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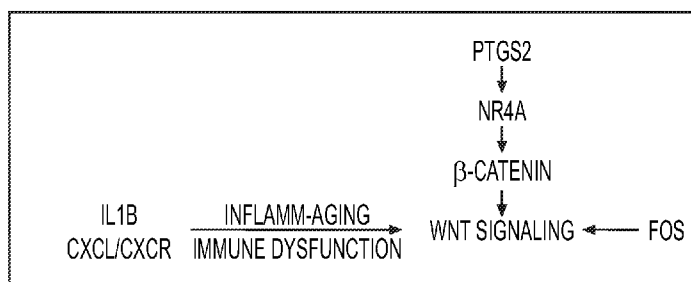


FIG. 2

(57) Abstract: Some embodiments of the invention include methods for treating melanoma in a subject. In other embodiments, the methods for treating melanoma in a subject comprise quantifying an RNA expression level for at least one biomarker in a sample (e.g., from a sentinel lymph node of the subject) and administering to the subject a treatment for melanoma (e.g., by administering to the subject immunotherapy, interferon, a BRAF inhibitor, a checkpoint inhibitor, a Wnt10b inhibitor, an IRAK3 inhibitor, or a combination thereof). In still other embodiments, the at least one biomarker can comprise FOS, NR4A, ITGB1, IRAK3, Wnt10b, or a combination thereof. Additional embodiments of the invention are also discussed herein.

METHODS FOR TREATING MELANOMA

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 63/086,613, filed October 2, 2020, entitled “Methods for Treating Diseases”
5 which is herein incorporated by reference in its entirety.

BACKGROUND

[0002] Melanoma is less common than some other skin cancers, but it is one of the more dangerous forms of skin cancer. To date, treatment of melanoma is limited. Accordingly, some embodiments of the present invention include treating
10 melanoma. Additional embodiments of the invention are also discussed herein.

SUMMARY

[0003] Some embodiments of the present invention include methods for treating melanoma in a subject, the method comprising quantifying an RNA expression level for at least one biomarker in a sample from a sentinel lymph node of
15 the subject, where the at least one biomarker comprises FOS, NR4A, ITGB1, IRAK3, Wnt10b, or a combination thereof, and administering to the subject immunotherapy, interferon, a BRAF inhibitor, a checkpoint inhibitor, a Wnt10b inhibitor, an IRAK3 inhibitor, or a combination thereof.

[0004] In some embodiments, the at least one biomarker comprises NR4A1,
20 NR4A2, NR4A3, or a combination thereof. In certain embodiments, the at least one biomarker comprises NR4A2, NR4A3, or both. In other embodiments, the at least one biomarker comprises NR4A, FOS, Wnt10b, or a combination thereof. In still other embodiments, the at least one biomarker comprises NR4A. In yet other

embodiments, the at least one biomarker comprises IRAK3. In certain embodiments, the at least one biomarker further comprises SFRP2, SFRP4, PTGS2, LINC00518, IL1B, or a combination thereof. In some embodiments, the at least one biomarker further comprises a biomarker listed in Table 2, Table 3, Table 4, Table 5, Table 6, Table 7, Table 8, Table 9, Table 10, Supp. Table 2, Supp. Table 3, Supp. Table 4, Supp. Table 5, Supp. Table 6, or a combination thereof. In other embodiments, the at least one biomarker further comprises ACVR1C, ACVR2A, ALCAM, ALKBH2, ATG7, ATP2B2, BACH2, BAGE, BCOR, BID, C1QBP, C3, C6, CASP9, CCL16, CCL18, CCL4, CD244, CD84, CD8A, CDK4, CDK6, CDKN1A, CLEC4C, CLEC7A, COL28A1, COMP, CTNNB1, CXL/CXCR, CXCL3, CXCL5, CXCR4, CYBB, DKK2, DLK1, DLL4, DNMT3A, DOCK9, DUSP1, ELK1, EPOR, ERCC2, ERCC6, ERGIC3, F2RL1, FANCB, FANCL, FOS, FOSB, FUT8, GADD45A, GRB14, GRIK2, HHEX, HLA-A, HLA-DMA, HLA-DMB, HLA-G, HSP90B1, HSPA1A, ICAM1, IDO1, IFIH1, IFITM1, IGF1R, IL1B, IL23R, IL6, INHBA, INPP5D, IRAK3, ITGB1, ITGBL1, JAM3, KLF4, KLRC4-KLRK1///KLRK1, LAMA5, LIF, LINC00354, LINC00518, LIX1, LOC100507516, LOC101928963, LOC105373225, MAGEA3, MAP2K2, MAP2K4, MAPK11, MAVS, MIF, MKX, MLANA, MME, MS4A6A, MST1R, MUC15, MX1, NCAM1, NFKBIZ, NKD1, NOD1, NOG, NR4A, NR4A1, NR4A2, NR4A3, NRCAM, PBRM1, PCNA, PIK3CB, PIK3R3, PLAUI, PLD1, PPP2R1A, PRKAR2A, PRUNE2, PSMB8, PTGS2, RAD50, RB1, RELA, RGS1, RNF152, RPS6KA5, RUNX1, S100B, SATB1, SFRP2, SFRP4, SLC13A5, SMAD2, SOS1, SPINK5, STK11, TBK1, TFDPI, TFPI2, TGFB3, TLR10, TLR6, TNC, TNFRSF10C, TNFRSF13B, UBB, WNT10B, WWC1, XRCC4, or a combination thereof.

[0005] In some embodiments, the quantifying is carried out using polymerase chain reaction, real-time polymerase chain reaction, reverse transcriptase polymerase chain reaction, real-time quantitative RT-PCR, microarray, NanoString, or a combination thereof. In other embodiments, the quantifying is carried out using
5 polymerase chain reaction, real-time polymerase chain reaction, reverse transcriptase polymerase chain reaction, real-time quantitative RT-PCR, or a combination thereof.

[0006] In certain embodiments, the subject is no more than about 30 years old, no more than about 40 years old, no more than about 50 years old, no more than about 60 years old, or no more than about 70 years old. In other embodiments, the subject is
10 at least about 40 years old, at least about 50 years old, at least about 60 years old, at least about 70 years old, or at least about 80 years old.

[0007] In some embodiments, the subject has a positive sentinel lymph node status.

[0008] In other embodiments, the quantifying is relative to a control sample
15 where the melanoma was without recurrence for at least about 5.0 years.

[0009] In yet other embodiments, the fold change in the RNA expression level, relative to a control sample where the melanoma was without recurrence for at least about 5.0 years, in one or more of the at least one biomarker is at least about 1.0, at least about 1.5, at least about 2.0, at least about 2.5, at least about 3.0, at least about
20 3.5, at least about 4.0, or at least about 4.5.

[0010] In some embodiments, the method further comprises assessing a clinicopathologic feature of the subject from which the sample was obtained. In certain embodiments, the method further comprises assessing a clinicopathologic feature of the subject from which the sample was obtained and the clinicopathologic

feature is age, gender, anatomic location, Breslow thickness, ulceration, sentinel lymph node status, or a combination thereof. In other embodiments, the method further comprises assessing a clinicopathologic feature of the subject from which the sample was obtained and the clinicopathologic feature is metastasis, age, lesion site, tumor burden, number of positive nodes, ulceration, tumor thickness, or a combination thereof.

[0011] In some embodiments, the subject has stage III melanoma.

[0012] In certain embodiments, the administering comprises administering interferon, interferon alfa-2b, or both. In other embodiments, the administering comprises administering a BRAF inhibitor, vemurafenib, dabrafenib, trametinib, encorafenib, or a combination thereof. In still other embodiments, the administering comprises administering a checkpoint inhibitor, a PD-1 inhibitor, nivolumab, pembrolizumab, cemiplimab, a PD-L1 inhibitor, atezolizumab, avelumab, durvalumab, a cytotoxic T-lymphocyte antigen 4 inhibitor, ipilimumab, or a combination thereof. In yet other embodiments, the administering comprises administering a Wnt10b inhibitor, DKN-01, CGX1321, ETC 1922159, or a combination thereof. In certain embodiments, (a) the administering comprises administering a Wnt10b inhibitor, DKN-01, CGX1321, ETC 1922159 or a combination thereof and (b) the subject is at least about 60 years old. In some embodiments, the administering comprises administering an IRAK3 inhibitor, pacritinib, thymoquinone, or a combination thereof. In other embodiments, (a) the administering comprises administering an IRAK3 inhibitor, pacritinib, thymoquinone, or a combination thereof and (b) the subject is no more than about 60 years old.

[0013] In certain embodiments, the administering only occurs if the fold change in the RNA expression level, relative to a control sample where the melanoma was without recurrence for at least about 5.0 years, in one or more of the at least one biomarker is at least about 1.0, at least about 1.5, at least about 2.0, at least about 2.5, at least about 3.0, at least about 3.5, at least about 4.0, or at least about 4.5.

[0014] In yet other embodiments, the administering comprises administering a Wnt10b inhibitor, DKN-01, CGX1321, ETC 1922159, or a combination thereof, and the administering only occurs if (a) the fold change in the RNA expression level, relative to a control sample where the melanoma was without recurrence for at least about 5.0 years, in one or more of the at least one biomarker is at least about 1.0, at least about 1.5, at least about 2.0, at least about 2.5, at least about 3.0, at least about 3.5, at least about 4.0, or at least about 4.5 and (b) the subject is at least about 60 years old.

[0015] In some embodiments, the administering comprises administering of IRAK3 inhibitor, pacritinib, thymoquinone, or a combination thereof, and the administering only occurs if (a) the fold change in the RNA expression level, relative to a control sample where the melanoma was without recurrence for at least about 5.0 years, in one or more of the at least one biomarker is at least about 1.0, at least about 1.5, at least about 2.0, at least about 2.5, at least about 3.0, at least about 3.5, at least about 4.0, or at least about 4.5 and (b) the subject is no more than about 60 years old.

[0016] In still other embodiments, the treating further comprises one or more of surgery, chemotherapy, radiation therapy, targeted therapy, or vaccine therapy.

[0017] In certain embodiments, the subject is a mammal, a primate, or a human. In other embodiments, the subject is a human.

[0018] In other embodiments, the method comprises quantifying an RNA expression level for at least one biomarker in a sample from a sentinel lymph node of the human, where the at least one biomarker comprises FOS, NR4A, Wnt10b, or a combination thereof, and administering to the human a Wnt10b inhibitor, DKN-01, CGX1321, ETC 1922159, or a combination thereof, and optionally one or more of immunotherapy, interferon, a BRAF inhibitor, a checkpoint inhibitor, or an IRAK3 inhibitor; the human is at least about 60 years old, and the administering only occurs if the fold change in the RNA expression level, relative to a control sample where the melanoma was without recurrence for at least about 5.0 years, in one or more of the at least one biomarker is at least about 1.5.

[0019] In still other embodiments, the method comprises quantifying an RNA expression level for at least one biomarker in a sample from a sentinel lymph node of the human, where the at least one biomarker comprises IRAK3 and administering to the human an IRAK3 inhibitor, pacritinib, thymoquinone, or a combination thereof, and optionally one or more of immunotherapy, interferon, a BRAF inhibitor, a checkpoint inhibitor, or a Wnt10b inhibitor; the human is no more than about 60 years old, and the administering only occurs if the fold change in the RNA expression level, relative to a control sample where the melanoma was without recurrence for at least about 5.0 years, in one or more of the at least one biomarker is at least about 1.5.

[0020] Other embodiments of the invention are also discussed herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The

invention may be better understood by reference to one or more of these drawings in combination with the description of specific embodiments presented herein.

[0022] **FIG. 1:** The network connection of the 156 DEGs in the older versus the younger patients by microarray T4 filter.

5 [0023] **FIG. 2:** A schematic model showing that the DEGs in the older melanoma patients by recurrence status converge at the Wnt signaling pathway.

DETAILED DESCRIPTION

[0024] Some embodiments of the invention include methods for treating
10 melanoma in a subject (e.g., primate or human), the method comprising quantifying an RNA expression level for at least one biomarker in a sample from a sentinel lymph node (SLN) of the subject and then treating the subject (e.g., by administering one or more molecules to the subject). In certain embodiments, the at least one biomarker comprises one or more of FOS, NR4A, ITGB1, IRAK3, or Wnt10b. In other
15 embodiments, treating the subject comprises administering to the subject with one or more of immunotherapy, interferon, a BRAF inhibitor, a checkpoint inhibitor, a Wnt10b inhibitor, or an IRAK3 inhibitor.

[0025] Any suitable method for RNA extraction can be used. In some
embodiments, RNA isolation can be performed using purification kit, buffer set and
20 protease from commercial manufacturers according to the manufacturer's instructions. In other embodiments, total RNA from cells in culture can be isolated using Qiagen RNeasy mini-columns. Numerous RNA isolation kits are commercially available and can be used in the methods of the invention.

[0026] The method for quantifying an RNA expression level can be any suitable method including but not limited to using a microarray, NanoString (e.g., NanoString technologies, Seattle, WA, USA), real-time polymerase chain reaction (RT-PCR), real time quantitative PCR (e.g., which measures PCR product accumulation through a dual-labeled fluorogenic probe), quantitative competitive PCR (e.g., where internal competitor for each target sequence is used for normalization), quantitative comparative PCR (e.g., which uses a normalization gene contained within the sample), or a combination thereof. In certain embodiments, the quantifying is carried out using polymerase chain reaction, real-time polymerase chain reaction, reverse transcriptase polymerase chain reaction, real-time quantitative RT-PCR, microarray, NanoString, or a combination thereof. In other embodiments, the quantifying is carried out using polymerase chain reaction, real-time polymerase chain reaction, reverse transcriptase polymerase chain reaction, real-time quantitative RT-PCR, or a combination thereof.

[0027] In some instances, one of the first steps in gene expression profiling by RT-PCR is the reverse transcription of the RNA template into cDNA, followed by amplification in a PCR reaction. Reverse transcriptases include, but are not limited to, avilo myeloblastosis virus reverse transcriptase (AMV-RT) and Moloney murine leukemia virus reverse transcriptase (MMLV-RT). The reverse transcription step is sometimes primed using specific primers, random hexamers, or oligo-dT primers, depending on the circumstances and the goal of expression profiling. For example, extracted RNA can be reverse-transcribed using a GeneAmp RNA PCR kit (Perkin Elmer, Calif., USA), following the manufacturer's instructions. The derived cDNA can then be used as a template in the subsequent PCR reaction.

[0028] Although the PCR step can use a variety of thermostable DNA-dependent DNA polymerases, it sometimes employs the Taq DNA polymerase, which has a 5'-3' nuclease activity but lacks a 3'-5' proofreading endonuclease activity.

TaqMan PCR sometimes utilizes the 5'-nuclease activity of Taq or Tth polymerase to

5 hydrolyze a hybridization probe bound to its target amplicon, but any enzyme with equivalent 5' nuclease activity can be used. Two oligonucleotide primers are used to generate an amplicon typical of a PCR reaction. A third oligonucleotide, or probe, is designed to detect nucleotide sequence located between the two PCR primers. The probe is non-extendible by Taq DNA polymerase enzyme, and is labeled with a
10 reporter fluorescent dye and a quencher fluorescent dye. Any laser-induced emission from the reporter dye is quenched by the quenching dye when the two dyes are located close together as they are on the probe. During the amplification reaction, the Taq DNA polymerase enzyme cleaves the probe in a template-dependent manner. The resultant probe fragments disassociate in solution, and signal from the released
15 reporter dye is free from the quenching effect of the second fluorophore. One molecule of reporter dye is liberated for each new molecule synthesized, and detection of the unquenched reporter dye provides the basis for quantitative interpretation of the data.

[0029] TaqManTM RT-PCR can be performed using commercially available
20 equipment, such as, for example, ABI PRISM 7700.TM. Sequence Detection System.TM. (Perkin-Elmer-Applied Biosystems, Foster City, Calif., USA), or Lightcycler (Roche Molecular Biochemicals, Mannheim, Germany). In one specific embodiment, the 5' nuclease procedure is run on a real-time quantitative PCR device such as the ABI PRISM 7700.TM. Sequence Detection System.TM. The system

consists of a thermocycler, laser, charge-coupled device (CCD), camera and computer. The system amplifies samples in a 96-well format on a thermocycler. During amplification, laser-induced fluorescent signal is collected in real-time through fiber optics cables for all 96 wells, and detected at the CCD. The system includes software for running the instrument and for analyzing the data.

[0030] 5'-Nuclease assay data are initially expressed as Ct, or the threshold cycle. As discussed above, fluorescence values are recorded during every cycle and represent the amount of product amplified to that point in the amplification reaction. The point when the fluorescent signal is first recorded as statistically significant is the threshold cycle (Ct).

[0031] To minimize errors and the effect of sample-to-sample variation, RT-PCR is sometimes performed using an internal standard. The ideal internal standard is expressed at a constant level among different tissues, and is unaffected by the experimental treatment. RNAs most frequently used to normalize patterns of gene expression are mRNAs for the housekeeping genes glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), Beta-2-microglobulin (B2M), and β -actin.

[0032] Another variation of the RT-PCR technique is the real time quantitative PCR, which measures PCR product accumulation through a dual-labeled fluorogenic probe (e.g., TaqMan.TM. probe). Real time PCR is compatible both with quantitative competitive PCR, where internal competitor for each target sequence is used for normalization, and with quantitative comparative PCR using a normalization gene contained within the sample, or a housekeeping gene for RT-PCR.

[0033] When conducting RT-PCR analysis, an initial step is the isolation of mRNA from the sample. The starting material is sometimes total RNA isolated from a

SLN. mRNA can be extracted, for example, from frozen or archived paraffin-embedded and fixed (e.g., formalin-fixed) tissue samples.

[0034] The route of administration for any of the treatments of the invention can be of any suitable route. Administration routes can be, but are not limited to the oral route, the parenteral route, the cutaneous route, the nasal route, the rectal route, the vaginal route, and the ocular route. In other embodiments, administration routes can be parenteral administration, a mucosal administration, intravenous administration, subcutaneous administration, topical administration, intradermal administration, oral administration, sublingual administration, intranasal administration, or intramuscular administration. The choice of administration route can depend on the molecule used for treatment (e.g., inhibitor), the physical and chemical properties of the molecule used for treatment, as well as the age and weight of the subject (e.g., human), the particular melanoma, the severity of the melanoma, and the stage of the melanoma. Of course, combinations of administration routes can be administered, as desired.

[0035] In some embodiments, melanoma is a tumor arising from the melanocytic system of the skin and other organs. In other embodiments, melanomas can include, for example, acral-lentiginous melanoma, amelanotic melanoma, benign juvenile melanoma, Cloudman's melanoma, S91 melanoma, Harding-Passey melanoma, juvenile melanoma, lentigo maligna melanoma, malignant melanoma, nodular melanoma subungual melanoma, and superficial spreading melanoma.

[0036] In some embodiments, the sample obtained from a sentinel lymph node refers to a sample that comprises a biomolecule and/or is derived from a sentinel lymph node of the animal. In certain embodiments, biomolecules can include, but are

not limited to total DNA, RNA, miRNA, mRNA, and polypeptides. The sample can be used for the detection of the presence and/or expression level of a biomolecule of interest (e.g., biomarker). Any suitable portion of the lymph node can be used with the methods (e.g., described herein), such as but not limited to biopsy, tissue, tissue
5 section, cell, group of cells, cell fragment, or cell product from the lymph node. In some embodiments, the sample can be provided as a frozen or fresh cell or tissue sample (e.g., paraffin-embedded tissue). In some embodiments, the sample can be provided as an extract (e.g., mRNA extracted from cell or tissue).

[0037] In certain embodiments, the sample obtained from the SLN, or the
10 SLN from which the sample is obtained, can be acquired at a time when sentinel nodes would be normally identified and removed, for example at or around the time of surgery to remove a primary melanoma. In some embodiments, it can be desirable to use a fresh sample, or a paraffin-embedded tissue sample. In other embodiments, it can be desirable to freeze or otherwise store for use at a later date. In still other
15 embodiments, it can be useful to process (e.g., extract) the sample, using a portion for immediate testing and/or saving a portion for use at a later date.

[0038] In some embodiments, the subject is an animal or is a vertebrate animal, such as but not limited to a warm-blooded vertebrate, a mammal, primate or a human. In certain embodiments, veterinary therapeutic uses are provided in
20 accordance with the presently disclosed subject matter. As such, the presently disclosed subject matter provides methods related to mammals such as humans, as well as those mammals (e.g., primates) of importance due to being endangered, such as Siberian tigers; of economic importance, such as animals raised by humans; and/or animals of social importance to humans, such as animals kept as pets or in zoos.

Examples of such animals include but are not limited to: carnivores (e.g., cats and dogs); swine (e.g., pigs, hogs, and wild boars); ruminants and/or ungulates (e.g., cattle, oxen, sheep, giraffes, deer, goats, bison, and camels); and horses (e.g., race horses). In some embodiments, the animal is a human.

5 [0039] In some embodiments, the subject has a single positive sentinel lymph node. In some embodiments, the subject is classified or diagnosed with stage III melanoma; classification with stage III melanoma can occur when there is a presence of at least one positive sentinel lymph node.

[0040] In some embodiments, the at least one biomarker for which RNA
10 expression level is quantified can be any suitable biomarker or set of biomarkers. In certain embodiments, the number of biomarkers quantified can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 27, 29, or 30. In some embodiments, the at least one biomarker comprises one or more of FOS, NR4A, ITGB1, IRAK3, or Wnt10b. In other embodiments, the at least one biomarker
15 comprises two or more of FOS (FBJ murine osteosarcoma viral oncogene homolog), NR4A (nuclear receptor subfamily 4, group A), or ITGB1 (Integrin subunit beta 1). In yet other embodiments, the at least one biomarker comprises NR4A1, NR4A2, NR4A3, or a combination thereof. In certain embodiments, the at least one biomarker comprises NR4A2, NR4A3, or both. In other embodiments, the at least one
20 biomarker comprises NR4A, FOS, Wn10b, or a combination thereof. In some embodiments, the at least one biomarker comprises NR4A. In other embodiments, the at least one biomarker comprises IRAK3. In still other embodiments, the at least one biomarker further comprises SFRP2, SFRP4, PTGS2, LINC00518, IL1B, or a combination thereof. In yet other embodiments, the at least one biomarker further

comprises a biomarker listed in Table 2, Table 3, Table 4, Table 5, Table 6, Table 7, Table 8, Table 9, Table 10, Supp. Table 2, Supp. Table 3, Supp. Table 4, Supp. Table 5, Supp. Table 6, or a combination thereof. In some embodiments, the at least one biomarker further comprises ACVR1C, ACVR2A, ALCAM, ALKBH2, ATG7,

5 ATP2B2, BACH2, BAGE, BCOR, BID, C1QBP, C3, C6, CASP9, CCL16, CCL18, CCL4, CD244, CD84, CD8A, CDK4, CDK6, CDKN1A, CLEC4C, CLEC7A, COL28A1, COMP, CTNNB1, CXL/CXCR, CXCL3, CXCL5, CXCR4, CYBB, DKK2, DLK1, DLL4, DNMT3A, DOCK9, DUSP1, ELK1, EPOR, ERCC2, ERCC6, ERGIC3, F2RL1, FANCB, FANCL, FOS, FOSB, FUT8, GADD45A, GRB14,

10 GRIK2, HHEX, HLA-A, HLA-DMA, HLA-DMB, HLA-G, HSP90B1, HSPA1A, ICAM1, IDO1, IFIH1, IFITM1, IGF1R, IL1B, IL23R, IL6, INHBA, INPP5D, IRAK3, ITGB1, ITGBL1, JAM3, KLF4, KLRC4-KLRK1///KLRK1, LAMA5, LIF, LINC00354, LINC00518, LIX1, LOC100507516, LOC101928963, LOC105373225, MAGEA3, MAP2K2, MAP2K4, MAPK11, MAVS, MIF, MKX, MLANA, MME,

15 MS4A6A, MST1R, MUC15, MX1, NCAM1, NFKBIZ, NKD1, NOD1, NOG, NR4A, NR4A1, NR4A2, NR4A3, NRCAM, PBRM1, PCNA, PIK3CB, PIK3R3, PLAU, PLD1, PPP2R1A, PRKAR2A, PRUNE2, PSMB8, PTGS2, RAD50, RB1, REL, RGS1, RNF152, RPS6KA5, RUNX1, S100B, SATB1, SFRP2, SFRP4, SLC13A5, SMAD2, SOS1, SPINK5, STK11, TBK1, TFDPI, TFPI2, TGFB3, TLR10, TLR6,

20 TNC, TNFRSF10C, TNFRSF13B, UBB, WNT10B, WWC1, XRCC4, or a combination thereof.

Table A - Abbreviations for Biomarkers

ACVR1C	activin A receptor, type IC
ACVR2A	activin A receptor, type IIA
ALCAM	activated leukocyte cell adhesion molecule
ALKBH2	alkB, alkylation repair homolog 2 (E. coli)

ATG7	autophagy related 7
ATP2B2	ATPase, Ca++ transporting, plasma membrane 2
BACH2	BTB and CNC homology 1, basic leucine zipper transcription factor 2
BAGE	B melanoma antigen
BCOR	BCL6 corepressor
BID	BH3 interacting domain death agonist
C1QBP	complement component 1, q subcomponent binding protein
C3	complement component 3
C6	complement component 6
CASP9	caspase 9, apoptosis-related cysteine peptidase
CCL16	chemokine (C-C motif) ligand 16
CCL18	chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)
CCL4	chemokine (C-C motif) ligand 4
CD244	CD244 molecule, natural killer cell receptor 2B4
CD84	CD84 molecule
CD8A	CD8a molecule
CDK4	cyclin-dependent kinase 4
CDK6	cyclin-dependent kinase 6
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)
CLEC4C	C-type lectin domain family 4, member C
CLEC7A	C-type lectin domain family 7, member A
COL28A1	collagen, type XXVIII, alpha 1
COMP	cartilage oligomeric matrix protein
CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa
CXL/CXCR	C-X-C Motif Chemokine Ligand
CXCL3	chemokine (C-X-C motif) ligand 3
CXCL5	chemokine (C-X-C motif) ligand 5
CXCR4	chemokine (C-X-C motif) receptor 4
CYBB	cytochrome b-245, beta polypeptide
DKK2	dickkopf WNT signaling pathway inhibitor 2
DLK1	delta-like 1 homolog (Drosophila)
DLL4	delta-like 4 (Drosophila)
DNMT3A	DNA (cytosine-5-)-methyltransferase 3 alpha
DOCK9	dedicator of cytokinesis 9
DUSP1	dual specificity phosphatase 1
ELK1	ELK1, member of ETS oncogene family
EPOR	erythropoietin receptor
ERCC2	excision repair cross-complementing rodent repair deficiency, complementation group 2
ERCC6	excision repair cross-complementing rodent repair deficiency, complementation group 6
ERGIC3	ERGIC and Golgi 3
F2RL1	coagulation factor II (thrombin) receptor-like 1
FANCB	Fanconi anemia, complementation group B
FANCL	Fanconi anemia, complementation group L

FOS	FBJ murine osteosarcoma viral oncogene homolog
FOSB	FBJ murine osteosarcoma viral oncogene homolog B
FUT8	fucosyltransferase 8 (alpha (1,6) fucosyltransferase)
GADD45A	growth arrest and DNA-damage-inducible, alpha
GRB14	growth factor receptor bound protein 14
GRIK2	glutamate receptor, ionotropic, kainate 2
HHEX	hematopoietically expressed homeobox
HLA-A	major histocompatibility complex, class I, A
HLA-DMA	major histocompatibility complex, class II, DM alpha
HLA-DMB	major histocompatibility complex, class II, DM beta
HLA-G	major histocompatibility complex, class I, G
HSP90B1	heat shock protein 90kDa beta (Grp94), member 1
HSPA1A	heat shock 70kDa protein 1A
ICAM1	intercellular adhesion molecule 1
IDO1	indoleamine 2,3-dioxygenase 1
IFIH1	interferon induced with helicase C domain 1
IFITM1	interferon induced transmembrane protein 1
IGF1R	insulin-like growth factor 1 receptor
IL1B	interleukin 1 beta
IL23R	interleukin 23 receptor
IL6	interleukin 6 (interferon, beta 2)
INHBA	inhibin beta A
INPP5D	inositol polyphosphate-5-phosphatase, 145kDa
IRAK3	interleukin-1 receptor-associated kinase 3
ITGB1	integrin subunit beta 1
ITGBL1	integrin beta like 1
JAM3	junctional adhesion molecule 3
KLF4	Kruppel-like factor 4 (gut)
KLRC4-KLRK1	KLRC4-KLRK1 read through /// killer cell lectin-like receptor subfamily K, member 1
LAMA5	laminin, alpha 5
LIF	leukemia inhibitory factor
LINC00354	long intergenic non-protein coding RNA 354
LINC00518	long intergenic non-protein coding RNA 518
LIX1	limb and CNS expressed 1
LOC100507516	uncharacterized LOC100507516
LOC101928963	uncharacterized LOC101928963
LOC105373225	uncharacterized LOC105373225
MAGEA3	melanoma antigen family A, 3
MAP2K2	mitogen-activated protein kinase kinase 2
MAP2K4	mitogen-activated protein kinase kinase 4
MAPK11	mitogen-activated protein kinase 11
MAVS	mitochondrial antiviral signaling protein
MIF	macrophage migration inhibitory factor (glycosylation-inhibiting factor)
MKX	mohawk homeobox
MLANA	melan-A

MME	membrane metallo-endorpeptidase
MS4A6A	membrane-spanning 4-domains, subfamily A, member 6A
MST1R	macrophage stimulating 1 receptor (c-met-related tyrosine kinase)
MUC15	mucin 15, cell surface associated
MX1	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)
NCAM1	neural cell adhesion molecule 1
NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta
NKD1	naked cuticle homolog 1 (Drosophila)
NOD1	nucleotide-binding oligomerization domain containing 1
NOG	noggin
NR4A	nuclear receptor subfamily 4A
NR4A1	nuclear receptor subfamily 4, group A, member 1
NR4A2	nuclear receptor subfamily 4, group A, member 2
NR4A3	nuclear receptor subfamily 4, group A, member 3
NRCAM	neuronal cell adhesion molecule
PBRM1	polybromo 1
PCNA	proliferating cell nuclear antigen
PIK3CB	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit beta
PIK3R3	phosphoinositide-3-kinase, regulatory subunit 3 (gamma)
PLAU	plasminogen activator, urokinase
PLD1	phospholipase D1, phosphatidylcholine-specific
PPP2R1A	protein phosphatase 2, regulatory subunit A, alpha
PRKAR2A	protein kinase, cAMP-dependent, regulatory, type II, alpha
PRUNE2	prune homolog 2 (Drosophila)
PSMB8	proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional peptidase 7)
PTGS2	Prostaglandin-Endoperoxide Synthase 2 (also named COX2)
RAD50	RAD50 homolog (S. cerevisiae)
RB1	retinoblastoma 1
RELA	v-rel reticuloendotheliosis viral oncogene homolog A (avian)
RGS1	Regulator of G-protein signaling 1
RNF152	ring finger protein 152
RPS6KA5	ribosomal protein S6 kinase, 90kDa, polypeptide 5
RUNX1	runt-related transcription factor 1
S100B	S100 calcium binding protein B
SATB1	SATB homeobox 1
SFRP2	secreted frizzled-related protein 2
SFRP4	secreted frizzled-related protein 4
SLC13A5	solute carrier family 13 (sodium-dependent citrate transporter), member 5
SMAD2	SMAD family member 2
SOS1	son of sevenless homolog 1 (Drosophila)
SPINK5	serine peptidase inhibitor, Kazal type 5

STK11	serine/threonine kinase 11
TBK1	TANK-binding kinase 1
TFDP1	transcription factor Dp-1
TFPI2	tissue factor pathway inhibitor 2
TGFB3	transforming growth factor, beta 3
TLR10	toll-like receptor 10
TLR6	toll-like receptor 6
TNC	tenascin C
TNFRSF10C	tumor necrosis factor receptor superfamily, member 10c, decoy without an intracellular domain
TNFRSF13B	tumor necrosis factor receptor superfamily, member 13B
UBB	ubiquitin B
WNT10B	wingless-type MMTV integration site family, member 10B
WWC1	WW and C2 domain containing 1
XRCC4	X-ray repair complementing defective repair in Chinese hamster cells 4

[0041] In some embodiments, the subject (e.g., human) is no more than about 30 years old, no more than about 40 years old, no more than about 50 years old, no more than about 60 years old, or no more than about 70 years old. In other

5 embodiments, the subject (e.g., human) is at least about 40 years old, at least about 50 years old, at least about 60 years old, at least about 70 years old, or at least about 80 years old. In certain embodiments, the subject is about 10, about 15, about 20, about 25, about 30, about 35, about 40, about 42, about 44, about 45, about 46, about 48, about 50, about 51, about 52, about 53, about 54, about 55, about 56, about 57, about

10 58, about 59, about 60, about 61, about 62, about 63, about 64, about 65, about 66, about 67, about 68, about 69, about 70, about 72, about 74, about 75, about 76, about 78, about 80, about 85, or about 90 years old.

[0042] In some embodiments, the subject (e.g., human) has a positive sentinel lymph node status.

15 [0043] The term “control sample” is used herein to refer to a reference to which a sample can be compared. In other embodiments, the control sample can be a

reference standard. In certain embodiments, a reference standard can be a manufactured control sample, for example, designed to include a predetermined presence or amount of one or more biomarkers to which a sample can be compared. In some embodiments, a reference standard can comprise a compilation about the presence and/or level of one or more biomarkers considered to be control values. In some embodiments, the control sample can be a sample obtained from a sentinel lymph node of a control subject. A “control subject” can be selected with consideration to the subject being tested. In some embodiments, a control subject can be a subject in which melanoma has not recurred for a period of about 3.0, about 4.0, about 5.0, about 6.0, about 7.0, about 8.0, about 9.0, about 10.0, or more years. In some embodiments, a control subject can be a subject which has been non-symptomatic for a period of about 3.0, about 4.0, about 5.0, about 6.0, about 7.0, about 8.0, about 9.0, about 10.0, or more years. In some embodiments, a control subject can be a subject which is free of melanoma. In some embodiments, the control sample can be an average or composite value based on analysis of a population of “control subjects.”

[0044] In other embodiments, the quantifying is relative to a control sample where the melanoma was without recurrence for at least about 5.0 years. In some embodiments, the control sample is obtained from a control subject (e.g., a human or a primate), where the control subject is the same type of animal as the subject (e.g., human or primate).

[0045] In some embodiments, the fold change in the RNA expression level, relative to a control sample where the melanoma was without recurrence for at least about 5.0 years, in one or more of the at least one biomarker can be at least about 1.0,

at least about 1.5, at least about 2.0, at least about 2.5, at least about 3.0, at least about 3.5, at least about 4.0, at least about 4.5, at least about 5.0, at least about 5.5, at least about 6.0, at least about 6.5, at least about 7.0, at least about 7.5, at least about 8.0, at least about 8.5, at least about 9.0, at least about 9.5, at least about 10.0, at least about 10.5, at least about 11.0, at least about 11.5, at least about 12.0, at least about 12.5, at least about 13.0, at least about 13.5, at least about 14.0, at least about 15.0, at least about 16.0, at least about 17.0, at least about 18.0, at least about 19.0, or at least about 20.0. In other embodiments, the fold change in the RNA expression level, relative to a control sample where the melanoma was without recurrence for at least about 5.0 years, in one or more of the at least one biomarker, can be no more than about -0.5, no more than about -1.0, no more than about -1.5, no more than about -2.0, no more than about -2.5, no more than about -3.0, no more than about -3.5, no more than about -4.0, no more than about -4.5, no more than about -5.0, or no more than about -6.0. In certain embodiments, the fold change in the RNA expression level, relative to a control sample where the melanoma was without recurrence for at least about 5.0 years, in one or more of the at least one biomarker, can be about -6.0, about -5.0, about -4.5, about -4.0, about -3.5, about -3.0, about -2.5, about -2.0, about -1.5, about -1.0, about -0.75, about -0.50, about -0.25, about 0.25, about 0.50, about 0.75, about 1.0, about 1.1, about 1.2, about 1.3, about 1.4, about 1.5, about 1.6, about 1.7, about 1.8, about 1.9, about 2.0, about 2.1, about 2.2, about 2.3, about 2.4, about 2.5, about 2.6, about 2.7, about 2.8, about 2.9, about 3.0, about 3.1, about 3.2, about 3.3, about 3.4, about 3.5, about 3.6, about 3.7, about 3.8, about 3.9, about 4.0, about 4.1, about 4.2, about 4.3, about 4.4, about 4.5, about 4.6, about 4.7, about 4.8, about 4.9, about 5.0, about 5.1, about 5.2, about 5.3, about 5.4, about 5.5, about 5.6, about 5.7, about

5.8, about 5.9, about 6.0, about 6.5, about 7.0, about 7.5, about 8.0, about 8.5, about 9.0, about 9.5, about 10.0, about 10.5, about 11.0, about 11.5, about 12.0, about 12.5, about 13.0, about 13.5, about 14.0, about 14.5, about 15.0, about 15.5, about 16.0, about 16.5, about 17.0, about 17.5, about 18.0, about 18.5, about 19.0, about 19.5, about 20.0, about 20.5, from about -0.25 to about -6.0, from about -0.25 to about -2.0, from about -0.25 to about -1.0, from about 1.0 to about 20.5, from about 1.5 to about 15.0, from about 2.0 to about 15.0, or from about 2.5 to about 13.0.

[0046] The fold changes for microarray are calculated as described in the Statistical Analysis section of the Materials and Methods in the Examples described herein; see also Hao et al. (2017) "Sentinel lymph node genes to predict prognosis in node-positive melanoma patients" Ann Surg Oncol., Vol. 24, No. 1, pp. 108-116 and Menefee et al. (2020) "Age-related transcriptome changes in melanoma patients with tumor-positive sentinel lymph nodes" AGING, Vol. 12, No. 24, pp. 24914-24939.

The fold changes for NanoString are calculated as described in the Statistical Analysis section of the Materials and Methods in the Examples described herein; see also Hao et al. (2017) "Sentinel lymph node genes to predict prognosis in node-positive melanoma patients" Ann Surg Oncol., Vol. 24, No. 1, pp. 108-116 and Menefee et al. (2020) "Age-related transcriptome changes in melanoma patients with tumor-positive sentinel lymph nodes" AGING, Vol. 12, No. 24, pp. 24914-24939. The fold changes for PCR are calculated with the $2^{-\Delta\Delta C_t}$ method; see also Hao et al. (2017) "Sentinel lymph node genes to predict prognosis in node-positive melanoma patients" Ann Surg Oncol., Vol. 24, No. 1, pp. 108-116 and Menefee et al. (2020) "Age-related transcriptome changes in melanoma patients with tumor-positive sentinel lymph nodes" AGING, Vol. 12, No. 24, pp. 24914-24939.

[0047] In some embodiments, the method further comprises assessing a clinicopathologic feature of the subject (e.g., human or primate) from which the sample was obtained. In certain embodiments, consideration of clinicopathologic features can in some cases increase specificity and sensitivity of the prognosis and/or the treatment. In other embodiments, the clinicopathologic feature of the subject (e.g., human) from which the sample was obtained and the clinicopathologic feature is age, gender, anatomic location, Breslow thickness, ulceration, or sentinel lymph node status, or a combination thereof. In certain embodiments, the clinicopathologic feature of the subject (e.g., human or primate) from which the sample was obtained and the clinicopathologic feature is metastasis, age, lesion site, tumor burden, number of positive nodes, ulceration, tumor thickness, or a combination thereof.

[0048] In some embodiments, the subject has stage III melanoma.

[0049] In some embodiments, treating the subject (e.g., human or primate) comprises administering to the subject (e.g., human) one or more of immunotherapy, interferon, a BRAF inhibitor (e.g., Proietti et al. (2020) "BRAF Inhibitors: Molecular Targeting and Immunomodulatory Actions" Cancers (Basel), Vol. 12, No. 7, Article 1823, 13 pages, which is herein incorporated by reference in its entirety), a checkpoint inhibitor (e.g., Darvin et al. (2018) "Immune checkpoint inhibitors: recent progress and potential biomarkers" Experimental & Molecular Medicine, Vol. 50, Article 165, 11 pages, which is herein incorporated by reference in its entirety), a Wnt10b inhibitor (e.g., Goldsberry et al. (2019) "A Review of the Role of Wnt in Cancer Immunomodulation" Cancers, Vol. 11, Article 771, 19 pages, which is herein incorporated by reference in its entirety), or an IRAK3 inhibitor (e.g., Singer et al. (2018) "Inhibition of interleukin-1 receptor-associated kinase 1 (IRAK1) as a

therapeutic strategy" Oncotarget, Vol. 9, No. 70, pp. 33416-33439, which is herein incorporated by reference in its entirety; Hossen et al. (2017) "Thymoquinone: An IRAK1 inhibitor with in vivo and in vitro antiinflammatory activities" Scientific Reports, Vol. 7, Article 42995, 12 pages, which is herein incorporated by reference in its entirety). In certain embodiments, treating the subject comprises administering to the subject one or more of a Wnt10b inhibitor or an IRAK3 inhibitor. In some embodiments, treating the subject (e.g., human) comprises administering to the subject one or more of immunotherapy, interferon-gamma, interferon alfa-2b, a BRAF inhibitor, vemurafenib, dabrafenib, trametinib, encorafenib, a checkpoint inhibitor, a PD-1 inhibitor, nivolumab, pembrolizumab, cemiplimab, a PD-L1 inhibitor, atezolizumab, avelumab, durvalumab, a cytotoxic T-lymphocyte antigen 4 (CTLA-4) inhibitor, ipilimumab, a Wnt10b inhibitor, DKN-01, CGX1321, ETC 1922159, an IRAK3 inhibitor, pacritinib, or thymoquinone. In other embodiments, the treating comprises administering interferon-gamma, interferon alfa-2b, or both. In yet other embodiments, the treating comprises administering a BRAF inhibitor, vemurafenib, dabrafenib, trametinib, encorafenib, or a combination thereof. In still other embodiments, the treating comprises administering a checkpoint inhibitor, a PD-1 inhibitor, nivolumab, pembrolizumab, cemiplimab, a PD-L1 inhibitor, atezolizumab, avelumab, durvalumab, a cytotoxic T-lymphocyte antigen 4 (CTLA-4) inhibitor, ipilimumab, or a combination thereof. In certain embodiments, the treating comprises administering a Wnt10b inhibitor, DKN-01, CGX1321, ETC 1922159, or a combination thereof. In some embodiments, the treating comprises administering an IRAK3 inhibitor, pacritinib, thymoquinone, or a combination thereof. In certain

embodiments, treating further comprises one or more of surgery, chemotherapy, radiation therapy, targeted therapy, or vaccine therapy.

[0050] In some embodiments, the treating comprises administering a Wnt10b inhibitor, DKN-01, CGX1321, ETC 1922159 or a combination thereof and the subject
5 (e.g., human) is at least about 40 years old, at least about 50 years old, at least about 60 years old, at least about 70 years old, at least about 80 years old, about 35, about 40, about 42, about 44, about 45, about 46, about 48, about 50, about 51, about 52, about 53, about 54, about 55, about 56, about 57, about 58, about 59, about 60, about 61, about 62, about 63, about 64, about 65, about 66, about 67, about 68, about 69,
10 about 70, about 72, about 74, about 75, about 76, about 78, about 80, about 85, or about 90 years old.

[0051] In some embodiments, the treating comprises administering an IRAK3 inhibitor, pacritinib, thymoquinone, or a combination thereof and the subject (e.g., human) is no more than about 30 years old, no more than about 40 years old, no more
15 than about 50 years old, no more than about 60 years old, no more than about 70 years old, about 10, about 15, about 20, about 25, about 30, about 35, about 40, about 42, about 44, about 45, about 46, about 48, about 50, about 51, about 52, about 53, about 54, about 55, about 56, about 57, about 58, about 59, about 60, about 61, about 62, about 63, about 64, about 65, about 66, about 67, about 68, about 69, about 70, about
20 72, about 74, about 75, about 76, about 78, or about 80 years old.

[0052] In some embodiments, the treating only occurs if the fold change in the RNA expression level, relative to a control sample where the melanoma was without recurrence for at least about 5.0 years, in one or more of the at least one biomarker is about -6.0, about -5.0, about -4.5, about -4.0, about -3.5, about -3.0, about -2.5, about

-2.0, about -1.5, about -1.0, about -0.75, about -0.50, about -0.25, about 0.25, about 0.50, about 0.75, about 1.0, about 1.1, about 1.2, about 1.3, about 1.4, about 1.5, about 1.6, about 1.7, about 1.8, about 1.9, about 2.0, about 2.1, about 2.2, about 2.3, about 2.4, about 2.5, about 2.6, about 2.7, about 2.8, about 2.9, about 3.0, about 3.1, about 3.2, about 3.3, about 3.4, about 3.5, about 3.6, about 3.7, about 3.8, about 3.9, about 4.0, about 4.1, about 4.2, about 4.3, about 4.4, about 4.5, about 4.6, about 4.7, about 4.8, about 4.9, about 5.0, about 5.1, about 5.2, about 5.3, about 5.4, about 5.5, about 5.6, about 5.7, about 5.8, about 5.9, about 6.0, about 6.5, about 7.0, about 7.5, about 8.0, about 8.5, about 9.0, about 9.5, about 10.0, about 10.5, about 11.0, about 11.5, about 12.0, about 12.5, about 13.0, about 13.5, about 14.0, about 14.5, about 15.0, about 15.5, about 16.0, about 16.5, about 17.0, about 17.5, about 18.0, about 18.5, about 19.0, about 19.5, about 20.0, about 20.5, from about -0.25 to about -6.0, from about -0.25 to about -2.0, from about -0.25 to about -1.0, from about 1.0 to about 20.5, from about 1.5 to about 15.0, from about 2.0 to about 15.0, or from about 2.5 to about 13.0.

[0053] In certain embodiments, the treating comprises administering a Wnt10b inhibitor, DKN-01, CGX1321, ETC 1922159, or a combination thereof, only occurs if (a) the fold change in the RNA expression level, relative to a control sample where the melanoma was without recurrence for at least about 5.0 years, in one or more of the at least one biomarker is about -6.0, about -5.0, about -4.5, about -4.0, about -3.5, about -3.0, about -2.5, about -2.0, about -1.5, about -1.0, about -0.75, about -0.50, about -0.25, about 0.25, about 0.50, about 0.75, about 1.0, about 1.1, about 1.2, about 1.3, about 1.4, about 1.5, about 1.6, about 1.7, about 1.8, about 1.9, about 2.0, about 2.1, about 2.2, about 2.3, about 2.4, about 2.5, about 2.6, about 2.7, about 2.8,

about 2.9, about 3.0, about 3.1, about 3.2, about 3.3, about 3.4, about 3.5, about 3.6,
 about 3.7, about 3.8, about 3.9, about 4.0, about 4.1, about 4.2, about 4.3, about 4.4,
 about 4.5, about 4.6, about 4.7, about 4.8, about 4.9, about 5.0, about 5.1, about 5.2,
 about 5.3, about 5.4, about 5.5, about 5.6, about 5.7, about 5.8, about 5.9, about 6.0,
 5 about 6.5, about 7.0, about 7.5, about 8.0, about 8.5, about 9.0, about 9.5, about 10.0,
 about 10.5, about 11.0, about 11.5, about 12.0, about 12.5, about 13.0, about 13.5,
 about 14.0, about 14.5, about 15.0, about 15.5, about 16.0, about 16.5, about 17.0,
 about 17.5, about 18.0, about 18.5, about 19.0, about 19.5, about 20.0, about 20.5,
 from about -0.25 to about -6.0, from about -0.25 to about -2.0, from about -0.25 to
 10 about -1.0, from about 1.0 to about 20.5, from about 1.5 to about 15.0, from about 2.0
 to about 15.0, or from about 2.5 to about 13.0 and (b) the subject is at least about 40
 years old, at least about 50 years old, at least about 60 years old, at least about 70
 years old, at least about 80 years old, about 35, about 40, about 42, about 44, about
 45, about 46, about 48, about 50, about 51, about 52, about 53, about 54, about 55,
 15 about 56, about 57, about 58, about 59, about 60, about 61, about 62, about 63, about
 64, about 65, about 66, about 67, about 68, about 69, about 70, about 72, about 74,
 about 75, about 76, about 78, about 80, about 85, or about 90 years old.

[0054] In certain embodiments, the treating comprises administering of
 IRAK3 inhibitor, pacritinib, thymoquinone, or a combination thereof, only occurs if
 20 (a) the fold change in the RNA expression level, relative to a control sample where
 the melanoma was without recurrence for at least about 5.0 years, in one or more of
 the at least one biomarker is about -6.0, about -5.0, about -4.5, about -4.0, about -3.5,
 about -3.0, about -2.5, about -2.0, about -1.5, about -1.0, about -0.75, about -0.50,
 about -0.25, about 0.25, about 0.50, about 0.75, about 1.0, about 1.1, about 1.2, about

1.3, about 1.4, about 1.5, about 1.6, about 1.7, about 1.8, about 1.9, about 2.0, about
 2.1, about 2.2, about 2.3, about 2.4, about 2.5, about 2.6, about 2.7, about 2.8, about
 2.9, about 3.0, about 3.1, about 3.2, about 3.3, about 3.4, about 3.5, about 3.6, about
 3.7, about 3.8, about 3.9, about 4.0, about 4.1, about 4.2, about 4.3, about 4.4, about
 5 4.5, about 4.6, about 4.7, about 4.8, about 4.9, about 5.0, about 5.1, about 5.2, about
 5.3, about 5.4, about 5.5, about 5.6, about 5.7, about 5.8, about 5.9, about 6.0, about
 6.5, about 7.0, about 7.5, about 8.0, about 8.5, about 9.0, about 9.5, about 10.0, about
 10.5, about 11.0, about 11.5, about 12.0, about 12.5, about 13.0, about 13.5, about
 14.0, about 14.5, about 15.0, about 15.5, about 16.0, about 16.5, about 17.0, about
 10 17.5, about 18.0, about 18.5, about 19.0, about 19.5, about 20.0, about 20.5, from
 about -0.25 to about -6.0, from about -0.25 to about -2.0, from about -0.25 to about -
 1.0, from about 1.0 to about 20.5, from about 1.5 to about 15.0, from about 2.0 to
 about 15.0, or from about 2.5 to about 13.0 and (b) the subject is no more than about
 30 years old, no more than about 40 years old, no more than about 50 years old, no
 15 more than about 60 years old, no more than about 70 years old, about 10, about 15,
 about 20, about 25, about 30, about 35, about 40, about 42, about 44, about 45, about
 46, about 48, about 50, about 51, about 52, about 53, about 54, about 55, about 56,
 about 57, about 58, about 59, about 60, about 61, about 62, about 63, about 64, about
 65, about 66, about 67, about 68, about 69, about 70, about 72, about 74, about 75,
 20 about 76, about 78, or about 80 years old.

[0055] In some embodiments, the method comprising quantifying an RNA
 expression level for at least one biomarker in a sample from a sentinel lymph node
 (SLN) of the human, where the at least one biomarker comprises one or more of FOS,
 NR4A, or Wnt10b, and treating the human with a Wnt10b inhibitor, DKN-01,

CGX1321, ETC 1922159, or a combination thereof, and optionally one or more of immunotherapy, interferon, a BRAF inhibitor, a checkpoint inhibitor, or an IRAK3 inhibitor. In certain aspects, the human is at least about 40 years old, at least about 50 years old, at least about 60 years old, at least about 70 years old, at least about 80 years old, about 35, about 40, about 42, about 44, about 45, about 46, about 48, about 50, about 51, about 52, about 53, about 54, about 55, about 56, about 57, about 58, about 59, about 60, about 61, about 62, about 63, about 64, about 65, about 66, about 67, about 68, about 69, about 70, about 72, about 74, about 75, about 76, about 78, about 80, about 85, or about 90 years old and the treating only occurs if the fold change in the RNA expression level, relative to a control sample where the melanoma was without recurrence for at least about 5.0 years, in one or more of the at least one biomarker is about -6.0, about -5.0, about -4.5, about -4.0, about -3.5, about -3.0, about -2.5, about -2.0, about -1.5, about -1.0, about -0.75, about -0.50, about -0.25, about 0.25, about 0.50, about 0.75, about 1.0, about 1.1, about 1.2, about 1.3, about 1.4, about 1.5, about 1.6, about 1.7, about 1.8, about 1.9, about 2.0, about 2.1, about 2.2, about 2.3, about 2.4, about 2.5, about 2.6, about 2.7, about 2.8, about 2.9, about 3.0, about 3.1, about 3.2, about 3.3, about 3.4, about 3.5, about 3.6, about 3.7, about 3.8, about 3.9, about 4.0, about 4.1, about 4.2, about 4.3, about 4.4, about 4.5, about 4.6, about 4.7, about 4.8, about 4.9, about 5.0, about 5.1, about 5.2, about 5.3, about 5.4, about 5.5, about 5.6, about 5.7, about 5.8, about 5.9, about 6.0, about 6.5, about 7.0, about 7.5, about 8.0, about 8.5, about 9.0, about 9.5, about 10.0, about 10.5, about 11.0, about 11.5, about 12.0, about 12.5, about 13.0, about 13.5, about 14.0, about 14.5, about 15.0, about 15.5, about 16.0, about 16.5, about 17.0, about 17.5, about 18.0, about 18.5, about 19.0, about 19.5, about 20.0, about 20.5, from about -0.25 to

about -6.0, from about -0.25 to about -2.0, from about -0.25 to about -1.0, from about 1.0 to about 20.5, from about 1.5 to about 15.0, from about 2.0 to about 15.0, or from about 2.5 to about 13.0.

[0056] In other embodiments, the method for treating melanoma in a human
5 comprises quantifying an RNA expression level for at least one biomarker in a sample from a sentinel lymph node (SLN) of the human, where the at least one biomarker comprises IRAK3 and administering to the human an IRAK3 inhibitor, pacritinib, thymoquinone, or a combination thereof, and optionally one or more of immunotherapy, interferon, a BRAF inhibitor, a checkpoint inhibitor, or a Wnt10b
10 inhibitor. In certain aspects of this methods, the human is no more than about 30 years old, no more than about 40 years old, no more than about 50 years old, no more than about 60 years old, no more than about 70 years old, about 10, about 15, about 20, about 25, about 30, about 35, about 40, about 42, about 44, about 45, about 46, about 48, about 50, about 51, about 52, about 53, about 54, about 55, about 56, about
15 57, about 58, about 59, about 60, about 61, about 62, about 63, about 64, about 65, about 66, about 67, about 68, about 69, about 70, about 72, about 74, about 75, about 76, about 78, or about 80 years old, and the treating only occurs if the fold change in the RNA expression level, relative to a control sample where the melanoma was without recurrence for at least about 5.0 years, in one or more of the at least one
20 biomarker is about -6.0, about -5.0, about -4.5, about -4.0, about -3.5, about -3.0, about -2.5, about -2.0, about -1.5, about -1.0, about -0.75, about -0.50, about -0.25, about 0.25, about 0.50, about 0.75, about 1.0, about 1.1, about 1.2, about 1.3, about 1.4, about 1.5, about 1.6, about 1.7, about 1.8, about 1.9, about 2.0, about 2.1, about 2.2, about 2.3, about 2.4, about 2.5, about 2.6, about 2.7, about 2.8, about 2.9, about

3.0, about 3.1, about 3.2, about 3.3, about 3.4, about 3.5, about 3.6, about 3.7, about
 3.8, about 3.9, about 4.0, about 4.1, about 4.2, about 4.3, about 4.4, about 4.5, about
 4.6, about 4.7, about 4.8, about 4.9, about 5.0, about 5.1, about 5.2, about 5.3, about
 5.4, about 5.5, about 5.6, about 5.7, about 5.8, about 5.9, about 6.0, about 6.5, about
 5 7.0, about 7.5, about 8.0, about 8.5, about 9.0, about 9.5, about 10.0, about 10.5, about
 11.0, about 11.5, about 12.0, about 12.5, about 13.0, about 13.5, about 14.0, about
 14.5, about 15.0, about 15.5, about 16.0, about 16.5, about 17.0, about 17.5, about
 18.0, about 18.5, about 19.0, about 19.5, about 20.0, about 20.5, from about -0.25 to
 about -6.0, from about -0.25 to about -2.0, from about -0.25 to about -1.0, from about
 10 1.0 to about 20.5, from about 1.5 to about 15.0, from about 2.0 to about 15.0, or from
 about 2.5 to about 13.0.

[0057] The presently-disclosed subject matter is further illustrated by the
 following specific but non-limiting examples. The following examples may include
 15 compilations of data that are representative of data gathered at various times during
 the course of development and experimentation related to the present invention.

EXAMPLES

20 [0058] Menefee et al. (2020) "Age-related transcriptome changes in
 melanoma patients with tumor-positive sentinel lymph nodes" AGING, Vol. 12, No.
 24, pp. 24914-24939, is herein incorporated by reference in its entirety. WO
 2013/172947 A1 to Hao et al. is herein incorporated by reference in its entirety. US
 Pat. Appl. No. 2021/0010090 A1 to Hao et al. is herein incorporated by reference in
 25 its entirety.

[0059] **Materials and Methods**

[0060] Patient selection

[0061] This study used two different technologies in three independent
5 datasets of RNA samples obtained from melanoma patients with positive SLNs to
identify age-related transcriptome changes in SLN and their association with
outcome.

[0062] Microarray analysis was performed in the first independent dataset to
assess 97 samples obtained from the Sunbelt Melanoma Trial (SMT). The samples
10 were randomly chosen from among 317 melanoma patients with positive SLNs. This
patient cohort has been described previously (Hao et al. (2017) "Sentinel lymph node
genes to predict prognosis in node-positive melanoma patients" Ann Surg Oncol.,
Vol. 24, No. 1, pp. 108-116, which is herein incorporated by reference in its entirety).
Thirty-nine patients experienced recurrence melanoma in this cohort, and fifty-eight
15 patients did not experience recurrence. Median follow-up was 93 months. This study
was approved by the institutional review boards (IRB) of each participating
institution. Clinicopathologic factors, recurrence, and survival data were collected
prospectively. Additional details of the SMT are described elsewhere (Hao et al.
(2017) "Sentinel lymph node genes to predict prognosis in node-positive melanoma
20 patients" Ann Surg Oncol., Vol. 24, No. 1, pp. 108-116); McMasters et al. (2016)
"Final results of the Sunbelt Melanoma Trial: a multi-institutional prospective
randomized phase III study evaluating the role of adjuvant high dose interferon alfa-
2b and completion lymph node dissection for patients staged by sentinel lymph node
biopsy" J Clin Oncol., Vol. 34, pp. 1079–1086).

[0063] NanoString analysis was applied to the second patient cohort, which included 12 patients with tumor-positive SLNs from the James Graham Brown Cancer Center Biorepository at University of Louisville. This study followed an approved IRB protocol. There were 6 patients who experienced recurrence (3 of each at age <60 and ≥ 60 years old) and 6 patients who did not experience recurrence (3 of each at age <60 and ≥ 60 years old). Median follow-up was 34 months.

[0064] The third independent dataset of 36 samples from the James Graham Brown Cancer Center Biorepository was used to validate the differentially expressed genes (DEGs). The SLN tissue was acquired from patients at the time of surgical treatment of cutaneous melanoma, including staging with SLN biopsy between 2003 and 2017. Median follow-up of this cohort was 33.2 months. Patient characteristics such as age and outcome from all three datasets are summarized in Supplementary Table 7.

[0065] Supplementary Table 7

Outcome	1 st microarray dataset		2 nd NanoString dataset		3 rd dataset qRT-PCR	
	< 60	≥ 60	< 60	≥ 60	< 60	≥ 60
No recurrence (recur ^{no})	51	7	3	3	9	13
Recurrence (recur ^{yes})	28	11	3	3	9	5

[0066] Definition of age groups

[0067] To ensure that we had a large enough sample size for a robust analysis, we grouped patients into two age groups. Patients were defined as being older if they were ≥ 60 years old (yr⁶⁰⁺). Patients were defined as being younger if they were <60 years old (yr⁶⁰⁻).

[0068] Microarray experiments

[0069] GeneChip Human HG-U133 plus 2.0 array (Affymetrix, Santa Clara, CA) was used in the first microarray dataset according to the manufacturer's

5 guidelines. Details of RNA isolation, microarray experiment, and quality control were described in detail previously (Hao et al. (2017) "Sentinel lymph node genes to predict prognosis in node-positive melanoma patients" Ann Surg Oncol., Vol. 24, No. 1, pp. 108-116). This set of microarray data is accessible through NCBI's Gene Expression Omnibus (GEO, <<www.ncbi.nlm.nih.gov/geo>>) by accession number
10 GSE 43081.

[0070] NanoString analysis of mRNA expression of immune panel genes and immune pathway panel genes

[0071] The second dataset of 12 RNA samples were isolated from fresh-
15 frozen human SLN tissues from melanoma patients using RNeasy Plus Mini Kit (Qiagen). RNA quality control/quantity assessment (QC/QA) was checked by Agilent bioanalyzer. The RNA concentration was measured by Qubit. Total RNA (100 ng per sample) were analyzed on the nCounter MAX system. Two gene expression assays were used: PanCancer immune profiling and PanCancer immune pathway profiling
20 (NanoString Technologies, Seattle, WA, USA). PanCancer immune profiling assay comprised 730 immune-related genes and 40 internal reference genes. Immune pathway profiling assay comprised 730 genes from 13 canonical pathways and 40 selected reference genes. Raw counts for each assay were collected using the NanoString data analysis software (nSolver).

[0072] Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

[0073] The third dataset of 36 RNA samples were isolated from fresh-frozen
 5 human SLN tissues from melanoma patients using RNeasy Plus Mini Kit (Qiagen). Total SLN RNA (1000 ng) from each sample was reverse-transcribed with the SuperScript III First-Strand Synthesis System. mRNA primers were purchased from Life Technologies (Carlsbad, CA). Quantitative RT-PCR reactions were completed on a 7500 Fast Real Time PCR system (Life Technologies). The relative quantity of the
 10 target mRNA was normalized to endogenous gene (B₂M). The fold changes (FC) of each mRNA in the qRT-PCR experiments were calculated with the $2^{-\Delta\Delta C_t}$ method.

[0074] Statistical analysis

[0075] For microarray analysis, a fold change outlier (FCO) filter was applied
 15 independently to reduce the dimension of the data before determining the DEGs between the two age groups (yr⁶⁰⁺ and yr⁶⁰⁻) as well as between patients with recurrence (recur^{yes}) and those without recurrence (recur^{no}) (Bolstad et al. (2003) "A comparison of normalization methods for high density oligonucleotide array data based on variance and bias" Bioinformatics, Vol. 19, pp. 185–193; Tusher et al.
 20 (2001) "Significance analysis of microarrays applied to the ionizing radiation response" Proc Natl Acad Sci USA., Vol. 98, pp. 5116–5121). For each of 54,675 probes on the array, the fold change (FC) was calculated and four filters (T1, T2, T3 and T4) were used. $T1 = \{\mu(FC) \pm 1.5\sigma(FC)\}$, $T2 = \{\mu(FC) \pm 2\sigma(FC)\}$, $T3 = \{\mu(FC) \pm 3\sigma(FC)\}$, and $T4 = \{\mu(FC) \pm 4\sigma(FC)\}$, where $\mu(FC)$ is the mean of fold changes (FC)

and σ is the standard deviation of FC from all 54,675 probes in the array. The genes that fell inside T1, T2, and T3 were filtered from the differential data. After filtering the data, a t-test for normal gene expression data and a Wilcoxon test for non-normal expression data were applied (Khan (2005) "ArrayVigil: a methodology for statistical comparison of gene signatures using segregated-one-tailed (SOT) Wilcoxon's signed-rank test" J Mol Biol., Vol. 345, pp. 645–649). The Benjamini-Hochberg method was employed to adjust the p values (Benjamini et al. (1995) "Controlling the false discovery rate: a practical and powerful approach to multiple testing" J R Stat Soc B., Vol. 57, pp. 289–300). When comparing the changes of the SLN gene expressions in the yr^{60+} versus yr^{60-} patients, a multivariable linear regression model was fitted for each gene about age (<60 or ≥ 60). The equation used is below:

[0076] Gene Expression = $\alpha + \beta_1 \text{ age}$, where $\text{age}=1$ if ≥ 60 years old and 0 otherwise.

[0077] The estimates and p values are presented by filter T2, T3, and T4.

15 When assessing the changes of the SLN gene expressions that are associated with $\text{recur}^{\text{yes}}$ versus recur^{no} in the yr^{60+} and yr^{60-} melanoma patients, a multivariable linear regression model was fitted for each gene of each sample about age (<60 years or ≥ 60), outcome ($\text{recur}^{\text{yes}}$ or recur^{no}), and the interaction of age and outcome. The equation used is below:

20 [0078] Gene Expression = $\alpha + \beta_1 \text{ age} + \beta_2 \text{ outcome} + \beta_3 \text{ age} * \text{outcome}$, where $\text{age}=1$ if ≥ 60 years old and 0 otherwise, $\text{outcome}=1$ if $\text{recur}^{\text{yes}}$ and 0 otherwise.

[0079] The estimate and p values are also presented by filter T2, T3, and T4.

Statistical Analysis System (SAS) was used to perform the regression analysis. p values of FC were calculated using ANOVA (Cary NC. (2003) The SAS system V9.

Cary, NC: SAS Institute Inc.; Gonen (2006) "Receiver operating characteristics (ROC) curves" In: Proceedings of the thirty-first annual SAS users group international conference, pp. 210–231).

[0080] For the NanoString results analysis, positive control normalization was performed by using gene expression data normalized to the mean of the positive control probes for each assay. RNA content normalization was performed by using gene expression data normalized to the geometric mean of housekeeping genes in the CodeSet. Raw data are also analyzed using the nSolver Advanced Analysis module. More information on the Advanced Analysis package can be found at

10 <<www.nanostring.com/products/nSolver>>.

[0081] Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, CA) was used for gene network and pathway analysis. The statistical score of a pathway is defined as $-\log(p \text{ value})$ from Fisher's exact test analysis.

15 [0082] Abbreviations

[0083] CI Confidence interval

[0084] DEGs Differentially expressed genes

[0085] FC Fold changes

[0086] FCO Fold change outlier

20 [0087] FOS FBJ murine osteosarcoma viral oncogene homolog

[0088] GEO Gene Expression Omnibus

[0089] IRAK3 Interleukin-1 receptor-associated kinase 3

[0090] ITGB1 Integrin subunit beta 1

[0091] ITGBL1 Integrin subunit beta like 1

	[0092]	NR4A2	Nuclear receptor subfamily 4, group A, member 2
	[0093]	PPAR	Peroxisome proliferator-activated receptor
	[0094]	QC/QA	Quality control/quantity assessment
	[0095]	qRT-PCR	Quantitative reverse transcriptase polymerase chain
5	reaction		
	[0096]	recur ^{no}	Without recurrence
	[0097]	recur ^{yes}	Recurrence
	[0098]	SLN	Sentinel lymph node
	[0099]	SMT	Sunbelt melanoma Trial
10	[00100]	TERT	Telomerase reverse transcriptase
	[00101]	Tregs	T regulatory cells
	[00102]	yr ⁶⁰⁻	<60 years old
	[00103]	yr ⁶⁰⁺	≥60 years old
15	[00104]	<u>Results</u>	
	[00105]	<u>Transcriptome changes in SLN genes in older patients (≥60 years old) versus younger patients (<60 years old) by microarray analysis</u>	
	[00106]	We were interested in comparing gene expression profiles in older versus younger patients and in assessing whether there was a correlation with	
20		melanoma recurrence. Therefore, we analyzed the first microarray dataset from 97 melanoma patients with positive SLNs from the Sunbelt Melanoma Trial (SMT) and evaluated the transcriptome changes of the SLN by two defined age groups: the older and the younger groups. Patients were defined as being older if they were ≥60 years old (yr ⁶⁰⁺). Patients were defined as being younger if they were <60 years old (yr ⁶⁰⁻).	

[00107] Table 1 lists the clinical data of the 97 melanoma patients grouped by age. In this dataset, there were no significant differences between the two age groups in primary tumor site, Breslow thickness, Clark level, or ulceration presence. However, in younger patients, the recurrence rate was significantly higher when

5 Breslow thickness was higher. In older patients, there were no significant differences in Breslow thickness, Clark level, and ulceration presence between groups of patients with recurrence (recur^{yes}) and those without recurrence (recur^{no}). Using microarray filter T3 and T4, we detected a total of 577 and 156 differentially expressed probe sets, in older versus the younger patients. Among them, there were 41 and 11

10 differentially expressed probe sets by filters T3 and T4 in the older versus younger groups ($p < 0.05$). Probe sets without defined gene names by annotation from Partek Genomics Suite software were removed from the lists. There were 7 differentially expressed genes (DEGs) in the yr⁶⁰⁺ group versus the yr⁶⁰⁻ group with a p value < 0.05 by T4 filter (Table 2). Among them, FBJ murine osteosarcoma viral oncogene

15 homolog (FOS) and nuclear receptor subfamily 4, group A, member 2 (NR4A2), were the two genes that had significant higher expression in the yr⁶⁰⁺ group than in the yr⁶⁰⁻ group. The DEGs between the yr⁶⁰⁻ and the yr⁶⁰⁺ group had various biological functions, including toll-like receptor signaling pathway transduction, adaptive and innate immune response, autophagy, and transcription regulation (Table 2). The

20 network connection of the 156 DEGs by T4 filter is shown in Fig. 1. The top canonical pathway that showed a difference in the yr⁶⁰⁻ and the yr⁶⁰⁺ group was the peroxisome proliferator-activated receptor (PPAR) signaling pathway, which had a close interaction with toll-like receptor signaling pathway (Supplementary Table 1) (Dana et al. (2020) "The effect of fenofibrate, a PPAR α activator on toll-like

receptor-4 signal transduction in melanoma both in vitro and in vivo" Clin Transl Oncol., Vol. 22, No. 4, pp. 486-494 ; Dana et al. (2019) "PPAR γ agonist, pioglitazone, suppresses melanoma cancer in mice by inhibiting TLR4 signaling" J Pharm Pharm Sci., Vol. 22, pp. 418-423). The list of all DEGs by T3 filter is listed in Supplementary Table 2.

Table 1. Clinical data of the first dataset (97 melanoma patients) grouped by age							
Variables	Age $\leq$ 60			Age >60			P Value for Age<60 vs Age$\geq$60
	No recurrence (N=51)	Recurrence (N=28)	P Value	No recurrence (N=7)	Recurrence (N=11)	P Value	
Gender			0.957			1.000 [†]	0.541
Female (%)	24 (47.1)	13 (46.4)		3 (42.9)	4 (36.4)		
Male (%)	27 (52.9)	15 (53.6)		4 (57.1)	7 (63.6)		
Primary Site			0.908 [†]			0.141 [†]	0.371 [†]
Head (%)	2 (3.9)	1 (3.6)					
Lower Extremity (%)	12 (23.5)	9 (32.1)		2 (28.6)	5 (45.5)		
Neck (%)	1 (2.0)	0 (0.0)		0 (0.0)	1 (9.1)		
Trunk (%)	28 (54.9)	13 (46.4)		4 (57.1)	2 (18.2)		
Upper Extremity (%)	8 (15.7)	5 (17.9)		0 (0.0)	3 (27.3)		
Breslow Thickness (mm)			0.006			0.837	0.362
Mean (95%CI)	2.5 (2.2 - 2.9)	3.9 (2.8 - 5.0)		2.6 (1.2 - 4.1)	2.5 (1.8 - 3.1)		
Median (min - max)	2.0 (1.0 - 6.0)	2.7 (1.5 - 13.0)		2.5 (1.2 - 6.8)	2.4 (1.1 - 4.4)		
Clark level			0.741 [†]			1.000 [†]	0.457 [†]
II/III (%)	7 (13.7)	3 (10.7)		2 (28.6)	2 (18.2)		
IV/V (%)	43 (84.3)	25 (89.3)		5 (71.4)	9 (81.8)		
Ulceration Present			0.255 [†]			0.430 [†]	0.268 [†]
NA (%)	0 (0.0)	1 (3.6)		1 (14.3)	0 (0.0)		
No (%)	34 (66.7)	15 (53.6)		3 (42.9)	5 (45.5)		

<u>Table 1.</u> Clinical data of the first dataset (97 melanoma patients) grouped by age							
Variables	Age ≤ 60			Age >60			P Value for Age<60 vs Age≥60
	No recurrence (N=51)	Recurrence (N=28)	P Value	No recurrence (N=7)	Recurrence (N=11)	P Value	
Yes (%)	17 (33.3)	12 (42.9)		2 (28.6)	6 (54.5)		
Time To FU (All Patients)			<.001			0.015	0.187
Mean (95%CI)	86.8 (80.8 - 92.7)	65.5 (53.3 - 77.7)		88.7 (73.0 - 104.5)	57.3 (42.3 - 72.2)		
Median (min - max)	92.0 (40.0 - 122.0)	58.5 (6.0 - 122.0)		94.0 (51.0 - 111.0)	57.0 (16.0 - 111.0)		

[00108] Table 2. The DEGs in the SLN in yr⁶⁰⁺ versus yr⁶⁰⁻ patients in the microarray dataset using T4 filter ($P<0.05$).

Gene symbol	Gene Name	Biological function	P value	Fold change
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	negative regulation of transcription from RNA polymerase II promoter	0.021	1.60
FOS	FBJ murine osteosarcoma viral oncogene homolog	toll-like receptor signaling pathway//MyD88-dependent and -independent toll-like receptor signaling pathway	0.0255	1.56
NR4A2	nuclear receptor subfamily 4, group A, member 2	negative regulation of transcription from RNA polymerase II promoter//response to hypoxia	0.0096	1.47
CLEC4C	C-type lectin domain family 4, member C	stimulatory C-type lectin receptor signaling pathway//adaptive and innate immune response	0.049	1.45
LIX1	limb and CNS expressed 1	autophagy//autophagosome maturation	0.0098	1.41
NRCAM	neuronal cell adhesion molecule	angiogenesis//neuron migration/cell adhesion	0.0008	1.40
GRB14	growth factor receptor bound	signal transduction	0.0135	0.79

	protein 14			
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[00109] Supplementary Table 1. Top canonical pathways that showed differences in yr⁶⁰⁺ versus yr⁶⁰⁻ patients in the first microarray dataset using T4 filter.

Pathway name	<i>p</i> - value	Overlap
PPAR Signaling	2.65E-04	4.0% (4/101)
Acute phase response signaling	2.20E-03	2.2% (4/178)
Melanocyte development and pigmentation signaling	3.13E-03	3.2% (3/95)
Coagulation system	5.18E-03	5.7% (2/35)
Cholecystokinin/Gastrin-mediated signaling	5.61E-03	2.6% (3/117)

5

[00110] Supplementary Table 2. Differentially expressed genes (DEGs) in the SLN in yr⁶⁰⁺ versus yr⁶⁰⁻ patients in the first microarray dataset using T3 filter.

Gene symbol	Gene Name	<i>P</i> value	Fold change
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	0.0208	1.5961
FOS	FBJ murine osteosarcoma viral oncogene homolog	0.0255	1.5558
DUSP1	dual specificity phosphatase 1	0.0453	1.5349
NR4A2	nuclear receptor subfamily 4, group A, member 2	0.0096	1.4719
IDO1	indoleamine 2,3-dioxygenase 1	0.0155	1.454
CLEC4C	C-type lectin domain family 4, member C	0.0486	1.4497
LIX1	limb and CNS expressed 1	0.0098	1.4118
CD8A	CD8a molecule	0.0003	1.4111
BACH2	BTB and CNC homology 1, basic leucine zipper transcription factor 2	0.003	1.3963
NRCAM	neuronal cell adhesion molecule	0.0008	1.3961
NOG	noggin	0.0027	1.3839
KLRC4-KLRK1 /// KLRK1	KLRC4-KLRK1 read through /// killer cell lectin-like receptor subfamily K, member 1	0.0011	1.3827
MS4A6A	membrane-spanning 4-domains, subfamily A, member 6A	0.0434	1.3709
KLF4	Kruppel-like factor 4 (gut)	0.0278	1.3662
SATB1	SATB homeobox 1	0.0322	1.3484
LOC101928963	uncharacterized LOC101928963	0.027	1.2614

GRIK2	glutamate receptor, ionotropic, kainate 2	0.0497	0.8748
MUC15	mucin 15, cell surface associated	0.0495	0.8681
DLK1	delta-like 1 homolog (Drosophila)	0.0339	0.8617
RNF152	ring finger protein 152	0.05	0.8483
ITGBL1	integrin beta like 1	0.03	0.8466
ERGIC3	ERGIC and golgi 3	0.0295	0.8456
INHBA	inhibin beta A	0.0461	0.8347
PRUNE2	prune homolog 2 (Drosophila)	0.0059	0.8323
LINC00354	long intergenic non-protein coding RNA 354	0.0292	0.8312
MKX	mohawk homeobox	0.0053	0.8308
WWC1	WW and C2 domain containing 1	0.0295	0.8228
LOC105373225	uncharacterized LOC105373225	0.029	0.8139
SLC13A5	solute carrier family 13 (sodium-dependent citrate transporter), member 5	0.018	0.7921
GRB14	growth factor receptor bound protein 14	0.0135	0.792
ATP2B2	ATPase, Ca++ transporting, plasma membrane 2	0.0295	0.791
COL28A1	collagen, type XXVIII, alpha 1	0.0092	0.7779
LOC100507516	uncharacterized LOC100507516	0.0223	0.7719
MLANA	melan-A	0.0486	0.7057

[00111] Transcriptome changes of immune genes and immune pathway genes in the SLNs of older versus younger patients as assessed by NanoString analysis

[00112] Immune cells are a component of lymph node structure. We then

5 focused on immune genes and immune pathways associated with both age groups and assessed by NanoString analysis. This analysis in the second dataset found that 12 immune-related genes were differentially expressed in SLNs in older versus younger patients (Table 3). There were 17 immune pathway-related genes in SLNs that were differentially expressed in yr⁶⁰⁺ versus yr⁶⁰⁻ patients (Table 4). Of note is that the

10 NR4A2 gene was found to be differentially expressed in yr⁶⁰⁺ versus yr⁶⁰⁻ patients from the first microarray dataset. The NR4A3 gene, which belongs to the same family members of NR4A2, was also found to have a higher fold change (FC) in yr⁶⁰⁺ patients, the *p* value is 0.0517 (last row in Table 4). The immune gene, integrin

subunit beta 1 (ITGB1), was found to be differentially expressed in yr⁶⁰⁺ versus the yr⁶⁰⁻ patients (Table 3). Integrin subunit beta like 1 (ITGBL1) was also found to be differentially expressed in yr⁶⁰⁺ versus yr⁶⁰⁻ patients by microarray analysis (Supplementary Table 2). The immune gene with the highest and lowest fold change in the yr⁶⁰⁺ versus the yr⁶⁰⁻ patient group was melanoma antigen family A, 3 (MAGEA3) and leukemia inhibitory factor (LIF) (fold change =2.87 and -1.16) (Table 3). Among the three highest fold changes of the immune pathway genes, two of them were secreted frizzled-related protein 2 and 4 (SFRP2 and SFRP4) (fold change =1.93 and 1.78) (Table 4). Both genes belong to the Wnt pathway.

[00113] NanoString results suggested that NR4A and ITGB1 genes are more highly expressed immune genes in older melanoma patients compared to their younger counterparts with lymph node metastasis. These genes, therefore, might be responsible for the age-related differences in response of SLN to the presence of nodal metastasis. The Wnt pathway might also be a relevant immune pathway associated with age-related immune response to melanoma metastasis to the SLN.

[00114] Table 3. Immune genes that were differentially expressed in the SLN in yr⁶⁰⁺ versus yr⁶⁰⁻ patients in the second dataset by NanoString analysis ($P<0.05$).

Gene symbol	Gene Name	P value	Fold change
MAGEA3	melanoma antigen family A, 3	0.0149	2.87
MME	membrane metallo-endopeptidase	0.0466	1.34
CD244	CD244 molecule, natural killer cell receptor 2B4	0.0453	1.07
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	0.0254	0.792
JAM3	junctional adhesion molecule 3	0.0405	0.628
ITGB1	integrin subunit beta 1	0.00565	0.564
ALCAM	activated leukocyte cell adhesion molecule	0.00272	0.489
MAVS	mitochondrial antiviral signaling protein	0.00886	0.303

IFIH1	interferon induced with helicase C domain 1	0.0118	-0.325
MX1	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	0.0493	-0.666
CXCL3	chemokine (C-X-C motif) ligand 3	0.0393	-0.851
LIF	leukemia inhibitory factor	0.0476	-1.16

[00115] Table 4. Immune pathway genes that are differentially expressed in the SLN in yr⁶⁰⁺ versus yr⁶⁰⁻ patients in the second dataset by NanoString analysis ($P < 0.05$)*.

Gene symbol	Gene Name	P value	Fold change
COMP	cartilage oligomeric matrix protein	0.0212	2.51
SFRP2	secreted frizzled-related protein 2	0.0428	1.93
SFRP4	secreted frizzled-related protein 4	0.0279	1.78
CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa	0.0247	0.832
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	0.0345	0.702
PLD1	phospholipase D1, phosphatidylcholine-specific	0.0088	0.661
TNC	tenascin C	0.0207	0.655
PBRM1	polybromo 1	0.00195	0.463
GADD45A	growth arrest and DNA-damage-inducible, alpha	0.00483	0.421
FUT8	fucosyltransferase 8 (alpha (1,6) fucosyltransferase)	0.0394	0.367
PIK3R3	phosphoinositide-3-kinase, regulatory subunit 3 (gamma)	0.0278	0.317
PRKAR2A	protein kinase, cAMP-dependent, regulatory, type II, alpha	0.0167	0.296
PIK3CB	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit beta	0.0454	0.283
FANCB	Fanconi anemia, complementation group B	0.0385	-0.589
ERCC2	excision repair cross-complementing rodent repair deficiency, complementation group 2	0.0176	-0.595
TNFRSF10C	tumor necrosis factor receptor superfamily, member 10c, decoy without an intracellular domain	0.0123	-0.677
CDK6	cyclin-dependent kinase 6	0.0308	-0.683
NR4A3	nuclear receptor subfamily 4, group A, member 3	0.0517	1.3

[00116] Transcriptome changes in SLN associated with recurrence in yr⁶⁰⁺ or yr⁶⁰⁻ melanoma patients by microarray analysis

[00117] After we compared the transcriptome changes in SLN genes between the yr⁶⁰⁺ and the yr⁶⁰⁻ melanoma patients, we studied whether there were any

5 differences between patients who experienced recurrence versus those who remained disease free. We also evaluated these results by age categories. A multivariable linear regression model was fitted for each gene of each sample about age (yr⁶⁰⁺ or yr⁶⁰⁻), outcome (recur^{yes} or recur^{no}), and the interaction of age and outcome in the first microarray dataset. There were 100 differentially expressed probe sets with a

10 statistically significant difference ($p < 0.05$) after adjusting either by age or outcome or the interaction of age and outcome using filter T4. Among them, there were 11 differentially expressed probe sets with a significant difference adjusting by the interaction of age and outcome ($p < 0.05$). Probe sets of the same gene were merged. There were 6 genes with statistically significant differences between groups (Table 5).

15 We further analyzed the mean and 95% confidence interval (CI) of these 6 DEGs (Table 5). Means (95% CI) without overlapped values between each group were italicized. The non-overlapped values implied that there were statistically significant differences between the two groups. For example, NR4A2 was differentially expressed in yr⁶⁰⁺ versus yr⁶⁰⁻ melanoma patients without recurrence. NR4A2 also

20 showed differences in yr⁶⁰⁺ patients with (recur^{yes}) versus those without recurrence (recur^{no}) (Table 5).

[00118] Table 5. Mean and 95% confidence interval (CI) of the DEGs adjusted by the interaction of age and outcome using a multivariable linear regression model in the first microarray dataset.

Variables	Total (N=97)	Young (<60 years old)		Old (≥ 60 years old)		P Value
		No recurrence (N=51)	Recurrence (N=28)	No recurrence (N=7)	Recurrence (N=11)	
NR4A2						<.001
Mean (95% CI)	6.0 (5.8 - 6.2)	6.0 (5.8 - 6.2)	5.6 (5.5 - 5.8)	7.6 (6.4 - 8.8)	5.7 (5.5 - 6.0)	
Mean $\pm$ SE	6.0 $\pm$ 0.1	6.0 $\pm$ 0.1	5.6 $\pm$ 0.1	7.6 $\pm$ 0.6	5.7 $\pm$ 0.1	
IL1B						<.001
Mean (95% CI)	6.5 (6.3 - 6.6)	6.5 (6.3 - 6.7)	6.2 (5.9 - 6.5)	7.8 (6.6 - 9.0)	6.0 (5.7 - 6.2)	
Mean $\pm$ SE	6.5 $\pm$ 0.1	6.5 $\pm$ 0.1	6.2 $\pm$ 0.1	7.8 $\pm$ 0.6	6.0 $\pm$ 0.1	
TFPI2						<.001
Mean (95%CI)	5.5 (5.4 - 5.7)	5.7 (5.5 - 5.9)	5.4 (5.1 - 5.8)	6.4 (5.8 - 7.0)	4.7 (4.2 - 5.1)	
Mean $\pm$ SE	5.5 $\pm$ 0.1	5.7 $\pm$ 0.1	5.4 $\pm$ 0.2	6.4 $\pm$ 0.3	4.7 $\pm$ 0.2	
CLEC7A						0.004
Mean (95%CI)	4.7 (4.5 - 4.9)	4.8 (4.6 - 5.1)	4.6 (4.3 - 4.9)	5.2 (4.4 - 6.0)	3.9 (3.5 - 4.3)	
Mean $\pm$ SE	4.7 $\pm$ 0.1	4.8 $\pm$ 0.1	4.6 $\pm$ 0.2	5.2 $\pm$ 0.4	3.9 $\pm$ 0.2	
PTGS2						0.001
Mean (95% CI)	6.1 (5.8 - 6.3)	6.2 (5.9 - 6.5)	5.7 (5.3 - 6.1)	7.6 (6.2 - 9.0)	5.5 (4.8 - 6.3)	
Mean $\pm$ SE	6.1 $\pm$ 0.1	6.2 $\pm$ 0.2	5.7 $\pm$ 0.2	7.6 $\pm$ 0.7	5.5 $\pm$ 0.4	
RGS1						<.001
Mean (95% CI)	5.9 (5.7 - 6.2)	6.2 (5.9 - 6.5)	5.5 (5.1 - 5.9)	7.2 (6.2 - 8.2)	5.1 (4.4 - 5.8)	
Mean $\pm$ SE	5.9 $\pm$ 0.1	6.2 $\pm$ 0.1	5.5 $\pm$ 0.2	7.2 $\pm$ 0.5	5.1 $\pm$ 0.4	

5 [00119] Transcriptome changes of immune genes and immune pathway genes in SLNs associated with recurrence in yr⁶⁰⁻ and yr⁶⁰⁺ melanoma patients by NanoString analysis

[00120] In the NanoString dataset, we first analyzed the differentially expressed immune genes between recur^{yes} and recur^{no} groups in younger melanoma patients (yr⁶⁰⁻). The results showed that there were 20 differentially expressed immune

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genes ($p < 0.05$) in this comparison (Supplementary Table 3). Selected differentially expressed immune genes between the recur^{yes} and recur^{no} patients with $p < 0.05$ and absolute fold change > 0.5 in the yr⁶⁰⁻ group were listed in Table 6. In yr⁶⁰⁻ patients with positive SLNs, highly expressed C6, interleukin 23 receptor (IL23R), B

5 melanoma antigen (BAGE), chemokine [C-C motif] ligand 16 (CCL16), and lower expression of S100 calcium binding protein B (S100B) were associated with recur^{yes} patients.

[00121] In older patients, there were 20 differentially expressed genes between the recur^{yes} and recur^{no} group ($p < 0.05$) (Supplementary Table 4). Table 7 lists the

10 selected differentially expressed immune genes by recurrence status in the yr⁶⁰⁺ melanoma patients with $p < 0.05$ and absolute fold change > 0.5 . In yr⁶⁰⁺ patients with positive SLNs, highly expressed FOS and CCL18 were associated with recur^{yes}.

[00122] When comparing the difference in the DEGs by recurrence status in both age groups, we found that MAPK11 was expressed more highly in the younger

15 melanoma patients in the recur^{yes} versus the recur^{no} group (FC=2.84) (Table 6). A similar family member, MAP2K4, had marginal expression in older patients in the recur^{yes} versus the recur^{no} group (FC=0.25) (Supplementary Table 4). CCL16 had a higher expression in the younger patient cohort in the recur^{yes} versus the recur^{no} group (FC=3.46) (Table 6). Another family member, CCL18, also had a higher expression in

20 older patients with recurrence (FC=1.8) (Table 7). C6 was more highly expressed in younger melanoma patients with recurrence (FC=4.28) (Table 6), while C3 had marginal expression in older patients with recurrence (FC=0.83) (Table 7).

[00123] In terms of immune pathway genes, there were 18 differentially expressed genes with $p < 0.05$ and absolute fold change > 0.5 in the younger patients

when comparing recur^{yes} versus recur^{no} (Table 8). A complete list of the DEGs with $p < 0.05$ is presented in Supplementary Table 5. In the group of older patients, there were 13 differentially expressed immune pathway genes with $p < 0.05$ and absolute fold change > 0.5 by recurrence status (Table 9). All the DEGs with $p < 0.05$ in the older group are listed in Supplementary Table 6. IRAK3 (interleukin-1 receptor-associated kinase 3) was the major immune pathway gene found in younger patients with recurrence (Table 8), while Wnt10b was the major pathway found in older patients with recurrence (Table 9). There were no overlapped immune pathway genes in either age group by recurrence status. These results suggested that, even though some immune genes have similar changes in older and younger patients, different pathways may be involved in recurrence in different age groups.

[00124] Supplementary Table 3. Differentially expressed immune genes in younger patients between the recur^{yes} and the recur^{no} group by NanoString analysis ($p < 0.05$).

Gene symbol	Gene Name	P value	Fold change
C6	complement component 6	0.00745	4.28
IL23R	interleukin 23 receptor	0.00545	3.64
BAGE	B melanoma antigen	0.0136	3.58
CCL16	chemokine (C-C motif) ligand 16	0.0168	3.46
SPINK5	serine peptidase inhibitor, Kazal type 5	0.0161	2.96
MAPK11	mitogen-activated protein kinase 11	0.00968	2.84
MST1R	macrophage stimulating 1 receptor (c-met-related tyrosine kinase)	0.00947	2.52
F2RL1	coagulation factor II (thrombin) receptor-like 1	0.00399	1.97
DOCK9	dedicator of cytokinesis 9	0.00847	1.61
IGF1R	insulin-like growth factor 1 receptor	0.0141	0.971
TBK1	TANK-binding kinase 1	0.0116	0.616
MAP2K2	mitogen-activated protein kinase kinase 2	0.00105	-0.306
HLA-A	major histocompatibility complex, class I, A	0.00051	-0.386
CCL4	chemokine (C-C motif) ligand 4	0.0165	-0.485

ICAM1	intercellular adhesion molecule 1	0.0143	-0.587
C1QBP	complement component 1, q subcomponent binding protein	0.0131	-0.9
PSMB8	proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional peptidase 7)	0.0121	-0.939
MIF	macrophage migration inhibitory factor (glycosylation-inhibiting factor)	0.016	-1.09
HLA-G	major histocompatibility complex, class I, G	0.00237	-1.18
S100B	S100 calcium binding protein B	0.00859	-5.64

[00125] Table 6. Selected differentially expressed immune genes between recur^{yes} and recur^{no} group in younger patients (yr⁶⁰⁺) by NanoString analysis ($p < 0.05$, absolute fold change > 0.5).

Gene symbol	Gene Name	P value	Fold change
C6	complement component 6	0.00745	4.28
IL23R	interleukin 23 receptor	0.00545	3.64
BAGE	B melanoma antigen	0.0136	3.58
CCL16	chemokine (C-C motif) ligand 16	0.0168	3.46
SPINK5	serine peptidase inhibitor, Kazal type 5	0.0161	2.96
MAPK11	mitogen-activated protein kinase 11	0.00968	2.84
MST1R	macrophage stimulating 1 receptor (c-met-related tyrosine kinase)	0.00947	2.52
F2RL1	coagulation factor II (thrombin) receptor-like 1	0.00399	1.97
DOCK9	dedicator of cytokinesis 9	0.00847	1.61
IGF1R	insulin-like growth factor 1 receptor	0.0141	0.971
TBK1	TANK-binding kinase 1	0.0116	0.616
ICAM1	intercellular adhesion molecule 1	0.0143	-0.587
C1QBP	complement component 1, q subcomponent binding protein	0.0131	-0.9
PSMB8	proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional peptidase 7)	0.0121	-0.939
MIF	macrophage migration inhibitory factor (glycosylation-inhibiting factor)	0.016	-1.09
HLA-G	major histocompatibility complex, class I, G	0.00237	-1.18
S100B	S100 calcium binding protein B	0.00859	-5.64

[00126] Supplementary Table 4. Differentially expressed immune genes in older patients between the recur^{yes} and the recur^{no} group by NanoString analysis ($p < 0.05$).

Gene symbol	Gene Name	P value	Fold change
FOS	FBJ murine osteosarcoma viral oncogene homolog	0.0221	1.9
CCL18	chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	0.00867	1.8
CXCR4	chemokine (C-X-C motif) receptor 4	0.0238	1.07
C3	complement component 3	0.00481	0.832
TLR10	toll-like receptor 10	0.0189	0.787
NOD1	nucleotide-binding oligomerization domain containing 1	0.00347	0.768
PLAU	plasminogen activator, urokinase	0.00371	0.741
CYBB	cytochrome b-245, beta polypeptide	0.00314	0.732
TLR6	toll-like receptor 6	0.013	0.626
HLA-DMA	major histocompatibility complex, class II, DM alpha	0.0192	0.606
TNFRSF13B	tumor necrosis factor receptor superfamily, member 13B	0.0191	0.555
CD84	CD84 molecule	0.014	0.504
ATG7	autophagy related 7	0.00748	0.486
HLA-DMB	major histocompatibility complex, class II, DM beta	0.0199	0.313
MAP2K4	mitogen-activated protein kinase kinase 4	0.00196	0.248
INPP5D	inositol polyphosphate-5-phosphatase, 145kDa	0.0117	0.216
ELK1	ELK1, member of ETS oncogene family	0.00172	-0.491
RELA	v-rel reticuloendotheliosis viral oncogene homolog A (avian)	0.0225	-0.52
IFITM1	interferon induced transmembrane protein 1	0.0176	-0.675
NCAM1	neural cell adhesion molecule 1	0.00896	-0.984

5 [00127] Table 7. Selected differentially expressed immune genes between the recur^{yes} and recur^{no} group in yr⁶⁰⁺ patients by NanoString analysis ($p < 0.05$, absolute fold change > 0.5).

Gene symbol	Gene Name	P value	Fold change
FOS	FBJ murine osteosarcoma viral oncogene homolog	0.0221	1.9
CCL18	chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	0.00867	1.8

CXCR4	chemokine (C-X-C motif) receptor 4	0.0238	1.07
C3	complement component 3	0.00481	0.832
TLR10	toll-like receptor 10	0.0189	0.787
NOD1	nucleotide-binding oligomerization domain containing 1	0.00347	0.768
PLAU	plasminogen activator, urokinase	0.00371	0.741
CYBB	cytochrome b-245, beta polypeptide	0.00314	0.732
TLR6	toll-like receptor 6	0.013	0.626
HLA-DMA	major histocompatibility complex, class II, DM alpha	0.0192	0.606
TNFRSF13B	tumor necrosis factor receptor superfamily, member 13B	0.0191	0.555
CD84	CD84 molecule	0.014	0.504
RELA	v-rel reticuloendotheliosis viral oncogene homolog A (avian)	0.0225	-0.52
IFITM1	interferon induced transmembrane protein 1	0.0176	-0.675
NCAM1	neural cell adhesion molecule 1	0.00896	-0.984

[00128] Table 8. Differentially expressed immune pathway genes in younger patients (yr⁶⁰⁻) between the recur^{yes} and recur^{no} group by NanoString analysis ($p < 0.05$, absolute fold change > 0.5).

Gene symbol	Gene Name	P value	Fold change
IRAK3	interleukin-1 receptor-associated kinase 3	0.00552	2.15
NKD1	naked cuticle homolog 1 (Drosophila)	0.00565	2.13
ACVR1C	activin A receptor, type IC	0.0111	1.9
SOS1	son of sevenless homolog 1 (Drosophila)	0.00681	1.42
EPOR	erythropoietin receptor	0.00773	1.32
ACVR2A	activin A receptor, type IIA	0.012	1.12
RAD50	RAD50 homolog (S. cerevisiae)	0.0101	0.83
SMAD2	SMAD family member 2	0.00233	0.799
DNMT3A	DNA (cytosine-5-)-methyltransferase 3 alpha	0.0113	0.745
RPS6KA5	ribosomal protein S6 kinase, 90kDa, polypeptide 5	0.00593	0.596
FANCL	Fanconi anemia, complementation group L	0.00732	-0.554
PPP2R1A	protein phosphatase 2, regulatory subunit A, alpha	0.00791	-0.679
RB1	retinoblastoma 1	0.0108	-0.84
UBB	ubiquitin B	0.0109	-0.849

CDK4	cyclin-dependent kinase 4	0.00456	-1.22
CASP9	caspase 9, apoptosis-related cysteine peptidase	0.00969	-1.22
HSP90B1	heat shock protein 90kDa beta (Grp94), member 1	0.0106	-1.25
PCNA	proliferating cell nuclear antigen	0.00314	-1.45

[00129] Supplementary Table 5. Differentially expressed immune pathway

genes in younger patients between the recur^{yes} and the recur^{no} group by NanoString analysis ($p < 0.05$).

Gene symbol	Gene Name	P value	Fold change
IRAK3	interleukin-1 receptor-associated kinase 3	0.00552	2.15
NKD1	naked cuticle homolog 1 (Drosophila)	0.00565	2.13
ACVR1C	activin A receptor, type IC	0.0111	1.9
SOS1	son of sevenless homolog 1 (Drosophila)	0.00681	1.42
EPOR	erythropoietin receptor	0.00773	1.32
ACVR2A	activin A receptor, type IIA	0.012	1.12
RAD50	RAD50 homolog (S. cerevisiae)	0.0101	0.83
SMAD2	SMAD family member 2	0.00233	0.799
DNMT3A	DNA (cytosine-5-)-methyltransferase 3 alpha	0.0113	0.745
RPS6KA5	ribosomal protein S6 kinase, 90kDa, polypeptide 5	0.00593	0.596
MAP2K2	mitogen-activated protein kinase kinase 2	0.00454	-0.363
PIK3R3	phosphoinositide-3-kinase, regulatory subunit 3 (gamma)	0.00981	-0.439
FANCL	Fanconi anemia, complementation group L	0.00732	-0.554
PPP2R1A	protein phosphatase 2, regulatory subunit A, alpha	0.00791	-0.679
RB1	retinoblastoma 1	0.0108	-0.84
UBB	ubiquitin B	0.0109	-0.849
CDK4	cyclin-dependent kinase 4	0.00456	-1.22
CASP9	caspase 9, apoptosis-related cysteine peptidase	0.00969	-1.22
HSP90B1	heat shock protein 90kDa beta (Grp94), member 1	0.0106	-1.25
PCNA	proliferating cell nuclear antigen	0.00314	-1.45

[00130] Table 9. Differentially expressed immune pathway genes in older patients (yr⁶⁰⁺) between the recur^{yes} and recur^{no} group by NanoString analysis ($p < 0.05$, absolute fold change > 0.5).

Gene symbol	Gene Name	P value	Fold change
WNT10B	wingless-type MMTV integration site family, member 10B	0.027	2.27
HSPA1A	heat shock 70kDa protein 1A	0.0283	2.04
FOS	FBJ murine osteosarcoma viral oncogene homolog	0.0219	1.96
DKK2	dickkopf WNT signaling pathway inhibitor 2	0.0247	1.7
IL6	interleukin 6 (interferon, beta 2)	0.00111	1.36
TGFB3	transforming growth factor, beta 3	0.0379	1.19
HHEX	hematopoietically expressed homeobox	0.0263	1.06
DLL4	delta-like 4 (Drosophila)	0.00752	0.883
XRCC4	X-ray repair complementing defective repair in Chinese hamster cells 4	0.0354	0.787
NR4A1	nuclear receptor subfamily 4, group A, member 1	0.0232	0.766
ALKBH2	alkB, alkylation repair homolog 2 (E. coli)	0.0384	0.752
PLAU	plasminogen activator, urokinase	0.00195	0.659
BID	BH3 interacting domain death agonist	0.0369	0.564

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[00131] Supplementary Table 6. Differentially expressed immune pathway genes in older patients between the recur^{yes} and the recur^{no} group by NanoString analysis ($p < 0.05$).

Gene symbol	Gene Name	P value	Fold change
WNT10B	wingless-type MMTV integration site family, member 10B	0.027	2.27
HSPA1A	heat shock 70kDa protein 1A	0.0283	2.04
FOS	FBJ murine osteosarcoma viral oncogene homolog	0.0219	1.96
DKK2	dickkopf WNT signaling pathway inhibitor 2	0.0247	1.7
IL6	interleukin 6 (interferon, beta 2)	0.00111	1.36
TGFB3	transforming growth factor, beta 3	0.0379	1.19
HHEX	hematopoietically expressed homeobox	0.0263	1.06
DLL4	delta-like 4 (Drosophila)	0.00752	0.883

XRCC4	X-ray repair complementing defective repair in Chinese hamster cells 4	0.0354	0.787
NR4A1	nuclear receptor subfamily 4, group A, member 1	0.0232	0.766
ALKBH2	alkB, alkylation repair homolog 2 (E. coli)	0.0384	0.752
PLAU	plasminogen activator, urokinase	0.00195	0.659
BID	BH3 interacting domain death agonist	0.0369	0.564
ERCC6	excision repair cross-complementing rodent repair deficiency, complementation group 6	0.0236	0.479
LAMA5	laminin, alpha 5	0.00181	0.467
NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	0.00751	0.418
RUNX1	runt-related transcription factor 1	0.0281	0.417
TFDP1	transcription factor Dp-1	0.0364	0.36
STK11	serine/threonine kinase 11	0.0186	0.312
BCOR	BCL6 corepressor	0.0379	-0.377

[00132] Verification of the DEGs in the third independent dataset by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

[00133] After using microarray and NanoString technologies to identify the
 5 DEGs in the yr⁶⁰⁻ and yr⁶⁰⁺ patients as well as the different outcome-associated age groups (recur^{yes} versus recur^{no}), we used qRT-PCR in another independent dataset to confirm the findings above. We selected genes that were differentially expressed in both microarray and NanoString analysis or had higher fold changes in either of the analysis. The results showed that FOS, NR4A2, PTGS2, LINC00518, IL1B, and
 10 Wnt10b were all highly expressed in older patients with recurrence (Table 10). These genes converged at the Wnt10b pathway (Fig. 2).

[00134] Table 10. qRT-PCR Validation of the DEGs in the third independent dataset (recur^{yes} versus recur^{no})

Gene name	Fold Change	
	Age <60	Age ≥ 60
FOS	+1.2	+12.6

NR4A2	+1.4	+4.2
PTGS2	+1.8	+3.5
LINC00518	+3.2	+9.0
IL1B	-1.2	+1.1
Wnt10b	+1.6	+2.9
CCL18	-1.7	+1.3
HSPA1A	-1.2	+1.3
NRCAM	+1.14	+2.0
CXCL5	+1.4	+1.6

[00135] **Discussion**

[00136] In this study, we used three independent datasets and two different technologies, microarray and NanoString, to identify the DEGs in SLNs that are associated with recurrence by age group. NanoString used a novel method of direct mRNA barcoding and digital detection of target molecules through the use of color-coded probe pairs. This new technology does not need reverse transcription and the downstream PCR amplification to assess the gene expression level. We selected an immune panel and an immune pathway panel for NanoString analysis to focus on immune-related gene changes in SLNs. The results showed that there was some overlap of DEGs (NR4A and FOS) that have been detected by both technologies. Those genes have been confirmed by PCR in an independent dataset. Some genes (PTGS2, IL1B, LINC00518, and Wnt10b) that have higher fold changes detected by either of the two technologies were also confirmed by PCR in an independent dataset. The two technologies complement each other. In combination with the three independent datasets used in this study, these data provide a higher standard of research integrity.

[00137] Our results showed that Wnt signaling and related genes in SLNs have changes that correlate with recurrence in older melanoma patients with SLN metastasis. We found that SFRP2 and SFRP4 has high fold change genes in older melanoma patients compared with their younger counterparts.

5 [00138] Currently, no reports have shown how the PTGS2-NR4A-Wnt network is associated with age-related immunity in melanoma. In our study, we found that Wnt10b was upregulated in older melanoma patients who experience recurrence. The upstream genes of PTGS2 and NR4A were also upregulated.

[00139] Our results showed that FOS was the gene with a high fold change in
10 recur^{yes} versus recur^{no} in older melanoma patients. Upregulated FOS might occur in conjunction with activated Wnt pathway to promote melanoma progression in older patients.

[00140] The headings used in the disclosure are not meant to suggest that all
15 disclosure relating to the heading is found within the section that starts with that heading. Disclosure for any subject may be found throughout the specification.

[00141] It is noted that terms like “preferably,” “commonly,” and “typically” are not used herein to limit the scope of the claimed invention or to imply that certain features are critical, essential, or even important to the structure or function of the
20 claimed invention. Rather, these terms are merely intended to highlight alternative or additional features that may or may not be utilized in a particular embodiment of the present invention.

[00142] As used in the disclosure, “a” or “an” means one or more than one, unless otherwise specified. As used in the claims, when used in conjunction with the

word “comprising” the words “a” or “an” means one or more than one, unless otherwise specified. As used in the disclosure or claims, “another” means at least a second or more, unless otherwise specified. As used in the disclosure, the phrases “such as”, “for example”, and “e.g.” mean “for example, but not limited to” in that the list following the term (“such as”, “for example”, or “e.g.”) provides some examples but the list is not necessarily a fully inclusive list. The word “comprising” means that the items following the word “comprising” may include additional unrecited elements or steps; that is, “comprising” does not exclude additional unrecited steps or elements.

[00143] Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term “about”. Accordingly, unless indicated to the contrary, the numerical parameters set forth in this specification and claims are approximations that can vary depending upon the desired properties sought to be obtained by the presently-disclosed subject matter.

[00144] As used herein, the term “about” when referring to a value or to an amount of mass, weight, time, volume, concentration or percentage is meant to encompass variations of in some embodiments $\pm 20\%$, in some embodiments $\pm 10\%$, in some embodiments $\pm 5\%$, in some embodiments $\pm 1\%$, in some embodiments $\pm 0.5\%$, and in some embodiments $\pm 0.1\%$ from the specified amount, as such variations are appropriate to perform the disclosed method.

[00145] Detailed descriptions of one or more embodiments are provided herein. It is to be understood, however, that the present invention may be embodied in various forms. Therefore, specific details disclosed herein (even if designated as preferred or

advantageous) are not to be interpreted as limiting, but rather are to be used as an illustrative basis for the claims and as a representative basis for teaching one skilled in the art to employ the present invention in any appropriate manner. Indeed, various modifications of the invention in addition to those described herein will become
5 apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

[00146] What is claimed is:

CLAIMS

1. A method for treating melanoma in a subject, the method comprising:
 - quantifying an RNA expression level for at least one biomarker in a sample from a sentinel lymph node of the subject, where the at least one biomarker comprises
 - 5 FOS, NR4A, ITGB1, IRAK3, Wnt10b, or a combination thereof, and
 - administering to the subject immunotherapy, interferon, a BRAF inhibitor, a checkpoint inhibitor, a Wnt10b inhibitor, an IRAK3 inhibitor, or a combination thereof.
- 10 2. The method of claim 1, wherein the at least one biomarker comprises NR4A1, NR4A2, NR4A3, or a combination thereof.
3. The method of claim 1 or claim 2, wherein the at least one biomarker comprises NR4A2, NR4A3, or both.
- 15 4. The method of any of the preceding claims, wherein the at least one biomarker comprises NR4A, FOS, Wnt10b, or a combination thereof.
5. The method of any of the preceding claims, wherein the at least one biomarker
- 20 comprises NR4A.
6. The method of any of the preceding claims, wherein the at least one biomarker comprises IRAK3.

7. The method of any of the preceding claims, wherein the at least one biomarker further comprises SFRP2, SFRP4, PTGS2, LINC00518, IL1B, or a combination thereof.
- 5 8. The method of any of the preceding claims, wherein the at least one biomarker further comprises a biomarker listed in Table 2, Table 3, Table 4, Table 5, Table 6, Table 7, Table 8, Table 9, Table 10, Supp. Table 2, Supp. Table 3, Supp. Table 4, Supp. Table 5, Supp. Table 6, or a combination thereof.
- 10 9. The method of any of the preceding claims, wherein the at least one biomarker further comprises ACVR1C, ACVR2A, ALCAM, ALKBH2, ATG7, ATP2B2, BACH2, BAGE, BCOR, BID, C1QBP, C3, C6, CASP9, CCL16, CCL18, CCL4, CD244, CD84, CD8A, CDK4, CDK6, CDKN1A, CLEC4C, CLEC7A, COL28A1, COMP, CTNNB1, CXL/CXCR, CXCL3, CXCL5, CXCR4, CYBB, DKK2, DLK1,
- 15 DLL4, DNMT3A, DOCK9, DUSP1, ELK1, EPOR, ERCC2, ERCC6, ERGIC3, F2RL1, FANCB, FANCL, FOS, FOSB, FUT8, GADD45A, GRB14, GRIK2, HHEX, HLA-A, HLA-DMA, HLA-DMB, HLA-G, HSP90B1, HSPA1A, ICAM1, IDO1, IFIH1, IFITM1, IGF1R, IL1B, IL23R, IL6, INHBA, INPP5D, IRAK3, ITGB1, ITGBL1, JAM3, KLF4, KLRC4-KLRK1///KLRK1, LAMA5, LIF, LINC00354,
- 20 LINC00518, LIX1, LOC100507516, LOC101928963, LOC105373225, MAGEA3, MAP2K2, MAP2K4, MAPK11, MAVS, MIF, MKX, MLANA, MME, MS4A6A, MST1R, MUC15, MX1, NCAM1, NFKBIZ, NKD1, NOD1, NOG, NR4A, NR4A1, NR4A2, NR4A3, NRCAM, PBRM1, PCNA, PIK3CB, PIK3R3, PLAUG, PLD1, PPP2R1A, PRKAR2A, PRUNE2, PSMB8, PTGS2, RAD50, RB1, RELA, RGS1,

RNF152, RPS6KA5, RUNX1, S100B, SATB1, SFRP2, SFRP4, SLC13A5, SMAD2, SOS1, SPINK5, STK11, TBK1, TFDPI, TFPI2, TGFB3, TLR10, TLR6, TNC, TNFRSF10C, TNFRSF13B, UBB, WNT10B, WWC1, XRCC4, or a combination thereof.

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10. The method of any of the preceding claims, wherein the quantifying is carried out using polymerase chain reaction, real-time polymerase chain reaction, reverse transcriptase polymerase chain reaction, real-time quantitative RT-PCR, microarray, NanoString, or a combination thereof.

10

11. The method of any of the preceding claims, wherein the quantifying is carried out using polymerase chain reaction, real-time polymerase chain reaction, reverse transcriptase polymerase chain reaction, real-time quantitative RT-PCR, or a combination thereof.

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12. The method of any of the preceding claims, wherein the subject is no more than about 30 years old, no more than about 40 years old, no more than about 50 years old, no more than about 60 years old, or no more than about 70 years old.

20

13. The method of any of the preceding claims, wherein the subject is at least about 40 years old, at least about 50 years old, at least about 60 years old, at least about 70 years old, or at least about 80 years old.

14. The method of any of the preceding claims, wherein the subject has a positive sentinel lymph node status.

15. The method of any of the preceding claims, wherein the quantifying is relative to a control sample where the melanoma was without recurrence for at least about 5.0 years.

16. The method of any of the preceding claims, wherein the fold change in the RNA expression level, relative to a control sample where the melanoma was without recurrence for at least about 5.0 years, in one or more of the at least one biomarker is at least about 1.0, at least about 1.5, at least about 2.0, at least about 2.5, at least about 3.0, at least about 3.5, at least about 4.0, or at least about 4.5.

17. The method of any of the preceding claims, wherein the method further comprises assessing a clinicopathologic feature of the subject from which the sample was obtained.

18. The method of any of the preceding claims, wherein the method further comprises assessing a clinicopathologic feature of the subject from which the sample was obtained and the clinicopathologic feature is age, gender, anatomic location, Breslow thickness, ulceration, sentinel lymph node status, or a combination thereof.

19. The method of any of the preceding claims, wherein the method further comprises assessing a clinicopathologic feature of the subject from which the sample

was obtained and the clinicopathologic feature is metastasis, age, lesion site, tumor burden, number of positive nodes, ulceration, tumor thickness, or a combination thereof.

5 20. The method of any of the preceding claims, wherein the subject has stage III melanoma.

21. The method of any of the preceding claims, wherein the administering comprises administering interferon-gamma, interferon alfa-2b, or both.

10

22. The method of any of the preceding claims, wherein the administering comprises administering a BRAF inhibitor, vemurafenib, dabrafenib, trametinib, encorafenib, or a combination thereof.

15 23. The method of any of the preceding claims, wherein the administering comprises administering a checkpoint inhibitor, a PD-1 inhibitor, nivolumab, pembrolizumab, cemiplimab, a PD-L1 inhibitor, atezolizumab, avelumab, durvalumab, a cytotoxic T-lymphocyte antigen 4 inhibitor, ipilimumab, or a combination thereof.

20 24. The method of any of the preceding claims, wherein the administering comprises administering a Wnt10b inhibitor, DKN-01, CGX1321, ETC 1922159, or a combination thereof.

25. The method of any of the preceding claims, wherein (a) the administering comprises administering a Wnt10b inhibitor, DKN-01, CGX1321, ETC 1922159 or a combination thereof and (b) the subject is at least about 60 years old.

5 26. The method of any of the preceding claims, wherein the administering comprises administering an IRAK3 inhibitor, pacritinib, thymoquinone, or a combination thereof.

10 27. The method of any of the preceding claims, wherein (a) the administering comprises administering an IRAK3 inhibitor, pacritinib, thymoquinone, or a combination thereof and (b) the subject is no more than about 60 years old.

15 28. The method any of the preceding claims, wherein the administering only occurs if the fold change in the RNA expression level, relative to a control sample where the melanoma was without recurrence for at least about 5.0 years, in one or more of the at least one biomarker is at least about 1.0, at least about 1.5, at least about 2.0, at least about 2.5, at least about 3.0, at least about 3.5, at least about 4.0, or at least about 4.5.

20 29. The method any of the preceding claims, wherein the administering comprises administering a Wnt10b inhibitor, DKN-01, CGX1321, ETC 1922159, or a combination thereof, and the administering only occurs if (a) the fold change in the RNA expression level, relative to a control sample where the melanoma was without recurrence for at least about 5.0 years, in one or more of the at least one biomarker is at least about 1.0, at least about 1.5, at least about 2.0, at least about 2.5, at least about

3.0, at least about 3.5, at least about 4.0, or at least about 4.5 and (b) the subject is at least about 60 years old.

30. The method any of the preceding claims, wherein the administering comprises
5 administering of IRAK3 inhibitor, pacritinib, thymoquinone, or a combination thereof, and the administering only occurs if (a) the fold change in the RNA expression level, relative to a control sample where the melanoma was without recurrence for at least about 5.0 years, in one or more of the at least one biomarker is at least about 1.0, at least about 1.5, at least about 2.0, at least about 2.5, at least about
10 3.0, at least about 3.5, at least about 4.0, or at least about 4.5 and (b) the subject is no more than about 60 years old.

31. The method any of the preceding claims, wherein the treating further comprises surgery, chemotherapy, radiation therapy, targeted therapy, vaccine therapy, or a
15 combination thereof.

32. The method any of the preceding claims, wherein the subject is a mammal, a primate, or a human.

20 33. The method any of the preceding claims, wherein the subject is a human.

34. A method for treating melanoma in a human, the method comprising:
- quantifying an RNA expression level for at least one biomarker in a sample
from a sentinel lymph node of the human, where the at least one biomarker comprises
25 FOS, NR4A, Wnt10b, or a combination thereof, and

- administering to the human a Wnt10b inhibitor, DKN-01, CGX1321, ETC 1922159, or a combination thereof, and optionally one or more of immunotherapy, interferon, a BRAF inhibitor, a checkpoint inhibitor, or an IRAK3 inhibitor;

wherein

- 5 - the human is at least about 60 years old, and
- the administering only occurs if the fold change in the RNA expression level, relative to a control sample where the melanoma was without recurrence for at least about 5.0 years, in one or more of the at least one biomarker is at least about 1.5.

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35. A method for treating melanoma in a human, the method comprising:

- quantifying an RNA expression level for at least one biomarker in a sample from a sentinel lymph node of the human, where the at least one biomarker comprises IRAK3, and

15

- administering to the human an IRAK3 inhibitor, pacritinib, thymoquinone, or a combination thereof, and optionally one or more of immunotherapy, interferon, a BRAF inhibitor, a checkpoint inhibitor, or a Wnt10b inhibitor;

wherein

- the human is no more than about 60 years old, and
- 20 - the administering only occurs if the fold change in the RNA expression level, relative to a control sample where the melanoma was without recurrence for at least about 5.0 years, in one or more of the at least one biomarker is at least about 1.5.

25

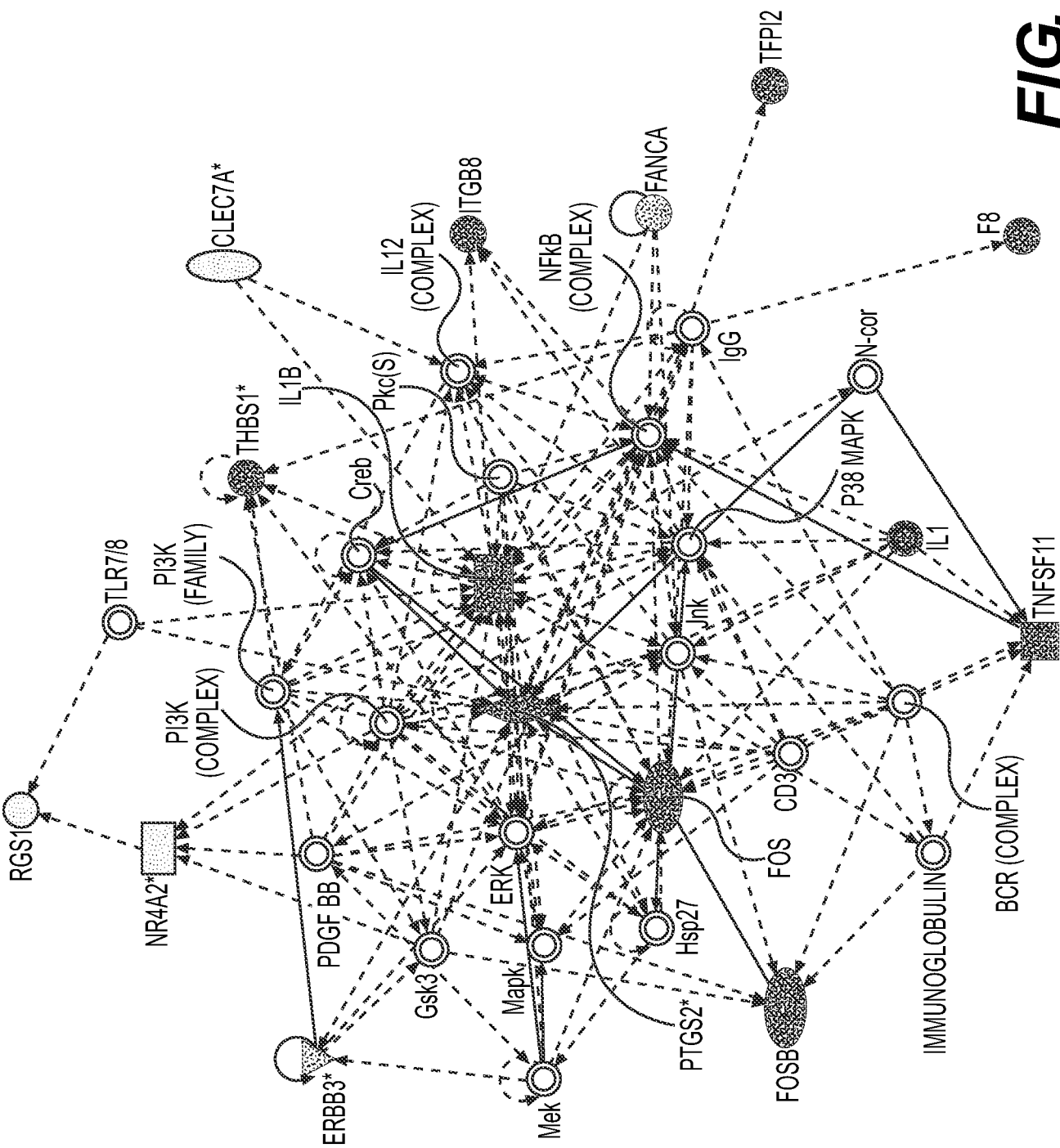


FIG. 1

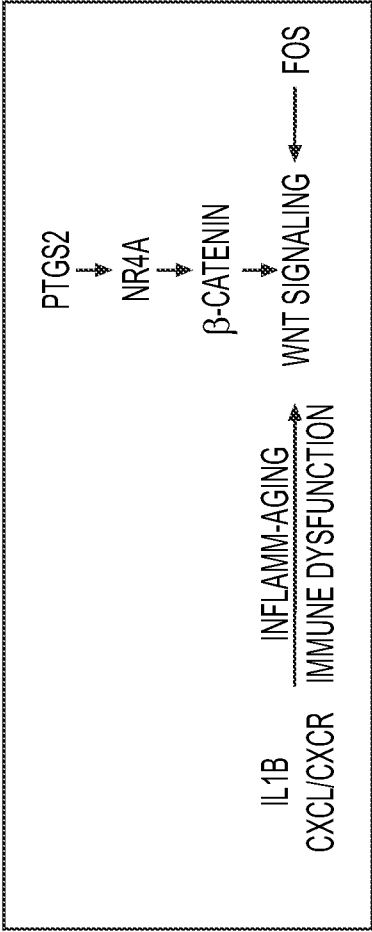


FIG. 2