Somatic Mutations in MAP3K5 Attenuate Its Proapoptotic Function in Melanoma through Increased Binding to Thioredoxin

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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, on analysis of our whole-genome and whole-exome sequencing data of 29 melanoma samples, we identified several genes that harbor recurrent nonsynonymous mutations. These included MAP3K5 (mitogen-activated protein kinase kinase kinase-5), 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 (mitogen-activated protein kinase kinase 4) 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.

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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 because of 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 United States alone, ~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 (Flaherty *et al.*, 2010; Chapman *et al.*, 2011). However, despite the success in identifying genetic alterations utilizing candidate gene approaches (Davies *et al.*, 2002; Curtin

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Abbreviations: FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAPK, mitogen-activated protein kinase; MAP3K5, mitogen-activated protein kinase kinase kinase-5; MEK, MAPK/ERK kinase; MKK4, mitogen-activated protein kinase kinase 4; ROS, reactive oxygen species

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et al., 2006; Prickett *et al.*, 2009) as well as whole-genome (Pleasance *et al.*, 2010; Turajlic *et al.*, 2011; Berger *et al.*, 2012) and whole-exome (Wei *et al.*, 2011; Krauthammer *et al.*, 2012; Nikolaev *et al.*, 2012; Stark *et al.*, 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.

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 (Dicker *et al.*, 1990; Davies *et al.*, 2002; Nikolaev *et al.*, 2011; Stark *et al.*, 2011) and large-scale cancer genetic studies support these findings (Dicker *et al.*, 1990; Davies *et al.*, 2002; Clark *et al.*, 2004; Marks *et al.*, 2008; Johannessen *et al.*, 2010; 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 MAP3K5 (mitogen-activated protein kinase kinase kinase-5), also known as apoptosis signal-regulating kinase 1 (ASK1), is a serine/threonine protein kinase that activates c-Jun N-terminal kinase and p38 (Yang *et al.*, 2010; Tzeng *et al.*, 2013) 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, 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, whereas 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 prodeath activity and increasing melanoma cell survival.

RESULTS AND DISCUSSION

Genetic analysis reveals a hotspot mutation in MAP3K5 (R256C)

Recent sequencing projects using Sanger/whole-exome/whole-genome techniques have implicated many different genes involved in melanomagenesis (Pleasance *et al.*, 2010; Turajlic *et al.*, 2011; Wei *et al.*, 2011; Berger *et al.*, 2012; Hodis *et al.*, 2012; Krauthammer *et al.*, 2012; Nikolaev *et al.*, 2012; Stark *et al.*, 2012). 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 reanalysis. 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.* (2012), indicating the potential importance of this gene in melanoma development, we focused our follow-up work on *MAP3K5*.

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 arginine-to-cysteine substitution at amino acid residue 256 of the protein (R256C) (Supplementary Figure S1 online). Furthermore, sequencing of the *MAP3K5* recurrent mutation in an independent cohort of 87 melanomas yielded a fifth sample with the same mutation (Supplementary Table S1 online). 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) (Supplementary Table S2 online contains a list of mutations occurring in *MAP3K5* from whole-exome and genome sequenced samples screened or reanalyzed in this study). In these data sets, 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 (Supplementary Figure S2 online), suggesting that 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 (The Cancer Genome Atlas) 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, with 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 an almost mutual exclusivity between the MAP3K5 recurrent mutation and BRAF V600E (*P*-value = 0.01016; see Supplementary Figure S3 online). Because of the frequency of the MAP3K5 R256C mutation in melanoma, we decided to investigate whether the mutation has a role in tumorigenesis.

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

MAP3K5 is stimulated via inflammatory cytokines (tumor necrosis factor-α, lipopolysaccharide, 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 MAPKs c-Jun N-terminal kinase and/or p38 via MKK4/7, leading to an increased propensity for cell death or apoptosis. A marker of MAP3K5 proapoptotic effect is the measure of the 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 pro-apoptotic 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 in both 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 with 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 with 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 proapoptotic 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 with mutant MAP3K5 (R256C) or empty vector (Figure 2a and 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 (Supplementary Figure S4A and B online). However, although Mel-STR pooled clones expressing wild-type or R256C in the presence of low serum (1%) also exhibited similar proliferation rates (Figure 2c), the 2183 (17T) pooled clones expressing R256C exhibited elevated proliferation rates compared with wild-type expressing cells (Figure 2d). These data suggest 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 whether melanoma cells with endogenous MAP3K5 mutations are dependent on MAP3K5 signaling for proliferation, we used small hairpin RNA (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 reverse transcription–PCR analysis, using *MAP3K5*-specific primers and *glyceraldehyde-3-phosphate dehydrogenase* (*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 proapoptotic 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 (Figure 4a) or endogenous Trx (Figure 4b). 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 prodeath pathways of p38 or c-Jun N-terminal kinase. Furthermore, mutation of MAP3K5 resulted in increased proliferation as well as anchorage-independent growth, further demonstrating attenuation of the prodeath MKK4/7 signaling pathway causing increased survival via MEK/MAPK activation.

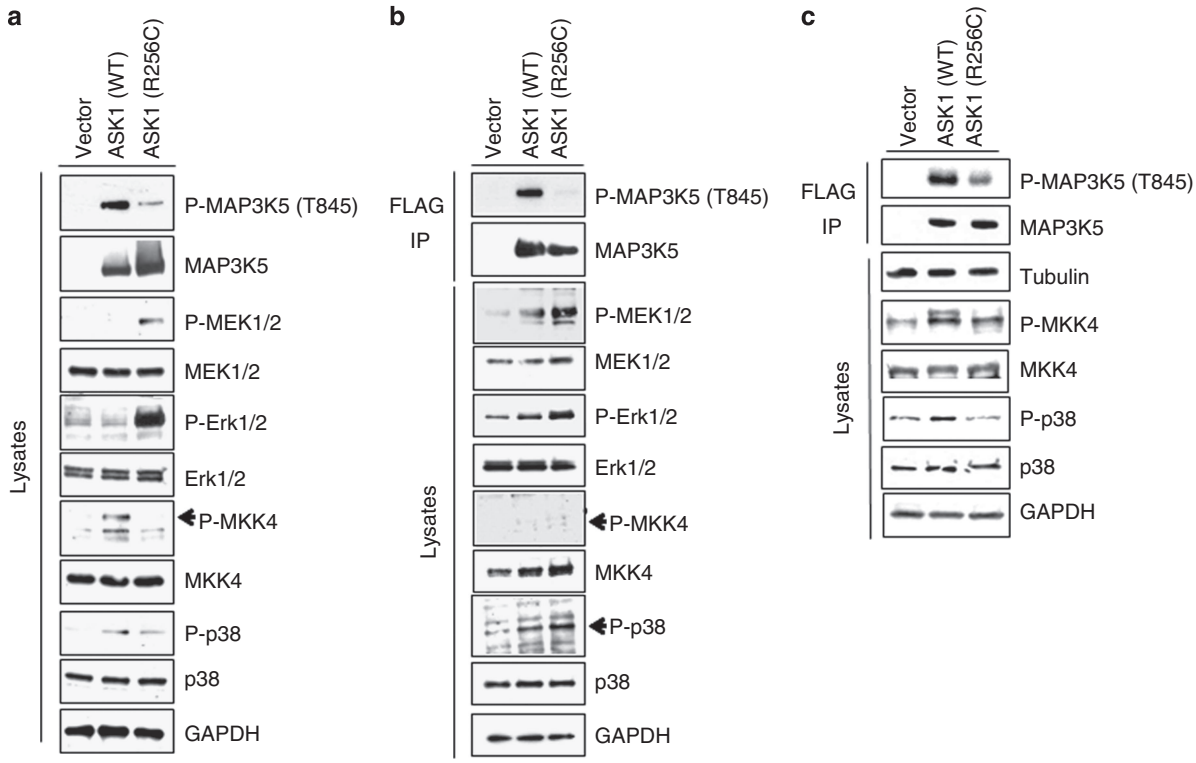

Figure 1. Effects of the mitogen-activated protein kinase kinase kinase-5 (MAP3K5; R256C) recurrent mutation on cell signaling. Lysates and immunoprecipitates from cells transiently or stably expressing wild-type or mutant MAP3K5 were analyzed for activation of MAP3K5 and its downstream effector molecules. (**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. ASK1, apoptosis signal-regulating kinase 1; ERK1/2, extracellular signal–regulated kinase1/2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IP, immunoprecipitation; MEK, MAPK/ERK kinase; MKK4, mitogen-activated protein kinase kinase 4; WT, wild type.

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 (Dicker *et al.*, 1990; Davies *et al.*, 2002). Recent genetic studies of *MEK1* and/or *MEK2* found them both to harbor recurrent somatic mutations resulting in increased MAPK1/2 (extracellular signal–regulated kinase1/2 (Erk1/2)) activation, proliferation, and cellular transformation (Marks *et al.*, 2008; Nikolaev *et al.*, 2011). Interestingly, hyper-activated forms of these kinases have increased sensitivity to inhibition using small-molecule inhibitors targeting the MAPK pathway, including PLX4032 (vemurafenib) (Bollag *et al.*, 2010; Flaherty *et al.*, 2010; Halaban *et al.*, 2010; Chapman *et al.*, 2011). 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 nonsynonymous somatic mutations of MAP3K5 in 8 of 85 melanomas (Stark *et al.*, 2012). However, in contrast to this study, the MAP3K5 mutations analyzed had little to no effect on downstream activation or suppression of c-Jun N-terminal kinase 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; whereas 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) (Supplementary Figure S5 online). Recent work by Kim *et al.* (2012) 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. 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 with the mid-regions of MAP3K5 demonstrates the complexity of this kinase, and may explain the differences observed in pathway stimulation.

Prodeath signaling pathways can be activated via many different stimuli such as tumor necrosis factor-α, H_2O_2, Fas ligand, or reduced serum levels (Liu *et al.*, 2000; Liu and Min, 2002; Shiizaki *et al.*, 2013). On activation of death receptors

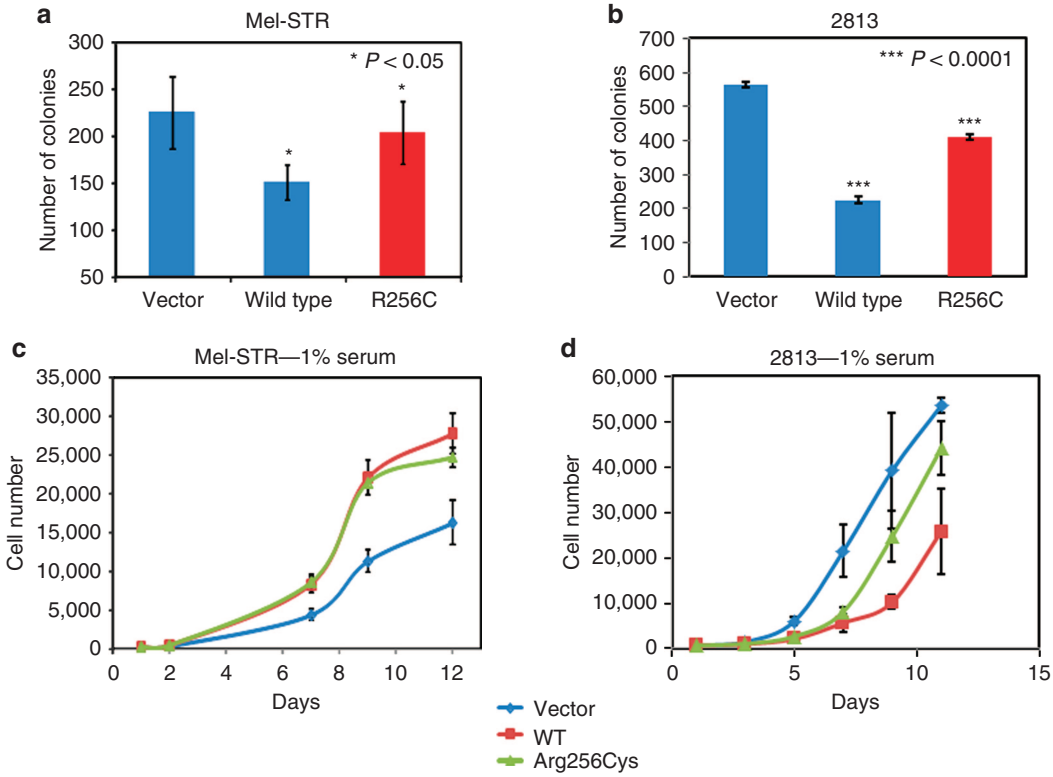

Figure 2. Effects of the mitogen-activated protein kinase kinase kinase-5 (MAP3K5; R256C) recurrent mutation on cell growth and proliferation. (**a**) Wild-type MAP3K5 suppresses growth in soft agar. Mel-STR and 2183 (17T) pooled MAP3K5 clones were seeded into soft agar to test for anchorage-independent growth. Mel-STR (WT, R256C, or empty vector) clones were grown for 10 days before harvesting, staining, and counting. (**b**) 2183 (17T) (WT, R256C, or empty vector) clones were grown for 10 days before 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 s.d. ($n=3$; *comparing WT or R256C with empty vector, ***comparing WT R256C to empty vector); *$P<0.05$ and ***$P<0.0001$ using an unpaired Student's *t*-test).

by these ligands, proapoptotic signals involving, e.g., the tumor necrosis factor receptor or 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 proapoptotic 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 with wild-type 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 prosurvival pathway being upregulated, 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 have been described previously (Palavalli *et al.*, 2009). All specimens are from an institutional review board–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

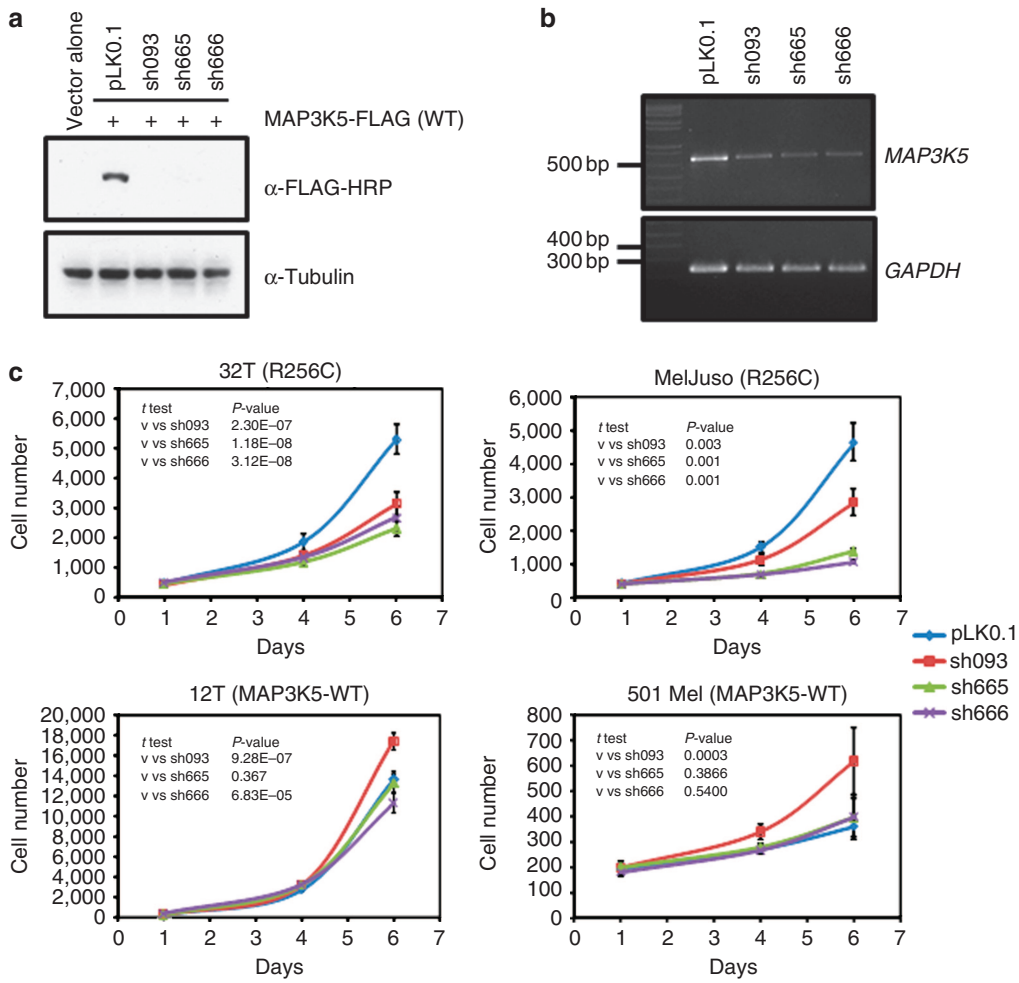

Figure 3. Effects of stable depletion of mitogen-activated protein kinase kinase kinase-5 (MAP3K5) on melanoma cell growth. The small hairpin RNA (shRNA)–mediated depletion of MAP3K5 was tested using transient transfection and immunoblotting of lysates or reverse transcription–PCR (RT–PCR) analysis of 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. Glyceraldehyde-3-phosphate dehydrogenase (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 (WT) 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 s.d. ($n=3$).

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. The 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 (Denver, CO). Tissue was collected at the University of Colorado Hospital, Anschutz Medical Campus (Aurora, CO), 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.* (2006). Tissues used for Validation set 2 of melanomas were obtained from optimum cutting temperature–embedded frozen clinical specimens from the Melanoma Informatics, Tissue Resource, and Pathology Core (MelCore) at The University of Texas MD Anderson Cancer Center (Houston, TX) 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 (Brisbane, QLD, Australia; 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; Pavey *et al.*, 2004; Dutton-Regester *et al.*, 2012) with informed patient consent under a protocol approved by the human research ethics committee of the Queensland Institute of Medical Research.

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

The MAP3K5 recurrent mutation was confirmed and further screened using primers listed in Supplementary Table S3 online in

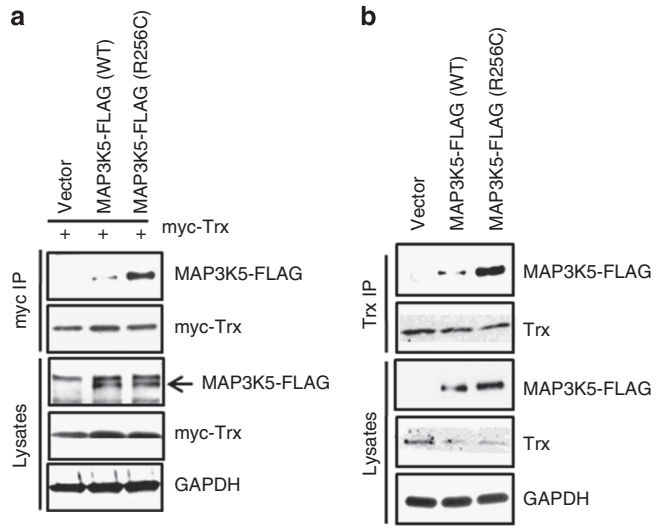

Figure 4. Somatic mutation in mitogen-activated protein kinase kinase kinase-5 (MAP3K5) at residue R256C causes increased binding of thioredoxin (TXN/Trx). MAP3K5 binds Trx in the absence of stimuli and mutation enhances binding. (**a**) HEK293 cells transiently transfected with MAP3K5-FLAG (wild type (WT), R256C, or vector (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. Immunoprecipitates were analyzed using the antibodies shown and lysates were probed with anti-GAPDH as an internal control. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IP, immunoprecipitation.

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:

$x=5,\ n=288,\ P=28.8e-6.$

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

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 (Huntsville, AL) with primers listed in Supplementary Table S4 online. The PCR products were cloned into the mammalian expression vectors pCDF-MCS2-EF1-Puro or pCDF-MCS2-EF1-Neo (Systems Biosciences, Mountain View, CA) or pcDNA3.1(−) (Invitrogen-Life Technologies, Grand Island, NY) via the *Xba*I and *Not*I restriction sites. MAP3K5 complementary DNA 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 S4 online. Thioredoxin (myc-Trx) wild type was purchased from Addgene (Cambridge, MA) (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) in a 6:1 ratio with DNA (μl/μg) using 2–5 μg of plasmid DNA.

Immunoprecipitation and western blotting

Transfected cells were gently washed 2 × in phosphate-buffered saline and then lysed using 1.0 ml 1% NP-40 lysis buffer (1% NP-40, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, Complete Protease Inhibitor tablet, EDTA-free (Roche, Indianapolis, IN), 1 μM 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 r.p.m. at 4 °C. Then, 800 μl of supernatant was immunoprecipitated overnight using 20 μl of anti-FLAG (M2) beads (Sigma-Aldrich, St Louis, MO) or 10 μl of anti-myc antibody with 30 μl of 50% slurry of Protein-A/G Sepharose beads (1 × phosphate-buffered saline). 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, Danvers, MA), anti-myc (SC-40; Santa Cruz Biotechnology, Dallas, TX), and anti-GAPDH (CB1001; Calbiochem-EMD Biosciences, Billerica, MA).

Pooled stable expression

To make lentivirus, *MAP3K5* constructs were cotransfected into HEK293T cells seeded at 1.5×10^{6} per T75 flask with pVSV-G and pFIV-34N (kind gifts from Todd Waldman, Georgetown University, Washington , DC) helper plasmids using Arrest-IN as described by the manufacturer. Virus-containing media were harvested 60 hours after transfection, filtered, aliquoted, and stored at −80 °C. Mel-STR cells (kind gift from Dr Robert Weinberg, Whitehead Institute, Cambridge, MA) were grown in RPMI-1640 (Lonza, Walkersville, MD) and supplemented with 10% FBS (HyClone, Logan, UT). The 2183 (17T) cells were maintained in RPMI-1640 and supplemented with 10% FBS. Cells were seeded at 1.5×10^{6} cells per T75 flask 24 hours before 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 hours by lysing cells in 50 µl 0.2% SDS per well and incubating for 2 hours at 37 °C before addition of 150 µl/well of SYBR Green I solution (1:750 SYBR Green I (Invitrogen-Molecular Probes, Carlsbad, CA) diluted in distilled H_2O). Plates were analyzed using a BMG Labtech FLOUstar Optima (Cary, NC).

Soft agar assay

Stable pooled MAP3K5 clones were plated in triplicate at 1,000 cells per well and in top plugs consisting of sterile 0.33% Bacto-Agar (BD, Sparks, MD) and 10% FBS (HyClone) in a 24-well plate. The lower plug contained sterile 0.5% Bacto-Agar and 10% FBS. After 2 weeks, the colonies were photographed and quantitated using ImageJ (NIH software, NIH, Bethesda, MD).

Lentiviral shRNA

Constructs for stable depletion of *MAP3K5* (cat no. RHS4533-NM_005923) were obtained from Open Biosystems 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 3 µg ml^{-1} puromycin containing normal medium for 3–5 days before determining knockdown 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, Frederick, MD). Total RNA was eluted in 30 µl diethylpyrocarbonate-treated distilled H_2O. A total of 1 µg of total RNA was used for single-strand complementary DNA synthesis using a SuperScript III First-Strand kit (Invitrogen Life Technologies, 18080-051). Complementary DNA was amplified using the oligo dT20 (Invirtogen-Life Technologies) primer supplied in the kit. To test for loss of *MAP3K5* message, we used 1 µl of complementary DNA in the PCR reaction with either *MAP3K5* primers or *GAPDH* primers (Supplementary Table S4 online).

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 per well and incubating for 2 hours at 37 °C before addition of 150 µl per well of SYBR Green I solution (1:750 SYBR Green I (Invitrogen-Molecular Probes) diluted in distilled H_2O). Plates were analyzed using a BMG Labtech FLOUstar Optima.

Data access

All somatic variants found in MAP3K5 present in our melanoma cohorts will be deposited to dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

TDP, SCJP, JJG, EHM, and YS designed the study; MAD, JEG, WR, SR, JJ, KD-R, NKH, and SAR collected and analyzed the melanoma samples; SCJP, JJG, XW, JKT, JCL, KD-R, NKH, NKH, and the NISC Comparative Sequencing Program analyzed the genetic data. TDP, BZ, and JJ performed the functional analyses. All authors contributed to the final version of the paper.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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