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(54) **Title:** RANBP2 AS MODIFIER OF THE PTEN/IGF PATHWAY AND METHODS OF USE

(57) **Abstract:** Human RANBP2 genes are identified as modulators of the PTEN/IGF pathway, and thus are therapeutic targets for disorders associated with defective PTEN/IGF function. Methods for identifying modulators of PTEN/IGF, comprising screening for agents that modulate the activity of RANBP2 are provided.

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# RANBP2 AS MODIFIER OF THE PTEN/IGF PATHWAY AND METHODS OF USE

## REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. provisional patent application 60/470,766 filed 5/14/2003. The contents of the prior applications are hereby incorporated in their entirety.

## BACKGROUND OF THE INVENTION

Somatic mutations in the PTEN (Phosphatase and Tensin homolog deleted on chromosome 10) gene are known to cause tumors in a variety of human tissues. In addition, germline mutations in PTEN are the cause of human diseases (Cowden disease and Bannayan-Zonana syndrome) associated with increased risk of breast and thyroid cancer (Nelen MR et al. (1997) Hum Mol Genet, 8:1383-1387; Liaw D et al. (1997) Nat Genet, 1:64-67; Marsh DJ et al. (1998) Hum Mol Genet, 3:507-515). PTEN is thought to act as a tumor suppressor by regulating several signaling pathways through the second messenger phosphatidylinositol 3,4,5 triphosphate (PIP3). PTEN dephosphorylates the D3 position of PIP3 and downregulates signaling events dependent on PIP3 levels (Maehama T and Dixon JE (1998) J Biol Chem, 22, 13375-8). In particular, pro-survival pathways downstream of the insulin-like growth factor (IGF) pathway are regulated by PTEN activity. Stimulation of the IGF pathway, or loss of PTEN function, elevates PIP3 levels and activates pro-survival pathways associated with tumorigenesis (Stambolic V et al. (1998) Cell, 95:29-39). Consistent with this model, elevated levels of insulin-like growth factors I and II correlate with increased risk of cancer (Yu H et al (1999) J Natl Cancer Inst 91:151-156) and poor prognosis (Takanami I et al, 1996, J Surg Oncol 61(3):205-8).

PTEN sequence is conserved in evolution, and exists in mouse (Hansen GM and Justice MJ (1998) Mamm Genome, 9(1):88-90), *Drosophila* (Goberdhan DC et al (1999) Genes and Dev, 24:3244-58; Huang H et al (1999) Development 23:5365-72), and *C. elegans* (Ogg S and Ruvkun G, (1998) Mol Cell, (6):887-93). Studies in these model organisms have helped to elucidate the role of PTEN in processes relevant to tumorigenesis. In Drosophila, the PTEN homolog (dPTEN) has been shown to regulate cell size, survival, and proliferation (Huang et al, *supra*; Goberdhan et al, *supra*; Gao X et al, 2000, 221:404-418). In mice, loss of PTEN function increases cancer susceptibility (Di

Cristofano A et al (1998) Nature Genetics, 19:348-355; Suzuki A et al (1998) Curr. Biol., 8:1169-78).

In addition, a member of the IGF/insulin receptor family exists in Drosophila and has been shown to respond to insulin stimulation (Fernandez-Almonacid R, and Rozen OM (1987) Mol Cell Bio, (8):2718-27). Similar to PTEN, studies in Drosophila (Brogiolo W et al (2001) Curr Biol, 11(4):213-21) and mouse (Moorehead RA et al (2003) Oncogene, 22(6):853-857) establish a conserved role for the IGF/insulin pathway in growth control.

RAN is a small GTP-binding protein of the RAS superfamily that is associated with the nuclear membrane and is thought to control a variety of cellular functions through its interactions with other proteins. This gene encodes a very large RAN-binding protein that immunolocalizes to the nuclear pore complex (Yokoyama, N, et al (1995) Nature 376: 184-188). The protein is a giant scaffold and mosaic cyclophilin-related nucleoporin implicated in the Ran-GTPase cycle. The encoded protein directly interacts with the E2 enzyme UBC9 and strongly enhances SUMO1 transfer from UBC9 to the SUMO1 target SP100 (Pichler, A, et al (2002) Cell 108: 109-120). These findings place sumoylation at the cytoplasmic filaments of the nuclear pore complex and suggest that, for some substrates, modification and nuclear import are linked events. This gene is partially duplicated in a gene cluster that lies in a hot spot for recombination on chromosome 2q. RANBP2 is differentially expressed in colon cancer (Dunican et al (2002) Oncogene 21:3253-3257).

The ability to manipulate the genomes of model organisms such as *Drosophila* provides a powerful means to analyze biochemical processes that, due to significant evolutionary conservation, have direct relevance to more complex vertebrate organisms. Due to a high level of gene and pathway conservation, the strong similarity of cellular processes, and the functional conservation of genes between these model organisms and mammals, identification of the involvement of novel genes in particular pathways and their functions in such model organisms can directly contribute to the understanding of the correlative pathways and methods of modulating them in mammals (see, for example, Mechler BM et al., 1985 EMBO J 4:1551-1557; Gateff E. 1982 Adv. Cancer Res. 37: 33-74; Watson KL., et al., 1994 J Cell Sci. 18: 19-33; Miklos GL, and Rubin GM. 1996 Cell 86:521-529; Wassarman DA, et al., 1995 Curr Opin Gen Dev 5: 44-50; and Booth DR. 1999 Cancer Metastasis Rev. 18: 261-284). For example, a genetic screen can be carried out in an invertebrate model organism or cell having underexpression (e.g. knockout) or

overexpression of a gene (referred to as a "genetic entry point") that yields a visible phenotype, such as altered cell growth. Additional genes are mutated in a random or targeted manner. When a gene mutation changes the original phenotype caused by the mutation in the genetic entry point, the gene is identified as a "modifier" involved in the same or overlapping pathway as the genetic entry point. When inactivation of either gene is not lethal, but inactivation of both genes results in reduced viability or death of the cell, tissue, or organism, the interaction is defined as "synthetic lethal" (Bender, A and Pringle J, (1991) Mol Cell Biol, 11:1295-1305; Hartman J et al, (2001) Science 291:1001-1004; US PAT No:6,489,127). In a synthetic lethal interaction, the modifier may also be identified as an "interactor". When the genetic entry point is an ortholog of a human gene implicated in a disease pathway, such as the IGF pathway, modifier genes can be identified that may be attractive candidate targets for novel therapeutics.

All references cited herein, including patents, patent applications, publications, and sequence information in referenced Genbank identifier numbers, are incorporated herein in their entireties.

## SUMMARY OF THE INVENTION

We have discovered genes that modify the PTEN/IGF pathway in *Drosophila* cells, and identified their human orthologs, hereinafter referred to as RAN Binding Protein 2 (RANBP2). The invention provides methods for utilizing these PTEN/IGF modifier genes and polypeptides to identify RANBP2-modulating agents that are candidate therapeutic agents that can be used in the treatment of disorders associated with defective or impaired PTEN/IGF function and/or RANBP2 function. Preferred RANBP2-modulating agents specifically bind to RANBP2 polypeptides and restore PTEN/IGF function. Other preferred RANBP2-modulating agents are nucleic acid modulators such as antisense oligomers and RNAi that repress RANBP2 gene expression or product activity by, for example, binding to and inhibiting the respective nucleic acid (i.e. DNA or mRNA).

RANBP2 modulating agents may be evaluated by any convenient *in vitro* or *in vivo* assay for molecular interaction with a RANBP2 polypeptide or nucleic acid. In one embodiment, candidate RANBP2 modulating agents are tested with an assay system comprising a RANBP2 polypeptide or nucleic acid. Agents that produce a change in the activity of the assay system relative to controls are identified as candidate PTEN/IGF modulating agents. The assay system may be cell-based or cell-free. RANBP2-

modulating agents include RANBP2 related proteins (e.g. dominant negative mutants, and biotherapeutics); RANBP2 -specific antibodies; RANBP2 -specific antisense oligomers and other nucleic acid modulators; and chemical agents that specifically bind to or interact with RANBP2 or compete with RANBP2 binding partner (e.g. by binding to a RANBP2 binding partner). In one specific embodiment, a small molecule modulator is identified using a binding assay. In specific embodiments, the screening assay system is selected from an apoptosis assay, a cell proliferation assay, an angiogenesis assay, and a hypoxic induction assay.

In another embodiment, candidate PTEN/IGF pathway modulating agents are further tested using a second assay system that detects changes in the PTEN/IGF pathway, such as angiogenic, apoptotic, or cell proliferation changes produced by the originally identified candidate agent or an agent derived from the original agent. The second assay system may use cultured cells or non-human animals. In specific embodiments, the secondary assay system uses non-human animals, including animals predetermined to have a disease or disorder implicating the PTEN/IGF pathway, such as an angiogenic, apoptotic, or cell proliferation disorder (e.g. cancer).

The invention further provides methods for modulating the RANBP2 function and/or the PTEN/IGF pathway in a mammalian cell by contacting the mammalian cell with an agent that specifically binds a RANBP2 polypeptide or nucleic acid. The agent may be a small molecule modulator, a nucleic acid modulator, or an antibody and may be administered to a mammalian animal predetermined to have a pathology associated with the PTEN/IGF pathway.

## DETAILED DESCRIPTION OF THE INVENTION

The PTEN co-RNAi plus insulin synthetic lethal screen was designed to identify modifier genes that are lethal or reduce proliferation in cells with a hyperstimulated IGF/insulin pathway, but not in normal cells. We created cells with a hyperstimulated IGF/insulin pathway by treatment with insulin and RNAi-mediated inactivation of dPTEN, the Drosophila homologue of the human tumor suppressor PTEN. In addition to identifying genes with synthetic lethal interactions in insulin-treated, PTEN-deficient cells, this screen identified genes that, when inactivated, preferentially reduced the viability of insulin-treated, PTEN-deficient cells relative to normal cells. The CG11856 gene was identified as having a synthetic interaction with the IGF pathway. Accordingly, vertebrate orthologs of CG11856, and preferably the human orthologs, RANBP2 genes (i.e., nucleic

acids and polypeptides) are attractive drug targets for the treatment of pathologies associated with a defective IGF signaling pathway, such as cancer.

In vitro and in vivo methods of assessing RANBP2 function are provided herein. Modulation of the RANBP2 or their respective binding partners is useful for understanding the association of the PTEN/IGF pathway and its members in normal and disease conditions and for developing diagnostics and therapeutic modalities for PTEN/IGF related pathologies. RANBP2-modulating agents that act by inhibiting or enhancing RANBP2 expression, directly or indirectly, for example, by affecting a RANBP2 function such as enzymatic (e.g., catalytic) or binding activity, can be identified using methods provided herein. RANBP2 modulating agents are useful in diagnosis, therapy and pharmaceutical development.

**Nucleic acids and polypeptides of the invention**

Sequences related to RANBP2 nucleic acids and polypeptides that can be used in the invention are disclosed in Genbank (referenced by Genbank identifier (GI) number) as GI#s 19718756 (SEQ ID NO:1), 924266 (SEQ ID NO:2), 10437982 (SEQ ID NO:3), 10438317 (SEQ ID NO:4), 10439992 (SEQ ID NO:5), and 624231 (SEQ ID NO:6) for nucleic acid, and GI# 6382079 (SEQ ID NO:7) for polypeptide sequences.

The term "RANBP2 polypeptide" refers to a full-length RANBP2 protein or a functionally active fragment or derivative thereof. A "functionally active" RANBP2 fragment or derivative exhibits one or more functional activities associated with a full-length, wild-type RANBP2 protein, such as antigenic or immunogenic activity, enzymatic activity, ability to bind natural cellular substrates, etc. The functional activity of RANBP2 proteins, derivatives and fragments can be assayed by various methods known to one skilled in the art (Current Protocols in Protein Science (1998) Coligan *et al.*, eds., John Wiley & Sons, Inc., Somerset, New Jersey) and as further discussed below. In one embodiment, a functionally active RANBP2 polypeptide is a RANBP2 derivative capable of rescuing defective endogenous RANBP2 activity, such as in cell based or animal assays; the rescuing derivative may be from the same or a different species. For purposes herein, functionally active fragments also include those fragments that comprise one or more structural domains of a RANBP2, such as a binding domain. Protein domains can be identified using the PFAM program (Bateman A., et al., Nucleic Acids Res, 1999, 27:260-2). For example, the RanBP1 domain (PFAM 00638) of RANBP2 from GI# 6382079 (SEQ ID NO:7) is located at approximately amino acid residues 1285 to 1406, 1581 to

1703, 1995 to 2112, and 2510 to 2637. Also, the zinc finger domain of the same protein (PFAM 00641) ) is located at approximately amino acid residues 1734 to 1763, and 1854 to 1883. Methods for obtaining RANBP2 polypeptides are also further described below. In some embodiments, preferred fragments are functionally active, domain-containing fragments comprising at least 25 contiguous amino acids, preferably at least 50, more preferably 75, and most preferably at least 100 contiguous amino acids of a RANBP2. In further preferred embodiments, the fragment comprises the entire functionally active domain.

The term "RANBP2 nucleic acid" refers to a DNA or RNA molecule that encodes a RANBP2 polypeptide. Preferably, the RANBP2 polypeptide or nucleic acid or fragment thereof is from a human, but can also be an ortholog, or derivative thereof with at least 70% sequence identity, preferably at least 80%, more preferably 85%, still more preferably 90%, and most preferably at least 95% sequence identity with human RANBP2. Methods of identifying orthlogs are known in the art. Normally, orthologs in different species retain the same function, due to presence of one or more protein motifs and/or 3-dimensional structures. Orthologs are generally identified by sequence homology analysis, such as BLAST analysis, usually using protein bait sequences. Sequences are assigned as a potential ortholog if the best hit sequence from the forward BLAST result retrieves the original query sequence in the reverse BLAST (Huynen MA and Bork P, Proc Natl Acad Sci (1998) 95:5849-5856; Huynen MA *et al.*, Genome Research (2000) 10:1204-1210). Programs for multiple sequence alignment, such as CLUSTAL (Thompson JD et al, 1994, Nucleic Acids Res 22:4673-4680) may be used to highlight conserved regions and/or residues of orthologous proteins and to generate phylogenetic trees. In a phylogenetic tree representing multiple homologous sequences from diverse species (e.g., retrieved through BLAST analysis), orthologous sequences from two species generally appear closest on the tree with respect to all other sequences from these two species. Structural threading or other analysis of protein folding (e.g., using software by ProCeryon, Biosciences, Salzburg, Austria) may also identify potential orthologs. In evolution, when a gene duplication event follows speciation, a single gene in one species, such as *Drosophila*, may correspond to multiple genes (paralogs) in another, such as human. As used herein, the term "orthologs" encompasses paralogs. As used herein, "percent (%) sequence identity" with respect to a subject sequence, or a specified portion of a subject sequence, is defined as the percentage of nucleotides or amino acids in the candidate derivative sequence identical with the nucleotides or amino acids in the subject

sequence (or specified portion thereof), after aligning the sequences and introducing gaps, if necessary to achieve the maximum percent sequence identity, as generated by the program WU-BLAST-2.0a19 (Altschul *et al.*, J. Mol. Biol. (1997) 215:403-410) with all the search parameters set to default values. The HSP S and HSP S2 parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched. A % identity value is determined by the number of matching identical nucleotides or amino acids divided by the sequence length for which the percent identity is being reported. "Percent (%) amino acid sequence similarity" is determined by doing the same calculation as for determining % amino acid sequence identity, but including conservative amino acid substitutions in addition to identical amino acids in the computation.

A conservative amino acid substitution is one in which an amino acid is substituted for another amino acid having similar properties such that the folding or activity of the protein is not significantly affected. Aromatic amino acids that can be substituted for each other are phenylalanine, tryptophan, and tyrosine; interchangeable hydrophobic amino acids are leucine, isoleucine, methionine, and valine; interchangeable polar amino acids are glutamine and asparagine; interchangeable basic amino acids are arginine, lysine and histidine; interchangeable acidic amino acids are aspartic acid and glutamic acid; and interchangeable small amino acids are alanine, serine, threonine, cysteine and glycine.

Alternatively, an alignment for nucleic acid sequences is provided by the local homology algorithm of Smith and Waterman (Smith and Waterman, 1981, Advances in Applied Mathematics 2:482-489; database: European Bioinformatics Institute; Smith and Waterman, 1981, J. of Molec.Biol., 147:195-197; Nicholas et al., 1998, "A Tutorial on Searching Sequence Databases and Sequence Scoring Methods" (www.psc.edu) and references cited therein.; W.R. Pearson, 1991, Genomics 11:635-650). This algorithm can be applied to amino acid sequences by using the scoring matrix developed by Dayhoff (Dayhoff: Atlas of Protein Sequences and Structure, M. O. Dayhoff ed., 5 suppl. 3:353-358, National Biomedical Research Foundation, Washington, D.C., USA), and normalized by Gribskov (Gribskov 1986 Nucl. Acids Res. 14(6):6745-6763). The Smith-Waterman algorithm may be employed where default parameters are used for scoring (for example, gap open penalty of 12, gap extension penalty of two). From the data generated, the "Match" value reflects "sequence identity."

Derivative nucleic acid molecules of the subject nucleic acid molecules include sequences that hybridize to the nucleic acid sequence of a RANBP2. The stringency of hybridization can be controlled by temperature, ionic strength, pH, and the presence of denaturing agents such as formamide during hybridization and washing. Conditions routinely used are set out in readily available procedure texts (*e.g.*, Current Protocol in Molecular Biology, Vol. 1, Chap. 2.10, John Wiley & Sons, Publishers (1994); Sambrook *et al.*, Molecular Cloning, Cold Spring Harbor (1989)). In some embodiments, a nucleic acid molecule of the invention is capable of hybridizing to a nucleic acid molecule containing the nucleotide sequence of a RANBP2 under high stringency hybridization conditions that are: prehybridization of filters containing nucleic acid for 8 hours to overnight at 65° C in a solution comprising 6X single strength citrate (SSC) (1X SSC is 0.15 M NaCl, 0.015 M Na citrate; pH 7.0), 5X Denhardt's solution, 0.05% sodium pyrophosphate and 100 μg/ml herring sperm DNA; hybridization for 18-20 hours at 65° C in a solution containing 6X SSC, 1X Denhardt's solution, 100 μg/ml yeast tRNA and 0.05% sodium pyrophosphate; and washing of filters at 65° C for 1h in a solution containing 0.1X SSC and 0.1% SDS (sodium dodecyl sulfate).

In other embodiments, moderately stringent hybridization conditions are used that are: pretreatment of filters containing nucleic acid for 6 h at 40° C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH7.5), 5mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 μg/ml denatured salmon sperm DNA; hybridization for 18-20h at 40° C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH7.5), 5mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 μg/ml salmon sperm DNA, and 10% (wt/vol) dextran sulfate; followed by washing twice for 1 hour at 55° C in a solution containing 2X SSC and 0.1% SDS.

Alternatively, low stringency conditions can be used that are: incubation for 8 hours to overnight at 37° C in a solution comprising 20% formamide, 5 x SSC, 50 mM sodium phosphate (pH 7.6), 5X Denhardt's solution, 10% dextran sulfate, and 20 μg/ml denatured sheared salmon sperm DNA; hybridization in the same buffer for 18 to 20 hours; and washing of filters in 1 x SSC at about 37° C for 1 hour.

**Isolation, Production, Expression, and Mis-expression of RANBP2 Nucleic Acids and Polypeptides**

RANBP2 nucleic acids and polypeptides are useful for identifying and testing agents that modulate RANBP2 function and for other applications related to the

involvement of RANBP2 in the PTEN/IGF pathway. RANBP2 nucleic acids and derivatives and orthologs thereof may be obtained using any available method. For instance, techniques for isolating cDNA or genomic DNA sequences of interest by screening DNA libraries or by using polymerase chain reaction (PCR) are well known in the art. In general, the particular use for the protein will dictate the particulars of expression, production, and purification methods. For instance, production of proteins for use in screening for modulating agents may require methods that preserve specific biological activities of these proteins, whereas production of proteins for antibody generation may require structural integrity of particular epitopes. Expression of proteins to be purified for screening or antibody production may require the addition of specific tags (*e.g.*, generation of fusion proteins). Overexpression of a RANBP2 protein for assays used to assess RANBP2 function, such as involvement in cell cycle regulation or hypoxic response, may require expression in eukaryotic cell lines capable of these cellular activities. Techniques for the expression, production, and purification of proteins are well known in the art; any suitable means therefore may be used (e.g., Higgins SJ and Hames BD (eds.) Protein Expression: A Practical Approach, Oxford University Press Inc., New York 1999; Stanbury PF et al., Principles of Fermentation Technology, 2nd edition, Elsevier Science, New York, 1995; Doonan S (ed.) Protein Purification Protocols, Humana Press, New Jersey, 1996; Coligan JE et al, Current Protocols in Protein Science (eds.), 1999, John Wiley & Sons, New York). In particular embodiments, recombinant RANBP2 is expressed in a cell line known to have defective PTEN/IGF function. The recombinant cells are used in cell-based screening assay systems of the invention, as described further below.

The nucleotide sequence encoding a RANBP2 polypeptide can be inserted into any appropriate expression vector. The necessary transcriptional and translational signals, including promoter/enhancer element, can derive from the native RANBP2 gene and/or its flanking regions or can be heterologous. A variety of host-vector expression systems may be utilized, such as mammalian cell systems infected with virus (*e.g.* vaccinia virus, adenovirus, *etc.*); insect cell systems infected with virus (*e.g.* baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage, plasmid, or cosmid DNA. An isolated host cell strain that modulates the expression of, modifies, and/or specifically processes the gene product may be used.

To detect expression of the RANBP2 gene product, the expression vector can comprise a promoter operably linked to a RANBP2 gene nucleic acid, one or more origins

of replication, and, one or more selectable markers (*e.g.* thymidine kinase activity, resistance to antibiotics, *etc.*). Alternatively, recombinant expression vectors can be identified by assaying for the expression of the RANBP2 gene product based on the physical or functional properties of the RANBP2 protein in *in vitro* assay systems (*e.g.* immunoassays).

The RANBP2 protein, fragment, or derivative may be optionally expressed as a fusion, or chimeric protein product (i.e. it is joined via a peptide bond to a heterologous protein sequence of a different protein), for example to facilitate purification or detection. A chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other using standard methods and expressing the chimeric product. A chimeric product may also be made by protein synthetic techniques, *e.g.* by use of a peptide synthesizer (Hunkapiller *et al.*, Nature (1984) 310:105-111).

Once a recombinant cell that expresses the RANBP2 gene sequence is identified, the gene product can be isolated and purified using standard methods (*e.g.* ion exchange, affinity, and gel exclusion chromatography; centrifugation; differential solubility; electrophoresis). Alternatively, native RANBP2 proteins can be purified from natural sources, by standard methods (*e.g.* immunoaffinity purification). Once a protein is obtained, it may be quantified and its activity measured by appropriate methods, such as immunoassay, bioassay, or other measurements of physical properties, such as crystallography.

The methods of this invention may also use cells that have been engineered for altered expression (mis-expression) of RANBP2 or other genes associated with the PTEN/IGF pathway. As used herein, mis-expression encompasses ectopic expression, over-expression, under-expression, and non-expression (*e.g.* by gene knock-out or blocking expression that would otherwise normally occur).

**Genetically modified animals**

Animal models that have been genetically modified to alter RANBP2 expression may be used in *in vivo* assays to test for activity of a candidate PTEN/IGF modulating agent, or to further assess the role of RANBP2 in a PTEN/IGF pathway process such as apoptosis or cell proliferation. Preferably, the altered RANBP2 expression results in a detectable phenotype, such as decreased or increased levels of cell proliferation, angiogenesis, or apoptosis compared to control animals having normal RANBP2

expression. The genetically modified animal may additionally have altered PTEN/IGF expression (e.g. PTEN/IGF knockout). Preferred genetically modified animals are mammals such as primates, rodents (preferably mice or rats), among others. Preferred non-mammalian species include zebrafish, *C. elegans*, and *Drosophila*. Preferred genetically modified animals are transgenic animals having a heterologous nucleic acid sequence present as an extrachromosomal element in a portion of its cells, i.e. mosaic animals (see, for example, techniques described by Jakobovits, 1994, Curr. Biol. 4:761-763.) or stably integrated into its germ line DNA (i.e., in the genomic sequence of most or all of its cells). Heterologous nucleic acid is introduced into the germ line of such transgenic animals by genetic manipulation of, for example, embryos or embryonic stem cells of the host animal.

Methods of making transgenic animals are well-known in the art (for transgenic mice see Brinster et al., Proc. Nat. Acad. Sci. USA 82: 4438-4442 (1985), U.S. Pat. Nos. 4,736,866 and 4,870,009, both by Leder et al., U.S. Pat. No. 4,873,191 by Wagner et al., and Hogan, B., Manipulating the Mouse Embryo, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (1986); for particle bombardment see U.S. Pat. No., 4,945,050, by Sandford *et al.*; for transgenic *Drosophila* see Rubin and Spradling, Science (1982) 218:348-53 and U.S. Pat. No. 4,670,388; for transgenic insects see Berghammer A.J. *et al.*, A Universal Marker for Transgenic Insects (1999) Nature 402:370-371; for transgenic Zebrafish see Lin S., Transgenic Zebrafish, Methods Mol Biol. (2000);136:375-3830); for microinjection procedures for fish, amphibian eggs and birds see Houdebine and Chourrout, Experientia (1991) 47:897-905; for transgenic rats see Hammer *et al.*, Cell (1990) 63:1099-1112; and for culturing of embryonic stem (ES) cells and the subsequent production of transgenic animals by the introduction of DNA into ES cells using methods such as electroporation, calcium phosphate/DNA precipitation and direct injection see, e.g., Teratocarcinomas and Embryonic Stem Cells, A Practical Approach, E. J. Robertson, ed., IRL Press (1987)). Clones of the nonhuman transgenic animals can be produced according to available methods (see Wilmut, I. *et al.* (1997) Nature 385:810-813; and PCT International Publication Nos. WO 97/07668 and WO 97/07669).

In one embodiment, the transgenic animal is a "knock-out" animal having a heterozygous or homozygous alteration in the sequence of an endogenous RANBP2 gene that results in a decrease of RANBP2 function, preferably such that RANBP2 expression is undetectable or insignificant. Knock-out animals are typically generated by homologous recombination with a vector comprising a transgene having at least a portion

of the gene to be knocked out. Typically a deletion, addition or substitution has been introduced into the transgene to functionally disrupt it. The transgene can be a human gene (e.g., from a human genomic clone) but more preferably is an ortholog of the human gene derived from the transgenic host species. For example, a mouse RANBP2 gene is used to construct a homologous recombination vector suitable for altering an endogenous RANBP2 gene in the mouse genome. Detailed methodologies for homologous recombination in mice are available (see Capecchi, Science (1989) 244:1288-1292; Joyner *et al.*, Nature (1989) 338:153-156). Procedures for the production of non-rodent transgenic mammals and other animals are also available (Houdebine and Chourrout, *supra*; Pursel *et al.*, Science (1989) 244:1281-1288; Simms *et al.*, Bio/Technology (1988) 6:179-183). In a preferred embodiment, knock-out animals, such as mice harboring a knockout of a specific gene, may be used to produce antibodies against the human counterpart of the gene that has been knocked out (Claesson MH et al., (1994) Scan J Immunol 40:257-264; Declerck PJ et al., (1995) J Biol Chem. 270:8397-400).

In another embodiment, the transgenic animal is a "knock-in" animal having an alteration in its genome that results in altered expression (e.g., increased (including ectopic) or decreased expression) of the RANBP2 gene, e.g., by introduction of additional copies of RANBP2, or by operatively inserting a regulatory sequence that provides for altered expression of an endogenous copy of the RANBP2 gene. Such regulatory sequences include inducible, tissue-specific, and constitutive promoters and enhancer elements. The knock-in can be homozygous or heterozygous.

Transgenic nonhuman animals can also be produced that contain selected systems allowing for regulated expression of the transgene. One example of such a system that may be produced is the cre/loxP recombinase system of bacteriophage P1 (Lakso *et al.*, PNAS (1992) 89:6232-6236; U.S. Pat. No. 4,959,317). If a cre/loxP recombinase system is used to regulate expression of the transgene, animals containing transgenes encoding both the Cre recombinase and a selected protein are required. Such animals can be provided through the construction of "double" transgenic animals, e.g., by mating two transgenic animals, one containing a transgene encoding a selected protein and the other containing a transgene encoding a recombinase. Another example of a recombinase system is the FLP recombinase system of Saccharomyces cerevisiae (O'Gorman et al. (1991) Science 251:1351-1355; U.S. Pat. No. 5,654,182). In a preferred embodiment, both Cre-LoxP and Flp-Frt are used in the same system to regulate expression of the

transgene, and for sequential deletion of vector sequences in the same cell (Sun X et al (2000) Nat Genet 25:83-6).

The genetically modified animals can be used in genetic studies to further elucidate the PTEN/IGF pathway, as animal models of disease and disorders implicating defective PTEN/IGF function, and for *in vivo* testing of candidate therapeutic agents, such as those identified in screens described below. The candidate therapeutic agents are administered to a genetically modified animal having altered RANBP2 function and phenotypic changes are compared with appropriate control animals such as genetically modified animals that receive placebo treatment, and/or animals with unaltered RANBP2 expression that receive candidate therapeutic agent.

In addition to the above-described genetically modified animals having altered RANBP2 function, animal models having defective PTEN/IGF function (and otherwise normal RANBP2 function), can be used in the methods of the present invention. For example, a mouse with defective PTEN function can be used to assess, *in vivo*, the activity of a candidate PTEN modulating agent identified in one of the *in vitro* assays described below. Transgenic mice with defective PTEN function have been described in literature (Di Cristofano et al, *supra*). Preferably, the candidate PTEN/IGF modulating agent when administered to a model system with cells defective in PTEN/IGF function, produces a detectable phenotypic change in the model system indicating that the PTEN/IGF function is restored, i.e., the cells exhibit normal cell cycle progression.

**Modulating Agents**

The invention provides methods to identify agents that interact with and/or modulate the function of RANBP2 and/or the PTEN/IGF pathway. Modulating agents identified by the methods are also part of the invention. Such agents are useful in a variety of diagnostic and therapeutic applications associated with the PTEN/IGF pathway, as well as in further analysis of the RANBP2 protein and its contribution to the PTEN/IGF pathway. Accordingly, the invention also provides methods for modulating the PTEN/IGF pathway comprising the step of specifically modulating RANBP2 activity by administering a RANBP2-interacting or -modulating agent.

As used herein, an "RANBP2-modulating agent" is any agent that modulates RANBP2 function, for example, an agent that interacts with RANBP2 to inhibit or enhance RANBP2 activity or otherwise affect normal RANBP2 function. RANBP2 function can be affected at any level, including transcription, protein expression, protein

localization, and cellular or extra-cellular activity. In a preferred embodiment, the RANBP2 - modulating agent specifically modulates the function of the RANBP2. The phrases "specific modulating agent", "specifically modulates", etc., are used herein to refer to modulating agents that directly bind to the RANBP2 polypeptide or nucleic acid, and preferably inhibit, enhance, or otherwise alter, the function of the RANBP2. These phrases also encompass modulating agents that alter the interaction of the RANBP2 with a binding partner, substrate, or cofactor (e.g. by binding to a binding partner of a RANBP2, or to a protein/binding partner complex, and altering RANBP2 function). In a further preferred embodiment, the RANBP2- modulating agent is a modulator of the PTEN/IGF pathway (e.g. it restores and/or upregulates PTEN/IGF function) and thus is also a PTEN/IGF-modulating agent.

Preferred RANBP2-modulating agents include small molecule compounds; RANBP2-interacting proteins, including antibodies and other biotherapeutics; and nucleic acid modulators such as antisense and RNA inhibitors. The modulating agents may be formulated in pharmaceutical compositions, for example, as compositions that may comprise other active ingredients, as in combination therapy, and/or suitable carriers or excipients. Techniques for formulation and administration of the compounds may be found in "Remington's Pharmaceutical Sciences" Mack Publishing Co., Easton, PA, 19th edition.

**Small molecule modulators**

Small molecules are often preferred to modulate function of proteins with enzymatic function, and/or containing protein interaction domains. Chemical agents, referred to in the art as "small molecule" compounds are typically organic, non-peptide molecules, having a molecular weight up to 10,000, preferably up to 5,000, more preferably up to 1,000, and most preferably up to 500 daltons. This class of modulators includes chemically synthesized molecules, for instance, compounds from combinatorial chemical libraries. Synthetic compounds may be rationally designed or identified based on known or inferred properties of the RANBP2 protein or may be identified by screening compound libraries. Alternative appropriate modulators of this class are natural products, particularly secondary metabolites from organisms such as plants or fungi, which can also be identified by screening compound libraries for RANBP2–modulating activity. Methods for generating and obtaining compounds are well known in the art (Schreiber SL, Science (2000) 151: 1964-1969; Radmann J and Gunther J, Science (2000) 151:1947-1948).

Small molecule modulators identified from screening assays, as described below, can be used as lead compounds from which candidate clinical compounds may be designed, optimized, and synthesized. Such clinical compounds may have utility in treating pathologies associated with the PTEN/IGF pathway. The activity of candidate small molecule modulating agents may be improved several-fold through iterative secondary functional validation, as further described below, structure determination, and candidate modulator modification and testing. Additionally, candidate clinical compounds are generated with specific regard to clinical and pharmacological properties. For example, the reagents may be derivatized and re-screened using *in vitro* and *in vivo* assays to optimize activity and minimize toxicity for pharmaceutical development.

**Protein Modulators**

Specific RANBP2-interacting proteins are useful in a variety of diagnostic and therapeutic applications related to the PTEN/IGF pathway and related disorders, as well as in validation assays for other RANBP2-modulating agents. In a preferred embodiment, RANBP2-interacting proteins affect normal RANBP2 function, including transcription, protein expression, protein localization, and cellular or extra-cellular activity. In another embodiment, RANBP2-interacting proteins are useful in detecting and providing information about the function of RANBP2 proteins, as is relevant to PTEN/IGF related disorders, such as cancer (e.g., for diagnostic means).

An RANBP2-interacting protein may be endogenous, i.e. one that naturally interacts genetically or biochemically with a RANBP2, such as a member of the RANBP2 pathway that modulates RANBP2 expression, localization, and/or activity. RANBP2-modulators include dominant negative forms of RANBP2-interacting proteins and of RANBP2 proteins themselves. Yeast two-hybrid and variant screens offer preferred methods for identifying endogenous RANBP2-interacting proteins (Finley, R. L. et al. (1996) in DNA Cloning-Expression Systems: A Practical Approach, eds. Glover D. & Hames B. D (Oxford University Press, Oxford, England), pp. 169-203; Fashema SF et al., Gene (2000) 250:1-14; Drees BL Curr Opin Chem Biol (1999) 3:64-70; Vidal M and Legrain P Nucleic Acids Res (1999) 27:919-29; and U.S. Pat. No. 5,928,868). Mass spectrometry is an alternative preferred method for the elucidation of protein complexes (reviewed in, e.g., Pandley A and Mann M, Nature (2000) 405:837-846; Yates JR 3rd, Trends Genet (2000) 16:5-8).

An RANBP2-interacting protein may be an exogenous protein, such as a RANBP2-specific antibody or a T-cell antigen receptor (see, e.g., Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory; Harlow and Lane (1999) Using antibodies: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press). RANBP2 antibodies are further discussed below.

In preferred embodiments, a RANBP2-interacting protein specifically binds a RANBP2 protein. In alternative preferred embodiments, a RANBP2-modulating agent binds a RANBP2 substrate, binding partner, or cofactor.

*Antibodies*

In another embodiment, the protein modulator is a RANBP2 specific antibody agonist or antagonist. The antibodies have therapeutic and diagnostic utilities, and can be used in screening assays to identify RANBP2 modulators. The antibodies can also be used in dissecting the portions of the RANBP2 pathway responsible for various cellular responses and in the general processing and maturation of the RANBP2.

Antibodies that specifically bind RANBP2 polypeptides can be generated using known methods. Preferably the antibody is specific to a mammalian ortholog of RANBP2 polypeptide, and more preferably, to human RANBP2. Antibodies may be polyclonal, monoclonal (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab').sub.2 fragments, fragments produced by a FAb expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. Epitopes of RANBP2 which are particularly antigenic can be selected, for example, by routine screening of RANBP2 polypeptides for antigenicity or by applying a theoretical method for selecting antigenic regions of a protein (Hopp and Wood (1981), Proc. Nati. Acad. Sci. U.S.A. 78:3824-28; Hopp and Wood, (1983) Mol. Immunol. 20:483-89; Sutcliffe et al., (1983) Science 219:660-66) to the amino acid sequence of a RANBP2. Monoclonal antibodies with affinities of $10^8$ $M^{-1}$ preferably $10^9$ $M^{-1}$ to $10^{10}$ $M^{-1}$, or stronger can be made by standard procedures as described (Harlow and Lane, *supra*; Goding (1986) Monoclonal Antibodies: Principles and Practice (2d ed) Academic Press, New York; and U.S. Pat. Nos. 4,381,292; 4,451,570; and 4,618,577). Antibodies may be generated against crude cell extracts of RANBP2 or substantially purified fragments thereof. If RANBP2 fragments are used, they preferably comprise at least 10, and more preferably, at least 20 contiguous amino acids of a RANBP2 protein. In a particular embodiment, RANBP2-specific antigens and/or immunogens are coupled to carrier

proteins that stimulate the immune response. For example, the subject polypeptides are covalently coupled to the keyhole limpet hemocyanin (KLH) carrier, and the conjugate is emulsified in Freund's complete adjuvant, which enhances the immune response. An appropriate immune system such as a laboratory rabbit or mouse is immunized according to conventional protocols.

The presence of RANBP2-specific antibodies is assayed by an appropriate assay such as a solid phase enzyme-linked immunosorbant assay (ELISA) using immobilized corresponding RANBP2 polypeptides. Other assays, such as radioimmunoassays or fluorescent assays might also be used.

Chimeric antibodies specific to RANBP2 polypeptides can be made that contain different portions from different animal species. For instance, a human immunoglobulin constant region may be linked to a variable region of a murine mAb, such that the antibody derives its biological activity from the human antibody, and its binding specificity from the murine fragment. Chimeric antibodies are produced by splicing together genes that encode the appropriate regions from each species (Morrison et al., Proc. Natl. Acad. Sci. (1984) 81:6851-6855; Neuberger et al., Nature (1984) 312:604-608; Takeda et al., Nature (1985) 31:452-454). Humanized antibodies, which are a form of chimeric antibodies, can be generated by grafting complementary-determining regions (CDRs) (Carlos, T. M., J. M. Harlan. 1994. Blood 84:2068-2101) of mouse antibodies into a background of human framework regions and constant regions by recombinant DNA technology (Riechmann LM, et al., 1988 Nature 323: 323-327). Humanized antibodies contain ~10% murine sequences and ~90% human sequences, and thus further reduce or eliminate immunogenicity, while retaining the antibody specificities (Co MS, and Queen C. 1991 Nature 351: 501-501; Morrison SL. 1992 Ann. Rev. Immun. 10:239-265). Humanized antibodies and methods of their production are well-known in the art (U.S. Pat. Nos. 5,530,101, 5,585,089, 5,693,762, and 6,180,370).

RANBP2-specific single chain antibodies which are recombinant, single chain polypeptides formed by linking the heavy and light chain fragments of the Fv regions via an amino acid bridge, can be produced by methods known in the art (U.S. Pat. No. 4,946,778; Bird, Science (1988) 242:423-426; Huston et al., Proc. Natl. Acad. Sci. USA (1988) 85:5879-5883; and Ward et al., Nature (1989) 334:544-546).

Other suitable techniques for antibody production involve in vitro exposure of lymphocytes to the antigenic polypeptides or alternatively to selection of libraries of antibodies in phage or similar vectors (Huse et al., Science (1989) 246:1275-1281). As

used herein, T-cell antigen receptors are included within the scope of antibody modulators (Harlow and Lane, 1988, *supra*).

The polypeptides and antibodies of the present invention may be used with or without modification. Frequently, antibodies will be labeled by joining, either covalently or non-covalently, a substance that provides for a detectable signal, or that is toxic to cells that express the targeted protein (Menard S, et al., Int J. Biol Markers (1989) 4:131-134). A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Suitable labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent moieties, fluorescent emitting lanthanide metals, chemiluminescent moieties, bioluminescent moieties, magnetic particles, and the like (U.S. Pat. Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241). Also, recombinant immunoglobulins may be produced (U.S. Pat. No. 4,816,567). Antibodies to cytoplasmic polypeptides may be delivered and reach their targets by conjugation with membrane-penetrating toxin proteins (U.S. Pat. No. 6,086,900).

When used therapeutically in a patient, the antibodies of the subject invention are typically administered parenterally, when possible at the target site, or intravenously. The therapeutically effective dose and dosage regimen is determined by clinical studies. Typically, the amount of antibody administered is in the range of about 0.1 mg/kg –to about 10 mg/kg of patient weight. For parenteral administration, the antibodies are formulated in a unit dosage injectable form (e.g., solution, suspension, emulsion) in association with a pharmaceutically acceptable vehicle. Such vehicles are inherently nontoxic and non-therapeutic. Examples are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Nonaqueous vehicles such as fixed oils, ethyl oleate, or liposome carriers may also be used. The vehicle may contain minor amounts of additives, such as buffers and preservatives, which enhance isotonicity and chemical stability or otherwise enhance therapeutic potential. The antibodies' concentrations in such vehicles are typically in the range of about 1 mg/ml to about10 mg/ml. Immunotherapeutic methods are further described in the literature (US Pat. No. 5,859,206; WO0073469).

**Nucleic Acid Modulators**

Other preferred RANBP2-modulating agents comprise nucleic acid molecules, such as antisense oligomers or double stranded RNA (dsRNA), which generally inhibit

RANBP2 activity. Preferred nucleic acid modulators interfere with the function of the RANBP2 nucleic acid such as DNA replication, transcription, translocation of the RANBP2 RNA to the site of protein translation, translation of protein from the RANBP2 RNA, splicing of the RANBP2 RNA to yield one or more mRNA species, or catalytic activity which may be engaged in or facilitated by the RANBP2 RNA.

In one embodiment, the antisense oligomer is an oligonucleotide that is sufficiently complementary to a RANBP2 mRNA to bind to and prevent translation, preferably by binding to the 5′ untranslated region. RANBP2-specific antisense oligonucleotides, preferably range from at least 6 to about 200 nucleotides. In some embodiments the oligonucleotide is preferably at least 10, 15, or 20 nucleotides in length. In other embodiments, the oligonucleotide is preferably less than 50, 40, or 30 nucleotides in length. The oligonucleotide can be DNA or RNA or a chimeric mixture or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone. The oligonucleotide may include other appending groups such as peptides, agents that facilitate transport across the cell membrane, hybridization-triggered cleavage agents, and intercalating agents.

In another embodiment, the antisense oligomer is a phosphothioate morpholino oligomer (PMO). PMOs are assembled from four different morpholino subunits, each of which contain one of four genetic bases (A, C, G, or T) linked to a six-membered morpholine ring. Polymers of these subunits are joined by non-ionic phosphodiamidate intersubunit linkages. Details of how to make and use PMOs and other antisense oligomers are well known in the art (e.g. see WO99/18193; Probst JC, Antisense Oligodeoxynucleotide and Ribozyme Design, Methods. (2000) 22(3):271-281; Summerton J, and Weller D. 1997 Antisense Nucleic Acid Drug Dev. :7:187-95; US Pat. No. 5,235,033; and US Pat No. 5,378,841).

Alternative preferred RANBP2 nucleic acid modulators are double-stranded RNA species mediating RNA interference (RNAi). 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. Methods relating to the use of RNAi to silence genes in *C. elegans*, *Drosophila*, plants, and humans are known in the art (Fire A, et al., 1998 Nature 391:806-811; Fire, A. Trends Genet. 15, 358-363 (1999); Sharp, P. A. RNA interference 2001. Genes Dev. 15, 485-490 (2001); Hammond, S. M., et al., Nature Rev. Genet. 2, 110-1119 (2001); Tuschl, T. Chem. Biochem. 2, 239-

245 (2001); Hamilton, A. et al., Science 286, 950-952 (1999); Hammond, S. M., et al., Nature 404, 293-296 (2000); Zamore, P. D., et al., Cell 101, 25-33 (2000); Bernstein, E., et al., Nature 409, 363-366 (2001); Elbashir, S. M., et al., Genes Dev. 15, 188-200 (2001); WO0129058; WO9932619; Elbashir SM, et al., 2001 Nature 411:494-498).

Nucleic acid modulators are commonly used as research reagents, diagnostics, and therapeutics. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used to elucidate the function of particular genes (see, for example, U.S. Pat. No. 6,165,790). Nucleic acid modulators are also used, for example, to distinguish between functions of various members of a biological pathway. For example, antisense oligomers have been employed as therapeutic moieties in the treatment of disease states in animals and man and have been demonstrated in numerous clinical trials to be safe and effective (Milligan JF, *et al*, Current Concepts in Antisense Drug Design, J Med Chem. (1993) 36:1923-1937; Tonkinson JL *et al.*, Antisense Oligodeoxynucleotides as Clinical Therapeutic Agents, Cancer Invest. (1996) 14:54-65). Accordingly, in one aspect of the invention, a RANBP2-specific nucleic acid modulator is used in an assay to further elucidate the role of the RANBP2 in the PTEN/IGF pathway, and/or its relationship to other members of the pathway. In another aspect of the invention, a RANBP2-specific antisense oligomer is used as a therapeutic agent for treatment of PTEN/IGF-related disease states.

**Assay Systems**

The invention provides assay systems and screening methods for identifying specific modulators of RANBP2 activity. As used herein, an "assay system" encompasses all the components required for performing and analyzing results of an assay that detects and/or measures a particular event. In general, primary assays are used to identify or confirm a modulator's specific biochemical or molecular effect with respect to the RANBP2 nucleic acid or protein. In general, secondary assays further assess the activity of a RANBP2 modulating agent identified by a primary assay and may confirm that the modulating agent affects RANBP2 in a manner relevant to the PTEN/IGF pathway. In some cases, RANBP2 modulators will be directly tested in a secondary assay.

In a preferred embodiment, the screening method comprises contacting a suitable assay system comprising a RANBP2 polypeptide or nucleic acid with a candidate agent under conditions whereby, but for the presence of the agent, the system provides a reference activity (e.g. binding activity), which is based on the particular molecular event

the screening method detects. A statistically significant difference between the agent-biased activity and the reference activity indicates that the candidate agent modulates RANBP2 activity, and hence the PTEN/IGF pathway. The RANBP2 polypeptide or nucleic acid used in the assay may comprise any of the nucleic acids or polypeptides described above.

**Primary Assays**

The type of modulator tested generally determines the type of primary assay.

***Primary assays for small molecule modulators***

For small molecule modulators, screening assays are used to identify candidate modulators. Screening assays may be cell-based or may use a cell-free system that recreates or retains the relevant biochemical reaction of the target protein (reviewed in Sittampalam GS *et al.*, Curr Opin Chem Biol (1997) 1:384-91 and accompanying references). As used herein the term "cell-based" refers to assays using live cells, dead cells, or a particular cellular fraction, such as a membrane, endoplasmic reticulum, or mitochondrial fraction. The term "cell free" encompasses assays using substantially purified protein (either endogenous or recombinantly produced), partially purified or crude cellular extracts. Screening assays may detect a variety of molecular events, including protein-DNA interactions, protein-protein interactions (*e.g.*, receptor-ligand binding), transcriptional activity (*e.g.*, using a reporter gene), enzymatic activity (*e.g.*, via a property of the substrate), activity of second messengers, immunogenicty and changes in cellular morphology or other cellular characteristics. Appropriate screening assays may use a wide range of detection methods including fluorescent, radioactive, colorimetric, spectrophotometric, and amperometric methods, to provide a read-out for the particular molecular event detected.

Cell-based screening assays usually require systems for recombinant expression of RANBP2 and any auxiliary proteins demanded by the particular assay. Appropriate methods for generating recombinant proteins produce sufficient quantities of proteins that retain their relevant biological activities and are of sufficient purity to optimize activity and assure assay reproducibility. Yeast two-hybrid and variant screens, and mass spectrometry provide preferred methods for determining protein-protein interactions and elucidation of protein complexes. In certain applications, when RANBP2-interacting proteins are used in screens to identify small molecule modulators, the binding specificity

of the interacting protein to the RANBP2 protein may be assayed by various known methods such as substrate processing (e.g. ability of the candidate RANBP2-specific binding agents to function as negative effectors in RANBP2-expressing cells), binding equilibrium constants (usually at least about $10^7$ $M^{-1}$, preferably at least about $10^8$ $M^{-1}$, more preferably at least about $10^9$ $M^{-1}$), and immunogenicity (e.g. ability to elicit RANBP2 specific antibody in a heterologous host such as a mouse, rat, goat or rabbit). For enzymes and receptors, binding may be assayed by, respectively, substrate and ligand processing.

The screening assay may measure a candidate agent's ability to specifically bind to or modulate activity of a RANBP2 polypeptide, a fusion protein thereof, or to cells or membranes bearing the polypeptide or fusion protein. The RANBP2 polypeptide can be full length or a fragment thereof that retains functional RANBP2 activity. The RANBP2 polypeptide may be fused to another polypeptide, such as a peptide tag for detection or anchoring, or to another tag. The RANBP2 polypeptide is preferably human RANBP2, or is an ortholog or derivative thereof as described above. In a preferred embodiment, the screening assay detects candidate agent-based modulation of RANBP2 interaction with a binding target, such as an endogenous or exogenous protein or other substrate that has RANBP2 –specific binding activity, and can be used to assess normal RANBP2 gene function.

Suitable assay formats that may be adapted to screen for RANBP2 modulators are known in the art. Preferred screening assays are high throughput or ultra high throughput and thus provide automated, cost-effective means of screening compound libraries for lead compounds (Fernandes PB, Curr Opin Chem Biol (1998) 2:597-603; Sundberg SA, Curr Opin Biotechnol 2000, 11:47-53). In one preferred embodiment, screening assays uses fluorescence technologies, including fluorescence polarization, time-resolved fluorescence, and fluorescence resonance energy transfer. These systems offer means to monitor protein-protein or DNA-protein interactions in which the intensity of the signal emitted from dye-labeled molecules depends upon their interactions with partner molecules (*e.g.*, Selvin PR, Nat Struct Biol (2000) 7:730-4; Fernandes PB, *supra*; Hertzberg RP and Pope AJ, Curr Opin Chem Biol (2000) 4:445-451).

A variety of suitable assay systems may be used to identify candidate RANBP2 and PTEN/IGF pathway modulators (.S. Pat. Nos. 5,550,019 and 6,133,437 (apoptosis assays); and U.S. Pat. Nos. 5,976,782, 6,225,118 and 6,444,434 (angiogenesis assays), among others). Specific preferred assays are described in more detail below.

**Apoptosis assays.** Assays for apoptosis may be performed by terminal deoxynucleotidyl transferase-mediated digoxigenin-11-dUTP nick end labeling (TUNEL) assay. The TUNEL assay is used to measure nuclear DNA fragmentation characteristic of apoptosis ( Lazebnik *et al.*, 1994, Nature 371, 346), by following the incorporation of fluorescein-dUTP (Yonehara *et al.*, 1989, J. Exp. Med. 169, 1747). Apoptosis may further be assayed by acridine orange staining of tissue culture cells (Lucas, R., et al., 1998, Blood 15:4730-41). Other cell-based apoptosis assays include the caspase-3/7 assay and the cell death nucleosome ELISA assay. The caspase 3/7 assay is based on the activation of the caspase cleavage activity as part of a cascade of events that occur during programmed cell death in many apoptotic pathways. In the caspase 3/7 assay (commercially available Apo-ONE™ Homogeneous Caspase-3/7 assay from Promega, cat# 67790), lysis buffer and caspase substrate are mixed and added to cells. The caspase substrate becomes fluorescent when cleaved by active caspase 3/7. The nucleosome ELISA assay is a general cell death assay known to those skilled in the art, and available commercially (Roche, Cat# 1774425). This assay is a quantitative sandwich-enzyme-immunoassay which uses monoclonal antibodies directed against DNA and histones respectively, thus specifically determining amount of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. Mono and oligonucleosomes are enriched in the cytoplasm during apoptosis due to the fact that DNA fragmentation occurs several hours before the plasma membrane breaks down, allowing for accumalation in the cytoplasm. Nucleosomes are not present in the cytoplasmic fraction of cells that are not undergoing apoptosis. An apoptosis assay system may comprise a cell that expresses a RANBP2, and that optionally has defective PTEN/IGF function (e.g. PTEN/IGF is over-expressed or under-expressed relative to wild-type cells). A test agent can be added to the apoptosis assay system and changes in induction of apoptosis relative to controls where no test agent is added, identify candidate PTEN/IGF modulating agents. In some embodiments of the invention, an apoptosis assay may be used as a secondary assay to test a candidate PTEN/IGF modulating agents that is initially identified using a cell-free assay system. An apoptosis assay may also be used to test whether RANBP2 function plays a direct role in apoptosis. For example, an apoptosis assay may be performed on cells that over- or under-express RANBP2 relative to wild type cells. Differences in apoptotic response compared to wild type cells suggests that the RANBP2 plays a direct role in the apoptotic response. Apoptosis assays are described further in US Pat. No. 6,133,437.

**Cell proliferation and cell cycle assays.** Cell proliferation may be assayed via bromodeoxyuridine (BRDU) incorporation. This assay identifies a cell population undergoing DNA synthesis by incorporation of BRDU into newly-synthesized DNA. Newly-synthesized DNA may then be detected using an anti-BRDU antibody (Hoshino *et al.*, 1986, Int. J. Cancer 38, 369; Campana *et al.*, 1988, J. Immunol. Meth. 107, 79), or by other means.

Cell proliferation is also assayed via phospho-histone H3 staining, which identifies a cell population undergoing mitosis by phosphorylation of histone H3. Phosphorylation of histone H3 at serine 10 is detected using an antibody specfic to the phosphorylated form of the serine 10 residue of histone H3. (Chadlee,D.N. 1995, J. Biol. Chem 270:20098-105). Cell Proliferation may also be examined using [$^3$H]-thymidine incorporation (Chen, J., 1996, Oncogene 13:1395-403; Jeoung, J., 1995, J. Biol. Chem. 270:18367-73). This assay allows for quantitative characterization of S-phase DNA syntheses. In this assay, cells synthesizing DNA will incorporate [$^3$H]-thymidine into newly synthesized DNA. Incorporation can then be measured by standard techniques such as by counting of radioisotope in a scintillation counter (e.g., Beckman LS 3800 Liquid Scintillation Counter). Another proliferation assay uses the dye Alamar Blue (available from Biosource International), which fluoresces when reduced in living cells and provides an indirect measurement of cell number (Voytik-Harbin SL et al., 1998, In Vitro Cell Dev Biol Anim 34:239-46). Yet another proliferation assay, the MTS assay, is based on in vitro cytotoxicity assessment of industrial chemicals, and uses the soluble tetrazolium salt, MTS. MTS assays are commercially available, for example, the Promega CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Cat.# G5421).

Cell proliferation may also be assayed by colony formation in soft agar (Sambrook et al., Molecular Cloning, Cold Spring Harbor (1989)). For example, cells transformed with RANBP2 are seeded in soft agar plates, and colonies are measured and counted after two weeks incubation.

Cell proliferation may also be assayed by measuring ATP levels as indicator of metabolically active cells. Such assays are commercially available, for example Cell Titer-Glo™, which is a luminescent homogeneous assay available from Promega.

Involvement of a gene in the cell cycle may be assayed by flow cytometry (Gray JW et al. (1986) Int J Radiat Biol Relat Stud Phys Chem Med 49:237-55). Cells transfected with a RANBP2 may be stained with propidium iodide and evaluated in a flow

cytometer (available from Becton Dickinson), which indicates accumulation of cells in different stages of the cell cycle.

Involvement of a gene in cell cycle may also be assayed by FOXO nuclear translocation assays. The FOXO family of transcription factors are mediators of various cellular functions including cell cycle progression and cell death, and are negatively regulated by activation of the PI3 kinase pathway. Akt phosphorylation of FOXO family members leads to FOXO sequestration in the cytoplasm and transcriptional inactivation (Medema, R. H et al (2000) Nature 404: 782-787). PTEN is a negative regulator of PI3 kinase pathway. Activation of PTEN, or loss of PI3 kinase or AKT, prevents phosphorylation of FOXO, leading to accumulation of FOXO in the nucleus, transcriptional activation of FOXO regulated genes, and apoptosis. Alternatively, loss of PTEN leads to pathway activation and cell survival (Nakamura, N. et al (2000) Mol Cell Biol 20: 8969-8982). FOXO translocation into the cytoplasm is used in assays and screens to identify members and/or modulators of the PTEN pathway. FOXO translocation assays using GFP or luciferase as detection reagents are known in the art (e.g., Zhang X et al (2002) J Biol Chem 277:45276-45284; and Li et al (2003) Mol Cell Biol 23:104-118).

Accordingly, a cell proliferation or cell cycle assay system may comprise a cell that expresses a RANBP2, and that optionally has defective PTEN/IGF function (e.g. PTEN/IGF is over-expressed or under-expressed relative to wild-type cells). A test agent can be added to the assay system and changes in cell proliferation or cell cycle relative to controls where no test agent is added, identify candidate PTEN/IGF modulating agents. In some embodiments of the invention, the cell proliferation or cell cycle assay may be used as a secondary assay to test a candidate PTEN/IGF modulating agents that is initially identified using another assay system such as a cell-free assay system. A cell proliferation assay may also be used to test whether RANBP2 function plays a direct role in cell proliferation or cell cycle. For example, a cell proliferation or cell cycle assay may be performed on cells that over- or under-express RANBP2 relative to wild type cells. Differences in proliferation or cell cycle compared to wild type cells suggests that the RANBP2 plays a direct role in cell proliferation or cell cycle.

**Angiogenesis.** Angiogenesis may be assayed using various human endothelial cell systems, such as umbilical vein, coronary artery, or dermal cells. Suitable assays include Alamar Blue based assays (available from Biosource International) to measure proliferation; migration assays using fluorescent molecules, such as the use of Becton

Dickinson Falcon HTS FluoroBlock cell culture inserts to measure migration of cells through membranes in presence or absence of angiogenesis enhancer or suppressors; and tubule formation assays based on the formation of tubular structures by endothelial cells on Matrigel® (Becton Dickinson). Accordingly, an angiogenesis assay system may comprise a cell that expresses a RANBP2, and that optionally has defective PTEN/IGF function (e.g. PTEN/IGF is over-expressed or under-expressed relative to wild-type cells). A test agent can be added to the angiogenesis assay system and changes in angiogenesis relative to controls where no test agent is added, identify candidate PTEN/IGF modulating agents. In some embodiments of the invention, the angiogenesis assay may be used as a secondary assay to test a candidate PTEN/IGF modulating agents that is initially identified using another assay system. An angiogenesis assay may also be used to test whether RANBP2 function plays a direct role in cell proliferation. For example, an angiogenesis assay may be performed on cells that over- or under-express RANBP2 relative to wild type cells. Differences in angiogenesis compared to wild type cells suggests that the RANBP2 plays a direct role in angiogenesis. U.S. Pat. Nos. 5,976,782, 6,225,118 and 6,444,434, among others, describe various angiogenesis assays.

**Hypoxic induction.** The alpha subunit of the transcription factor, hypoxia inducible factor-1 (HIF-1), is upregulated in tumor cells following exposure to hypoxia in vitro. Under hypoxic conditions, HIF-1 stimulates the expression of genes known to be important in tumour cell survival, such as those encoding glyolytic enzymes and VEGF. Induction of such genes by hypoxic conditions may be assayed by growing cells transfected with RANBP2 in hypoxic conditions (such as with 0.1% O2, 5% CO2, and balance N2, generated in a Napco 7001 incubator (Precision Scientific)) and normoxic conditions, followed by assessment of gene activity or expression by Taqman®. For example, a hypoxic induction assay system may comprise a cell that expresses a RANBP2, and that optionally has defective PTEN/IGF function (e.g. PTEN/IGF is over-expressed or under-expressed relative to wild-type cells). A test agent can be added to the hypoxic induction assay system and changes in hypoxic response relative to controls where no test agent is added, identify candidate PTEN/IGF modulating agents. In some embodiments of the invention, the hypoxic induction assay may be used as a secondary assay to test a candidate PTEN/IGF modulating agents that is initially identified using another assay system. A hypoxic induction assay may also be used to test whether RANBP2 function plays a direct role in the hypoxic response. For example, a hypoxic induction assay may

be performed on cells that over- or under-express RANBP2 relative to wild type cells. Differences in hypoxic response compared to wild type cells suggests that the RANBP2 plays a direct role in hypoxic induction.

**Cell adhesion.** Cell adhesion assays measure adhesion of cells to purified adhesion proteins, or adhesion of cells to each other, in presence or absence of candidate modulating agents. Cell-protein adhesion assays measure the ability of agents to modulate the adhesion of cells to purified proteins. For example, recombinant proteins are produced, diluted to 2.5g/mL in PBS, and used to coat the wells of a microtiter plate. The wells used for negative control are not coated. Coated wells are then washed, blocked with 1% BSA, and washed again. Compounds are diluted to 2× final test concentration and added to the blocked, coated wells. Cells are then added to the wells, and the unbound cells are washed off. Retained cells are labeled directly on the plate by adding a membrane-permeable fluorescent dye, such as calcein-AM, and the signal is quantified in a fluorescent microplate reader.

Cell-cell adhesion assays measure the ability of agents to modulate binding of cell adhesion proteins with their native ligands. These assays use cells that naturally or recombinantly express the adhesion protein of choice. In an exemplary assay, cells expressing the cell adhesion protein are plated in wells of a multiwell plate. Cells expressing the ligand are labeled with a membrane-permeable fluorescent dye, such as BCECF , and allowed to adhere to the monolayers in the presence of candidate agents. Unbound cells are washed off, and bound cells are detected using a fluorescence plate reader.

High-throughput cell adhesion assays have also been described. In one such assay, small molecule ligands and peptides are bound to the surface of microscope slides using a microarray spotter, intact cells are then contacted with the slides, and unbound cells are washed off. In this assay, not only the binding specificity of the peptides and modulators against cell lines are determined, but also the functional cell signaling of attached cells using immunofluorescence techniques in situ on the microchip is measured (Falsey JR et al., Bioconjug Chem. 2001 May-Jun;12(3):346-53).

**Tubulogenesis.** Tubulogenesis assays monitor the ability of cultured cells, generally endothelial cells, to form tubular structures on a matrix substrate, which generally simulates the environment of the extracellular matrix. Exemplary substrates

include Matrigel™ (Becton Dickinson), an extract of basement membrane proteins containing laminin, collagen IV, and heparin sulfate proteoglycan, which is liquid at 4° C and forms a solid gel at 37° C. Other suitable matrices comprise extracellular components such as collagen, fibronectin, and/or fibrin. Cells are stimulated with a pro-angiogenic stimulant, and their ability to form tubules is detected by imaging. Tubules can generally be detected after an overnight incubation with stimuli, but longer or shorter time frames may also be used. Tube formation assays are well known in the art (e.g., Jones MK et al., 1999, Nature Medicine 5:1418-1423). These assays have traditionally involved stimulation with serum or with the growth factors FGF or VEGF. Serum represents an undefined source of growth factors. In a preferred embodiment, the assay is performed with cells cultured in serum free medium, in order to control which process or pathway a candidate agent modulates. Moreover, we have found that different target genes respond differently to stimulation with different pro-angiogenic agents, including inflammatory angiogenic factors such as TNF-alpa. Thus, in a further preferred embodiment, a tubulogenesis assay system comprises testing a RANBP2's response to a variety of factors, such as FGF, VEGF, phorbol myristate acetate (PMA), TNF-alpha, ephrin, etc.

**Cell Migration.** An invasion/migration assay (also called a migration assay) tests the ability of cells to overcome a physical barrier and to migrate towards pro-angiogenic signals. Migration assays are known in the art (e.g., Paik JH et al., 2001, J Biol Chem 276:11830-11837). In a typical experimental set-up, cultured endothelial cells are seeded onto a matrix-coated porous lamina, with pore sizes generally smaller than typical cell size. The matrix generally simulates the environment of the extracellular matrix, as described above. The lamina is typically a membrane, such as the transwell polycarbonate membrane (Corning Costar Corporation, Cambridge, MA), and is generally part of an upper chamber that is in fluid contact with a lower chamber containing pro-angiogenic stimuli. Migration is generally assayed after an overnight incubation with stimuli, but longer or shorter time frames may also be used. Migration is assessed as the number of cells that crossed the lamina, and may be detected by staining cells with hemotoxylin solution (VWR Scientific, South San Francisco, CA), or by any other method for determining cell number. In another exemplary set up, cells are fluorescently labeled and migration is detected using fluorescent readings, for instance using the Falcon HTS FluoroBlok (Becton Dickinson). While some migration is observed in the absence of stimulus, migration is greatly increased in response to pro-angiogenic factors. As

described above, a preferred assay system for migration/invasion assays comprises testing a RANBP2's response to a variety of pro-angiogenic factors, including tumor angiogenic and inflammatory angiogenic agents, and culturing the cells in serum free medium.

**Sprouting assay.** A sprouting assay is a three-dimensional *in vitro* angiogenesis assay that uses a cell-number defined spheroid aggregation of endothelial cells ("spheroid"), embedded in a collagen gel-based matrix. The spheroid can serve as a starting point for the sprouting of capillary-like structures by invasion into the extracellular matrix (termed "cell sprouting") and the subsequent formation of complex anastomosing networks (Korff and Augustin, 1999, J Cell Sci 112:3249-58). In an exemplary experimental set-up, spheroids are prepared by pipetting 400 human umbilical vein endothelial cells into individual wells of a nonadhesive 96-well plates to allow overnight spheroidal aggregation (Korff and Augustin: J Cell Biol 143: 1341-52, 1998). Spheroids are harvested and seeded in 900$\mu$l of methocel-collagen solution and pipetted into individual wells of a 24 well plate to allow collagen gel polymerization. Test agents are added after 30 min by pipetting 100 $\mu$l of 10-fold concentrated working dilution of the test substances on top of the gel. Plates are incubated at 37°C for 24h. Dishes are fixed at the end of the experimental incubation period by addition of paraformaldehyde. Sprouting intensity of endothelial cells can be quantitated by an automated image analysis system to determine the cumulative sprout length per spheroid.

***Primary assays for antibody modulators***

For antibody modulators, appropriate primary assays test is a binding assay that tests the antibody's affinity to and specificity for the RANBP2 protein. Methods for testing antibody affinity and specificity are well known in the art (Harlow and Lane, 1988, 1999, *supra*). The enzyme-linked immunosorbant assay (ELISA) is a preferred method for detecting RANBP2-specific antibodies; others include FACS assays, radioimmunoassays, and fluorescent assays.

In some cases, screening assays described for small molecule modulators may also be used to test antibody modulators.

***Primary assays for nucleic acid modulators***

For nucleic acid modulators, primary assays may test the ability of the nucleic acid modulator to inhibit or enhance RANBP2 gene expression, preferably mRNA expression.

In general, expression analysis comprises comparing RANBP2 expression in like populations of cells (*e.g.*, two pools of cells that endogenously or recombinantly express RANBP2) in the presence and absence of the nucleic acid modulator. Methods for analyzing mRNA and protein expression are well known in the art. For instance, Northern blotting, slot blotting, ribonuclease protection, quantitative RT-PCR (*e.g.*, using the TaqMan®, PE Applied Biosystems), or microarray analysis may be used to confirm that RANBP2 mRNA expression is reduced in cells treated with the nucleic acid modulator (*e.g.*, Current Protocols in Molecular Biology (1994) Ausubel FM *et al.*, *eds.*, John Wiley & Sons, Inc., chapter 4; Freeman WM *et al.*, Biotechniques (1999) 26:112-125; Kallioniemi OP, Ann Med 2001, 33:142-147; Blohm DH and Guiseppi-Elie, A Curr Opin Biotechnol 2001, 12:41-47). Protein expression may also be monitored. Proteins are most commonly detected with specific antibodies or antisera directed against either the RANBP2 protein or specific peptides. A variety of means including Western blotting, ELISA, or in situ detection, are available (Harlow E and Lane D, 1988 and 1999, *supra*).

In some cases, screening assays described for small molecule modulators, particularly in assay systems that involve RANBP2 mRNA expression, may also be used to test nucleic acid modulators.

**Secondary Assays**

Secondary assays may be used to further assess the activity of RANBP2-modulating agent identified by any of the above methods to confirm that the modulating agent affects RANBP2 in a manner relevant to the PTEN/IGF pathway. As used herein, RANBP2-modulating agents encompass candidate clinical compounds or other agents derived from previously identified modulating agent. Secondary assays can also be used to test the activity of a modulating agent on a particular genetic or biochemical pathway or to test the specificity of the modulating agent's interaction with RANBP2.

Secondary assays generally compare like populations of cells or animals (*e.g.*, two pools of cells or animals that endogenously or recombinantly express RANBP2) in the presence and absence of the candidate modulator. In general, such assays test whether treatment of cells or animals with a candidate RANBP2–modulating agent results in changes in the PTEN/IGF pathway in comparison to untreated (or mock- or placebo-treated) cells or animals. Certain assays use "sensitized genetic backgrounds", which, as used herein, describe cells or animals engineered for altered expression of genes in the PTEN/IGF or interacting pathways.

*Cell-based assays*

Cell based assays may detect endogenous PTEN/IGF pathway activity or may rely on recombinant expression of PTEN/IGF pathway components. Any of the aforementioned assays may be used in this cell-based format. Candidate modulators are typically added to the cell media but may also be injected into cells or delivered by any other efficacious means.

*Animal Assays*

A variety of non-human animal models of normal or defective PTEN/IGF pathway may be used to test candidate RANBP2 modulators. Models for defective PTEN/IGF pathway typically use genetically modified animals that have been engineered to mis-express (*e.g.*, over-express or lack expression in) genes involved in the PTEN/IGF pathway. Assays generally require systemic delivery of the candidate modulators, such as by oral administration, injection, etc.

In a preferred embodiment, PTEN/IGF pathway activity is assessed by monitoring neovascularization and angiogenesis. Animal models with defective and normal PTEN/IGF are used to test the candidate modulator's affect on RANBP2 in Matrigel® assays. Matrigel® is an extract of basement membrane proteins, and is composed primarily of laminin, collagen IV, and heparin sulfate proteoglycan. It is provided as a sterile liquid at 4° C, but rapidly forms a solid gel at 37° C. Liquid Matrigel® is mixed with various angiogenic agents, such as bFGF and VEGF, or with human tumor cells which over-express the RANBP2. The mixture is then injected subcutaneously(SC) into female athymic nude mice (Taconic, Germantown, NY) to support an intense vascular response. Mice with Matrigel® pellets may be dosed via oral (PO), intraperitoneal (IP), or intravenous (IV) routes with the candidate modulator. Mice are euthanized 5 - 12 days post-injection, and the Matrigel® pellet is harvested for hemoglobin analysis (Sigma plasma hemoglobin kit). Hemoglobin content of the gel is found to correlate the degree of neovascularization in the gel.

In another preferred embodiment, the effect of the candidate modulator on RANBP2 is assessed via tumorigenicity assays. Tumor xenograft assays are known in the art (see, e.g., Ogawa K et al., 2000, Oncogene 19:6043-6052). Xenografts are typically implanted SC into female athymic mice, 6-7 week old, as single cell suspensions either from a pre-existing tumor or from *in vitro* culture. The tumors which express the RANBP2 endogenously are injected in the flank, 1 x $10^5$ to 1 x $10^7$ cells per mouse in a

volume of 100 μL using a 27gauge needle. Mice are then ear tagged and tumors are measured twice weekly. Candidate modulator treatment is initiated on the day the mean tumor weight reaches 100 mg. Candidate modulator is delivered IV, SC, IP, or PO by bolus administration. Depending upon the pharmacokinetics of each unique candidate modulator, dosing can be performed multiple times per day. The tumor weight is assessed by measuring perpendicular diameters with a caliper and calculated by multiplying the measurements of diameters in two dimensions. At the end of the experiment, the excised tumors maybe utilized for biomarker identification or further analyses. For immunohistochemistry staining, xenograft tumors are fixed in 4% paraformaldehyde, 0.1M phosphate, pH 7.2, for 6 hours at 4°C, immersed in 30% sucrose in PBS, and rapidly frozen in isopentane cooled with liquid nitrogen.

In another preferred embodiment, tumorogenicity is monitored using a hollow fiber assay, which is described in U.S. Pat No. US 5,698,413. Briefly, the method comprises implanting into a laboratory animal a biocompatible, semi-permeable encapsulation device containing target cells, treating the laboratory animal with a candidate modulating agent, and evaluating the target cells for reaction to the candidate modulator. Implanted cells are generally human cells from a pre-existing tumor or a tumor cell line. After an appropriate period of time, generally around six days, the implanted samples are harvested for evaluation of the candidate modulator. Tumorogenicity and modulator efficacy may be evaluated by assaying the quantity of viable cells present in the macrocapsule, which can be determined by tests known in the art, for example, MTT dye conversion assay, neutral red dye uptake, trypan blue staining, viable cell counts, the number of colonies formed in soft agar, the capacity of the cells to recover and replicate in vitro, etc.

In another preferred embodiment, a tumorogenicity assay use a transgenic animal, usually a mouse, carrying a dominant oncogene or tumor suppressor gene knockout under the control of tissue specific regulatory sequences; these assays are generally referred to as transgenic tumor assays. In a preferred application, tumor development in the transgenic model is well characterized or is controlled. In an exemplary model, the "RIP1-Tag2" transgene, comprising the SV40 large T-antigen oncogene under control of the insulin gene regulatory regions is expressed in pancreatic beta cells and results in islet cell carcinomas (Hanahan D, 1985, Nature 315:115-122; Parangi S et al, 1996, Proc Natl Acad Sci USA 93: 2002-2007; Bergers G et al, 1999, Science 284:808-812). An "angiogenic switch," occurs at approximately five weeks, as normally quiescent capillaries in a subset of hyperproliferative islets become angiogenic. The RIP1-TAG2 mice die by age 14

weeks. Candidate modulators may be administered at a variety of stages, including just prior to the angiogenic switch (e.g., for a model of tumor prevention), during the growth of small tumors (e.g., for a model of intervention), or during the growth of large and/or invasive tumors (e.g., for a model of regression). Tumorogenicity and modulator efficacy can be evaluating life-span extension and/or tumor characteristics, including number of tumors, tumor size, tumor morphology, vessel density, apoptotic index, etc.

**Diagnostic and therapeutic uses**

Specific RANBP2-modulating agents are useful in a variety of diagnostic and therapeutic applications where disease or disease prognosis is related to defects in the PTEN/IGF pathway, such as angiogenic, apoptotic, or cell proliferation disorders. Accordingly, the invention also provides methods for modulating the PTEN/IGF pathway in a cell, preferably a cell pre-determined to have defective or impaired PTEN/IGF function (e.g. due to overexpression, underexpression, or misexpression of PTEN/IGF, or due to gene mutations), comprising the step of administering an agent to the cell that specifically modulates RANBP2 activity. Preferably, the modulating agent produces a detectable phenotypic change in the cell indicating that the PTEN/IGF function is restored. The phrase "function is restored", and equivalents, as used herein, means that the desired phenotype is achieved, or is brought closer to normal compared to untreated cells. For example, with restored PTEN/IGF function, cell proliferation and/or progression through cell cycle may normalize, or be brought closer to normal relative to untreated cells. The invention also provides methods for treating disorders or disease associated with impaired PTEN/IGF function by administering a therapeutically effective amount of a RANBP2 -modulating agent that modulates the PTEN/IGF pathway. The invention further provides methods for modulating RANBP2 function in a cell, preferably a cell pre-determined to have defective or impaired RANBP2 function, by administering a RANBP2 -modulating agent. Additionally, the invention provides a method for treating disorders or disease associated with impaired RANBP2 function by administering a therapeutically effective amount of a RANBP2 -modulating agent.

The discovery that RANBP2 is implicated in PTEN/IGF pathway provides for a variety of methods that can be employed for the diagnostic and prognostic evaluation of diseases and disorders involving defects in the PTEN/IGF pathway and for the identification of subjects having a predisposition to such diseases and disorders.

Various expression analysis methods can be used to diagnose whether RANBP2 expression occurs in a particular sample, including Northern blotting, slot blotting, ribonuclease protection, quantitative RT-PCR, and microarray analysis. (*e.g.*, Current Protocols in Molecular Biology (1994) Ausubel FM *et al.*, *eds.*, John Wiley & Sons, Inc., chapter 4; Freeman WM *et al.*, Biotechniques (1999) 26:112-125; Kallioniemi OP, Ann Med 2001, 33:142-147; Blohm and Guiseppi-Elie, Curr Opin Biotechnol 2001, 12:41-47). Tissues having a disease or disorder implicating defective PTEN/IGF signaling that express a RANBP2, are identified as amenable to treatment with a RANBP2 modulating agent. In a preferred application, the PTEN/IGF defective tissue overexpresses a RANBP2 relative to normal tissue. For example, a Northern blot analysis of mRNA from tumor and normal cell lines, or from tumor and matching normal tissue samples from the same patient, using full or partial RANBP2 cDNA sequences as probes, can determine whether particular tumors express or overexpress RANBP2. Alternatively, the TaqMan® is used for quantitative RT-PCR analysis of RANBP2 expression in cell lines, normal tissues and tumor samples (PE Applied Biosystems).

Various other diagnostic methods may be performed, for example, utilizing reagents such as the RANBP2 oligonucleotides, and antibodies directed against a RANBP2, as described above for: (1) the detection of the presence of RANBP2 gene mutations, or the detection of either over- or under-expression of RANBP2 mRNA relative to the non-disorder state; (2) the detection of either an over- or an under-abundance of RANBP2 gene product relative to the non-disorder state; and (3) the detection of perturbations or abnormalities in the signal transduction pathway mediated by RANBP2.

Kits for detecting expression of RANBP2 in various samples, comprising at least one antibody specific to RANBP2, all reagents and/or devices suitable for the detection of antibodies, the immobilization of antibodies, and the like, and instructions for using such kits in diagnosis or therapy are also provided.

Thus, in a specific embodiment, the invention is drawn to a method for diagnosing a disease or disorder in a patient that is associated with alterations in RANBP2 expression, the method comprising: a) obtaining a biological sample from the patient; b) contacting the sample with a probe for RANBP2 expression; c) comparing results from step (b) with a control; and d) determining whether step (c) indicates a likelihood of the disease or disorder. Preferably, the disease is cancer, most preferably pancreatic cancer. The probe may be either DNA or protein, including an antibody.

## EXAMPLES

The following experimental section and examples are offered by way of illustration and not by way of limitation.

I. Drosophila PTEN/IGF synthetic cell lethal screen

RNA interference (RNAi) was used to create dPTEN-deficient cultured Drosophila cells (Schneider S2 cells (Schneider, I. (1972) J. Embryol. Exp. Morph. 27, 363), adapted to serum-free media, from Invitrogen Corp., Carlsbad, CA). Cells were treated for 3 days with dPTEN double stranded RNA (dsRNA) or a control dsRNA representing sequences from a Renilla luciferase cDNA. After a 3 day dsRNA pretreatment, 1 $\mu$M bovine insulin was added to cells treated with dPTEN dsRNA to provide additional stimulation of the IGF/insulin pathway. PTEN-deficient, insulin-stimulated cells and control cells were plated in 384-well format and dsRNA representing approximately 6000 different Drosophila genes were added to individual wells. A cell proliferation assay (AqueousOne™ assay – Promega Corp, Madison, WI) was used to quantify cell viability after 96-hours incubation. For each of the greater than 6000 dsRNA sequences tested in this manner, cell viability data was obtained on dPTEN-deficient, insulin-stimulated cells (insulin and dPTEN dsRNA-treated) and control cells (Renilla luciferase dsRNA-treated). Comparison of this data for each dsRNA identified dsRNA sequences that preferentially reduced the viability of insulin and dPTEN dsRNA treated cells. CG11856 reduced the viability of insulin and dPTEN dsRNA treated cells. Orthologs of the CG11856 are referred to herein as RANBP2.

BLAST analysis (Altschul et al., *supra*) was employed to identify orthologs of Drosophila CG11856. For example, representative sequence from RANBP2, GI# 6382079 (SEQ ID NO:7), shares 27% amino acid identity with CG11856.

Various domains, signals, and functional subunits in proteins were analyzed using the PSORT (Nakai K., and Horton P., Trends Biochem Sci, 1999, 24:34-6; Kenta Nakai, Protein sorting signals and prediction of subcellular localization, Adv. Protein Chem. 54, 277-344 (2000)), PFAM (Bateman A., et al., Nucleic Acids Res, 1999, 27:260-2), SMART (Ponting CP, et al., SMART: identification and annotation of domains from signaling and extracellular protein sequences. Nucleic Acids Res. 1999 Jan 1;27(1):229-32), TM-HMM (Erik L.L. Sonnhammer, Gunnar von Heijne, and Anders Krogh: A hidden Markov model for predicting transmembrane helices in protein sequences. In Proc. of Sixth Int. Conf. on Intelligent Systems for Molecular Biology, p 175-182 Ed J. Glasgow,

T. Littlejohn, F. Major, R. Lathrop, D. Sankoff, and C. Sensen Menlo Park, CA: AAAI Press, 1998), and clust (Remm M, and Sonnhammer E. Classification of transmembrane protein families in the Caenorhabditis elegans genome and identification of human orthologs. Genome Res. 2000 Nov;10(11):1679-89) programs. For example, the RanBP1 domain (PFAM 00638) of RANBP2 from GI# 6382079 (SEQ ID NO:7) is located at approximately amino acid residues 1285 to 1406, 1581 to 1703, 1995 to 2112, and 2510 to 2637. Also, the zinc finger domain of the same protein (PFAM 00641) is located at approximately amino acid residues 1734 to 1763, and 1854 to 1883.

II. High-Throughput In Vitro Fluorescence Polarization Assay

Fluorescently-labeled RANBP2 peptide/substrate are added to each well of a 96-well microtiter plate, along with a test agent in a test buffer (10 mM HEPES, 10 mM NaCl, 6 mM magnesium chloride, pH 7.6). Changes in fluorescence polarization, determined by using a Fluorolite FPM-2 Fluorescence Polarization Microtiter System (Dynatech Laboratories, Inc), relative to control values indicates the test compound is a candidate modifier of RANBP2 activity.

III. High-Throughput In Vitro Binding Assay.

$^{33}$P-labeled RANBP2 peptide is added in an assay buffer (100 mM KCl, 20 mM HEPES pH 7.6, 1 mM $MgCl_2$, 1% glycerol, 0.5% NP-40, 50 mM beta-mercaptoethanol, 1 mg/ml BSA, cocktail of protease inhibitors) along with a test agent to the wells of a Neutralite-avidin coated assay plate and incubated at 25°C for 1 hour. Biotinylated substrate is then added to each well and incubated for 1 hour. Reactions are stopped by washing with PBS, and counted in a scintillation counter. Test agents that cause a difference in activity relative to control without test agent are identified as candidate PTEN/IGF modulating agents.

IV. Immunoprecipitations and Immunoblotting

For coprecipitation of transfected proteins, $3 \times 10^6$ appropriate recombinant cells containing the RANBP2 proteins are plated on 10-cm dishes and transfected on the following day with expression constructs. The total amount of DNA is kept constant in each transfection by adding empty vector. After 24 h, cells are collected, washed once with phosphate-buffered saline and lysed for 20 min on ice in 1 ml of lysis buffer containing 50 mM Hepes, pH 7.9, 250 mM NaCl, 20 mM -glycerophosphate, 1 mM

sodium orthovanadate, 5 mM p-nitrophenyl phosphate, 2 mM dithiothreitol, protease inhibitors (complete, Roche Molecular Biochemicals), and 1% Nonidet P-40. Cellular debris is removed by centrifugation twice at 15,000 × g for 15 min. The cell lysate is incubated with 25 μl of M2 beads (Sigma) for 2 h at 4 °C with gentle rocking.

After extensive washing with lysis buffer, proteins bound to the beads are solubilized by boiling in SDS sample buffer, fractionated by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane and blotted with the indicated antibodies. The reactive bands are visualized with horseradish peroxidase coupled to the appropriate secondary antibodies and the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham Pharmacia Biotech).

V. Expression analysis

All cell lines used in the following experiments are NCI (National Cancer Institute) lines, and are available from ATCC (American Type Culture Collection, Manassas, VA 20110-2209). Normal and tumor tissues were obtained from Impath, UC Davis, Clontech, Stratagene, Ardais, Genome Collaborative, and Ambion.

TaqMan® analysis was used to assess expression levels of the disclosed genes in various samples.

RNA was extracted from each tissue sample using Qiagen (Valencia, CA) RNeasy kits, following manufacturer's protocols, to a final concentration of 50ng/μl. Single stranded cDNA was then synthesized by reverse transcribing the RNA samples using random hexamers and 500ng of total RNA per reaction, following protocol 4304965 of Applied Biosystems (Foster City, CA).

Primers for expression analysis using TaqMan® assay (Applied Biosystems, Foster City, CA) were prepared according to the TaqMan® protocols, and the following criteria: a) primer pairs were designed to span introns to eliminate genomic contamination, and b) each primer pair produced only one product. Expression analysis was performed using a 7900HT instrument.

TaqMan® reactions were carried out following manufacturer's protocols, in 25 μl total volume for 96-well plates and 10 μl total volume for 384-well plates, using 300nM primer and 250 nM probe, and approximately 25ng of cDNA. The standard curve for result analysis was prepared using a universal pool of human cDNA samples, which is a mixture of cDNAs from a wide variety of tissues so that the chance that a target will be

present in appreciable amounts is good. The raw data were normalized using 18S rRNA (universally expressed in all tissues and cells).

For each expression analysis, tumor tissue samples were compared with matched normal tissues from the same patient. A gene was considered overexpressed in a tumor when the level of expression of the gene was 2 fold or higher in the tumor compared with its matched normal sample. In cases where normal tissue was not available, a universal pool of cDNA samples was used instead. In these cases, a gene was considered overexpressed in a tumor sample when the difference of expression levels between a tumor sample and the average of all normal samples from the same tissue type was greater than 2 times the standard deviation of all normal samples (i.e., Tumor – average(all normal samples) > 2 x STDEV(all normal samples) ).

RANBP2 (SEQ ID NO:1) was overexpressed in 50% of pancreatic cancer samples (12 matched tumor sets). A modulator identified by an assay described herein can be further validated for therapeutic effect by administration to a tumor in which the gene is overexpressed. A decrease in tumor growth confirms therapeutic utility of the modulator. Prior to treating a patient with the modulator, the likelihood that the patient will respond to treatment can be diagnosed by obtaining a tumor sample from the patient, and assaying for expression of the gene targeted by the modulator. The expression data for the gene(s) can also be used as a diagnostic marker for disease progression. The assay can be performed by expression analysis as described above, by antibody directed to the gene target, or by any other available detection method.

VI. RANBP2 functional assays

RNAi experiments were carried out to knock down expression of RANBP2 (SEQ ID NO:1) in various cell lines using small interfering RNAs (siRNA, Elbashir et al, *supra*).

Effect of RANBP2 RNAi on cell proliferation and growth. BrdU and Cell Titer-Glo™ assays, as described above, were employed to study the effects of decreased RANBP2 expression on cell proliferation. The results of these experiments indicated that RNAi of RANBP2 decreases proliferation in 231T breast cancer and HCT116 colon cancer, and PC3 prostate cancer cells. MTS cell proliferation assay, as described above, was also employed to study the effects of decreased RANBP2 expression on cell proliferation. The results of this experiment indicated that RNAi of RANBP2 decreased proliferation in PC3 prostate cancer and A549 lung cancer cells. Standard colony growth

assays, as described above, were employed to study the effects of decreased RANBP2 expression on cell growth. Results indicated a decrease in cell growth in A549 lung cancer, PC3 prostate cancer, HCT116 colon cancer, SW480 colon cancer, and RD1 Rhabdomyosarcoma cells.

Effect of RANBP2 RNAi on apoptosis. Nucleosome ELISA apoptosis assay, as described above, was employed to study the effects of decreased RANBP2 expression on apoptosis. RANBP2 RNAi caused apoptosis in A549 lung cancer cells.

RANBP2 FOXO nuclear translocation assays. FOXO nuclear translocation assays, as described above, were employed to assess involvement of RANBP2 in the PTEN/IGF pathway. In these experiments, cells with reduced expression of RANBP2 by RNAi were transiently transfected with a plasmid expressing GFP-tagged FOXO. Automated imaging of cellular components, such as nucleus and cytoplasm were then carried out to assess translocation of FOXO. Results indicated that reduced expression of RANBP2 caused nuclear retention of FOXO, similar to lack of AKT. These results suggest involvement of RANBP2 in the PTEN/IGF pathway.

WHAT IS CLAIMED IS:

1. A method of identifying a candidate PTEN/IGF pathway modulating agent, said method comprising the steps of:

(a) providing an assay system comprising a RANBP2 polypeptide or nucleic acid;

(b) contacting the assay system with a test agent under conditions whereby, but for the presence of the test agent, the system provides a reference activity; and

(c) detecting a test agent-biased activity of the assay system, wherein a difference between the test agent-biased activity and the reference activity identifies the test agent as a candidate PTEN/IGF pathway modulating agent.

2. The method of claim 1 wherein the assay system comprises cultured cells that express the RANBP2 polypeptide.

3. The method of claim 2 wherein the cultured cells additionally have defective PTEN/IGF function.

4. The method of claim 1 wherein the assay system includes a screening assay comprising a RANBP2 polypeptide, and the candidate test agent is a small molecule modulator.

5. The method of claim 4 wherein the assay is a binding assay.

6. The method of claim 1 wherein the assay system is selected from the group consisting of an apoptosis assay system, a cell proliferation assay system, an angiogenesis assay system, and a hypoxic induction assay system.

7. The method of claim 1 wherein the assay system includes a binding assay comprising a RANBP2 polypeptide and the candidate test agent is an antibody.

8. The method of claim 1 wherein the assay system includes an expression assay comprising a RANBP2 nucleic acid and the candidate test agent is a nucleic acid modulator.

9. The method of claim 8 wherein the nucleic acid modulator is an antisense oligomer.

10. The method of claim 8 wherein the nucleic acid modulator is a PMO.

11. The method of claim 1 additionally comprising:

(d) administering the candidate PTEN/IGF pathway modulating agent identified in (c) to a model system comprising cells defective in PTEN/IGF function and, detecting a phenotypic change in the model system that indicates that the PTEN/IGF function is restored.

12. The method of claim 11 wherein the model system is a mouse model with defective PTEN/IGF function.

13. A method for modulating a PTEN/IGF pathway of a cell comprising contacting a cell defective in PTEN/IGF function with a candidate modulator that specifically binds to a RANBP2 polypeptide, whereby PTEN/IGF function is restored.

14. The method of claim 13 wherein the candidate modulator is administered to a vertebrate animal predetermined to have a disease or disorder resulting from a defect in PTEN/IGF function.

15. The method of claim 13 wherein the candidate modulator is selected from the group consisting of an antibody and a small molecule.

16. The method of claim 1, comprising the additional steps of:

(e) providing a secondary assay system comprising cultured cells or a non-human animal expressing RANBP2 ,

(f) contacting the secondary assay system with the test agent of (b) or an agent derived therefrom under conditions whereby, but for the presence of the test agent or agent derived therefrom, the system provides a reference activity; and

(g) detecting an agent-biased activity of the second assay system,

wherein a difference between the agent-biased activity and the reference activity of the second assay system confirms the test agent or agent derived therefrom as a candidate PTEN/IGF pathway modulating agent,

and wherein the second assay detects an agent-biased change in the PTEN/IGF pathway.

17. The method of claim 16 wherein the secondary assay system comprises cultured cells.

18. The method of claim 16 wherein the secondary assay system comprises a non-human animal.

19. The method of claim 18 wherein the non-human animal mis-expresses a PTEN/IGF pathway gene.

20. A method of modulating PTEN/IGF pathway in a mammalian cell comprising contacting the cell with an agent that specifically binds a RANBP2 polypeptide or nucleic acid.

21. The method of claim 20 wherein the agent is administered to a mammalian animal predetermined to have a pathology associated with the PTEN/IGF pathway.

22. The method of claim 20 wherein the agent is a small molecule modulator, a nucleic acid modulator, or an antibody.

23. A method for diagnosing a disease in a patient comprising:

(a) obtaining a biological sample from the patient;

(b) contacting the sample with a probe for RANBP2 expression;

(c) comparing results from step (b) with a control;

(d) determining whether step (c) indicates a likelihood of disease.

24. The method of claim 23 wherein said disease is cancer.

25. The method according to claim 24, wherein said cancer is a pancreatic cancer.

**PATENT COOPERATION TREATY**
**IN THE UNITED STATES RECEIVING OFFICE**

International Application of Exelixis, Inc.

Int. Appl. No.: New

Int. Filing Date: Herewith

For RANBP2 AS MODIFIER OF THE PTEN/IGF PATHWAY AND METHODS OF USE

Attorney Docket No: EX04-037C-PC

## STATEMENT CONCERNING NUCLEOTIDE AND/OR AMINO ACID SEQUENCE LISTING

Mail Stop PCT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Officer:

In compliance with WIPO Standard ST.25, the application submitted herewith is accompanied by a diskette containing SEQ ID NOS. 1-7 in computer readable form under Section 208 and Annex C of the Administrative Instructions. The computer readable Sequence Listing was prepared through the use of the software program "PatentIn" provided by the Patent and Trademark Office. The sequence data recorded on the enclosed diskette are identical to that of the written sequence listing submitted herewith and does not go beyond the disclosure of the international application as filed.

Respectfully submitted,

Dated: 13 May 2004

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SEQUENCE LISTING

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Ile Cys Thr Tyr Ile Asn Val Gln Glu Arg Asp Pro Lys Ala His Arg
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Phe Leu Gly Leu Leu Tyr Glu Leu Glu Glu Asn Thr Asp Lys Ala Val
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Glu Cys Tyr Arg Arg Ser Val Glu Leu Asn Pro Thr Gln Lys Asp Leu
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Val Leu Lys Ile Ala Glu Leu Leu Cys Lys Asn Asp Val Thr Asp Gly
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115 120 125

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Pro Ile Asp Pro Leu Phe Lys His Phe His Ser Val Asp Ile Gln Ala
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Ser Ile Lys Ser Val Val Ser Tyr Trp Asn Leu Ala Leu Ile Phe His
675 680 685

Arg Lys Ala Glu Asp Ile Glu Asn Asp Ala Leu Ser Pro Glu Glu Gln
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755 760 765

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770 775 780

Tyr Ser Leu Ser Pro Ser Lys Ser Tyr Lys Tyr Ser Pro Lys Thr Pro
785 790 795 800

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Pro Thr Lys Gly Pro Val Tyr Gly Met Asn Arg Leu Pro Pro Gln Gln
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1880 1885 1890

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2405 2410 2415

Leu Ala Val Arg Phe Lys Leu Gln Asp Val Ala Asp Ser Phe Lys
2420 2425 2430

Lys Ile Phe Asp Glu Ala Lys Thr Ala Gln Glu Lys Asp Ser Leu
2435 2440 2445

Ile Thr Pro His Val Ser Arg Ser Ser Thr Pro Arg Glu Ser Pro
2450 2455 2460

Cys Gly Lys Ile Ala Val Ala Val Leu Glu Glu Thr Thr Arg Glu
2465 2470 2475

Arg Thr Asp Val Ile Gln Gly Asp Asp Val Ala Asp Ala Thr Ser
2480 2485 2490

Glu Val Glu Val Ser Ser Thr Ser Glu Thr Thr Pro Lys Ala Val
2495 2500 2505

Val Ser Pro Pro Lys Phe Val Phe Gly Ser Glu Ser Val Lys Ser
2510 2515 2520

Ile Phe Ser Ser Glu Lys Ser Lys Pro Phe Ala Phe Gly Asn Ser
2525 2530 2535

Ser Ala Thr Gly Ser Leu Phe Gly Phe Ser Phe Asn Ala Pro Leu
2540 2545 2550

Lys Ser Asn Asn Ser Glu Thr Ser Ser Val Ala Gln Ser Gly Ser
2555 2560 2565

Glu Ser Lys Val Glu Pro Lys Lys Cys Glu Leu Ser Lys Asn Ser
2570 2575 2580

Asp Ile Glu Gln Ser Ser Asp Ser Lys Val Lys Asn Leu Phe Ala
2585 2590 2595

Ser Phe Pro Thr Glu Glu Ser Ser Ile Asn Tyr Thr Phe Lys Thr
2600 2605 2610

Pro Glu Lys Ala Lys Glu Lys Lys Lys Pro Glu Asp Ser Pro Ser
2615 2620 2625

Asp Asp Asp Val Leu Ile Val Tyr Glu Leu Thr Pro Thr Ala Glu
2630 2635 2640

Gln Lys Ala Leu Ala Thr Lys Leu Lys Leu Pro Pro Thr Phe Phe
2645 2650 2655

Cys Tyr Lys Asn Arg Pro Asp Tyr Val Ser Glu Glu Glu Glu Asp
2660 2665 2670

Asp Glu Asp Phe Glu Thr Ala Val Lys Lys Leu Asn Gly Lys Leu
2675 2680 2685

Tyr Leu Asp Gly Ser Glu Lys Cys Arg Pro Leu Glu Glu Asn Thr
2690 2695 2700

Ala Asp Asn Glu Lys Glu Cys Ile Ile Val Trp Glu Lys Lys Pro
2705 2710 2715

Thr Val Glu Glu Lys Ala Lys Ala Asp Thr Leu Lys Leu Pro Pro
2720 2725 2730

Thr Phe Phe Cys Gly Val Cys Ser Asp Thr Asp Glu Asp Asn Gly
2735 2740 2745

Asn Gly Glu Asp Phe Gln Ser Glu Leu Gln Lys Val Gln Glu Ala
2750 2755 2760

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Gln Lys  Ser Gln Thr Glu Glu  Ile Thr Ser Thr Thr  Asp Ser Val
    2765                 2770                 2775

Tyr Thr  Gly Gly Thr Glu Val  Met Val Pro Ser Phe  Cys Lys Ser
    2780                 2785                 2790

Glu Glu  Pro Asp Ser Ile Thr  Lys Ser Ile Ser Ser  Pro Ser Val
    2795                 2800                 2805

Ser Ser  Glu Thr Met Asp Lys  Pro Val Asp Leu Ser  Thr Arg Lys
    2810                 2815                 2820

Glu Ile  Asp Thr Asp Ser Thr  Ser Gln Gly Glu Ser  Lys Ile Val
    2825                 2830                 2835

Ser Phe  Gly Phe Gly Ser Ser  Thr Gly Leu Ser Phe  Ala Asp Leu
    2840                 2845                 2850

Ala Ser  Ser Asn Ser Gly Asp  Phe Ala Phe Gly Ser  Lys Asp Lys
    2855                 2860                 2865

Asn Phe  Gln Trp Ala Asn Thr  Gly Ala Ala Val Phe  Gly Thr Gln
    2870                 2875                 2880

Ser Val  Gly Thr Gln Ser Ala  Gly Lys Val Gly Glu  Asp Glu Asp
    2885                 2890                 2895

Gly Ser  Asp Glu Glu Val Val  His Asn Glu Asp Ile  His Phe Glu
    2900                 2905                 2910

Pro Ile  Val Ser Leu Pro Glu  Val Glu Val Lys Ser  Gly Glu Glu
    2915                 2920                 2925

Asp Glu  Glu Ile Leu Phe Lys  Glu Arg Ala Lys Leu  Tyr Arg Trp
    2930                 2935                 2940

Asp Arg  Asp Val Ser Gln Trp  Lys Glu Arg Gly Val  Gly Asp Ile
    2945                 2950                 2955

Lys Ile  Leu Trp His Thr Met  Lys Asn Tyr Tyr Arg  Ile Leu Met
    2960                 2965                 2970

Arg Arg  Asp Gln Val Phe Lys  Val Cys Ala Asn His  Val Ile Thr
    2975                 2980                 2985
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Lys Thr Met Glu Leu Lys Pro Leu Asn Val Ser Asn Asn Ala Leu
2990 2995 3000

Val Trp Thr Ala Ser Asp Tyr Ala Asp Gly Glu Ala Lys Val Glu
3005 3010 3015

Gln Leu Ala Val Arg Phe Lys Thr Lys Glu Val Ala Asp Cys Phe
3020 3025 3030

Lys Lys Thr Phe Glu Glu Cys Gln Gln Asn Leu Met Lys Leu Gln
3035 3040 3045

Lys Gly His Val Ser Leu Ala Ala Glu Leu Ser Lys Glu Thr Asn
3050 3055 3060

Pro Val Val Phe Phe Asp Val Cys Ala Asp Gly Glu Pro Leu Gly
3065 3070 3075

Arg Ile Thr Met Glu Leu Phe Ser Asn Ile Val Pro Arg Thr Ala
3080 3085 3090

Glu Asn Phe Arg Ala Leu Cys Thr Gly Glu Lys Gly Phe Gly Phe
3095 3100 3105

Lys Asn Ser Ile Phe His Arg Val Ile Pro Asp Phe Val Cys Gln
3110 3115 3120

Gly Gly Asp Ile Thr Lys His Asp Gly Thr Gly Gly Gln Ser Ile
3125 3130 3135

Tyr Gly Asp Lys Phe Glu Asp Glu Asn Phe Asp Val Lys His Thr
3140 3145 3150

Gly Pro Gly Leu Leu Ser Met Ala Asn Gln Gly Gln Asn Thr Asn
3155 3160 3165

Asn Ser Gln Phe Val Ile Thr Leu Lys Lys Ala Glu His Leu Asp
3170 3175 3180

Phe Lys His Val Val Phe Gly Phe Val Lys Asp Gly Met Asp Thr
3185 3190 3195

Val Lys Lys Ile Glu Ser Phe Gly Ser Pro Lys Gly Ser Val Cys
3200 3205 3210

Arg Arg Ile Thr Ile Thr Glu Cys Gly Gln Ile
3215 3220