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(54) **Title:** RECURRENT MUTATION IN MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE 5 (MAP3K5) FOR DIAGNOSING AND TREATING MELANOMA

(57) **Abstract:** Described herein is an analysis of whole-genome and whole-exome data to identify recurrent somatically mutated genes in melanoma. The analysis identified a recurrent somatic mutation in the gene encoding mitogen- activated protein kinase kinase kinase 5 (MAP3K5) in 5 of 288 tumors. Functional analysis of the MAP3K5 mutation (C766T) revealed increased activation of the MEK/MAPK pathway, increased binding to thioredoxin (Trx), increased anchorage- independent growth and migration of melanoma cells. Thus, provided herein are methods of diagnosing, prognosing and treating subjects with melanoma by detecting the presence or absence of the C766T mutation in the MAP3K5 gene.



RECURRENT MUTATION IN MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE 5 (MAP3K5) FOR DIAGNOSING AND TREATING MELANOMA

CROSS REFERENCE TO RELATED APPLICATIONS

5 This application claims the benefit of U.S. Provisional Application No. 61/708,785, filed October 2, 2012, which is herein incorporated by reference in its entirety.

FIELD

10 This disclosure concerns the identification of a mutation in the mitogen-activated kinase kinase kinase 5 (MAP3K5) gene, and its use in the diagnosis, prognosis and treatment of melanoma.

BACKGROUND

15 In the United States one in four deaths occurs as a result of cancer. Despite this striking statistic, overall death rates are decreasing, largely due to improved diagnosis, treatment and prevention strategies. Even with these improvements, there are still a number of cancers whose incidence rates continue to rise. Melanoma falls within this category; in the United States alone approximately 76,250 new diagnoses and 9,180 deaths are predicted for 2012 (Siegel *et al.*, *CA Cancer J Clin* 62(1):10-29, 2012). For these reasons, further understanding of the molecular
20 pathogenesis of this often lethal disease is needed.

 The development and progression of melanoma can be attributed to the acquisition of somatic aberrations. Targeting these mutations through use of molecularly based targeted drugs, such as the use of Vemurafenib/Dabrafenib in BRAF mutant tumors, has recently led to significant clinical responses in metastatic melanoma (Flaherty *et al.*, *N Engl J Med* 363(9):809-819, 2010; Chapman *et al.*, *N Engl J Med* 364(26):2507-2516, 2011). However, despite the success in
25 identifying genetic alterations utilizing candidate gene approaches (Davies *et al.*, *Nature* 417(6892):949-954, 2002; Curtin *et al.*, *J Clin Oncol* 24(26):4340-4346, 2006; Prickett *et al.*, *Nat Genet* 41(10):1127-1132, 2009) as well as whole-genome (Pleasance *et al.*, *Nature* 463(7278):191-196, 2010; Turajlic *et al.*, *Genome Res* 22(2):196-207, 2012; Berger *et al.*, *Nature* 485(7399):502-
30 506, 2012) and whole exome (Wei *et al.*, *Nat Genet* 43(5):442-446, 2011; Nikolaev *et al.*, *Nat Genet* 44(2):133-139, 2012; Stark *et al.*, *Nat Genet* 44(2):165-169, 2012) sequencing, there remain a significant number of patients with advanced melanoma without a targetable mutation. Further identification of alterations in new genes represents an ongoing urgent need.

The mitogen-activated protein kinase (MAPK) pathway regulates cellular processes such as proliferation, survival, and migration (Robinson and Cobb, *Curr Opin Cell Biol* 9(2):180-186, 1997). Further potentiation of these signaling molecules, through either amplification or somatic mutations, plays a major role in tumorigenesis (Dicker *et al.*, *Genes Chromosomes Cancer* 1(4):257-269, 1990; Davies *et al.*, *Nature* 417(6892):949-954, 2002; Nikolaev *et al.*, *Nat Genet* 44(2):133-139, 2012; Stark *et al.*, *Nat Genet* 44(2):165-169, 2012). Large-scale cancer genetic studies also support this statement (Dicker *et al.*, *Genes Chromosomes Cancer* 1(4):257-269, 1990; Davies *et al.*, *Nature* 417(6892):949-954, 2002; Clark *et al.*, *Genes Chromosomes Cancer* 41(2):99-108, 2004; Marks *et al.*, *Cancer Res* 68(14):5524-5528, 2008; Johannessen *et al.*, *Nature* 468(7326):968-972, 2010; Nikolaev *et al.*, *Nat Genet* 44(2):133-139, 2012; Stark *et al.*, *Nat Genet* 44(2):165-169, 2012). Recently, whole-exome studies of melanoma found the MAPK family members *MAP2K1* (MEK1) and *MAP2K2* (MEK2) to harbor recurrent somatic mutations leading to increased signal transduction, proliferation and cellular transformation, suggesting that further investigation of mutational activation of the MAPK pathway in melanoma is warranted (Marks *et al.*, *Cancer Res* 68(14):5524-5528, 2008; Nikolaev *et al.*, *Nat Genet* 44(2):133-139, 2012).

The MAP kinase kinase kinase-5 (MAP3K5), also known as apoptosis signal-regulating kinase 1 (ASK1), is a serine/threonine protein kinase that activates JNK and p38 (Tzeng *et al.*, *Biochem Pharmacol* 85:531-540, 2013; Yang *et al.*, *Microbes Infect* 12:643-651, 2010) via activation of MAPK kinase-4/7 (MKK4/7). MAP3K5 can be activated in response to stress signals, including H₂O₂, tumor necrosis factor- α (TNF α) or reduced serum levels (Tzeng *et al.*, *Biochem Pharmacol* 85:531-540, 2013). It has been shown that a molecular target of reactive oxygen species (ROS), thioredoxin (TXN/Trx), is an inhibitor of MAP3K5 (Saitoh *et al.*, *EMBO J* 17:2596-2606, 1998). Trx binds to the N-terminus of MAP3K5 attenuating its kinase activity as well as downstream apoptotic signaling mechanisms (Saitoh *et al.*, *EMBO J* 17:2596-2606, 1998). Oxidation via ROS disrupts binding of Trx to MAP3K5, resulting in apoptosis. Interestingly, normal melanocytes are known to scavenge ROS while melanoma cells contain structurally abnormal melanocytes that generate free radicals (Fruehauf and Trapp, *Expert Rev Anticancer Ther* 8:1751-1757, 2008; Gidanian *et al.*, *Photochem Photobiol* 84:556-564, 2008). As a result, a mutation in MAP3K5 in melanoma cells that strengthens the interaction with Trx could lead to evasion of cell death and thus increased survival in the face of excessive amounts of ROS.

SUMMARY

Disclosed herein is the identification of a recurrent somatic mutation in the gene encoding MAP3K5 by analysis of whole-genome and whole-exome data. Functional analysis of the MAP3K5 mutation (C766T) revealed increased activation of the MEK/MAPK pathway, increased binding to thioredoxin (Trx), increased anchorage-independent growth and increased migration of melanoma cells.

Provided herein is a method of diagnosing a subject as having melanoma, or susceptible to developing melanoma, by detecting the presence of a C766T mutation in the MAP3K5 gene (SEQ ID NO: 1) in a sample obtained from the subject and diagnosing the subject as having melanoma or susceptible to developing melanoma if the C766T mutation is detected. Also provided is a method of determining the prognosis of a subject diagnosed with melanoma by detecting the presence of a C766T mutation in the MAP3K5 gene (SEQ ID NO: 1) in a sample obtained from the subject and determining that the subject has a poor prognosis if the C766T mutation is detected. In some embodiments, detecting the presence of the C766T mutation includes genotyping a sample obtained from the subject at the position of the C766T mutation. In some embodiments, the method further includes providing an appropriate therapy, such as administration of a MAPK pathway inhibitor to the subject, administration of a Trx/thioredoxin reductase (TrxR) inhibitor to the subject, or instituting precautionary measures such as increased surveillance for melanoma development or avoidance of melanoma precipitating agents, such as ultraviolet radiation.

Further provided is a method of treating a subject diagnosed with melanoma, by selecting a subject in whom a C766T mutation in the MAP3K5 gene of the subject is present, or has been determined to be present, and administering an inhibitor of the MAPK pathway or an inhibitor of Trx/TrxR to the subject in whom the C766T mutation is present. In some embodiments, the method further includes detecting the presence of the C766T mutation in the subject. In some examples, detecting the presence of the C766T mutation comprises genotyping a sample obtained from the subject at the position of the C766T mutation.

The foregoing and other objects, features, and advantages of the invention will become more apparent from the following detailed description, which proceeds with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A -1C are immunoblots showing the effects of the MAP3K5 (R256C) recurrent mutation on cell signaling. Lysates and immunoprecipitates from cells transiently or stably expressing wild-type or mutant MAP3K5 were analyzed for activation of MAP3K5 and its downstream effector molecules. (FIG. 1A) HEK293T cells were transiently transfected with wild-type MAP3K5 (WT), mutant MAP3K5 (R256C) or empty vector as control. Lysates were generated and immunoblotted with the indicated antibodies. (FIG. 1B) Mel-STR and (FIG. 1C) 2183 (17T) clones stably expressing MAP3K5 (WT, R256C, or empty vector) were tested for increased signaling downstream of MAP3K5. Lysates were immunoprecipitated with anti-FLAG (M2) beads or directly analyzed via SDS-PAGE. Immunoblots were probed with the indicated antibodies. In each case, anti-GAPDH was used as a loading control.

FIGS. 2A-2D are graphs showing the effects of the MAP3K5 (R256C) recurrent mutation on cell growth and cell migration. (FIG. 2A) Wild-type MAP3K5 suppresses growth in soft agar. Mel-STR and 2183 (17T) pooled MAP3K5 clones were seeded into soft agar to test for anchorage-independent growth. Mel-STR (WT, R256C or empty vector) clones were grown for 10 days prior to harvesting, staining and counting. (FIG. 2B) 2183 (17T) (WT, R256C or empty vector) clones were grown for 10 days prior to harvesting, staining and counting. Mel-STR or 2183 (17T) pooled clones were seeded in 96-well plates in various serum concentrations to assess for differences in growth properties on cells expressing either WT MAP3K5 or R256C. (FIG. 2C) Mel-STR (WT, R256C or empty vector) clones were seeded in 96-well plates in the presence of 1% serum and grown for 9-14 days. SYBRTM Green was used to determine cell counts per day harvested. (FIG. 2D) 2183 (17T) (WT, R256C or empty vector) clones were seeded in 96-well plates in the presence of 1% serum and grown for 9-14 days. SYBRTM Green was used to determine cell counts per day harvested. Graphs are averages of three parallel experiments with standard deviations (n=3; (* comparing WT or R256C to empty vector, ** comparing WT to R256C); * p<0.01 using an unpaired student's t test).

FIGS. 3A-3C show the effects of stable depletion of MAP3K5 on melanoma cell growth. shRNA-mediated depletion of MAP3K5 was tested using transient transfection and immunoblotting of lysates or RT-PCR analysis of mRNA from melanoma cells depleted of endogenous MAP3K5. (FIG. 3A) Lysates from HEK293T transiently transfected with MAP3K5-FLAG and either one of three MAP3K5-specific shRNAs or empty vector were immunoblotted using the indicated antibodies to show specificity. (FIG. 3B) mRNA from the 501Mel melanoma cell line was tested for stable depletion of MAP3K5 using RT-PCR analysis. GAPDH was used as

a loading control. (FIG. 3C) Depletion of MAP3K5 decreases proliferation of melanoma cells with mutant MAP3K5. Melanoma cells harboring either wild-type or mutant MAP3K5 were depleted of MAP3K5 and seeded in 96-well plates to assess for differences in growth properties. The cells were harvested and tested for proliferation using SYBRTM Green I. Microsoft Excel was used to analyze experiments and generate graphs that are averages of three parallel experiments with standard deviations. n=3; * p<0.01, using an unpaired student's t test.

FIGS. 4A and 4B are graphs showing the effect of R256C MAP3K5 on melanoma cell growth and proliferation. (FIG. 4A) Mel-STR (WT, R256C or empty vector) clones were seeded in 96-well plates in the presence of 10% serum and grown for 9-14 days. SYBRTM Green was used to determine cell counts per day harvested. (FIG. 4B) 2183 (17T) (WT, R256C or empty vector) clones were seeded in 96-well plates in the presence of 10% serum and grown for 9-14 days. SYBRTM Green was used to determine cell counts per day harvested. Graphs are averages of three parallel experiments with standard deviations (n=3; (* comparing WT or R256C to empty vector, ** comparing WT to R256C); * p<0.01 using an unpaired student's t test).

FIGS. 5A and 5B are immunoblots showing the somatic mutation in MAP3K5 at residue R256C causes increased binding of thioredoxin (Trx). MAP3K5 binds Trx in the absence of stimuli and the R256C mutation enhances binding. (FIG. 5A) HEK293 cells transiently transfected with MAP3K5-FLAG (WT, R256C, vector) and myc-Trx were analyzed for MAP3K5:Trx complex formation by co-immunoprecipitation with anti-myc. (FIG. 5B) 2183 melanoma stable pooled clones expressing MAP3K5-FLAG (WT, R256C, or empty vector) were analyzed for MAP3K5:Trx complex formation by co-immunoprecipitation with anti-Trx. Immunoprecipitates were analyzed using the antibodies shown and lysates were probed with anti-GAPDH as an internal control.

SEQUENCE LISTING

The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids, as defined in 37 C.F.R. 1.822. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand. The Sequence Listing is submitted as an ASCII text file, created on September 12, 2013, 21.5 KB, which is incorporated by reference herein. In the accompanying sequence listing:

SEQ ID NOs: 1 and 2 are the nucleotide and amino acid sequences, respectively, of human MAP3K5 deposited under GenBankTM Accession No. NM_005923.

SEQ ID NOs: 3-8 are nucleotide sequences of MAP3K5 primers used for non-synonymous recurrent mutation confirmation.

SEQ ID NOs: 9-16 are nucleotide sequences of MAP3K5 primers used for plasmid construction and RT-PCR.

5

DETAILED DESCRIPTION

I. Abbreviations

	ASK1	apoptosis signal-regulating kinase 1
	cDNA	complementary DNA
10	ERK	extracellular signal-regulated protein kinase
	FBS	fetal bovine serum
	MAP3K5	mitogen-activated protein kinase kinase kinase 5
	MAPK	mitogen-activated protein kinase
	MEK	MAPK/ERK kinase
15	OCT	optimum cutting temperature
	ROS	reactive oxygen species
	RT-PCR	reverse transcriptase polymerase chain reaction
	SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
	shRNA	short hairpin RNA
20	TNF	tumor necrosis factor
	Trx	thioredoxin
	TrxR	thioredoxin reductase
	WT	wild-type

25 II. Terms and Methods

Unless otherwise noted, technical terms are used according to conventional usage.

Definitions of common terms in molecular biology may be found in Benjamin Lewin, *Genes V*, published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew *et al.* (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

In order to facilitate review of the various embodiments of the disclosure, the following explanations of specific terms are provided:

Administration: The introduction of a composition into a subject by a chosen route. For example, if the chosen route is intravenous, the composition is administered by introducing the composition into a vein of the subject. Exemplary routes of administration include, but are not limited to, intradermal, intramuscular, intraperitoneal, parenteral, intravenous, subcutaneous, vaginal, rectal, intranasal, inhalation and oral.

Biological Sample: A biological specimen containing genomic DNA, RNA, protein, or combinations thereof, obtained from a subject. Examples include, but are not limited to, peripheral blood, urine, saliva, tissue biopsy (such as skin tissue), surgical specimen, and autopsy material. In one example, a sample includes a biopsy of a melanoma tumor or a sample of normal tissue, such as skin tissue (from a subject not afflicted with a known disease or disorder, such as a cancer-free subject).

Clinical outcome: Refers to the health status of a patient following treatment for a disease or disorder (such as melanoma), or in the absence of treatment. Clinical outcomes include, but are not limited to, a decrease in incidence of melanoma, an increase in the length of time until death, a decrease in the length of time until death, an increase in the chance of survival, an increase in the risk of death, survival, disease-free survival, chronic disease, metastasis, advanced or aggressive disease, disease recurrence, death, and favorable or poor response to therapy.

Decrease in survival: As used herein, “decrease in survival” refers to a decrease in the length of time before death of a patient, or an increase in the risk of death for the patient. A decrease in survival also can refer to a decrease in the average time to death in a group, such as a group of patients diagnosed with melanoma.

Detecting a mutation: As used herein, detecting a mutation in a biological sample refers to identifying the mutation, for example, by genotyping a nucleic acid in the sample by detecting a mutation in the DNA or a DNA-dependent molecule such as an RNA or protein.

Diagnosing: Refers to the process of identifying the nature or cause of a disease or disorder.

Extracellular signal-regulated protein kinase (ERK): ERK proteins are members of the MAPK pathway. ERK proteins include ERK1 (MAPK3), ERK2 (MAPK1), ERK3 (MAPK6), ERK4 (MAPK4), ERK5 (MAPK7), ERK6 (MAPK12), ERK7 (MAPK15) and ERK8.

Genotyping: Determining differences in the genotype of an individual using biological assays.

Hybridization: Oligonucleotides and their analogs hybridize by hydrogen bonding, which includes Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between

complementary bases. Generally, nucleic acid consists of nitrogenous bases that are either pyrimidines (cytosine (C), uracil (U), and thymine (T)) or purines (adenine (A) and guanine (G)). These nitrogenous bases form hydrogen bonds between a pyrimidine and a purine, and the bonding of the pyrimidine to the purine is referred to as “base pairing.” More specifically, A will hydrogen
5 bond to T or U, and G will bond to C. “Complementary” refers to the base pairing that occurs between two distinct nucleic acid sequences or two distinct regions of the same nucleic acid sequence.

“**Specifically hybridizable**” and “specifically complementary” are terms that indicate a sufficient degree of complementarity such that stable and specific binding occurs between the
10 oligonucleotide (or its analog) and the DNA or RNA target. The oligonucleotide or oligonucleotide analog need not be 100% complementary to its target sequence to be specifically hybridizable. An oligonucleotide or analog is specifically hybridizable when binding of the oligonucleotide or analog to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA, and there is a sufficient degree of complementarity to avoid non-specific binding of the
15 oligonucleotide or analog to non-target sequences under conditions where specific binding is desired, for example under physiological conditions in the case of *in vivo* assays or systems. Such binding is referred to as specific hybridization.

Hybridization conditions resulting in particular degrees of stringency will vary depending upon the nature of the hybridization method of choice and the composition and length of the
20 hybridizing nucleic acid sequences. Generally, the temperature of hybridization and the ionic strength (especially the Na⁺ and/or Mg⁺⁺ concentration) of the hybridization buffer will determine the stringency of hybridization, though wash times also influence stringency. Calculations regarding hybridization conditions required for attaining particular degrees of stringency are discussed by Sambrook *et al.* (ed.), *Molecular Cloning: A Laboratory Manual*, 2nd ed., vol. 1-3,
25 Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, chapters 9 and 11; and Ausubel *et al. Short Protocols in Molecular Biology*, 4th ed., John Wiley & Sons, Inc., 1999.

For purposes of the present disclosure, “stringent conditions” encompass conditions under which hybridization will only occur if there is less than 25% mismatch between the hybridization molecule and the target sequence. “Stringent conditions” may be broken down into particular
30 levels of stringency for more precise definition. Thus, as used herein, “moderate stringency” conditions are those under which molecules with more than 25% sequence mismatch will not hybridize; conditions of “medium stringency” are those under which molecules with more than 15% mismatch will not hybridize, and conditions of “high stringency” are those under which

sequences with more than 10% mismatch will not hybridize. Conditions of “very high stringency” are those under which sequences with more than 6% mismatch will not hybridize.

Inhibitor: As used herein, an “inhibitor” refers to any compound that is capable of reducing or altering the expression or activity of a target molecule (such as a nucleic acid molecule or a protein). In some embodiments, the inhibitor is an inhibitor of the MAPK pathway, such as an inhibitor of MEK and/or ERK. In other embodiments, the inhibitor is an inhibitor of Trx and/or TrxR.

Melanoma: A form of cancer that originates in melanocytes (cells that make the pigment melanin). Melanocytes are found primarily in the skin, but are also present in the bowel and eye.

As used herein, “melanoma” refers to any stage of melanoma, or any subtype of melanoma, such as superficial spreading melanoma, nodular melanoma, acral lentiginous melanoma, lentigo maligna, melanoma-in-situ, mucosal melanoma and uveal melanoma.

Metastasis: Refers to the spread of cancer cells from the original tumor to other sites in the body.

Mitogen-activated protein kinase kinase kinase 5 (MAP3K5): A member of the MAP kinase kinase kinase family (MAPKKK or MEKK). MAPKK kinase/MEKK phosphorylates and activates its downstream protein kinase, MAPK kinase/MEK, which in turn activates MAPK. The kinases of these signaling cascades are highly conserved, and homologs exist in yeast, drosophila, and mammalian cells. MAP3K5 contains 1,374 amino acids with all 11 kinase subdomains. Northern blot analysis shows that the MAP3K5 transcript is abundantly expressed in human heart and pancreas. The MAP3K5 protein phosphorylates and activates MKK4 (aliases SERK1, MAPKK4) *in vitro*, and activates c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK). MAP3K5 is also known as apoptosis signal-regulating kinase 1 (ASK1), MAPKKK5 and MEKK5.

MAP3K5 sequences are publically available. For example, GenBank Accession No. NM_005923.3 provides the nucleotide and amino acid sequences of human MAP3K5 (also set forth herein as SEQ ID NO: 1 and SEQ ID NO: 2, respectively). The NCBI CCDS database also provides nucleotide and amino acid sequences for human MAP3K5 under CCDS ID 5179. The GenBank Accession numbers and CCDS ID numbers listed above and disclosed herein are incorporated by reference as they appear in the database as of August 3, 2012.

Mitogen-activated protein kinase (MAPK) pathway: A cellular signaling pathway that regulates cellular processes such as proliferation, survival, and migration (Robinson and Cobb, *Curr Opin Cell Biol* 9(2):180-186, 1997). Mammals express at least four distinctly regulated

groups of MAPKs, extracellular signal-related kinases (ERK)-1/2, Jun amino-terminal kinases (JNK1/2/3), p38 proteins (p38alpha/beta/gamma/delta) and ERK5, which are activated by specific MAPKKs: MEK1/2 for ERK1/2, MKK3/6 for the p38, MKK4/7 (JNKK1/2) for the JNKs, and MEK5 for ERK5. Each MAPKK can be activated by more than one MAPKKK, increasing the complexity and diversity of MAPK signaling. Each MAPK pathway contains a three-tiered kinase cascade comprising a MAP kinase kinase kinase (MAPKKK, MAP3K, MEKK or MKKK), a MAP kinase kinase (MAPKK, MAP2K, MEK or MKK) and the MAPK. This three-tier module mediates ultrasensitive switch-like responses to stimuli. Frequently, a MAPKKK kinase (MAPKKKK, MAP4K or MKKKK) activates the MAPKKK. The MAPKKKK or MAPKKK can be linked to the plasma membrane, for example, through association with a small GTPase or lipid (Qi and Elion, *J Cell Sci* 118(Pt 16):3569-3572, 2005).

MAPK/ERK Kinase (MEK): MEK proteins are members of the MAPK family. MEK proteins include MEK1 (MAPKK1 or MAP2K1), MEK2 (MAPKK2 or MAP2K2), MEK3 (MAPKK3 or MAP2K3), MEK4 (MAPKK4 or MAP2K4), MEK5 (MAPKK5 or MAP2K5), MEK6 (MAPKK6 or MAP2K6) and MEK7 (MAPKK7 or MAP2K7).

Mutation: Any change of the DNA sequence within a gene or chromosome. In some instances, a mutation will alter a characteristic or trait (phenotype), but this is not always the case. Types of mutations include base substitution point mutations (*e.g.*, transitions or transversions), deletions and insertions. Synonymous mutations are mutations in a gene that do not result in a change in the encoded amino acid sequence. Non-synonymous mutations are mutations in a gene that result in change in amino acid sequences, such as an amino acid substitution or introduction of a stop codon. Missense mutations are those that introduce a different amino acid into the sequence of the encoded protein; nonsense mutations are those that introduce a new stop codon. In the case of insertions or deletions, mutations can be in-frame (not changing the frame of the overall sequence) or frame shift mutations, which may result in the misreading of a large number of codons (and often leads to abnormal termination of the encoded product due to the presence of a stop codon in the alternative frame).

The term “mutation” specifically encompasses variations that arise through somatic mutation, for instance those that are found only in disease cells (such as cancer cells), but not constitutionally, in a given individual. Examples of such somatically-acquired variations include the point mutations that frequently result in altered function of various genes that are involved in development of cancers. This term also encompasses DNA alterations that are present

constitutionally, that alter the function of the encoded protein in a readily demonstrable manner, and that can be inherited by the children of an affected individual.

Patient: As used herein, the term “patient” includes human and non-human animals. “Patient” and “subject” are used interchangeably herein.

5 **Pharmaceutically acceptable vehicles:** The pharmaceutically acceptable carriers (vehicles) useful in this disclosure are conventional. *Remington’s Pharmaceutical Sciences*, by E. W. Martin, Mack Publishing Co., Easton, PA, 15th Edition (1975), describes compositions and formulations suitable for pharmaceutical delivery of one or more therapeutic compounds, molecules or agents.

10 In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. For solid compositions (for example, powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can
15 include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. In addition to biologically-neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

20 **Preventing, treating or ameliorating a disease:** “Preventing” or “inhibiting” a disease (such as melanoma) refers to inhibiting the full development of a disease. “Treating” refers to a therapeutic intervention that ameliorates a sign or symptom of a disease or pathological condition after it has begun to develop. “Ameliorating” refers to the reduction in the number or severity of signs or symptoms of a disease.

25 **Prognosis:** The likelihood of the clinical outcome for a subject afflicted with a specific disease or disorder. With regard to cancer, the prognosis is a representation of the likelihood (probability) that the subject will survive (such as for one, two, three, four or five years) and/or respond to anti-melanoma treatment, and/or the likelihood (probability) that the tumor will metastasize. A “poor prognosis” indicates a greater than 50% chance that the subject will not
30 survive to a specified time point (such as one, two, three, four or five years), and/or a greater than 50% chance that the tumor will metastasize. In several examples, a poor prognosis indicates that there is a greater than 60%, 70%, 80%, or 90% chance that the subject will not survive and/or a greater than 60%, 70%, 80% or 90% chance that the tumor will metastasize. Conversely, a “good

prognosis” indicates a greater than 50% chance that the subject will survive to a specified time point (such as one, two, three, four or five years), and/or a greater than 50% chance that the tumor will not metastasize. In several examples, a good prognosis indicates that there is a greater than 60%, 70%, 80%, or 90% chance that the subject will survive and/or a greater than 60%, 70%, 80% or 90% chance that the tumor will not metastasize.

Small molecule inhibitor: A molecule, typically with a molecular weight less than about 1000 Daltons, or in some embodiments, less than about 500 Daltons, wherein the molecule is capable of inhibiting, to some measurable extent, an activity of a target molecule.

Somatic mutation: An acquired mutation that occurs in a somatic cell (as opposed to a germ cell).

Subject: Living multi-cellular vertebrate organisms, a category that includes both human and non-human mammals. In some embodiments, the subject is a human subject.

Therapeutic agent: A chemical compound, small molecule, or other composition, such as an antisense compound, antibody, peptide or nucleic acid molecule capable of inducing a desired therapeutic or prophylactic effect when properly administered to a subject. For example, therapeutic agents for melanoma include agents that prevent or inhibit development or metastasis of melanoma. In some embodiments, the therapeutic agent is an inhibitor of the MAPK pathway, such as an inhibitor of ERK or MEK, or an inhibitor of Trx/TrxR.

Therapeutically effective amount: A quantity of a specific substance sufficient to achieve a desired effect in a subject being treated. For example, a therapeutically effective amount of a therapeutic agent to treat melanoma can refer to the amount necessary to inhibit tumor growth, decrease tumor volume, inhibit tumor metastasis, or prolong survival.

Therapy: The mode of treatment or care of a patient. In some cases, therapy refers to administration of a therapeutic agent. In some embodiments herein, therapy includes administration of an inhibitor of the MAPK pathway or an inhibitor of Trx/TrxR. In other examples, therapy includes surgery, such as surgical resection of a melanoma tumor, chemotherapy, radiation therapy, administration of a second therapeutic agent, or any combination thereof. In other examples the therapy is institution of precautionary or prophylactic measures to monitor or detect development of melanoma, such as examination of the skin at regular or shorter intervals for early detection of melanoma (for example before it has become metastatic) and can still be cured by surgical resection of the melanoma lesion. Alternatively, the precautionary measures include advising the subject to minimize or prevent contact with agents (such as exposure to ultraviolet radiation) that are associated with the development

of melanoma, or avoiding such agents, for example by application of ultraviolet radiation blocking agents (such as sunscreen) to the skin.

Thioredoxin (Trx): A redox-sensitive protein exhibiting pleiotropic cellular effects, including control of proliferation, redox states and apoptosis. Trx functions as a protective cellular antioxidant and a regulator of transcription factor activity (Mukherjee and Martin, *Br J Radiol* S57-S68, 2008). This protein is capable of scavenging reactive oxygen species and directly inhibiting pro-apoptotic proteins such as MAP3K5 (Tonissen and Di Trapani, *Mol Nutr Food Res* 53(1):87-103, 2009). The Trx protein binds the N-terminus of MAP3K5, attenuating its kinase activity and downstream apoptotic signaling mechanisms (Saitoh *et al.*, *EMBO J* 17:2596-2606, 1998). Trx is also known to be upregulated in some types of cancer and is thought to protect cancer cells from oxidative stress. A number of specific inhibitors of Trx and Trx reductase are known in the art (see, *e.g.*, Mukherjee and Martin, *Br J Radiol* S57-S68, 2008).

Thioredoxin reductase (TrxR): An enzyme that catalyzes the reduction of Trx. TrxR and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) recycle Trx from its inactive oxidized form (which contains a disulfide bond) to its active reduced form containing thiol (SH) groups.

Tumor, neoplasia, malignancy or cancer: A neoplasm is an abnormal growth of tissue or cells that results from excessive cell division. Neoplastic growth can produce a tumor. The amount of a tumor in an individual is the “**tumor burden**” which can be measured as the number, volume, or weight of the tumor. A tumor that does not metastasize is referred to as “**benign**.” A tumor that invades the surrounding tissue and/or can metastasize is referred to as “**malignant**.” A “**non-cancerous tissue**” is a tissue from the same organ wherein the malignant neoplasm formed, but does not have the characteristic pathology of the neoplasm. Generally, noncancerous tissue appears histologically normal. A “**normal tissue**” is tissue from an organ, wherein the organ is not affected by cancer or another disease or disorder of that organ. A “**cancer-free**” subject has not been diagnosed with a cancer of that organ and does not have detectable cancer.

Vector: A nucleic acid molecule as introduced into a host cell, thereby producing a transformed host cell. A vector may include nucleic acid sequences that permit it to replicate in a host cell, such as an origin of replication (DNA sequences that participate in initiating DNA synthesis). A vector may also include one or more selectable marker genes and other genetic elements known in the art.

Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure

belongs. The singular terms “a,” “an,” and “the” include plural referents unless context clearly indicates otherwise. “Comprising A or B” means including A, or B, or A and B. It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description.

5 Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, suitable methods and materials are described below.

All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including explanations of terms, will control. In addition, the materials, methods, and examples are

10 illustrative only and not intended to be limiting.

III. Introduction

Described herein is an analysis of whole-genome and whole-exome data to identify recurrent somatically mutated genes in melanoma. In particular, disclosed herein is the finding that
15 the gene encoding mitogen-activated protein kinase kinase kinase-5 (MAP3K5) harbored a recurrent somatic mutation (C766T → R256C) in 5 of 288 tumors analyzed. Functional analysis of the MAP3K5 mutation revealed increased activation of the MEK/MAPK pathway, increased binding to Trx, increased anchorage-independent growth and increased migration of melanoma cells. The presence of the R256C mutation in MAP3K5 converts the pro-death kinase into a pro-
20 survival kinase, increasing the likelihood of cancer cell survival. Thus, the presence of the C766T mutation in the MAP3K5 gene represents a potential target for the design of cancer therapies for the treatment of melanoma, as well as for the diagnosis and prognosis of melanoma.

IV. Overview of Several Embodiments

25 Provided herein is a method of diagnosing a subject as having melanoma, or susceptible to developing melanoma, by detecting the presence of a C766T mutation in the MAP3K5 gene (SEQ ID NO: 1); and diagnosing the subject as having melanoma or susceptible to developing melanoma if the C766T mutation is detected. In particular examples, the mutation is detected in a sample of skin tissue or melanoma tumor obtained from the subject.

30 In some embodiments, the diagnostic method comprises obtaining a skin biopsy from a subject with a suspicious skin lesion (such as a mole); detecting the presence of a C766T mutation in the MAP3K5 gene (SEQ ID NO: 1) of the skin biopsy; and diagnosing the subject as having melanoma or susceptible to developing melanoma if the C766T mutation is detected in the skin

biopsy. In some examples, the method further includes determining that additional surgery is required to broaden the margins around the skin biopsy, and/or performing additional surgery to broaden the margins around the skin biopsy.

Also provided is a method of determining the prognosis of a subject diagnosed with melanoma by detecting the presence of a C766T mutation in the MAP3K5 gene (SEQ ID NO: 1); and determining that the subject has a poor prognosis if the C766T mutation is detected, for example if the mutation is detected in a sample of skin tissue or melanoma tumor obtained from the subject. A poor prognosis refers to any negative clinical outcome. For example, in some embodiments, a poor prognosis is an increase in the likelihood of death. In some embodiments, a poor prognosis is an increase in the likelihood of metastasis of the melanoma. In other embodiments, a poor prognosis refers to failure to respond to therapy, such as radiation therapy or chemotherapy.

In some embodiments of the diagnostic and prognostic methods, the method further includes providing an appropriate therapy to the subject. The appropriate therapy can include, for example, surgical removal of tumor tissue, radiation therapy, chemotherapy, administration of a mitogen-activated protein kinase (MAPK) pathway inhibitor, administration of a Trx/TrxR inhibitor, or any other therapy appropriate for the treatment of melanoma as determined by a medical practitioner, or any combination of therapies. In other examples, the therapy is institution of precautionary or prophylactic measures to monitor or detect development of melanoma, such as examination of the skin at regular or shorter intervals for early detection of melanoma. The precautionary measures can also include advising the subject to minimize contact with agents that are associated with the development of melanoma, such as ultraviolet radiation, or avoiding such agents, for example by application of ultraviolet radiation blocking agents (such as sunscreen) to the skin.

In some embodiments, the MAPK pathway inhibitor is an inhibitor of extracellular signal-regulated protein kinase (ERK), MAPK/ERK kinase (MEK), or both. In some examples, the MAPK pathway inhibitor is a small molecule. As discussed below in section VI, MAPK pathway inhibitors, including ERK and MEK inhibitors are well known in the art. Exemplary MEK inhibitors are provided herein in Table 1.

In some embodiments, the Trx/TrxR inhibitor is a specific inhibitor of Trx or TrxR. Exemplary specific Trx/TrxR inhibitors include, but are not limited to, diaryl chalcogenides, n-decyl 2-imidazolyl disulphide (VII-2), 1-methylpropyl 2-imidazolyl disulphide (IV-2), PMX464, pleurotin, naphthoquinone spiroketal compounds (*e.g.*, palmarumycin CP1, PX-916), organotellurium antioxidants, GTI-2601 (a phosphorothioate antisense oligonucleotide), 1,2-

[bis(1,2-benzisoselenazolone-3(2H)-ketone)]ethane (BBSKE) and auranofin. In other embodiments, the Trx/TrxR inhibitor is a non-specific inhibitor of Trx or TrxR. Non-specific Trx/TrxR inhibitors include, for example, nitrosoureas, antitumor quinoid compounds, suberoylanilide hydroxamic acid (SAHA) and motexafin gadolinium (see, *e.g.*, Mukherjee and Martin, *Br J Radiol* S57-S68, 2008; Peng *et al.*, *J Zhejiang Univ Sci B* 9(1):16-21, 2008; Cox *et al.*, *Biochem Pharmacol* 76(9):1097-1109, 2008; and U.S. Patent Application Publication Nos. 2012/0226031, 2009/0232827 and 2009/0131511). In some embodiments, the Trx/TrxR inhibitor is a small molecule.

In some embodiments of the diagnostic and prognostic methods disclosed herein, the method further includes providing a test output (*i.e.*, the result of the test to detect the mutation in MAP3K5) to a user (such as a physician or health care worker, the patient or laboratory personnel). In particular examples, the output includes the presence or absence of the mutation, a diagnosis, a treatment recommendation, or any combination thereof. Examples of such output include a printout or display screen that reports the output by displaying it to a clinician or technician. Other examples are electronic medical record reports or other records that include the output in a form discernible to the clinician or technician.

Also provided is a method of treating a subject diagnosed with melanoma, by selecting a subject in whom a C766T mutation in the MAP3K5 gene of the subject is present and administering an inhibitor of the MAPK pathway or an inhibitor of Trx/TrxR to the subject in whom the C766T mutation is present. In some embodiments, the method further includes detecting the presence or absence of the C766T mutation in the subject. In some embodiments, the MAPK pathway inhibitor is an inhibitor of ERK, MEK, or both. In some examples, the MAPK pathway inhibitor or Trx/TrxR inhibitor is a small molecule. MAPK pathway inhibitors, including ERK and MEK inhibitors, as well as Trx/TrxR inhibitors, are well known in the art. Exemplary MEK inhibitors are provided herein in Table 1 (see section VI below). Exemplary Trx/Trx inhibitors are discussed above.

Methods of treatment can further include, for example, surgical removal of tumor tissue, radiation therapy, chemotherapy, or any other therapy appropriate for the treatment of melanoma as determined by a medical practitioner, or any combination of therapies. In other examples, the therapy is institution of precautionary or prophylactic measures to monitor or detect development of melanoma, such as examination of the skin at regular or shorter intervals for early detection of melanoma. The precautionary measures can also include advising the subject to minimize contact with agents that are associated with the development of melanoma, such as ultraviolet radiation, or

avoiding such agents, for example by application of ultraviolet radiation blocking agents (such as sunscreen) to the skin.

In some embodiments of the disclosed methods, detecting the presence or absence of the C766T mutation comprises obtaining a biological sample from the subject, and detecting the presence or absence of the C766T mutation in DNA or RNA contained in the sample. In the context of the present disclosure “obtaining a biological sample” includes either directly collecting the sample from the subject, or obtaining the sample from a laboratory or service provider that has collected the sample from the subject. A sample “obtained from a subject” is a sample acquired by similar means.

For detection or genotyping of a MAP3K5 mutation, such as the C766T mutation, nucleic acid (such as DNA or RNA) can be isolated from a biological sample according to well-known methods. In some embodiments, the biological sample is a tissue sample, such as a skin sample or a tumor tissue sample. In other embodiments, the biological sample is a fluid sample, such as blood. For example, nucleic acid can be isolated from cells obtained from a blood sample. In some embodiments, the biological sample is obtained from a patient diagnosed with melanoma, at risk for developing melanoma, or suspected of having melanoma. In some embodiments, the biological sample is obtained from a control subject.

Methods of detecting or genotyping mutations in a gene are well known in the art. Detection or genotyping of the C766T mutation in MAP3K5 can be accomplished using any suitable technique, such as those described in detail in section V below. For example, MAP3K5-specific primers can be used to amplify nucleic acid from a biological sample (such as a skin sample, tumor tissue sample or blood sample). The amplified molecule can then be sequenced and compared to a reference MAP3K5 sequence (such as SEQ ID NO: 1). Alternatively, the sequence of the amplified molecule can be compared with MAP3K5 from a control sample, such as a non-cancerous tissue sample. MAP3K5 amplification primers and sequencing primers can be designed according to well-known methods. Examples of MAP3K5 primers are shown in Table 2 and Table 3. Other suitable primers can be designed using publically available MAP3K5 nucleic acid sequences, according to well-known procedures.

In some embodiments of the methods disclosed herein, detecting or genotyping the presence or absence of the C766T mutation comprises DNA amplification, such as DNA amplification by PCR (or RT-PCR) using MAP3K5-specific primers. In particular examples, the MAP3K5-specific primers include at least one primer shown in Table 2 or Table 3. In some examples, the method further includes sequencing the amplified DNA to detect the C766T mutation.

V. Methods of Detecting or Genotyping Mutations in MAP3K5

Methods of detecting mutations in genes of interest, for example by genotyping a biological sample from a subject, are known in the art and exemplary methods are described below. Although
5 detection of mutations in the MAP3K5 gene is exemplified herein, the techniques described can be applied to other genes and proteins, including other genes identified as mutated in melanoma and disclosed herein (such as those listed in Table 4).

Detecting or genotyping mutations in MAP3K5 can be accomplished using any technique known in the art. For example, the presence or absence of a MAP3K5 mutation can be determined
10 by conventional methods such as gene or RNA detection methods (for example, DNA sequencing, oligonucleotide hybridization, polymerase chain reaction (PCR) amplification with primers specific to the mutation), or protein detection methods (for example, immunoassays or biochemical assays to identify a mutated MAP3K5 protein). Generally, the nucleic acid sequence of the MAP3K5 gene or RNA in a sample can be detected by any suitable method or technique of detecting gene
15 sequence. Such methods include, but are not limited to, PCR, reverse transcriptase-PCR (RT-PCR), *in situ* PCR, *in situ* hybridization, Southern blot, Northern blot, sequence analysis, microarray analysis, or other DNA/RNA hybridization platforms.

Identifying point mutations in target nucleic acids can be accomplished by molecular cloning of the target nucleic acid molecules and sequencing the nucleic acid molecules using
20 techniques well known in the art. Alternatively, amplification techniques such as PCR can be used to amplify target nucleic acid sequences directly from a genomic DNA preparation from a tumor tissue or cell sample. The nucleic acid sequence of the amplified molecules can then be determined to identify mutations. Representative primer pairs that can be used to amplify MAP3K5 nucleic acid from a biological sample are listed in Table 2 and Table 3. However, design and selection of
25 appropriate primers is well within the abilities of one of ordinary skill in the art.

Ligase chain reaction (Wu *et al.*, *Genomics* 4:560-569, 1989) and allele-specific PCR (Ruano and Kidd, *Nucleic Acids Res.* 17:8392, 1989) can also be used to amplify target nucleic acid sequences. Amplification by allele-specific PCR uses primers that hybridize at their 3' ends to a particular target nucleic acid mutation. If the particular mutation is not present, an amplification
30 product is not observed. Amplification Refractory Mutation System can also be used to detect mutations in nucleic acid sequences (U.S. Patent No. 5,595,890; Newton *et al.*, *Nucleic Acids Res.* 17:2503-2516, 1989). Insertions and deletions of genes can also be detected by cloning, sequencing and amplification. In addition, restriction fragment length polymorphism probes for the

gene or surrounding marker genes can be used to score alteration of an allele or an insertion in a polymorphic fragment. Single stranded conformation polymorphism analysis can also be used to detect base change variants of an allele (Orita *et al.*, *Proc. Natl. Acad. Sci. USA* 86:2766-2770, 1989). Other known techniques for detecting insertions and deletions can also be used with the claimed methods.

Mismatch detection can be used to detect point mutations in a target nucleic acid molecule, such as MAP3K5. Mismatches are hybridized nucleic acid duplexes which are not 100% complementary. The lack of total complementarity can be due to deletions, insertions, inversions, substitutions or frameshift mutations. An example of a mismatch cleavage technique is the RNase protection method, which is described in detail in Winter *et al.* (*Proc. Natl. Acad. Sci. USA* 82:7575-7579, 1985) and Myers *et al.* (*Science* 230:1242-1246, 1985). For example, detection of mutations in MAP3K5 can involve the use of a labeled riboprobe that is complementary to wild-type MAP3K5. The riboprobe and nucleic acid molecule to be tested (for example, obtained from a tumor sample) are annealed (hybridized) together and subsequently digested with the enzyme RNase A, which is able to detect mismatches in a duplex RNA structure. If a mismatch is detected by RNase A, it cleaves at the site of the mismatch. Thus, when the annealed RNA preparation is separated on an electrophoretic gel matrix, if a mismatch has been detected and cleaved by RNase A, an RNA product will be seen which is smaller than the full-length duplex RNA for the riboprobe and the mRNA or DNA. The riboprobe need not be the full length of the target nucleic acid mRNA or gene, but can a portion of the target nucleic acid, provided it encompasses the position suspected of being mutated. If the riboprobe comprises only a segment of the target nucleic acid mRNA or gene, it may be desirable to use a number of these probes to screen the whole target nucleic acid sequence for mismatches if desired.

In a similar manner, DNA probes can be used to detect mismatches, for example through enzymatic or chemical cleavage (Cotton *et al.*, *Proc. Natl. Acad. Sci. USA* 85: 4397, 1988; Shenk *et al.*, *Proc. Natl. Acad. Sci. USA* 72:989, 1975). Alternatively, mismatches can be detected by shifts in the electrophoretic mobility of mismatched duplexes relative to matched duplexes (Cariello, *Human Genetics* 42:726, 1988). With riboprobes or DNA probes, the target nucleic acid mRNA or DNA which may contain a mutation can be amplified before hybridization. Changes in target nucleic acid DNA can also be detected using Southern hybridization, especially if the changes are gross rearrangements, such as deletions and insertions.

Amplified nucleic acid sequences can also be screened using allele-specific probes. These probes are nucleic acid oligomers, each of which contains a region of the target nucleic acid gene

harboring a known mutation. For example, one oligomer may be about 30 nucleotides in length, corresponding to a portion of the target gene sequence. By use of a battery of such allele-specific probes, target nucleic acid amplification products can be screened to identify the presence of a previously identified mutation in the target gene. Hybridization of allele-specific probes with
5 amplified target nucleic acid sequences can be performed, for example, on a nylon filter. Hybridization to a particular probe under stringent hybridization conditions indicates the presence of the same mutation in the tumor tissue as in the allele-specific probe.

Gene-specific primers are useful for determination of the nucleotide sequence of a target nucleic acid molecule using nucleic acid amplification techniques such as the polymerase chain
10 reaction. Pairs of single stranded DNA primers can be annealed to sequences within or surrounding the target nucleic acid sequence in order to prime amplification of the target sequence. Allele-specific primers can also be used. Such primers anneal only to particular mutant target sequence, and thus will only amplify a product in the presence of the mutant target sequence as a template. In order to facilitate subsequent cloning of amplified sequences, primers may have restriction enzyme
15 site sequences appended to their ends. Such enzymes and sites are well known in the art. The primers themselves can be synthesized using techniques which are well known in the art. Generally, the primers can be made using oligonucleotide synthesizing machines which are commercially available. Design of particular primers is well within the skill of the art. In addition, exemplary MAP3K5 primers are provided in Table 2 and Table 3.

20 Nucleic acid probes that hybridize with a MAP3K5 nucleic acid molecule, such as a wild-type MAP3K5 nucleic acid molecule or a mutant MAP3K5 nucleic acid molecule are useful for a number of purposes. They can be used in Southern hybridization to genomic DNA and in RNase protection assays for detecting point mutations. The probes can also be used to detect target nucleic acid amplification products. MAP3K5 probes can also be used to detect mismatches with
25 the wild type gene or mRNA using other techniques. Mismatches can be detected using either enzymes (*e.g.*, S1 nuclease), chemicals (*e.g.*, hydroxylamine or osmium tetroxide and piperidine), or changes in electrophoretic mobility of mismatched hybrids as compared to totally matched hybrids (Novack *et al.*, *Proc. Natl. Acad. Sci. USA* 83:586, 1986).

Mutations in nucleic acid molecules can also be detected by screening for alterations of the
30 corresponding protein. For example, monoclonal antibodies immunoreactive with a target gene product can be used to screen a tissue, for example an antibody that is known to bind to a particular mutated position of the gene product (protein). For example, a suitable antibody may be one that binds to a deleted exon or that binds to a conformational epitope comprising a deleted portion of

the target protein. Lack of cognate antigen would indicate a mutation. Such immunological assays can be accomplished using any convenient format known in the art, such as Western blot, immunohistochemical assay and ELISA. In some embodiments, the MAP3K5 amino acid mutation is R256C (SEQ ID NO: 2).

5 Mutations in a gene or encoded protein can be evaluated using any technique described above, or any other method known in the art. For example, mutations in a gene or corresponding mRNA can be detected by direct sequencing of a nucleic acid molecule, detection of an amplification product, microarray analysis or any other DNA/RNA hybridization platform. For detection of mutant proteins, an immunoassay, biochemical assay or microarray can be used.

10 Any suitable output device or format can be used to transmit the information obtained from the technique used to detect gene or protein mutations. For example, the output device can be a visual output device, such as a computer screen, a printed piece of paper or a written piece of paper. In other examples, the output device can be an auditory output device, such as a speaker. In other examples, the output device is a printer. In some cases, the data is recorded in a patient's electronic
15 medical record. In some embodiments, the results of the test used to identify a mutation are provided to a user (such as a clinician or other health care worker, laboratory personnel, or patient) in a perceivable output that provides information about the results of the test. In some examples, the output is communicated to the user, for example by providing an output via physical, audible or electronic means (for example, by mail, telephone, facsimile transmission, e-mail or
20 communication to an electronic medical record).

In some examples, the output is accompanied by guidelines for interpreting the data, for example, an indication of the likelihood of diagnosis of melanoma. The guidelines need not specify whether melanoma is present or absent, although it may include such a diagnosis. In other examples, the output can provide a recommended therapeutic regimen. For instance, based on the
25 presence of the C766T mutation in the MAP3K5 gene, the output can recommend treatment with an inhibitor of the MAPK pathway, alone or in combination with other standard cancer treatments, such as surgery, radiation therapy, chemotherapy, or any combination thereof. In some examples, the test may include determination of other clinical information (such as determining the presence or absence of mutations in other genes).

30 VI. Inhibitors of the MAPK Pathway

As disclosed herein, the R256C MAP3K5 mutation (resulting from the C766T mutation in the MAP3K5 gene) induces activation of the MAPK pathway, as evidenced by an increase in

phosphorylated MEK1/2 and ERK1/2, increased anchorage-independent growth, and increased cell migration. This data indicates that subjects with cancers (for example, melanoma) harboring the C766T mutation in the MAP3K5 gene are candidates for treatment with inhibitors of the MAPK pathway. In some embodiments of the methods disclosed herein, a subject with melanoma is administered a MAPK pathway inhibitor, such as an inhibitor of MEK or ERK. In some cases, the MAPK pathway inhibitor is a small molecule inhibitor. A number of MEK and/or ERK inhibitors are known in the art (see, for example, Messersmith *et al.*, *Clin Adv Hematol Oncol* 4(11):831-836, 2006; and U.S. Patent Application Publication Nos. 2009/0118324; 2011/0086837; 2006/0154990; 2006/0079494; 2010/0249096; 2003/0195241; 2003/0225151; and 2011/0189192). In addition, exemplary small molecule MEK inhibitors are listed below in Table 1.

Table 1. Small Molecule MEK Inhibitors

Compound Name	Alternative Name(s)	Description
CI-1040	PD184352	Oral MEK1/2 inhibitor; inhibits ERK1 and ERK2 phosphorylation
PD0325901		Orally bioactive MEK inhibitor; selectively binds to and inhibits MEK; a derivative of CI-1040
AZD6244	ARRY-142886 Selumetinib	Highly potent and specific MEK1/2 inhibitor; orally bioavailable
XL518	GDC-0973	Selective, orally bioavailable inhibitor of MEK; binds to and inhibits kinase activity of MEK
U0126		Selective inhibitor of MEK1/2; inhibits kinase activity
PD98059		Selective inhibitor of MEK1/2
BIX02189		Selective inhibitor of MEK5 and ERK5
AS703026		Highly selective and potent noncompetitive inhibitor of MEK1/2
GSK1120212	JTP-74057 Trametinib	Orally bioavailable inhibitor of MEK; specifically binds to and inhibits MEK1/2
TAK-733		Highly potent and selective MEK allosteric site inhibitor
Honokiol		A biphenolic compound present in the cones, bark and leaves of <i>Magnolia grandiflora</i>
AZD8330	ARRY-424704	Orally active, selective MEK inhibitor
PD318088		Competitive allosteric inhibitor of MEK1/2
BIX02188		Potent and selective inhibitor of MEK5
RDEA119	BAY 869766	Potent, selective allosteric inhibitor of MEK1/2

Compound Name	Alternative Name(s)	Description
ARRY-162		Orally active, selective and potent inhibitor of MEK1/2
ARRY-300		Small molecule inhibitor of MEK

The methods provided herein contemplate the use of any MEK inhibitor listed above, or any other inhibitor of the MAPK pathway, such as any MEK or ERK inhibitor.

- 5 The following examples are provided to illustrate certain particular features and/or embodiments. These examples should not be construed to limit the disclosure to the particular features or embodiments described.

EXAMPLES

10 **Example 1: Materials and Methods**

This example describes the experimental procedures for the studies described in Example 2.

Tumor tissues

- 15 Tissue and melanoma cell lines used for the Discovery and Prevalence Screen in this study were described previously (Palavalli *et al.*, *Nat Genet* 41(5):518-520, 2009). Tissues used for validation set 1 were fresh frozen melanoma tumors. DNA was isolated from enriched macrodissected tumor isolates. Tissue processing and storage were previously described by Morente *et al.* (Morente *et al.*, *Eur J Cancer* 42(16):2684-2691, 2006). Tissues used for validation set 2 of melanomas were obtained from Optimum Cutting Temperature (OCT)-embedded frozen clinical specimens. DNA isolation from the tumor-enriched isolates has been described previously (Davies *et al.*, *Clin Cancer Res* 15(24):7538-7546, 2009). Tissue was further collected and cell lines established (41 stage III and 46 stage IV (AJCC) early passage metastatic melanoma cell lines) as described previously (Castellano *et al.*, *Cancer Res* 57(21):4868-4875, 1997; Pavey *et al.*, *Oncogene* 23(23):4060-4067, 2004; Dutton-Regester *et al.*, *Genes Chromosomes Cancer* 51(5):452-461, 2012).
- 20
- 25

PCR, sequencing and mutational analysis of melanoma samples

- Genes identified to harbor recurrent mutations were confirmed and further screened using gene-specific primer sets in an additional 172 melanoma samples (MAP3K5 primer sets are listed in Table 2). Mutational analysis, confirmation and determination of somatic status were carried out
- 30

as previously described (Palavalli *et al.*, *Nat Genet* 41(5):518-520, 2009; Prickett *et al.*, *Nat Genet* 41(10):1127-1132, 2009).

Table 2. MAP3K5 Primers used for non-synonymous recurrent mutation confirmation

Primer Name	Location	Sequence	SEQ ID NO:
JG-122 For	Chr 6 137061120	GTAAAACGACGGCCAGTaaagtcctaattatttgaagtatgcagt	3
JG-122 Rev	Chr 6 137061706	ttgctgatttcaagttgctgtt	4
JG-123 For	Chr 6 137061199	GTAAAACGACGGCCAGTtggggaaatttcattgtcg	5
JG-123 Rev	Chr 6 137061670	tttccccctagttcttagctg	6
JG-124 For	Chr 6 137061185	GTAAAACGACGGCCAGTggaaatttcattgtcgttaggtc	7
JG-124 Rev	Chr 6 137061667	gaggataataaacattttccccta	8

Statistical calculation of the likelihood of a recurrent mutation

The probability of a specific base mutated at 5/288 is calculated using the binomial distribution assuming a background mutation rate of 11.4 mut/Mb employing the following values and formula:

$$x=5$$

$$n=288$$

$$p=11.4e-6$$

$$F(x; n, p) = \Pr(X \leq x) = \sum_{i=0}^x \binom{n}{i} p^i (1-p)^{n-i}$$

This is then corrected for multiple comparisons to arrive at the probability of any base mutated at

5/288 in the study by using a conservative Bonferroni correction such that the number is multiplied by the number of coding bases sequenced.

Construction of wild-type and mutant MAP3K5 expression vectors

Human *MAP3K5* (NM_005923.3) was cloned by PCR as previously described (Palavalli *et al.*, *Nat Genet* 41(5):518-520, 2009) using clones (#6007002-MAP3K5) purchased from Open Biosystems with primers listed in Table 3. The PCR products were cloned into the mammalian expression vectors pCDF-MCS2-EF1-Puro™ or pCDF-MCS2-EF1-Neo™ (Systems Biosciences, Inc., Mountain View, CA) or pcDNA3.1(-) (Invitrogen) via the XbaI and NotI restriction sites. *MAP3K5* cDNA contains a FLAG epitope tag in frame at the C-terminus. Point mutations were introduced as previously described (Prickett *et al.*, *Nat Genet* 41(10):1127-1132, 2009) using the primers found in Table 3. Thioredoxin (myc-Trx) wild-type was purchased from Addgene (Plasmid #21614).

Table 3. Primers used for plasmid construction and RT-PCR

Cloning primers for MAP3K5 wild type into pCDF1		
Forward	gctgtctagagccaccatgagcacggaggcggacgagggcatc	SEQ ID NO: 9
Reverse	atcagcggccgcttactatcgtcgtcatccttgtaatcagctctgtttgttc	SEQ ID NO: 10
Site-directed mutagenesis primers for Arg256Cys in MAP3K5		
Forward	acccatctgcttacctctgtggattgtttattcaactttgaagg	SEQ ID NO: 11
Reverse	caaaagttgaataaaacaatccacaagaggtgaagcagat	SEQ ID NO: 12
MAP3K5 RT-PCR N-terminal		
Forward	ctgggaactacacctttgttc	SEQ ID NO: 13
Reverse	gtagattcgaccaactaggc	SEQ ID NO: 14
MAP3K5 RT-PCR C-terminal		
Forward	ctaattggaatctttagctcag	SEQ ID NO: 15
Reverse	ccattcactctcagccagtcg	SEQ ID NO: 16

Cell culture and transient expression

- 5 HEK293T cells were purchased from ATCC (Manassas, VA) and maintained in complete RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). HEK293T cells were transfected with Arrest-IN reagent (Open Biosystems) at a 6:1 ratio with DNA (μl: μg) using 2-5 μg of plasmid DNA.

10 Immunoprecipitation and Western Blotting

- Transfected cells were gently washed 2X in PBS and then lysed using 1.0 ml 1% NP-40 lysis buffer (1% NP-40, 50mM Tris-HCl pH 7.5, 150mM NaCl, Complete Protease Inhibitor tablet, EDTA-free (Roche, Indianapolis, IN), 1μM sodium orthovanadate, 1 mM sodium fluoride, and 0.1% β-mercaptoethanol) per T-75 flask for 20 minutes on ice. Lysed cells were scraped and
- 15 transferred into a 1.5 mL microcentrifuge tube. Extracts were centrifuged for 10 minutes at 14,000 rpm at 4°C. Supernatant (800 μl) was immunoprecipitated overnight using 20 μl of anti-FLAG (M2) beads (Sigma-Aldrich) or 10 μl of anti-myc antibody with 30 μl of 50% slurry of Protein-A/G Sepharose beads (1X PBS). The immunoprecipitates were washed and subjected to SDS-PAGE and western blotting as previously described (Palavalli *et al.*, *Nat Genet* 41(5):518-520, 2009).
- 20 Primary antibodies used in the signal transduction pathway analysis were anti-MAP3K5 (#3762), anti-P-MAP3K5 (S83) (#3761), anti-P-MAP3K5 (S967) (#3764), anti-P-ERK1/2 (T202/Y204) (#9101), anti-ERK1/2 (#9102), anti-P-MEK1/2 (S217/221) (#9121), anti-MEK1/2 (#9122), anti-P-

p38 (T180/Y182) (#9211), anti-p38 (#9212), anti-P-MKK4 (T261) (#9151), anti-MKK4 (#9152) (Cell Signaling), anti-Trx (#2429) (Cell Signaling), anti-myc (#SC-40) (Santa Cruz), and anti-GAPDH (#CB1001) (Calbiochem-EMD Biosciences).

5 Pooled stable expression

To make lentivirus, *MAP3K5* constructs were co-transfected into HEK 293T cells seeded at 1.5×10^6 per T-75 flask with pVSV-G and pFIV-34N helper plasmids using Arrest-IN as described by the manufacturer. Virus-containing media was harvested 60 hours after transfection, filtered, aliquoted and stored at -80°C . Mel-STR cells (which harbor wild-type BRAF and mutant NRAS) were grown in RPMI-1640 (Lonza, Walkersville, MD) and supplemented with 10% FBS (HyClone, Logan, UT). A375 cells were purchased from National Cancer Institute, Division of Cancer Treatment, Developmental Therapeutics Program, Frederick, MD and maintained in RPMI-1640 and supplemented with 10% FBS. Mel-STR or A375 (which harbor V600E BRAF and wild-type NRAS) cells were seeded at 1.5×10^6 cells per T75 flask 24 hours prior to infection.

Lentivirus for *MAP3K5* (wild-type or R256C point mutant) and empty vector control were used to infect both Mel-STR or A375 cells as previously described (Prickett *et al.*, *Nat Genet* 41(10):1127-1132, 2009). Stable expression of MAP3K5 proteins (wild-type and mutant) was determined by immunoprecipitation and SDS-PAGE analysis followed by immunoblotting with anti-MAP3K5 and anti-GAPDH to show equivalent expression among pools.

Proliferation assays

To examine growth potential, pooled A375 and Mel-STR MAP3K5 clones were seeded into 96 well plates at 300 cells per well in either 1%, 2.5% or 10% serum-containing medium and incubated for 13-17 days. Samples were analyzed every 48 hours by lysing cells in 50 μl 0.2% SDS/well and incubating for 2 hours at 37°C prior to addition of 150 μl /well of SYBRTM Green I solution (1:750 SYBRTM Green I (Invitrogen-Molecular Probes-Carlsbad, CA) diluted in dH₂O). Plates were analyzed using a BMG Labtech FLOUstar Optima.

Soft agar assay

Mel-STR or A375 pooled MAP3K5 clones were plated in triplicate at 1000 cells/well and in top plugs consisting of sterile 0.33% Bacto-Agar (BD, Sparks, MD) and 10% FBS (HyClone, Logan, UT) in a 24-well plate. The lower plug contained sterile 0.5% Bacto-Agar and 10% FBS. After two weeks, the colonies were photographed and quantitated using ImageJ (NIH software).

Migration assays

Mel-STR or A375 pooled clones were seeded into pre-conditioned migration wells (8.0 μ m – BD Biocoat, BD Biosciences) at 15,000 cells per well in serum-free medium in the top chamber and incubated for 16-18 hours with complete serum containing medium in the bottom chamber prior to harvesting. Inserts were fixed and stained using Hema 3 Stat Pack as per manufacturers protocol. Inserts were analyzed and counted for cells migrated per field view and quantitated using ImageJ (NIH software).

Lentiviral shRNA

Constructs for stable depletion of MAP3K5 (cat# RHS4533-NM_005923) were obtained from Open Biosystems (Huntsville, AL) and were confirmed to efficiently knockdown MAP3K5 at the protein level. Lentiviral stocks were prepared as previously described (Prickett *et al.*, *Nat Genet* 41(10):1127-1132, 2009). Melanoma cell lines (24T, 32T, Mel-Juso, 12T, 501Mel and A375) were infected with shRNA lentiviruses for each condition (vector and two different *MAP3K5* specific shRNAs). Selection of stable pooled clones was done in the presence of 3 μ g/ml puromycin containing normal medium for 3-5 days prior to determining knock-down efficiency. Stably infected pooled clones were tested in functional assays.

Reverse Transcription PCR

Total RNA was extracted from pooled clones of melanoma cells 32T, MelJuso, 12T (2197), and 501Mel stably knocked down for endogenous *MAP3K5* following the manufacturer's protocol for the RNeasyTM Mini Kit (QIAGEN #74101). Total RNA was eluted in 30 μ L diethylpyrocarbonate (DEPC)-treated distilled H₂O. A total of 1 μ g of total RNA was used for single-strand complementary DNA (cDNA) synthesis using a SuperScript III First Strand kit (Invitrogen #18080-051). cDNA was amplified using the oligo dT20 primer supplied in the kit. To test for loss of *MAP3K5* message, 1 μ L of cDNA was used in the PCR reaction with either *MAP3K5* primers (Table 3) or *GAPDH* primers.

Proliferation assays of stable knockdown cells

To examine growth potential, pooled 32T, MelJuso, 12T (2197), and 501Mel *MAP3K5* stably depleted clones were seeded into 96-well plates at 500 cells per well in either 1%, 2.5% or 10% serum-containing medium and incubated for 6-8 days. Samples were analyzed every 2-3 days

by lysing cells in 50 µl 0.2% SDS/well and incubating for 2 hours at 37°C prior to addition of 150 µl/well of SYBR™ Green I solution (1:750 SYBR™ Green I (Invitrogen-Molecular Probes-Carlsbad, CA) diluted in dH₂O). Plates were analyzed using a BMG Labtech FLOUstar Optima.

5 **Example 2: Whole-genome sequencing identifies a novel non-synonymous recurrent mutation in MAP3K5 in melanoma**

This example describes the identification and characterization of a C766T mutation in the MAP3K5 transcript, which results in expression of a mutant (R256C) MAP3K5 protein and increased melanoma cell proliferation, anchorage-independent growth and cell migration.

10

Genetic analysis reveals a hot-spot mutation in MAP3K5 (R256C)

Recent sequencing projects using Sanger/whole-exome/whole-genome techniques have implicated many different genes involved in tumorigenesis (Stark *et al.*, *Nat Genet* 44(2):165-169, 2012; Wei *et al.*, *Nat Genet* 43(5):442-446, 2011; Nikolaev *et al.*, *Nat Genet* 44(2):133-139, 2012; 15 Pleasance *et al.*, *Nature* 463(7278):191-196, 2010; Berger *et al.*, *Nature* 485(7399):502-506, 2012; Hodis *et al.*, *Cell* 150:251-263, 2012; Krauthammer *et al.*, *Nat Genet* 44(9):1006-1014, 2012; Turajlic *et al.*, *Genome Res* 22(2):196-207, 2012). To further understanding of the molecular changes that underlie melanoma, and in order to identify potential novel druggable targets, the sequencing data was searched for recurrent (*i.e.* hotspot) mutations in genes that are permeable to 20 small molecule inhibition, such as serine/threonine kinases.

To further understand the molecular changes that underlie melanoma, the coding regions of 29 melanoma samples and corresponding normal DNA were comprehensively analyzed. To search for novel recurrent mutations, this study looked for alterations that occurred in two or more of the 29 samples subjected to whole-genome or whole-exome sequencing. This analysis identified the 25 previously described *BRAF* (V600E) alteration in 16 out of the 29 samples and the *TRRAP* (S722F) substitution in six samples (Davies *et al.*, *Nature* 417(6892):949-954, 2002; Wei *et al.*, *Nat Genet* 43(5):442-446, 2011). In addition to these *BRAF* and *TRRAP* mutations, 35 more genes were found to harbor a non-synonymous recurrent mutation (Table 4). Further screening of the novel non-synonymous hotspot mutations in an additional 172 melanomas identified 13 novel genes to have 30 more than two non-synonymous recurring mutations. SIFT analysis predicts that 40% of the alterations would affect protein function (Table 4). In *MAP3K5*, four cases harbored an identical mutation. The recurring mutation in *MAP3K5* was a cytosine to thymine change at position 766 of the transcript (NM_005923.3), leading to an arginine to a cysteine substitution at amino acid

residue 256 of the protein (R256C). Further screening of the *MAP3K5* hotspot mutation in an independent cohort of 87 melanomas found a 5th sample with the same mutation. Given the potential importance of *MAP3K5* in the development of melanoma (Stark *et al.*, *Nat Genet* 44:165-169, 2011), further studies were carried out to study this gene.

5 An additional interrogation of data from two recently published melanoma exome studies identified numerous mutations occurring in *MAP3K5* (Hodis *et al.*, *Cell* 150:251-263, 2012; Krauthammer *et al.*, *Nat Genet* 44(9):1006-1014, 2012). In these datasets the *MAP3K5* hotspot mutation occurred in 2/121 cases or 0/147 cases, respectively. The probability for the occurrence of this recurrent alteration is significantly low ($p < 3.14E-13$; binomial distribution followed with
10 Bonferroni correction ($p < 1.57E-5$)) and the affected residue is highly conserved suggesting it has been selected for during tumor development.

Table 4. Recurrent mutations identified in melanoma

Gene name	Tumor name	Amino acid change	Screen	SIFT score	SIFT median
BRAF	1T	V600E	Whole Exome	0	2.81
	5T		Whole Exome		
	9T		Whole Exome		
	22T		Whole Exome		
	35T		Whole Exome		
	51T		Whole Exome		
	14T		Whole Genome		
	23T		Whole Genome		
	26T		Whole Genome		
	56T		Whole Genome		
	64T		Whole Genome		
	81T		Whole Genome		
	88T		Whole Genome		
	8T		Whole Genome		
	colo-829		Whole Genome		
	91T		Whole Exome		
CD1C	12T	R89C	Whole Genome	0	2.84
	21T		Prevalence screen		
	32T		Whole Genome		
CDH6	130T	S524L	Whole Exome	0.57	2.86
	55T		Whole Exome		
CSF2RB	09T	G423E	Whole Exome	0	2.8
	55T		Whole Exome		
	131T		Prevalence screen		
D10S102 (HNRNPA3)	24T	G228E	Whole Exome	0.33	2.93
	7T		Whole Genome		
DCC	12T	G55E	Whole Exome	0.46	2.87
	18T		Whole Exome		
DNAH5	55T	E3499K	Whole Exome	0	2.88
	24T		Whole Exome		
DNAH7	23T	S2911F	Whole Exome	0.01	2.78
	60T		Whole Exome		
EMBP1 LOC647121	12T	S306L	Whole Genome	0.11	2.85
	81T		Whole Genome		
ERBB4	55T	E452K	Whole Exome	0.06	2.8
	7T		Whole Genome		
FAM154B	55T	P278S	Whole Exome	0.24	2.81
	12T		Whole Exome		
FAM184A	24T	S288L	Whole Exome	0.32	3.33
	130T		Whole Exome		
	76T		Prevalence screen		
HTN3	14T	S21L	Whole Exome	N/A	N/A
	24T		Whole Exome		
	74T		Prevalence screen		
IL32	55T	(+13 splice site) exon 3	Whole Exome	N/A	N/A
	8T		Whole Genome		
KCNT2	14T	N995S	Whole Exome	0.41	3.51
	1T		Whole Exome		
KIF17	55T	E888K	Whole Exome	0.33	3.12
	18T		Whole Genome		

Gene name	Tumor name	Amino acid change	Screen	SIFT score	SIFT median
LRRN3	12T 24T	E362K	Whole Exome Whole Exome	0.29	2.99
MAP3K5	24T 32T MB1113 Mel-Juso CO25	R256C	Whole Exome Whole Genome Prevalence screen Prevalence screen QIMR	0	2.87
MGAM	17T 32T	E218K	Whole Genome Whole Genome	0.39	2.76
NOS1	24T 60T	S771L	Whole Exome Whole Exome	0	2.86
OR13C8	01T 6T 48T 81T	G233E	Whole Exome Prevalence screen Prevalence screen Whole Genome	0	2.83
OR4K14	12T 23T	M200I	Whole Exome Whole Exome	0.39	2.75
PLCH1	1T 24T	Q303X	Whole Exome Whole Exome	N/A	N/A
PTK2B	17T 56T MB1160_T	R429C	Whole Genome Whole Genome Prevalence screen	0	2.81
RLBP1L1 (CLVS1)	55T 88T	P8S	Whole Exome Whole Genome	0.46	3.35
SIPA1L2	18T 7T B17	S1472L	Whole Exome Whole Genome Prevalence screen	0.16	3.28
SLC17A5	12T 18T	R364C	Whole Exome Whole Exome	0	2.76
SLC44A5	5T 130T	G204E	Whole Exome Whole Exome	0.02	3.16
SP140	64T 7T	R500K	Whole Exome Whole Genome	0.14	2.94
TAS2R60	17T 43T 120T	M213I	Whole Genome Whole Exome Prevalence screen	1	2.86
THBS4	55T 7T	G513R	Whole Exome Whole Genome	0.06	2.81
TRRAP	63T 91T 96T 106T 119T A375	S722F	Whole Exome Whole Exome Prevalence screen Prevalence screen Prevalence screen Prevalence screen	0	2.71
UGT1A9	88T 12T D24	P149S	Whole Genome Whole Exome Prevalence screen	0	2.75
USH2A	81T 88T	D1217N	Whole Genome Whole Genome	0.53	3.02
XIRP1	108T 7T	E361K	Whole Exome Whole Genome	0.68	3.54
ZNF804A	55T 7T MB929	E150K	Whole Exome Whole Genome Prevalence screen	0.02	3.48
ZNF831	43T 91T MB1160_T	S1474F	Whole Exome Whole Exome Prevalence screen	0	4.32

Samples used in whole genome and exome capture were obtained from the Surgery Branch at National Cancer Institute. Prevalence samples were obtained from the Surgery Branch National Cancer Institute, The Division of Medical Oncology, University of Colorado Denver School of Medicine, and the Department of Melanoma Medical Oncology, The University of Texas MD Anderson Cancer Center. A375 and Mel-Juso are commercially available melanoma cell line. Listed *BRAF* mutations are from the whole exome whole genome study only. Based on genome build hg18 (NCBI 36.1). Number of samples investigated: whole genome whole exome capture, $n = 21$; prevalence screen, $n = 180$, QIMR, Sample from Oncogenomics Laboratory, Queensland Institute of Medical Research, $n=87$. N/A indicates mutation unscored using SIFT algorithm.

In order to fully assess the mutual exclusivity with the recurrent mutation identified herein and BRAF, two published exome studies (Hodis *et al.*, *Cell* 150:251-263, 2012; and Krauthammer *et al.*, *Nat Genet* 44(9):1006-1014, 2012), as well as publically available TCGA cutaneous melanoma exome data (available online at tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm), were studied. As study disclosed herein focused exclusively on cutaneous malignant melanoma, all uveal, acral, and mucosal melanoma samples were removed from the published exome studies. This analysis left 435 exome samples to review and revealed 4 samples to contain the recurrent R256C. Of these 435 samples, 215 contained the BRAF V600E mutation and only a single sample contained both the recurrent MAP3K5 mutation and the recurrent BRAF mutation. When these studies were combined with the samples for the current study, 723 total samples were assessed for these two positions. Of the 723 samples, 9 contained the recurrent MAP3K5 mutation, 366 contained the recurrent BRAF mutation, and a single sample contained both mutations. Using the combination of additional exome data and the fisher's exact test for the samples from the current study indicates a mutual exclusivity between the MAP3K5 recurrent mutation and BRAF V600E (p-value =0.01016) (Table 5). Due to the frequency of the MAP3K5 R256C mutation in melanoma, further studies were carried out to investigate if the mutation has a role in tumorigenesis.

Table 5. MAP3K5 mutual exclusivity with BRAF

Samples sequenced	Samples with MAP3K5 R256C mutation	Samples with BRAF (V600) mutation	Samples with BRAF and MAP3K5 mutations	Study
288	5	151	0	Current study
435	4	215	1	Published exome studies
723	9	366	1	Combined

Expression of MAP3K5 (R256C) in melanoma cells leads to suppression of pro-apoptotic signaling and increased anchorage independent growth

MAP3K5 is stimulated via inflammatory cytokines (IL-6, LPS, or TNF- α) or ultraviolet light via direct activation of its upstream cognate receptors (Hattori *et al.*, *Cell Commun Signal* 7:9, 2009). Upon activation, MAP3K5 stimulates the stress-induced mitogen-activated protein kinases p38 and/or JNK leading to increased propensity for cell death or apoptosis. A marker of MAP3K5 pro-apoptotic or pro-survival effect is the measure of phosphorylation-state of MAP3K5 (Hattori *et al.*, *Cell Commun Signal* 7:9, 2009). Phosphorylation of MAP3K5 on Thr845, a critical residue on

the activation loop, is required for its activation (Tobiume *et al.*, *J Cell Physiol* 191(1):95-104, 2002).

To test the effects of mutant MAP3K5 on its activity as well as its downstream substrates, vector control, wild-type or mutant (R256C) MAP3K5 were transiently expressed in HEK293T cells and stable pooled clones expressing the same constructs were established in Mel-STR or 2183 (17T) melanoma cells that are wild type for BRAF, but express mutant NRAS (similar to what was observed in the genetic screen). Similar levels of expression of MAP3K5 protein were observed in the HEK293T, Mel-STR and 2183 (17T) cells. MAP3K5 activation was tested by using site specific phospho-antibodies to MAP3K5. Expression of mutant MAP3K5 (R256C) in HEK293T, Mel-STR or 2183 cells resulted in suppression of phosphorylation of Thr845 in the MAP3K5 activation loop compared to cells expressing wild-type MAP3K5 (FIGS. 1A-1C). As shown in FIG. 1, phospho-MKK4 and phospho-p38 signals are reduced in the mutant MAP3K5 compared to the wild-type in both the transient expression and stably expressed pooled clones. These results suggest that somatic mutation of MAP3K5 (R256C) causes melanoma cells expressing mutant MAP3K5 (R256C) to evade MAP3K5-dependent stress induced cell death signals by suppressing its pro-apoptotic activity.

To examine the effects of MAP3K5 mutation on cell growth, Mel-STR or 2183 (17T) stable pooled clones expressing vector control, wild-type or mutant (R256C) MAP3K5 were tested for anchorage-independent growth or growth on plastic. The melanoma cell lines Mel-STR and 2183 (17T) were selected because they both express wild-type MAP3K5 and are wild-type for BRAF. When assessing growth in soft agar, Mel-STR and 2183 (17T) clones expressing wild-type MAP3K5 showed reduced colony formation compared to mutant MAP3K5 (R256C) or empty vector (FIGS. 2A-2B). Next, growth on plastic was examined and it was determined that in the presence of normal serum levels (10%), both Mel-STR and 2183 (17T) pooled clones expressing MAP3K5 wild-type or R256C exhibited similar proliferation rates (FIGS. 4A-4B). However, while Mel-STR pooled clones expressing wild-type or R256C in the presence of low serum (1%) also exhibited similar proliferation rates (FIG. 2C), 2183 (17T) pooled clones expressing R256C exhibited elevated proliferation rates compared to wild-type expressing cells (FIG. 2D). This data suggests that expression of wild-type MAP3K5 may induce a pro-apoptotic signal in melanoma cells resulting in reduced anchorage-independent growth and proliferation.

MAP3K5 acts as a tumor suppressor in melanoma cells as determined by stable shRNA depletion

To assess if melanoma cells with endogenous MAP3K5 mutations are dependent on MAP3K5 signaling for proliferation and migration, shRNA was used to stably knock-down MAP3K5 in melanoma cells harboring either wild-type *MAP3K5* (501Mel and 12T) or mutant *MAP3K5* (32T and Mel-Juso). Specific targeting of MAP3K5 was confirmed by transient transfection in HEK293T cells (FIG. 3A) and immunoblotting, as well as by RT-PCR analysis using *MAP3K5* specific primers and *GAPDH* as a loading control (FIG. 3B). The shRNA had little to no effect on cells harboring wild-type MAP3K5, but significantly reduced the growth of cells harboring mutant forms of MAP3K5 (FIG. 3C). Taken together, these results demonstrate that the recurrent somatic mutation of MAP3K5 is essential for cellular proliferation, anchorage-independent growth and cell migration, a phenotypic hallmark of invasive malignant melanoma cells.

Attenuation of MAP3K5 pro-apoptotic phenotype by increased binding of Trx to mutant MAP3K5 (R256C)

To determine the mechanism responsible for attenuation of MAP3K5 induced apoptosis by the R256C mutation, the ability of transiently expressed or stably expressed wild-type or mutant (R256C) to bind the MAP3K5 inhibitor thioredoxin (TXN/Trx) was tested in HEK293 or 2183 (17T) cells, respectively. As shown in FIG. 5, wild-type MAP3K5-FLAG bound transiently expressed myc-Trx (FIG. 5A) or endogenous Trx (FIG. 5B). However, mutant MAP3K5-FLAG bound myc-Trx or Trx significantly better in both the transient system and stable pooled clones. These results suggest a potential mechanism for how cancer cells harboring the R256C MAP3K5 mutation are able to avert apoptosis, leading to progression of melanoma tumorigenesis.

Discussion

Disclosed herein is the identification of non-synonymous recurrent somatic mutations in melanoma whole-genome and whole-exomes. Some of the mutations found in this study were previously reported, including *BRAF* and *TRRAP* (Davies *et al.*, *Nature* 417(6892):949-954, 2002; Wei *et al.*, *Nat Genet* 43(5):442-446, 2011). However, a recurrent somatic mutation in MAP3K5 at R256C has not been previously reported. Functional analysis revealed that transient expression of the mutant form of MAP3K5 resulted in increased migration compared to wild-type MAP3K5 as

well as increased anchorage-independent growth. These results demonstrate a mutation affecting survival via MEK-MAPK activation. All the cases expressing mutant MAP3K5 harbor wild-type BRAF, apart from sample 32T which contains the non-activating BRAF (L597Q) mutation, potentially representing an alternative mechanism for MEK-MAPK pathway activation in the absence of mutant BRAF (V600E).

Components of the MAPK pathway including BRAF and NRAS have been reported previously to be mutated in melanoma causing phenotypes similar to the ones described above (Dicker *et al.*, *Genes Chromosomes Cancer* 1(4):257-269, 1990; Davies *et al.*, *Nature* 417(6892):949-954, 2002). Recent genetic studies of *MEK1* and/or *MEK2* found them both to harbor recurrent somatic mutations resulting in increased MAPK1/2 (Erk1/2) activation, proliferation and cellular transformation (Marks *et al.*, *Cancer Res* 68(14):5524-5528, 2008; Nikolaev *et al.*, *Nat Genet* 44(2):133-139, 2012). Hyperactivated forms of these kinases are hypersensitive to inhibition using small molecule inhibitors such as PLX4032 (vemurafenib) (Bollag *et al.*, *Nature* 467(7315):596-599, 2010; Flaherty *et al.*, *N Engl J Med* 363(9):809-819, 2010; Halaban *et al.*, *Pigment Cell Melanoma Res* 23(2):190-200, 2010; Chapman *et al.*, *N Engl J Med* 364(26):2507-2516, 2011). Tumors harboring these mutations show increased sensitivity in the presence of such inhibitors only to become resistant overtime (Alcala and Flaherty, *Clin Cancer Res* 18(1):33-39, 2012), via acquisition of somatic mutations or utilization of different RAF isoforms, allowing the tumors to evade cell death, emphasizing the need to identify additional inhibitors as well as novel drug targets.

A recent exome sequencing study of melanoma cell lines identified additional non-synonymous somatic mutations in MAP3K5 which were dispersed throughout the protein (Stark *et al.*, *Nat Genet* 44(2):165-169, 2012). Similar to the current study, the MAP3K5 mutations analyzed were shown to have little to no effect on downstream activation of JNK or p38. However, in contrast to the observations disclosed herein for the MAP3K5 R256C mutation, the mutations identified in the Stark *et al.* study reduced MEK-MAPK pathway activity. The differences observed may be due to the location of the mutated residues in the protein structure. The R256C mutation lies closer to the N-terminal region of the protein whereas the mutations previously checked were closer the mid-region of the kinase (amino acids E663 and I780), near the kinase domain. Recent work by Kim *et al.*, demonstrated that the N- and C-terminal regions of the MAP3K5 protein are important for binding to the transforming growth factor- β -activated kinase 1

(TAK1)-TAK1 binding protein 1 (TAB1) complex (Kim *et al.*, *J Biol Chem* 287(5):3381-3391, 2012). This complex formation negatively regulates MAP3K5 activity. The mid-region of MAP3K5, amino acid residues 278-945 did not associate with the TAK1-TAB1 complex and thus could not be negatively regulated. The importance of the N- and C-terminal regions compared to mid-regions of MAP3K5 demonstrate the complexity of this kinase, and may explain the differences observed in pathway stimulation.

Pro-death signaling pathways can be activated via many different stimuli, such as TNF α , H₂O₂, Fas ligand, or reduced serum levels (Shiizaki *et al.*, *Adv Biol Regul* 53:135-144, 2013; Liu *et al.*, *Molecular and cellular biology* 20:2198-2208, 2000; Liu and Min, *Circ Res* 90:1259-1266, 2002). Upon activation of death receptors by these ligands, pro-apoptotic signals involving, for example, the TNF receptor or reactive oxygen species (ROS), cause increased potentiation of stress-induced MAPK signaling resulting in programmed cell death (Tonissen and Di Trapani, *Mol Nutr Food Res* 53:87-103, 2009). MAP3K5, under normal conditions can be activated by loss of binding complex formation with its cytoplasmic inhibitor, thioredoxin (TXN/Trx) (Saitoh *et al.*, *EMBO J* 17:2596-2606, 1998). Trx binds the N-terminal region of MAP3K5 causing inhibition of the pro-apoptotic kinase. The binding between these molecules utilizes two highly conserved cysteine residues in Trx (Cys32 and Cys35) (Mahmood *et al.*, *Antioxid Redox Signal* (Epub), February 26, 2013). Trx inhibits MAP3K5-mediated apoptosis after reduction of Cys32 and Cys35 resulting in increased binding. The fact that increased binding of Trx to the MAP3K5 R256C, compared to WT MAP3K5, was observed suggests a way for evasion of programmed cell death signals that induce apoptosis. Inhibition of apoptosis via this mechanism would result in a more proliferative and pro-survival pathway being up-regulated, increasing the probability of tumorigenesis and/or metastases.

The current genetic study of 29 melanoma whole-genomes and whole-exomes allowed for the identification of a novel recurrently mutated gene. Provided herein is functional evidence that the MAP3K5 hotspot mutation is an activating event that potentiates the MEK-MAPK pathway and promotes the proliferation and migration of melanoma cells, thus providing a therapeutic target. The data disclosed herein point to subpopulations of individuals whose tumors are dependent on MAPK signaling, thus further emphasizing the importance of targeting this pathway in melanoma patients (Romano *et al.*, *Lancet Oncol* 12(9):913-922, 2011).

In view of the many possible embodiments to which the principles of the disclosed invention may be applied, it should be recognized that the illustrated embodiments are only preferred examples of the invention and should not be taken as limiting the scope of the invention. Rather, the scope of the invention is defined by the following claims. We therefore claim as our
5 invention all that comes within the scope and spirit of these claims.

CLAIMS

1. A method of diagnosing a subject as having melanoma or susceptible to developing melanoma, or a method of determining the prognosis of a subject diagnosed with melanoma,
5 comprising:

detecting the presence of a C766T mutation in the mitogen-activated kinase kinase kinase 5 (MAP3K5) gene (SEQ ID NO: 1) in a sample obtained from the subject; and

diagnosing the subject as having melanoma or susceptible to developing melanoma, or determining that the subject has a poor prognosis, if the C766T mutation is detected.

2. The method of claim 1, further comprising providing an appropriate therapy to the subject in whom the C766T mutation is detected.

3. The method of claim 2, wherein the appropriate therapy comprises surgical removal
15 of tumor tissue, radiation therapy, chemotherapy, administration of a mitogen-activated protein kinase (MAPK) pathway inhibitor, administration of a thioredoxin/thioredoxin reductase (Trx/TrxR) inhibitor, or any combination thereof.

4. The method of claim 3, wherein the MAPK pathway inhibitor is an inhibitor of
20 extracellular signal-regulated protein kinase (ERK), MAPK/ERK kinase (MEK), or both.

5. The method of claim 3, wherein the MAPK pathway inhibitor or the Trx/TrxR inhibitor is a small molecule.

6. The method of any one of claims 1-5, wherein detecting the presence of the C766T
25 mutation comprises obtaining a biological sample from the subject, and detecting the presence of the C766T mutation in DNA or RNA contained in the sample.

7. The method of any one of claims 1-5, wherein detecting the presence of the C766T
30 mutation comprises genotyping a sample obtained from the subject at the position of the C766T mutation.

8. A method of treating a subject diagnosed with melanoma, comprising:
selecting a subject in whom a C766T mutation in the MAP3K5 gene of the subject is
present; and
administering an inhibitor of the MAPK pathway or an inhibitor of Trx/TrxR to the subject
5 in whom the C766T mutation is present.

9. The method of claim 8, further comprising genotyping a sample obtained from the
subject at the position of the C766T mutation.

10. The method of claim 8, further comprising detecting the presence of the C766T
mutation in a sample obtained from the subject.

11. The method of claim 10, wherein detecting the presence of the C766T mutation
comprises obtaining a biological sample from the subject, and detecting the presence of the C766T
15 mutation in DNA or RNA contained in the sample.

12. The method of claim 10, wherein detecting the presence of the C766T mutation
comprises genotyping a sample obtained from the subject at the position of the C766T mutation.

13. The method of any one of claims 8-12, wherein the MAPK pathway inhibitor is an
inhibitor of ERK, MEK or both.

14. The method of any one of claims 8-13, wherein the MAPK pathway inhibitor or the
Trx/TrxR inhibitor is a small molecule.

25

15. The method of any one of claims 1-7 and 10-12, wherein detecting the presence or
absence of the C766T mutation comprises DNA amplification.

16. The method of any one of claims 7, 9 and 12, wherein genotyping a sample obtained
30 from the subject at the position of the C766T mutation comprises DNA amplification.

17. The method of claim 15 or claim 16, wherein DNA amplification comprises PCR using MAP3K5-specific primers.

18. The method of claim 17, wherein the MAP3K5-specific PCR primers include at least one primer set forth as any one of SEQ ID NOs: 3-16.

19. The method of any one of claims 15-18, further comprising sequencing the amplified DNA to detect or genotype the C766T mutation.

20. The method of any one of claims 1-7 and 9-19, wherein the sample comprises skin tissue or tumor tissue.

21. The method of claim 1, comprising obtaining a skin biopsy from a subject with a suspicious skin lesion; detecting the presence of a C766T mutation in the MAP3K5 gene (SEQ ID NO: 1) of the skin biopsy; and diagnosing the subject as having melanoma or susceptible to developing melanoma if the C766T mutation is detected in the skin biopsy.

22. The method of claim 21, further comprising determining that additional surgery is required to broaden the margins around the skin biopsy.

23. The method of claim 21 or claim 22, comprising performing additional surgery to broaden the margins around the skin biopsy.

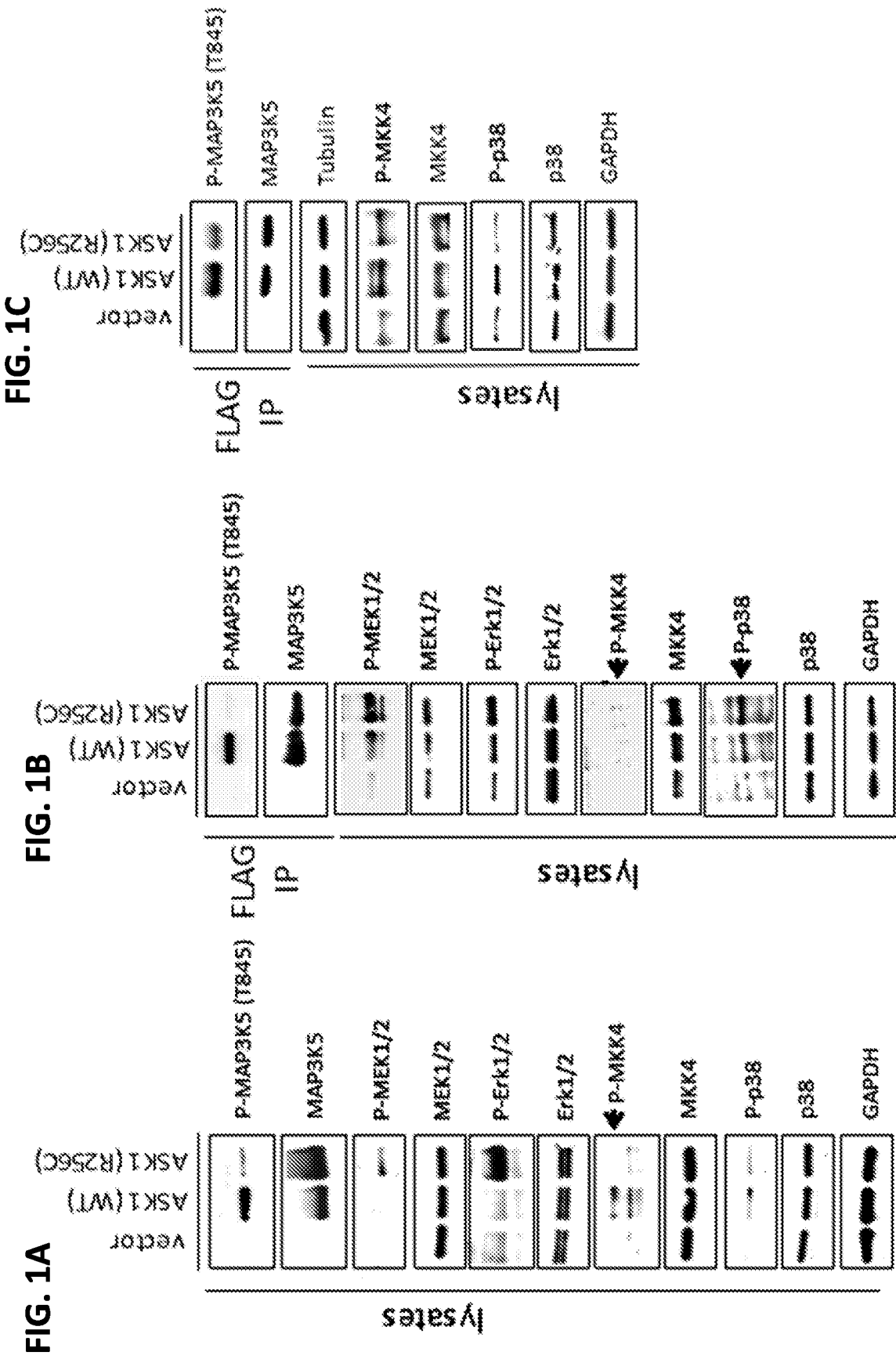


FIG. 2A

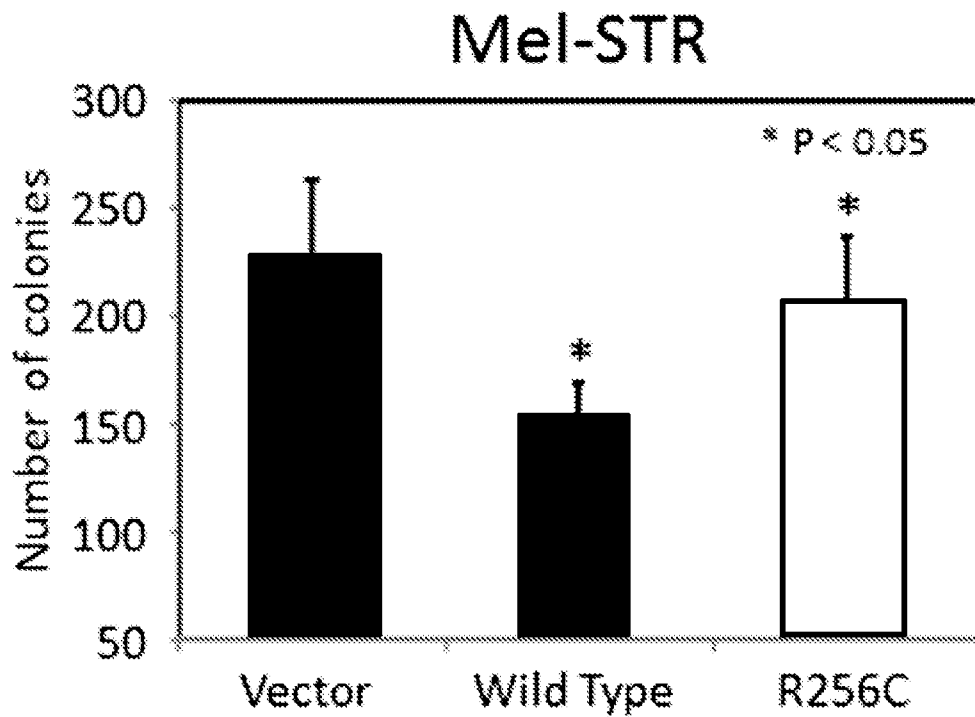


FIG. 2B

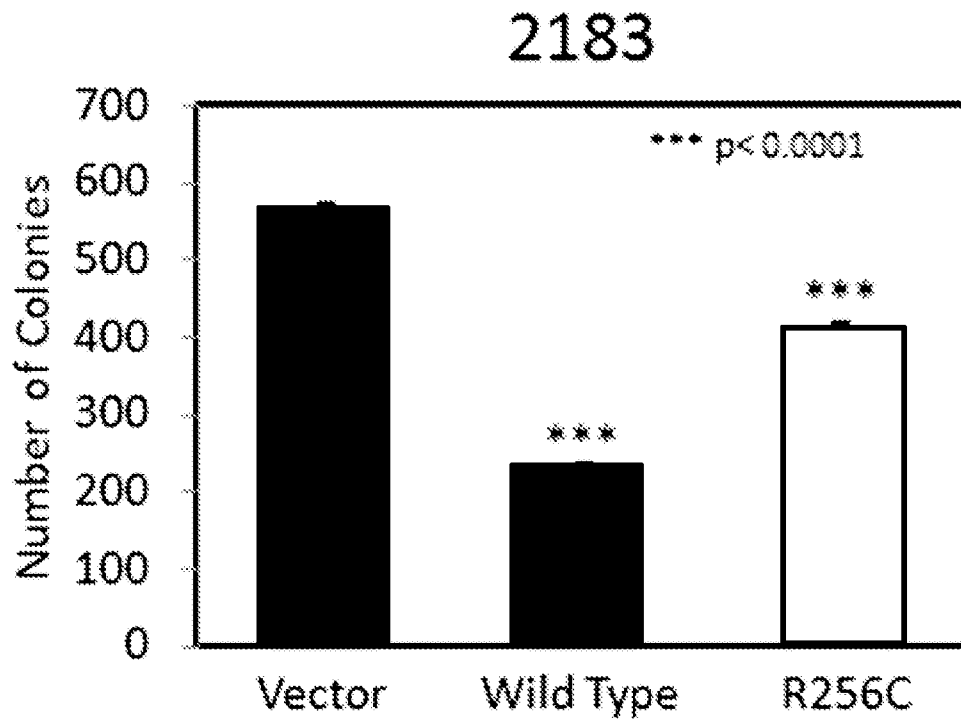


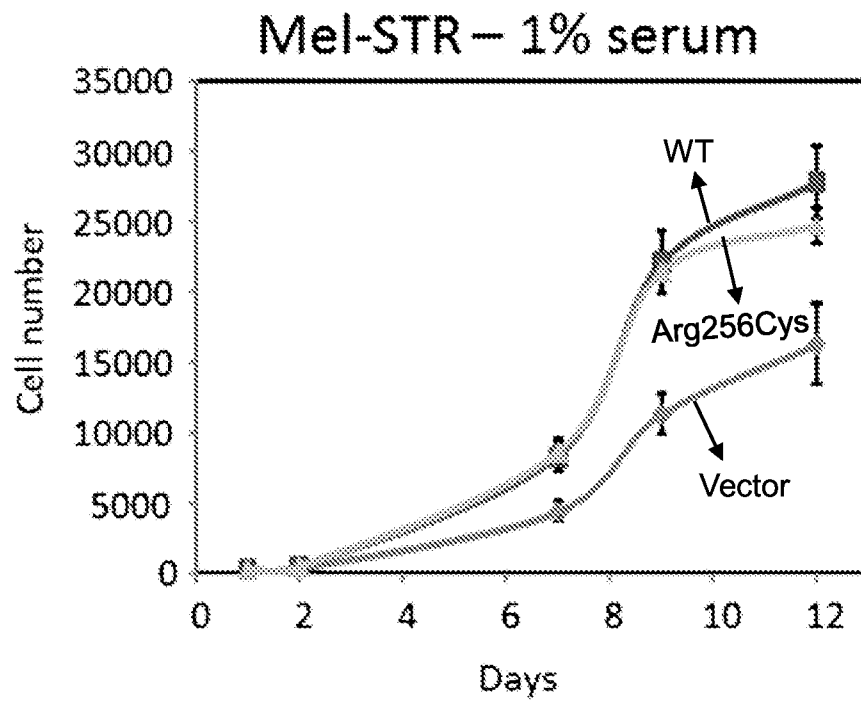
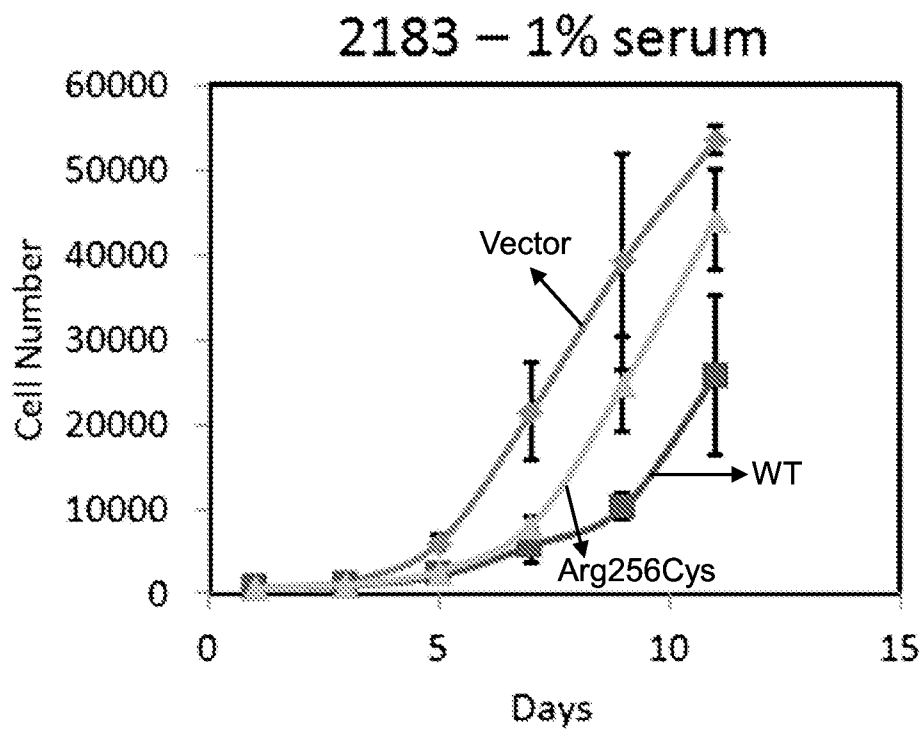
FIG. 2C**FIG. 2D**

FIG. 3A

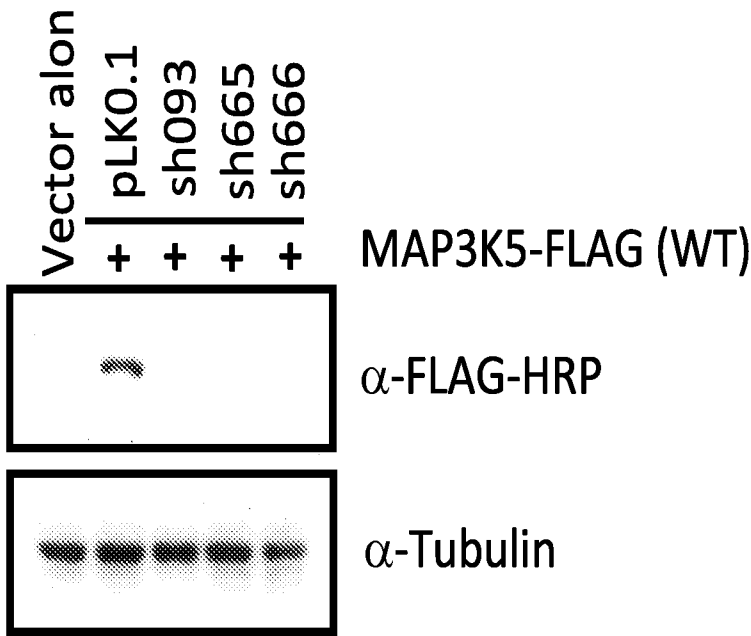


FIG. 3B

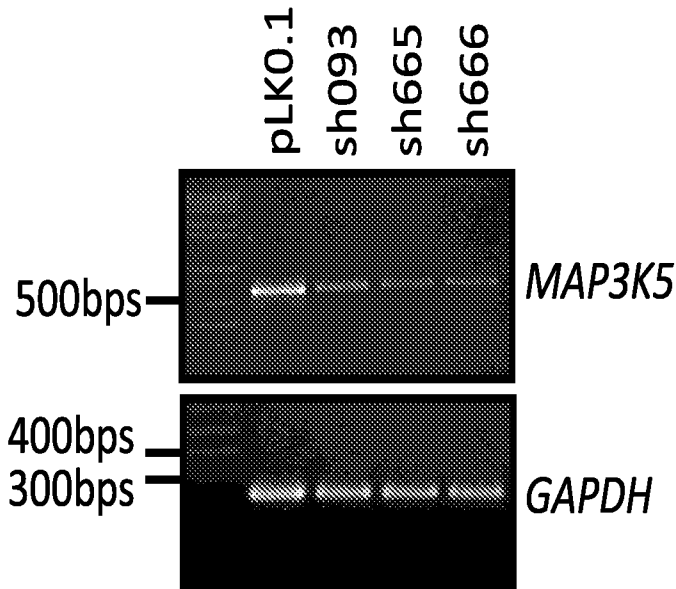


FIG. 3C

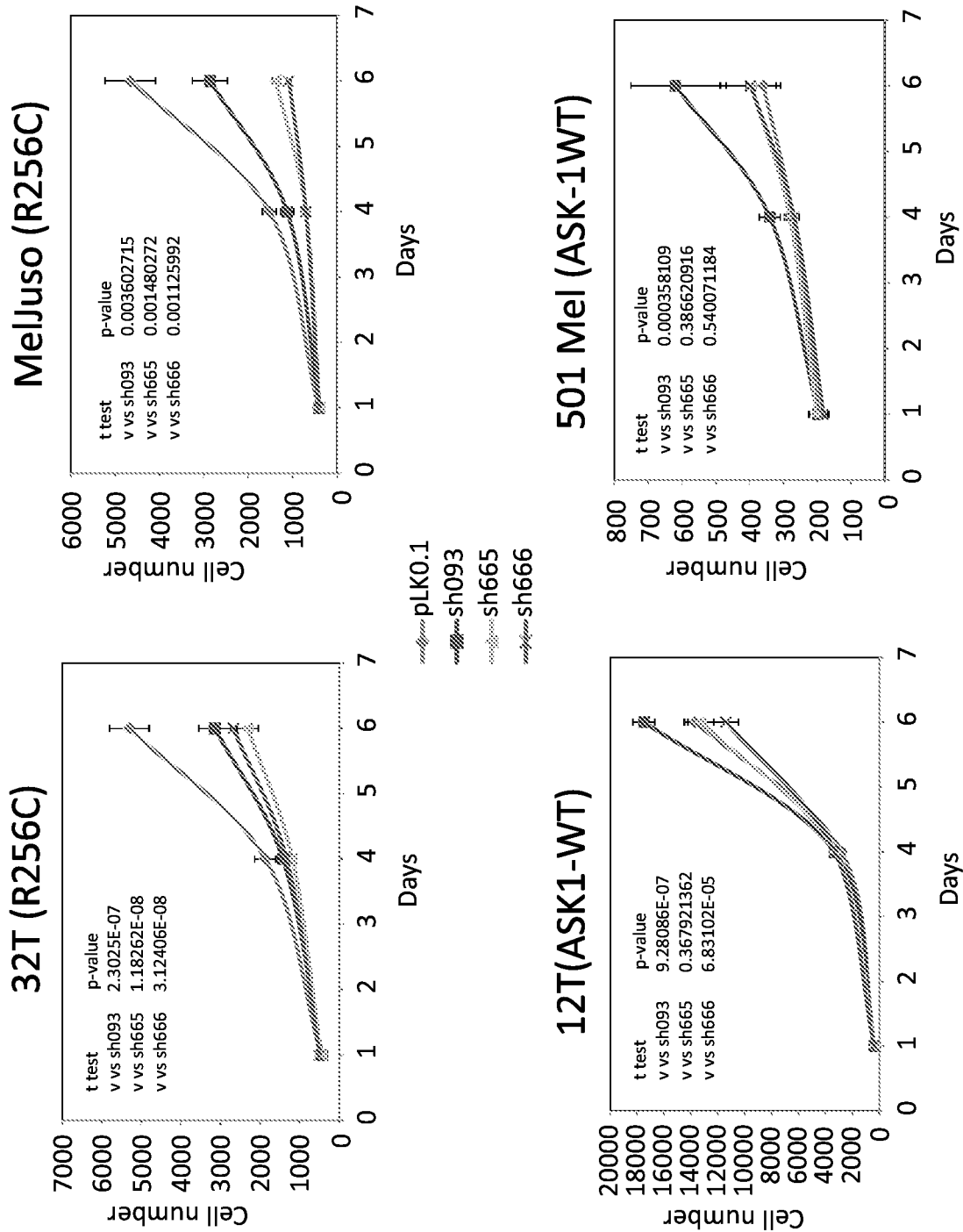


FIG. 4A

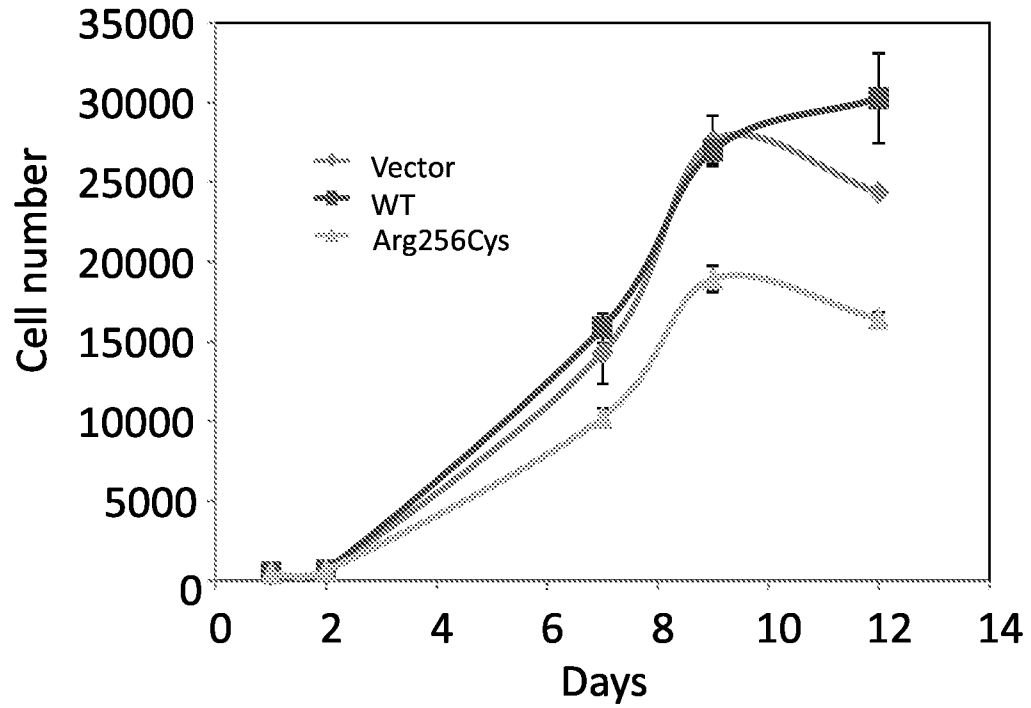


FIG. 4B

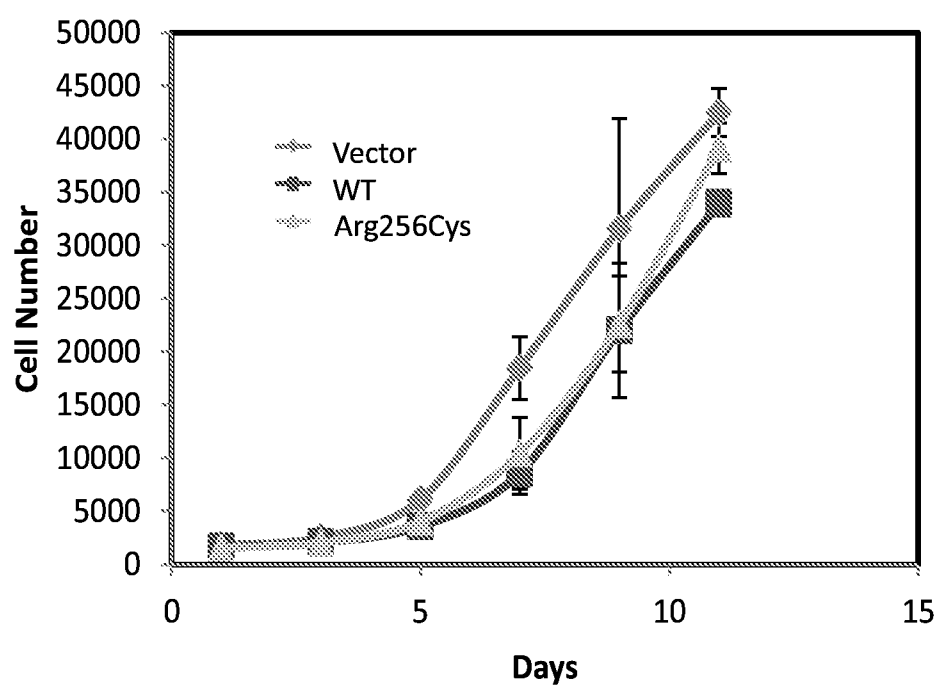


FIG. 5A

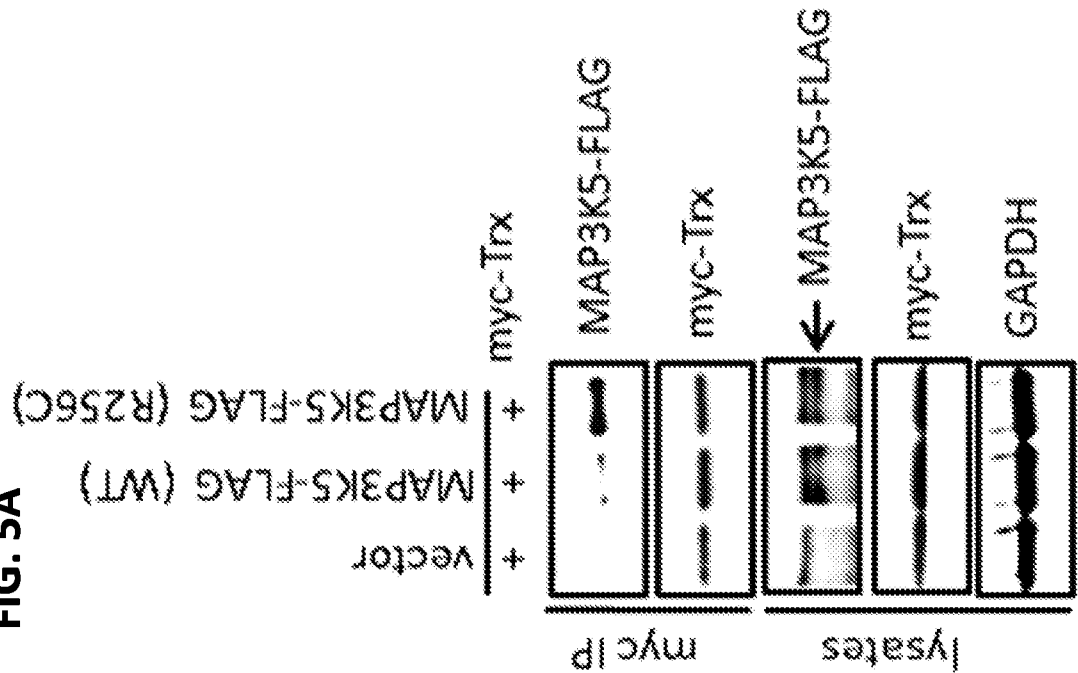
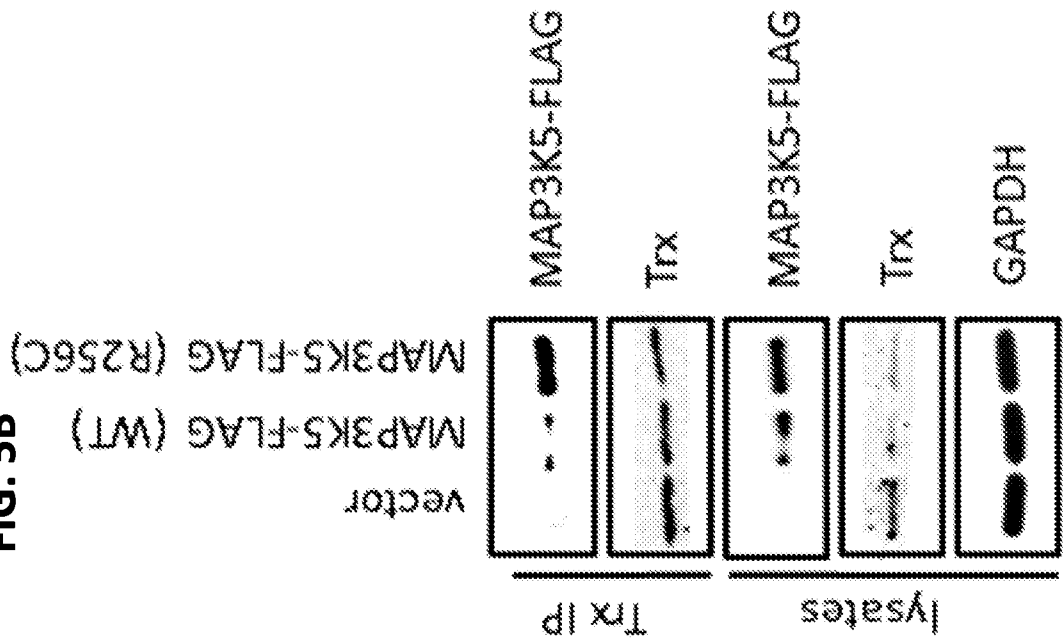


FIG. 5B



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/063077

A. CLASSIFICATION OF SUBJECT MATTER

G01N 33/50 (2006.01) C12Q 1/48 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, Epodoc, Medline, HCAplus, Biosis, Embase. Search terms: MAP3K5, ASK1, MEKK5, C766T, R256C, melanoma and like terms

Inventor search on patentscope, Google Scholar

Genome Quest search of mutated SEQ ID NO 1 & 2, Blast search of NP_005914.1

Google Scholar, search terms 'MAP3K5', melanoma and like terms

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	



Further documents are listed in the continuation of Box C



See patent family annex

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search
7 November 2013

Date of mailing of the international search report
08 November 2013

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INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/US2013/063077
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	STARK, M. S., et al. 'Frequent somatic MAP3K5 and MAP3K9 mutations in metastatic melanoma identified by exome sequencing'. Nature Genetics, epub December 2011, vol. 44, no. 2, pages 165-169. whole document	1-23
A	KRAUTHAMMER, M., et al. 'Exome sequencing identifies recurrent somatic RAC1 mutations in melanoma'. Nature Genetics September 2012, vol. 44, no. 9, pages 1006-1014. supplementary figure 5, supplementary table 6	1-23
A	PRICKETT, T. D., et al. 'Somatic mutations in MAP3K5 attenuate its proapoptotic function in melanoma through increased binding to thioredoxin'. Journal of Investigative Dermatology, 2013, doi:10.1038/jid.2013.365 (advance online publication, available 10 Oct 2013) whole document	1-23

Form PCT/ISA/210 (fifth sheet) (July 2009)