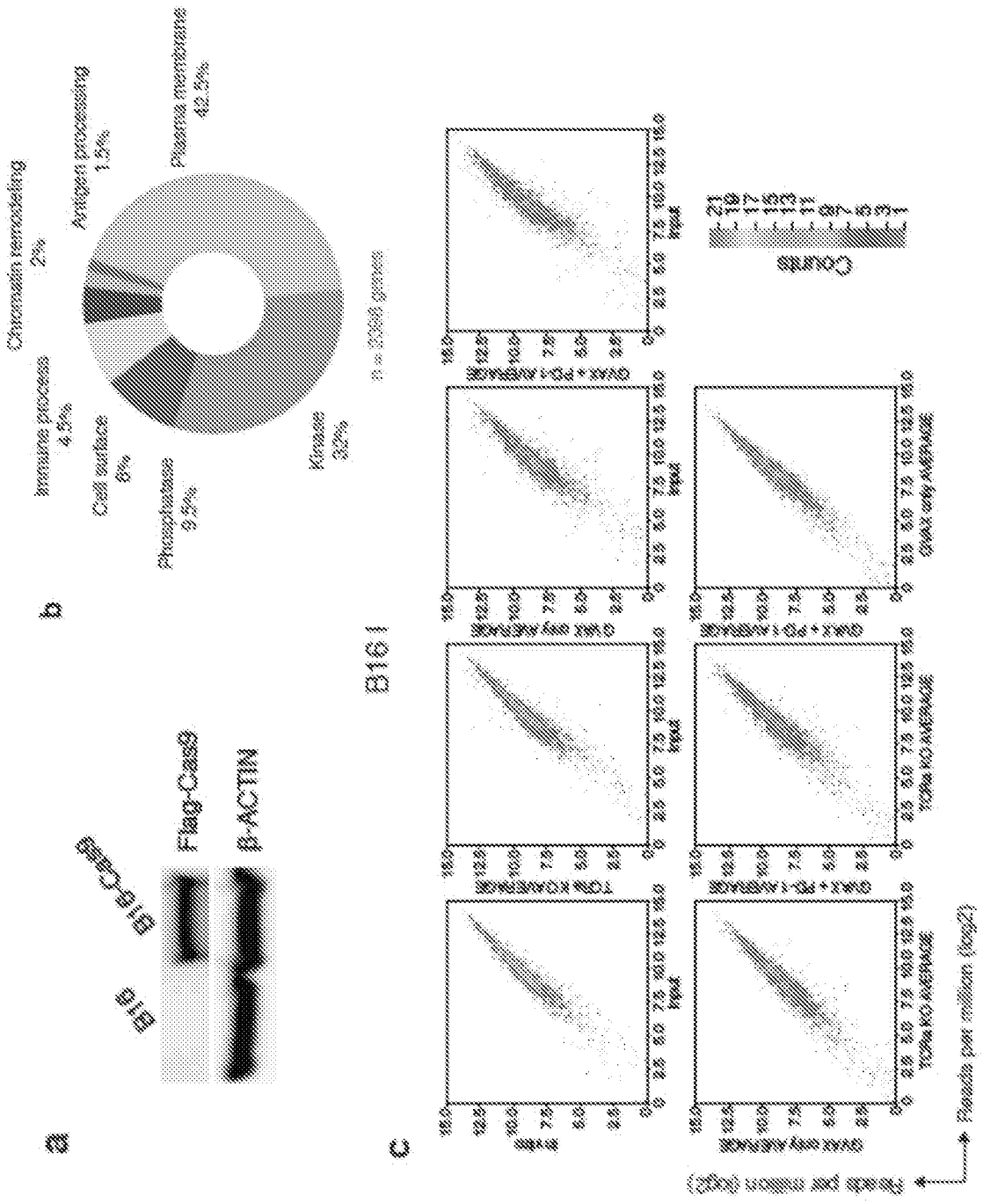

a
Bio
Bio-Cas9
Flag-Cas9
β-ACTIN
b
Chromatin remodeling 2%
Immune process 4.5%
Cell surface 6%
Phosphatase 9.5%
Kinase 32%
Antigen processing 1.5%
Plasma membrane 42.5%
n = 3366 genes
c
B16 I
Input
TCRa KO AVERAGE
Gene only AVERAGE
Gene + PD-1 AVERAGE
Counts
Reads per million (log2)

Figure 2 (cont.)

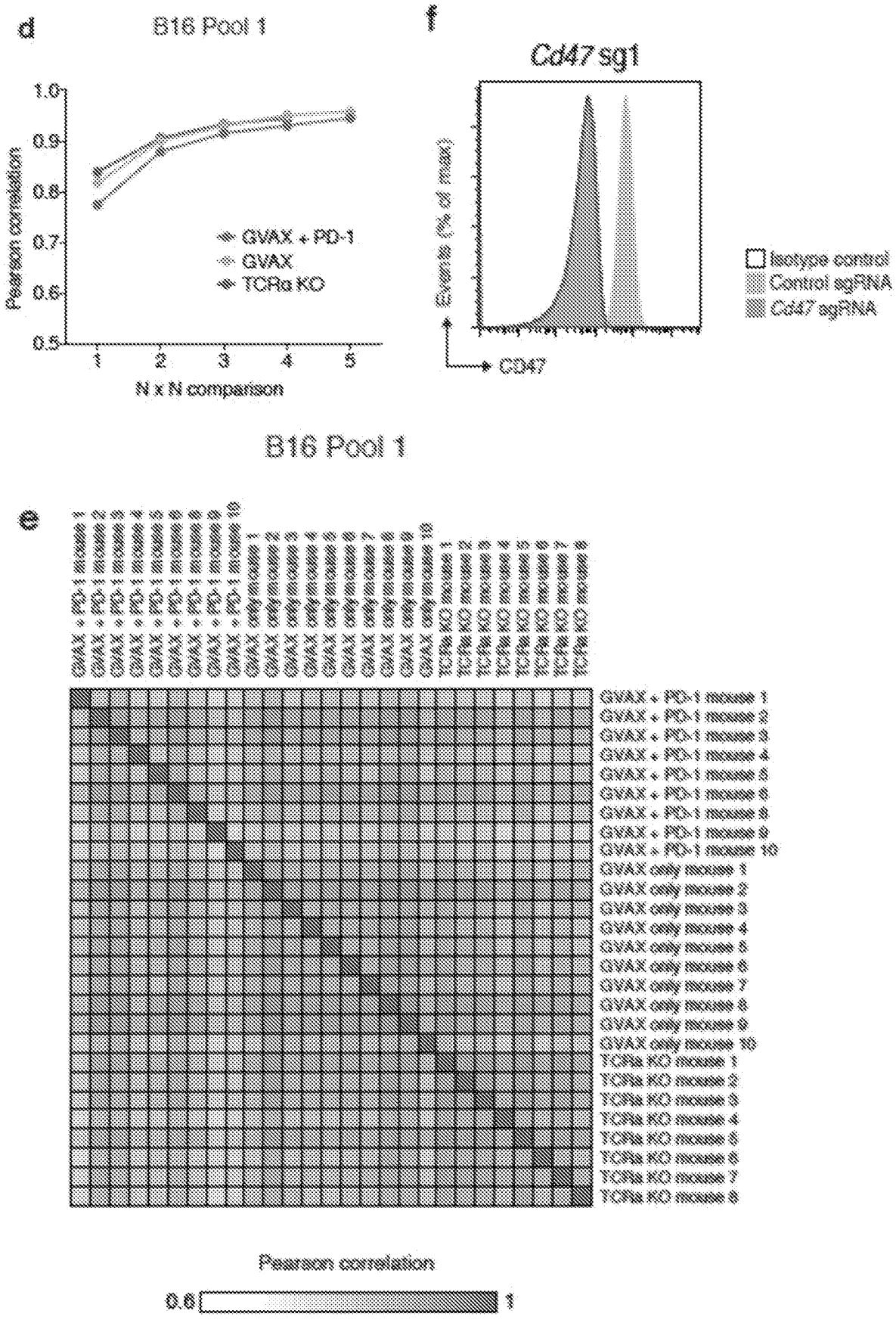


Figure 3

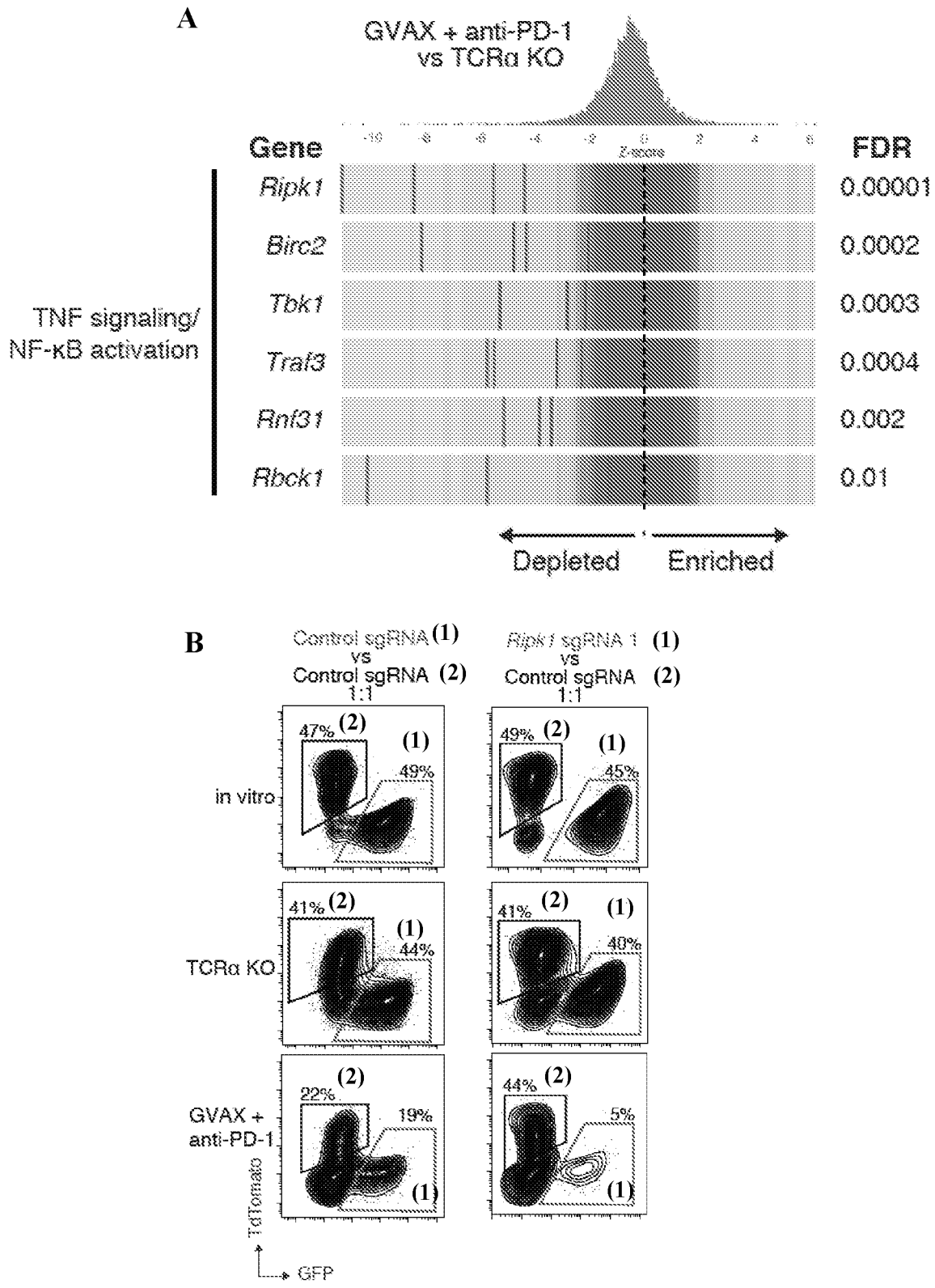
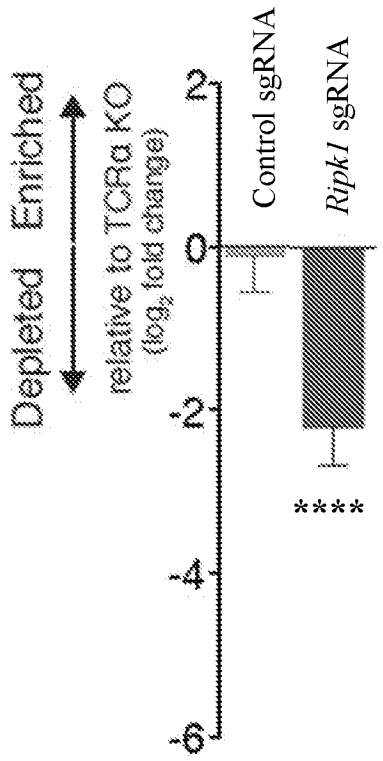

A
GVAX + anti-PD-1
vs TCRα KO
Gene
FDR
Ripk1
Birc2
Tbk1
Traf3
Rnf31
Rbck1
0.00001
0.0002
0.0003
0.0004
0.002
0.01
TNF signaling/
NF-κB activation
Depleted
Enriched
B
Control sgRNA (1)
vs
Control sgRNA (2)
1:1
Ripk1 sgRNA 1 (1)
vs
Control sgRNA (2)
1:1
in vitro
TCRα KO
GVAX +
anti-PD-1
47%
49%
41%
44%
22%
19%
49%
45%
41%
40%
44%
5%
TdTomato
GFP

Figure 3 (cont.)

C



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/042241

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61P 35/00; C12N 5/09; C12Q 1/68; G01N 33/50; G01N 33/574 (2018.01)

CPC - C12Q 1/6886; C12Q 2600/106; C12Q 2600/158; G01N 33/574; G01N 2333/4703; G01N 2800/50 (2018.08)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC - 424/9.2; 435/6; 435/375; 435/7.23 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2016/0122829 A1 (DANA-FARBER CANCER INSTITUTE, INC.) 05 May 2016 (05.05.2016) entire document	1-3, 57-60, 63-67
Y	WO 2017/022962 A1 (CATHOLIC UNIVERSITY OF KOREA INDUSTRY ACADEMIC COOPERATION FOUNDATION) 09 February 2017 (09.02.2017) entire document; see machine translation	1-3, 29-31, 57-60, 63-67
Y	US 2007/0105133 A1 (CLARKE et al) 10 May 2007 (10.05.2007) entire document	29-31, 57-60, 63-67
Y	US 2014/0010825 A1 (RAVINDRANATH et al) 09 January 2014 (09.01.2014) entire document	29-31
A	WO 2016/023918 A1 (MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN E.V.) 18 February 2016 (18.02.2016) entire document	1-3, 29-31, 57-60, 63-67
P, A	MANGUSO et al. "In vivo CRISPR screening identifies Ptpn2 as a cancer immunotherapy target," Nature, 19 July 2017 (19.07.2017), Vol. 547, Pgs. 413-418. entire document	1-3, 29-31, 57-60, 63-67

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
04 December 2018	26 DEC 2018
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/042241

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☒ furnished subsequent to the international filing date for the purposes of international search only:

      ☒ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NOs: 1-12, 14, 16, 18, 21, 23, 25, 27, 29, and 31 were searched.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/042241

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-28, 32-56, 61, 62, 68-75
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
See extra sheet(s).

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-3, 29-31, 57-60, 63-67 to the extent that they read on biomarker RIPK1 of SEQ ID NO:1 and/or SEQ ID NO:2.

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/042241

Continued from Box No. III Observations where unity of invention is lacking

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/042241

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees need to be paid.

Group I+: claims 1-3, 29-31, 57-60, and 63-67 are drawn to biomarkers, and methods comprising the same.

The first invention of Group I+ is restricted to a biomarker, and methods comprising the same, wherein the biomarker is selected to be RIPK1 of SEQ ID NO:2 (encoded by SEQ ID NO:1). It is believed that claims 1-3, 29-31, 57-60, and 63-67 read on this first named invention and thus these claims will be searched without fee to the extent that they read on biomarker RIPK1 of SEQ ID NO:1 and/or SEQ ID NO:2.

Applicant is invited to elect additional biomarkers, each with specified SEQ ID NO, to be searched in a specific combination by paying an additional fee for each set of election. An exemplary election would be a biomarker, and methods comprising the same, wherein the biomarker is selected to be BIRC2 of SEQ ID NO:9 (encoded by SEQ ID NO:8). Additional biomarkers will be searched upon the payment of additional fees. Applicants must specify the claims that read on any additional elected inventions. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined.

The inventions listed in Groups I+ do not relate to a single general inventive concept under PCT Rule 13.1, because under PCT Rule 13.2 they lack the same or corresponding special technical features for the following reasons:

The Groups I+ formulas do not share a significant structural element responsible for treating cancer, requiring the selection of alternatives for the biomarker, where "one or more biomarkers listed in Table 1 or a fragment thereof".

Additionally, even if Groups I+ were considered to share the technical features of a method of treating a subject afflicted with a cancer comprising administering to the subject a therapeutically effective amount of an agent that inhibits the copy number, the expression level, and/or the activity of one or more biomarkers or a fragment thereof, in combination with an immunotherapy; method of killing cancer cells comprising contacting the cancer cells with an agent that inhibits the copy number, the expression level, and/or the activity of one or more biomarkers or a fragment thereof, in combination with an immunotherapy; a method of determining whether a subject afflicted with a cancer or at risk for developing a cancer would benefit from inhibiting the copy number, amount, and/or activity of at least one biomarker, the method comprising: a) obtaining a biological sample from the subject; b) determining the copy number, amount, and/or activity of at least one biomarker; c) determining the copy number, amount, and/or activity of the at least one biomarker in a control; and d) comparing the copy number, amount, and/or activity of the at least one biomarker detected in steps b) and c); wherein the presence of, or a significant increase in, the copy number, amount, and/or activity of, the at least one biomarker in the subject sample relative to the control copy number, amount, and/or activity of the at least one biomarker indicates that the subject afflicted with the cancer or at risk for developing the cancer would benefit from inhibiting the copy number, amount, and/or activity of the at least one biomarker; a method for predicting the clinical outcome of a subject afflicted with a cancer expressing one or more biomarkers or a fragment thereof to treatment with an immunotherapy, the method comprising: a) determining the copy number, amount, and/or activity of at least one biomarker in a subject sample; b) determining the copy number, amount, and/or activity of the at least one biomarker in a control having a good clinical outcome; and c) comparing the copy number, amount, and/or activity of the at least one biomarker in the subject sample and in the control; wherein the presence of, or a significant increase in, the copy number, amount, and/or activity of, the at least one biomarker in the subject sample as compared to the copy number, amount and/or activity in the control, is an indication that the subject has a poor clinical outcome; a method for monitoring the progression of a cancer in a subject, wherein the subject is administered a therapeutically effective amount of an agent that inhibits the copy number, amount, and/or activity of at least one biomarker and an immunotherapy, the method comprising: a) detecting in a subject sample at a first point in time the copy number, amount, and/or activity of at least one biomarker; b) repeating step a) at a subsequent point in time; and c) comparing the amount or activity of at least one biomarker detected in steps a) and b) to monitor the progression of the cancer in the subject; a method of assessing the efficacy of an agent that inhibits the copy number, amount, and/or activity of at least one biomarker and an immunotherapy for treating a cancer in a subject, comprising: a) detecting in a subject sample at a first point in time the copy number, amount, and/or or activity of at least one biomarker; b) repeating step a) during at least one subsequent point in time after administration of the agent and the immunotherapy; and c) comparing the copy number, amount, and/or activity detected in steps a) and b), wherein the absence of, or a significant decrease in, the copy number, amount, and/or activity of, the at least one biomarker, in the subsequent sample as compared to the copy number, amount, and/or activity in the sample at the first point in time, indicates that the agent and immunotherapy treats the cancer in the subject; these shared technical features do not represent a contribution over the prior art.

Specifically, US 2016/0122829 A1 to Dana Farber Cancer Institute, Inc. discloses a method of treating a subject afflicted with a cancer (The present invention relates to methods for identifying, assessing, preventing, and treating cancer, Abstract) comprising administering to the subject a therapeutically effective amount of an agent that inhibits the copy number, the expression level, and/or the activity of one or more biomarkers or a fragment thereof (the method comprises administering an agent that downregulates the copy number, level of expression, or level of activity of one or more biomarkers, Para. [0023]), in combination with an immunotherapy (treatment with one or more modulators of PD-1 and/or PD-L1 levels is provided, Para. [0018]); method of killing cancer cells comprising contacting the cancer cells with an agent that inhibits the copy number, the expression level, and/or the activity of one or more biomarkers or a fragment thereof (the method comprises administering an agent that downregulates the copy number, level of expression, or level of activity of one or more biomarkers, Para. [0023]; cancer cell proliferation inhibition, induction of cancer cell apoptosis, enhancement of cancer cell susceptibility to chemotherapeutic agents, Para. [0174]); a method of determining whether a subject afflicted with a cancer or at risk for developing a cancer would benefit from inhibiting the copy number, amount, and/or activity of at least one biomarker (a method of determining whether a subject afflicted with a head, neck, or lung cancer or at risk for developing a head, neck, or lung cancer would benefit from modulating PD-1 and/or PD-L1 levels is provided, Para. [0015]), the method comprising: a) obtaining a biological sample from the subject (obtaining a biological sample from the subject:, Para. [0015]); b) determining the copy number, amount, and/or activity of at least one biomarker (determining the copy number, level of expression, or level of activity of one or more biomarkers, Para. [0015]); c) determining the copy number, amount, and/or activity of the at least one biomarker in a control; and d) comparing the copy number, amount, and/or activity of the at least one biomarker detected in steps b) and c) (determining the copy number, level of expression, or level of activity of the one or more biomarkers in a control; and d) comparing the copy number, level of expression, or level of activity of said one or more biomarkers detected in steps b) and c), Para. [0015]); wherein the presence of, or a

significant increase in, the copy number, amount, and/or activity of, the at least one biomarker in the subject sample relative to the control copy number, amount, and/or activity of the at least one biomarker indicates that the subject afflicted with the cancer or at risk for developing the cancer would benefit from inhibiting the copy number, amount, and/or activity of the at least one biomarker (wherein a significant increase in the copy number, level of expression, or level of activity of the one or more biomarkers in the subject sample relative to the control copy number, level of expression, or level of activity of the one or more biomarkers indicates that the subject afflicted with the head, neck, or lung cancer or at risk for developing the head, neck, or lung cancer would benefit from modulating PD-L1 and/or PD-L1 levels, Para. [0015]); a method for predicting the clinical outcome of a subject afflicted with a cancer expressing one or more biomarkers or a fragment thereof to treatment with an immunotherapy (aspect, a method for stratifying subjects afflicted with a head, neck, or lung cancer according to predicted clinical outcome of treatment with one or more modulators of PD-1 and/or PD-L1 levels is provided, Para. [0018]), the method comprising: a) determining the copy number, amount, and/or activity of at least one biomarker in a subject sample (determining the copy number, level of expression, or level of activity of one or more biomarkers listed in Table 1 or a fragment thereof in a subject sample, Para. [0018]); b) determining the copy number, amount, and/or activity of the at least one biomarker in a control having a good clinical outcome (determining the copy number, level of expression, or level of activity of the one or more biomarkers in a control sample, Para. [0018]); and c) comparing the copy number, amount, and/or activity of the at least one biomarker in the subject sample and in the control; wherein the presence of, or a significant increase in, the copy number, amount, and/or activity of, the at least one biomarker in the subject sample as compared to the copy number, amount and/or activity in the control, is an indication that the subject has a poor clinical outcome (comparing the copy number, level of expression, or level of activity of said one or more biomarkers detected in steps a) and b); wherein a significant modulation in the copy number, level of expression, or level of activity of the one or more biomarkers in the subject sample relative to the normal copy number, level of expression, or level of activity of the one or more biomarkers in the control sample predicts the clinical outcome of the patient to treatment with one or more modulators of PD-1 and/or PD-L levels, Para. [0018]); a method for monitoring the progression of a cancer in a subject (a method for monitoring the progression of a head, neck, or lung cancer in a subject is provided, Para. [0016]), wherein the subject is administered a therapeutically effective amount of an agent that inhibits the copy number, amount, and/or activity of at least one biomarker and an immunotherapy (detecting in a subject sample at a first point in time the copy number, level of expression, or level of activity of one or more biomarkers, Para. [0016]), the method comprising: a) detecting in a subject sample at a first point in time the copy number, amount, and/or activity of at least one biomarker; b) repeating step a) at a subsequent point in time; and c) comparing the amount or activity of at least one biomarker detected in steps a) and b) to monitor the progression of the cancer in the subject (repeating step a) at a subsequent point in time: and c) comparing the copy number, level of expression, or level of activity of said one or more biomarkers detected in steps a) and b) to monitor the progression of the head, neck, or lung cancer, wherein the one or more biomarkers comprises soluble PD-L1, Para. [0016]; to determine appropriate threshold values, a particular cancer therapeutic regimen can be administered to a population of subjects and the outcome can be correlated to copy number, level of expression, level of activity, etc. of one or more biomarkers, Para. [0093]); a method of assessing the efficacy of an agent (a method of determining the efficacy of a therapy for inhibiting a head, neck, or lung cancer in a subject is provided, Para. [0020]) that inhibits the copy number, amount, and/or activity of at least one biomarker and an immunotherapy for treating a cancer in a subject (wherein the method comprises: a) determining the copy number, level of expression, or level of activity of one or more biomarkers listed in Table 1 or a fragment thereof in a first sample obtained from the subject prior to providing at least a portion of the therapy to the subject, Para. [0020]), comprising: a) detecting in a subject sample at a first point in time the copy number, amount, and/or or activity of at least one biomarker (wherein the method comprises: a) determining the copy number, level of expression, or level of activity of one or more biomarkers listed in Table 1 or a fragment thereof in a first sample obtained from the subject prior to providing at least a portion of the therapy to the subject, Para. [0020]); b) repeating step a) during at least one subsequent point in time after administration of the agent and the immunotherapy; and c) comparing the copy number, amount, and/or activity detected in steps a) and b), wherein the absence of, or a significant decrease in, the copy number, amount, and/or activity of, the at least one biomarker, in the subsequent sample as compared to the copy number, amount, and/or activity in the sample at the first point in time (b) repeating step a) at a subsequent point in time: and c) comparing the copy number, level of expression, or level of activity of said one or more biomarkers detected in steps a) and b) to monitor the progression of the head, neck, or lung cancer, wherein the one or more biomarkers comprises soluble PD-L1, Para. [0016]; comparing the copy number, level of expression, or level of activity of the one or more biomarkers in the first and second samples, wherein a significantly decreased copy number, level of expression, or level of activity of the one or more biomarkers in the second sample, relative to the first sample, is an indication that the therapy as efficacious for inhibiting the head, neck, or lung cancer in the subject. In one embodiment, the one or more biomarkers comprises soluble PD-L1, Para. [0020]), indicates that the agent and immunotherapy treats the cancer in the subject (is an indication that the therapy as efficacious for inhibiting the head, neck, or lung cancer in the subject, Para. [0020]).

The inventions listed in Groups I+ therefore lack unity under Rule 13 because they do not share a same or corresponding special technical features.