Somatic mutations in MAP3K5 attenuate its pro-apoptotic function in melanoma through increased binding to Thioredoxin

Todd D. Prickett[1,#], **Brad Zerlanko**[1,#], **Jared J. Gartner**[1], **Stephen C. J. Parker**[1], **Ken Dutton-Regester**[2], **Jimmy C. Lin**[3], **Jamie K. Teer**[4,15], **Xiaomu Wei**[1], **Jiji Jiang**[1], **NISC Comparative Sequencing Program**[5], **Guo Chen**[6,7], **Michael A. Davies**[6,7], **Jeffrey E. Gershenwald**[8,9], **William Robinson**[10], **Steven Robinson**[10], **Nicholas K. Hayward**[2], **Steven, A. Rosenberg**[11], **Elliott H. Margulies**[12,13], and **Yardena Samuels**[1,14,*]

[1] The Cancer Genetics Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, Maryland, 20892, USA

[2] Oncogenomics Laboratory, Queensland Institute of Medical Research, Herston, Brisbane, Queensland, Australia

[3] Division of Laboratory and Genomic Medicine, Department of Pathology and Immunology, Washington University School of Medicine, St Louis, MO, 63110, USA

[4] Genetic Disease Research Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, Maryland, 20892, USA

[5] NIH Intramural Sequencing Center, National Human Genome Research Institute, National Institutes of Health, Bethesda, Maryland, 20852, USA

[6] Department of Melanoma Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas, 77030, USA

[7] Department of Systems Biology, The University of Texas MD Anderson Cancer Center, Houston, Texas, 77030, USA

*To whom correspondence should be addressed: Yardena Samuels Department of Molecular Cell Biology Weizmann Institute of Science Wolfson building, room 534 POB 26 234 Herzl St. Rehovot, 76100, Israel Phone: +972- 8-934-3631 Yardena.samuels@weizmann.ac.il.
[13]Present Address: Illumina Cambridge Ltd, Chesterford Research Park, Little Chesterford, Saffron Walden, Essex, CB10 1XL, UK
[14]Present Address: The Weizmann Institute of Science Department of Molecular Cell Biology, 234 Herzl St. Rehovot, 76100, Israel
[15]Present Address: H. Lee Moffitt Cancer Center and Research Institute Tampa, FL 33612, USA
#These authors contributed equally to this work

Author contributions

T.D.P, S.C.J.P, J.J.G, E.H.M., C.K.S, A.A.K, and Y.S. designed the study; K.S.H, M.A.D, J.E.G., W.R, S.R. JJ, U.K.B. K.D., N.K.H. and S.A.R. collected and analyzed the melanoma samples; S.C.J. P., J.J.G, X.W, J.K.T, J.C.L, K.D., N.K.H., K.K., Y.G., N.K., V.G., H.C., R. K, and the NISC Comparative Sequencing Program, analyzed the genetic data. T.D.P, B.Z., and J.J., performed the functional analyses. All authors contributed to the final version of the paper.

Conflict of Interest

The authors state no conflict of interest.

[8] Department of Surgical Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas, 77030, USA

[9] Department of Cancer Biology, The University of Texas MD Anderson Cancer Center, Houston, Texas, 77030, USA

[10] Division of Medical Oncology, University of Colorado School of Medicine, Aurora, CO 80045, USA

[11] The Surgery Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, 20892, USA

[12] Genome Informatics Section, Genome Technology Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, Maryland, 20892, USA

Abstract

Patients with advanced metastatic melanoma have poor prognosis and the genetics underlying its pathogenesis are poorly understood. High throughput sequencing has allowed comprehensive discovery of somatic mutations in cancer samples. Here, upon analysis of our whole-genome and whole-exome sequencing data of 29 melanoma samples we identified several genes that harbor recurrent non-synonymous mutations. These included MAP3K5, which in a prevalence screen of 288 melanomas was found to harbor a R256C substitution in 5 cases. All MAP3K5 mutated samples were wild-type for BRAF, suggesting a mutual exclusivity for these mutations. Functional analysis of the MAP3K5 R256C mutation revealed attenuation of MKK4 activation through increased binding of the inhibitory protein thioredoxin (TXN/TRX-1/Trx); resulting in increased proliferation and anchorage-independent growth of melanoma cells. This mutation represents a potential target for the design of new therapies to treat melanoma.

Introduction

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 and treatment strategies for a subset of patients. Even with these improvements there are still a number of cancers whose incidence rates continue to rise. Melanoma falls within this category; in the US alone approximately 76,690 new diagnoses and 9,480 deaths are predicted for 2013 (Siegel et al., 2012, 2013). For these reasons, further understanding of the molecular pathogenesis of this potentially 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 has recently led to significant clinical responses in metastatic melanoma, such as the use of vemurafenib or dabrafenib in BRAF mutant tumors (Chapman et al., 2011; Flaherty et al., 2010). However, despite the success in identifying genetic alterations utilizing candidate gene approaches (Curtin et al., 2006; Davies et al., 2002; Prickett et al., 2009) as well as whole-genome (Berger et al., 2012; Pleasance et al., 2010; Turajlic et al., 2011) and whole-exome (Krauthammer et al., 2012; Nikolaev et al., 2012; Stark et al., 2012; Wei et al., 2011) 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, 1997). Further potentiation of these signaling molecules through either amplification or somatic mutations play a major role in tumorigenesis (Davies et al., 2002; Dicker et al., 1990; Nikolaev et al., 2011; Stark et al., 2011)and large-scale cancer genetic studies support these findings (Clark et al., 2004; Davies et al., 2002; Dicker et al., 1990; Johannessen *et al.*, 2010; Marks et al., 2008; Nikolaev et al., 2011; Stark et al., 2012). Recently, large-scale genetic 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., 2008; Nikolaev et al., 2011).

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., 2013; Yang et al., 2010) via activation of MAPK kinase-4/7 (MKK4/7). MAP3K5 can be activated in response to stress signals, including H_2O_2, tumor necrosis factor-α (TNFα) or reduced serum levels (Tzeng et al., 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., 1998). Trx binds to the N-terminus of MAP3K5 attenuating its kinase activity as well as downstream apoptotic signaling mechanisms (Saitoh et al., 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, 2008; Gidanian et al., 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.

Here we analyzed whole-genome and whole-exome data to identify recurrent somatically mutated genes in melanoma. One of the most interesting findings in this study was that the gene encoding *MAP3K5* harbored a recurrent somatic mutation (R256C) in 5/288 tumors that was found to be mutually exclusive with *BRAF* mutations. Through functional analysis we demonstrated the importance of this mutation on MAP3K5 activity, by reducing the protein's pro-death activity and increasing melanoma cell survival.

Results and Discussion

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., 2012); (Wei et al., 2011);(Nikolaev et al., 2012);(Berger et al., 2012);(Pleasance et al., 2010);(Hodis et al., 2012; Krauthammer et al., 2012; Turajlic et al., 2011). To further our understanding of the molecular changes that underlie melanoma, and to identify potential drug targets, we

searched our sequencing data for recurrent mutations in genes that are permeable to small molecule inhibition such as serine/threonine kinases.

We comprehensively analyzed the coding regions of 29 melanoma samples and corresponding normal DNA. To search for recurrent mutations, we looked for alterations that occurred in two or more of the 29 samples subjected to whole-exome/whole-genome sequencing re-analysis. From this analysis we identified the previously described *BRAF* (V600E) alteration in 16 out of the 29 samples and the *TRRAP* (S722F) substitution (Davies et al., 2002; Wei et al., 2011). We found two samples with the same mutation in *MAP3K5*, and given the recent report by Stark, et. al., indicating the potential importance of this gene in melanoma development we focussed our follow up work on *MAP3K5* (Stark, Woods et al. 2012).

Screening for the R256C alteration in MAP3K5 in an additional 172 melanomas identified an additional four cases with the mutation - 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). (Figure S1). Furthermore, sequencing of the *MAP3K5* recurrent mutation in an independent cohort of 87 melanomas yielded a 5th sample with the same mutation (Table S1). An additional interrogation of data from two recently published melanoma exome studies identified numerous mutations occurring in *MAP3K5* (Hodis et al., 2012; Krauthammer et al., 2012) (Table S2 contains a list of mutations occurring in *MAP3K5* from whole-exome and genome sequenced samples screened or re-analyzed in this study). 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 Bonferroni correction(p<1.57E-5)) and the affected residue is highly conserved (Figure S2) suggesting it has been selected for during tumor development.

In order to fully assess the mutual exclusivity with our recurrent mutation and BRAF we reviewed two published exome studies (Hodis et al., 2012; Krauthammer et al., 2012) as well as TCGA cutaneous melanoma exome data publically available at https://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm. As our study focused exclusively on cutaneous malignant melanoma we removed all uveal, acral, and mucosal melanoma samples from the published exome studies. This analysis left us with 435 exome samples to review and revealed 4 samples to contain the recurrent p.R256C. Of these 435 samples 215 contained the BRAF p.V600E mutation and only a single sample contained both the recurrent MAP3K5 mutation and the recurrent BRAF mutation. When these studies are combined with our samples we then have 723 total samples assessed for these two positions, 9 containing the recurrent MAP3K5 mutation, 366 containing the recurrent BRAF mutation and a single sample containing both mutations. Using the combination of additional exome data and our samples a fisher's exact test indicates a mutual exclusivity between the MAP3K5 recurrent mutation and BRAF V600E (p-value =0.01016). (Figure S3). Due to the frequency of the MAP3K5 R256C mutation in melanoma, we decided to investigate if the mutation has a role in tumorigenesis.

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 (TNFα, LPS, or IL-6), reactive oxygen species (H_2O_2), or UV light via direct activation of its upstream cognate receptors (Hattori et al., 2009). Upon activation, MAP3K5 stimulates the stress-induced mitogen-activated protein kinases JNK and/or p38 via MKK4/7 leading to an increased propensity for cell death or apoptosis. A marker of MAP3K5 pro-apoptotic effect is the measure of phosphorylation-state of MAP3K5 (Hattori et al., 2009). Phosphorylation of MAP3K5 on Thr845, a critical residue in the activation loop, is required for its activation of proapoptotic signaling (Tobiume et al., 2002). To test the effects of mutant MAP3K5 on its activity as well as its downstream substrates, we transiently expressed vector control, wild-type or mutant (R256C) MAP3K5 in HEK293T cells as well as established stable pooled clones expressing the same constructs in Mel-STR or 2183 (17T) melanoma cells that are wild type BRAF but express mutant NRAS similar to what was observed in our genetic screen. We observed similar levels of expression of MAP3K5 protein both in the HEK293T, Mel-STR and 2183 (17T) cells. We tested for MAP3K5 activation by using site specific phospho-antibodies to MAP3K5. Expression of mutant MAP3K5 (R256C) in HEK293T, Mel-STR or 2813 cells resulted in phosphorylation of Thr845 in the MAP3K5 activation loop being suppressed compared to cells expressing wild-type MAP3K5 (Figure 1A-C). As seen in Figure 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) may cause 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, we tested Mel-STR or 2183 (17T) stable pooled clones expressing vector control, wild-type or mutant (R256C) MAP3K5 for anchorage-independent growth or growth on plastic. We selected the melanoma cell lines Mel-STR and 2183 (17T), as 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 (Figure 2A-B). We next examined growth on plastic and found 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 (Figure S4A-B). However, while Mel-STR pooled clones expressing wild-type or R256C in the presence of low serum (1%) also exhibited similar proliferation rates (Figure 2C), 2183 (17T) pooled clones expressing R256C exhibited elevated proliferation rates compared to wild-type expressing cells (Figure 2D). This data suggests that expression of wild-type MAP3K5 may induce a proapoptotic 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 determine if melanoma cells with endogenous MAP3K5 mutations are dependent on MAP3K5 signaling for proliferation we used shRNA 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 (Figure 3A) and immunoblotting as well as by RT-PCR analysis, using *MAP3K5* specific primers and *GAPDH* as a loading control (Figure 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 (Figure 3C). Taken together, our results demonstrate that the recurrent somatic mutation of MAP3K5 is essential for cellular proliferation and anchorage-independent growth, a phenotypic hallmark of invasive malignant melanoma cells.

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

Finally, to determine the mechanism responsible for attenuation of MAP3K5 induced apoptosis by the R256C mutation, we tested the ability of transiently expressed or stably expressed wild-type or mutant (R256C) to bind the MAP3K5 inhibitor thioredoxin (TXN/ Trx) in HEK293 or 2183 (17T) cells, respectively. As seen in Figure 4, wild-type MAP3K5-FLAG binds transiently expressed myc-Trx (A) or endogenous Trx (B). However, mutant MAP3K5-FLAG binds 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.

Here we report the identification of a recurrent somatic mutation in MAP3K5 at R256C that is predominately mutually exclusive to *BRAF* mutation. Functional analysis revealed that transient expression of the mutant form of MAP3K5 caused an attenuation of the pro-death pathways of p38 or JNK. Furthermore, mutation of MAP3K5 resulted in increased proliferation as well as anchorage-independent growth, further demonstrating attenuation of the pro-death MKK4/7 signaling pathway causing increased survival via MEK-MAPK activation.

Components of the MAPK pathway including BRAF and NRAS have been reported previously to be mutated in melanoma and lead to phenotypes similar to the ones described above (Davies et al., 2002; Dicker et al., 1990). 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., 2008; Nikolaev et al., 2011). Interestingly, hyperactivated forms of these kinases have increased sensitivity to inhibition using small molecule inhibitors targeting the MAPK pathway, including PLX4032 (vemurafenib) (Bollag et al., 2010; Chapman et al., 2011; Flaherty et al., 2010; Halaban et al., 2010). However, responses to these targeted drugs are often short lived, with resistance occurring through a variety of mechanisms including the acquisition of secondary somatic mutation events or utilization of different RAF isoforms (Alcala and Flaherty, 2012). Thus, there is a strong need to identify additional inhibitors as well as drug targets.

Recently, an exome sequencing analysis of melanoma cell lines identified gene-wide non-synonymous somatic mutations of MAP3K5 in 8 of 85 melanomas (Stark et al., 2012).

However, in contrast to our current study, the MAP3K5 mutations analyzed had little to no effect on downstream activation or suppression of JNK or p38, nor did we observe a negative effect on the MEK-MAPK pathway for the MAP3K5 R256C mutation. These differences may be accounted for by the location of the mutated residues throughout the protein analyzed in the respective studies; while the R256C mutation lies close to the N-terminal region of the protein, the mutations previously investigated are located centrally near the kinase domain (amino acids E663 and I780) (Figure S5). Recent work by Kim et al. demonstrated that the N- and C-terminal regions of the MAP3K5 (ASK1) protein are important for binding to the transforming growth factor-β-activated kinase 1 (TAK1)-TAK1 binding protein 1 (TAB1) complex (Kim et al., 2012). This complex formation negatively regulates MAP3K5 activity, thus attenuating the proapoptotic signal. 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 demonstrates 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 TNFα, H_2O_2, Fas ligand, or reduced serum levels (Shiizaki et al., 2013);(Liu et al., 2000; Liu and Min, 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, 2009). MAP3K5, under normal conditions can be activated by loss of binding complex formation with its cytoplasmic inhibitor, thioredoxin (TXN/Trx) (Saitoh et al., 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., 2013). Trx inhibits MAP3K5-mediated apoptosis after reduction of Cys32 and Cys35 resulting in increased binding. The fact that we observed increased binding of Trx to the MAP3K5 R256C compared to WT MAP3K5, 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.

Our study of melanoma genomes and exomes identified a recurrently mutated gene that is mutually exclusive with BRAF. We provide functional evidence that the MAP3K5 hotspot mutation is an inactivating event that leads to the evasion of apoptotic pathways and promotes the colony forming abilities of melanoma cells, thus providing a potential therapeutic target for the population of patients who are unresponsive to therapies that target BRAF. Although additional studies will be required to translate our findings to the clinic, our data 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., 2011).

Materials and Methods

Tumor tissues

Tissue and melanoma cell lines used for the Discovery and Prevalence Screen in this study were described previously (Palavalli et al., 2009). All specimens are from an IRB approved study and written informed consents have been obtained for all specimens. The Office of Human Subjects Research determined that Federal regulations for the protection of human subjects approved this study. All metastatic melanoma samples and peripheral blood collected and sequenced in this study were done under protocols approved by the Institutional Review Board of National Institutes of Health-National Cancer Institute. "Declaration of Helsinki" Principles were followed and patients gave their written informed consent before enrollment and sample collection. Tissues used for Validation set 1 were fresh frozen melanoma tumors obtained from the University of Colorado Denver Skin Cancer Biorepository, Division of Medical Oncology. Tissue was collected at University of Colorado Hospital, Anschutz Medical Campus, under Institutional Review Board protocols. DNA was isolated from enriched macrodissected tumor isolates as previously described http://www.riedlab.nci.nih.gov. Tissue processing and storage was previously described by Morente et al (Morente et al., 2006). Tissues used for Validation set 2 of melanomas, were obtained from Optimum Cutting Temperature (OCT)–embedded frozen clinical specimens from the Melanoma Informatics, Tissue Resource, and Pathology Core (MelCore) at The University of Texas MD Anderson Cancer Center under Institutional Review Board-approved protocols. DNA isolation from the tumor-enriched isolates has been described previously (Davies *et al.*, 2009). Additional tissue was collected and cell lines established at Queensland Institute of Medical Research (41 stage III and 46 stage IV (AJCC) early passage metastatic melanoma cell lines). All cell lines were established as described previously (Castellano et al., 1997; Dutton-Regester K, 2012; Pavey et al., 2004) with informed patient consent under a protocol approved by the Queensland Institute of Medical Research Human Research Ethics Committee.

PCR, sequencing and *MAP3K5* mutational analysis of melanoma samples

The MAP3K5 recurrent mutation was confirmed and further screened using primers listed in Table S3 in an additional 172 melanoma samples. Mutational analysis, confirmation and determination of somatic status were carried out as previously described (Palavalli et al., 2009; Prickett et al., 2009).

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 28.8 mut/Mb (dipyrimidine mutation rate) employing the following values and formula:

x=5

n=288

p=28.8e-6

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

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 expression vectors

Human *MAP3K5* (NM_005923.3) were cloned by PCR as previously described (Palavalli et al., 2009) using clones (#6007002-MAP3K5) purchased from Open Biosystems with primers listed in Table S4. 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 methods (Prickett et al., 2009) using the primers found in Supplementary Table 4. Thioredoxin (myc-Trx) wild-type was purchased from Addgene (Plasmid #21614).

Cell culture and transient expression

HEK293 and HEK293T cells were purchased from ATCC (Manassas, VA) and maintained in complete RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS). HEK293 and 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.

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 transferred into a 1.5 mL microcentrifuge tube. Extracts were centrifuged for 10 minutes at 14,000 rpm at 4°C. 800 μl of supernatant was immunoprecipitated overnight using 20 μl of anti-FLAG (M2) beads (Sigma-Aldrich) or 10 μl of anti-myc antibody with 30μl of 50% slurry of Protein-A/G Sepharose beads (1× PBS). The immunoprecipitates were washed and subjected to SDS-PAGE and western blotting as previously described (Palavalli et al., 2009). Primary antibodies used in our 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), anti-Trx (#2429) (Cell Signaling), anti-myc (#SC-40) (Santa Cruz), and anti-GAPDH (#CB1001) (Calbiochem-EMD Biosciences).

Pooled stable expression

To make lentivirus, *MAP3K5* constructs were co-transfected into HEK 293T cells seeded at 1.5×10^6 per T75 flask with pVSV-G and pFIV-34N (kind gifts from Todd Waldman, Georgetown University) helper plasmids using Arrest-IN as described by the manufacturer. Virus-containing media was harvested 60hr after transfection, filtered, aliquoted and stored at −80°C. Mel-STR cells (kind gift from Dr. Weinberg) were grown in RPMI-1640 (Lonza, Walkersville, MD) and supplemented with 10% fetal bovine serum (HyClone, Logan, UT). 2183 (17T) were maintained in RPMI-1640 and supplemented with 10% FBS. Cells were seeded at 1.5×10^6 cells per T75 flask 24 hr prior to infection. Lentivirus for *MAP3K5* (wild-type or R256C point mutant) and empty vector control were used to infect both Mel-STR and A375 cells as previously described (Prickett et al., 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 clones were seeded into 96 well plates at 300 cells per well in 1%, 2.5% or 10% serum-containing medium and incubated for 13-17 days. Samples were analyzed every 48 hr by lysing cells in 50 μl 0.2% SDS/well and incubating for 2 hour 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 dH_2O). Plates were analyzed using a BMG Labtech FLOUstar Optima.

Soft agar assay

Stable 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% fetal bovine serum (HyClone, Logan, UT) in a 24-well plate. The lower plug contained sterile 0.5% Bacto-Agar and 10% fetal bovine serum. After two weeks, the colonies were photographed 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., 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 3μg/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 stably knocked down for endogenous *MAP3K5* following the manufacturer's protocol for the RNeasy Mini Kit (QIAGEN #74101). Total RNA was eluted in 30 μL diethylpyrocarbonate (DEPC)-treated distilled

H2O. A total of 1 μg 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 olido dT20 primer supplied in the kit. To test for loss of *MAP3K5* message, we used 1 μL of cDNA in the PCR reaction with either *MAP3K5* primers or *GAPDH* primers (Table S4).

Proliferation assays of stable knockdown cells

To examine growth potential stably depleted clones were seeded into 96 well plates at 500 cells per well in 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 hour 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 dH_20). Plates were analyzed using a BMG Labtech FLOUstar Optima.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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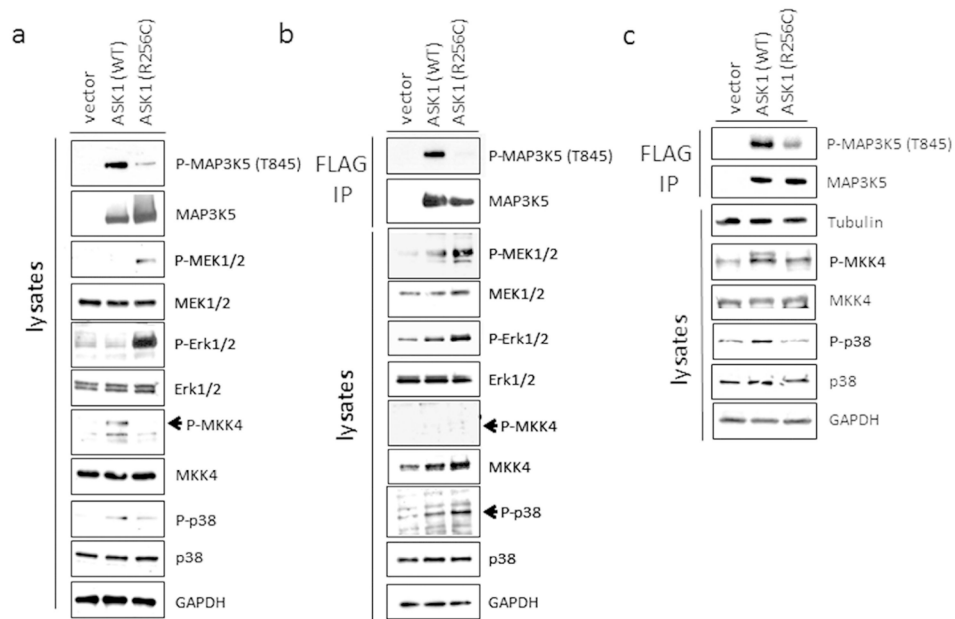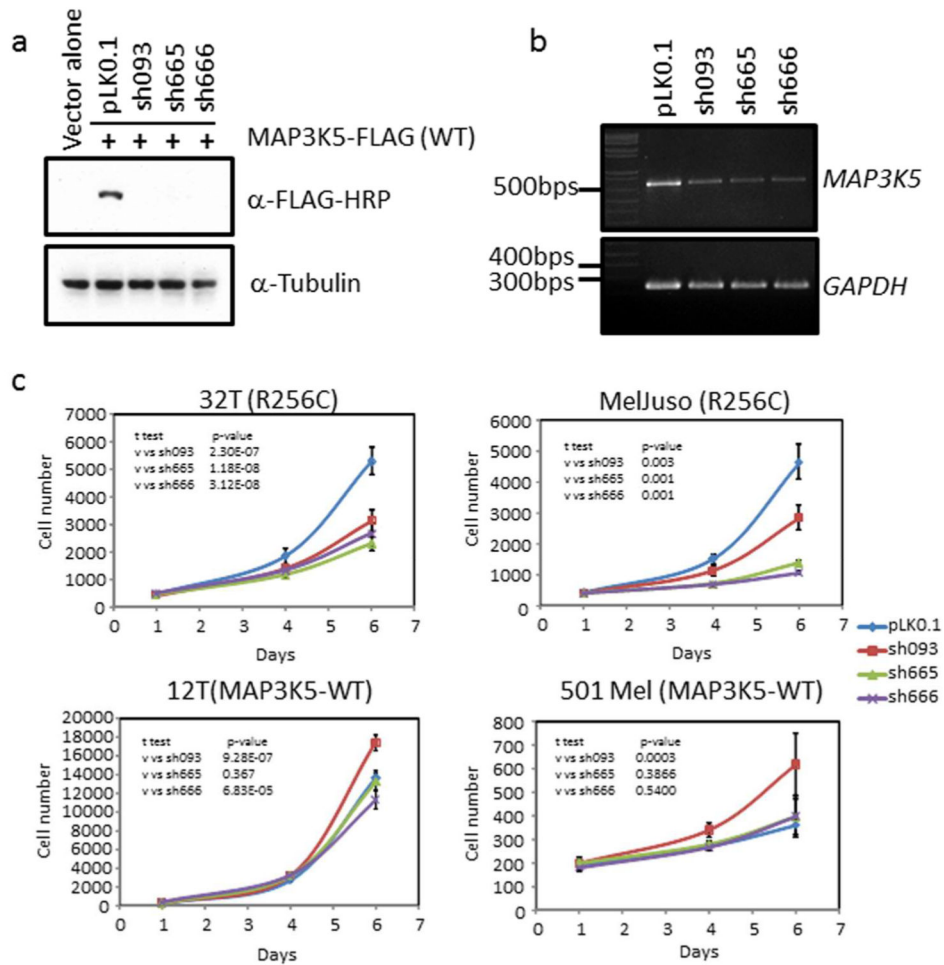
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Figure 1. Effects of the MAP3K5 (R256C) recurrent mutation on cell signaling
Lysates and immunoprecipitates from cells transiently or stably expressing wild-type or
mutant MAP3K5 were analyzed for activation of MAP3K5 and its downstream effector
molecules. A. HEK293T cells were transiently transfected with wild-type MAP3K5 (WT) or
mutant MAP3K5 (R256C) or empty vector as control. Lysates were generated and
immunoblotted with the indicated antibodies. B. Mel-STR and C. 2183 (17T) stably
expressing MAP3K5 (WT, R256C, or empty vector) clones were tested for increasing
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.
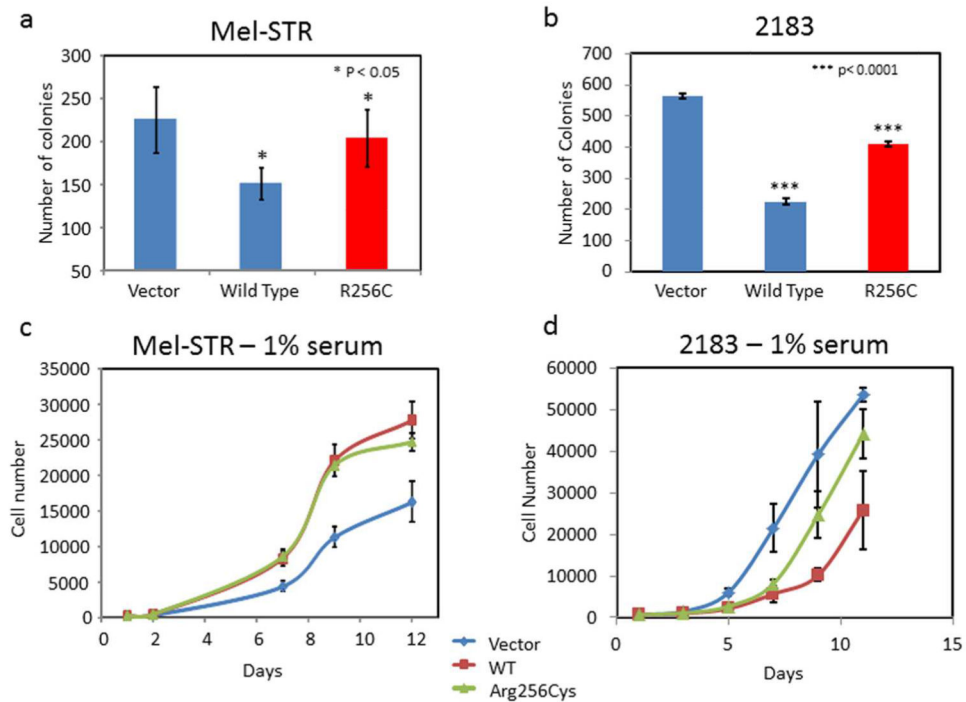
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Figure 2. Effects of the MAP3K5 (R256C) recurrent mutation on cell growth and proliferation
A. Wild-type MAP3K5 suppresses growth in soft agar. Mel-STR and 2183 (17T) pooled
MAP3K5 clones were seeded into soft agar to test for anchorage-independent growth. Mel-
STR (WT, R256C or empty vector) clones were grown for 10 days prior to harvesting,
staining and counting. B. 2183 (17T) (WT, R256C or empty vector) clones were grown for
10 days prior to harvesting, staining and counting. NIH ImageJ and Microsoft Excel were
used to analyze experiments. 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. C. 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.
SYBR Green was used to determine cell counts per day harvested. D. 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. SYBR Green was used to determine cell counts per day harvested.
NIH ImageJ and Microsoft Excel were used to analyze experiments. 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).

106x110mm (300 x 300 DPI)

Figure 3. 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. A. 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. B. 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. C. 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 SYBR 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).
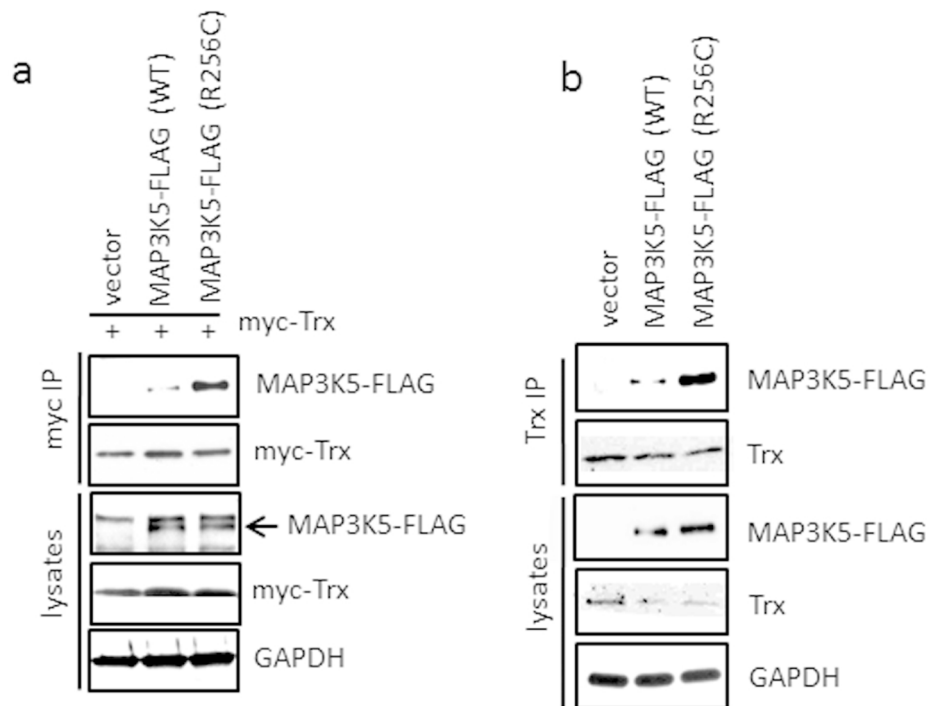
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Figure 4. Somatic mutation in MAP3K5 at residue R256C causes increased binding of Trx
MAP3K5 binds Trx in the absence of stimuli and mutation enhances binding. A. HEK293
cells transiently transfected with MAP3K5-FLAG (WT, R256C, vec) and myc-Trx were
analyzed for MAP3K5:Trx complex formation by co-immunoprecipitation with anti-myc. B.
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. Immunoprecipiates were analyzed using the antibodies shown and lyastes were
probed with anti-GAPDH as an internal control.