Targeting TBK1 Inhibits Migration and Resistance to MEK Inhibitors in Mutant NRAS Melanoma

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Abstract

Melanoma is a devastating form of skin cancer with limited therapeutic options. Fifteen to 20% of patients with melanoma have an activating mutation in the GTPase, NRAS. The major downstream effectors of RAS are RAFs (ARAF, BRAF, and CRAF), phosphoinositide 3-kinase (PI3K), and the Ras guanine exchange factors (RaGGEF). TANK-binding kinase 1 (TBK1) is an atypical IkB kinase family member that acts downstream of RasGEFs. Whereas many studies have analyzed RAF and PI3K signaling in mutant NRAS melanoma, the role of RasGEF/Ral is understudied and TBK1 has not been examined. To address this, TBK1 was modulated with knockdown approaches and targeted therapies to determine the role of TBK1 in motility, apoptosis, and signaling. In melanoma, NRAS overexpression increased TBK1 phosphorylation. TBK1 depletion inhibited migration and invasion, whereas its constitutive overexpression led to an increase in invasion. In three-dimensional systems that mimic the dermal microenvironment, TBK1 depletion or inhibition cooperated with MEK inhibitors to promote apoptosis, particularly in the context of MEK-insensitive mutant NRAS. This effect was absent in melanoma cells that are wild-type for NRAS. These results suggest the utility of TBK1 inhibitors as part of a treatment regimen for patients with mutant NRAS melanoma, for whom there are no current effective therapies.

Implications: TBK1 promotes the malignant properties of NRAS-mutant melanoma and its targeting, in combination with MEK, promotes apoptosis, thus providing a potential novel targeted therapeutic option.

Introduction

Melanoma is the deadliest form of skin cancer and its incidence is increasing at a greater rate than other cancers (1). It is notorious for its propensity to migrate and invade as well as its resistance to apoptosis. If melanoma is confined to expansion within the epidermis, it is easily cured with surgical intervention (2, 3). However, without early diagnosis, the cells can invade through the basement membrane and expand through deeper dermal layers. The mechanisms underlying melanoma invasion from the epidermis into the dermis remain poorly characterized. In addition to its aggressive nature, melanoma is also notorious for its evasion of apoptosis, a property that has made treatment extremely difficult. Conventional chemotherapies, such as alkylating agents, antibiotics, taxanes, and platinum drugs, do not elicit a significant therapeutic benefit (4).

Fifteen to 20% of patients with melanoma have mutations in NRAS, most frequently Q61R/K/L that trap the protein in a GTP-bound state, leading to its constitutive activation (5, 6). In addition, patients with mutant BRAF melanoma who have been treated with the RAF inhibitor vemurafenib often gain resistance through acquisition of a NRAS mutation or selection for cells with a coexisting NRAS mutation, which permits maintenance of MEK–ERK1/2 pathway activation in the presence of RAF inhibitors (7). While there are no targeted therapies that are FDA-approved for patients with melanoma with a primary mutation in NRAS or those that develop it secondarily, one form of targeted therapy that has been explored are MEK inhibitors. In contrast to findings in mutant BRAF V600E/K melanomas (8), phase I to III studies of MEK inhibitors in mutant NRAS melanomas have not shown consistent clinical efficacy (9, 10). The underlying basis for the varied response is not known. NRAS activates multiple effector pathways and some mutant NRAS melanoma cells may be less reliant on MEK–ERK1/2 signaling. The major downstream effectors of RAS are RAFs (ARAF, BRAF, CRAF), phosphoinositide 3-kinase (PI3K), and the Ras guanine exchange factors (RaGGEF; ref. 11). In mutant NRAS melanoma cells that are resistant to MEK inhibitor treatment, PI3K and AKT activity may be important compensatory pathways. Recent evidence has suggested that RalA/B–mediated pathways may also play a key role in mutant NRAS melanomas (12, 13).
RaLB signals, in part, by interacting with Sec5, a component of the exocyst complex involved in the maintenance of cell polarity, cell motility, and cytokinesis (14). Sec5 directly recruits and activates TANK-binding kinase 1 (TBK1), an atypical IKB kinase family member (15). Thus, TBK1 is activated downstream of RaLB and has been implicated in the phosphorylation of AKT at both Thr308 and Ser473 (16, 17). Furthermore, TBK1 has been implicated in oncogenesis as its depletion reduces the tumorigenic potential of mutant KRAS-transformed fibroblasts (14), mutant KRAS non–small cell lung carcinoma cell lines (16), and mutant KRAS pancreatic cancer cells (17). Given its role in KRAS-mediated oncogenesis, we examined the role of TBK1 in the malignant properties of mutant NRAS melanoma.

Here, we show that TBK1 is regulated by activated NRAS expression in melanoma cells. In mutant NRAS cells, TBK1 depletion leads to a decrease in tumor cell migration and invasion. In contrast, the expression of a constitutively active form of TBK1 led to an increase in tumor cell invasion. In three-dimensional (3D) collagen cultures, the combination of TBK1 and MEK inhibition cooperated to enhance apoptosis in mutant NRAS melanoma cells as well as vemurafenib-resistant mutant BRAF melanoma cells harboring secondary mutation in NRAS. This effect was absent in melanoma cell lines that are wild-type for NRAS. These results suggest that TBK1 promotes melanoma cell motility and is a potential target as part of a combination regimen for a subset of patients with melanoma with no current effective therapy options.

Materials and Methods

Cell culture

SKMel30 and SKMel173 were kindly donated by Dr. David Solit (Memorial Sloan-Kettering Cancer Center, New York, NY). WM852 and WM3670 were purchased from Coriell Institute (Camden, NJ). Other WM melanoma lines were kindly donated by Dr. Meenhard Herlyn (Wistar Institute, Philadelphia, PA). Additional SKMel cell lines were purchased from ATCC. WM cell lines, 1205Lu, SKMel28, and SBd2 were cultured in MCDB 153 medium containing 20% Leibovitz L-15 medium, 2% FBS, and 0.2% sodium bicarbonate (WM medium). WM793-Res #5 and WM793-Res #12 were cultured in WM medium in the presence of 5 mmol/L PLX4720 (19). SKMel2 cells were cultured in minimum essential medium (MEM) with 10% FBS. SKMel30, SKMel32, SKMel173, and SKMel207 were cultured in RPMI-1640 with 10% FBS. SKMel3 cells were cultured in McCoy’s 5A medium containing 15% FBS. SKMel30, SKMel32, SKMel173, and SKMel207 were cultured in RPMI-1640 with 10% FBS. SKMel3 cells were cultured in MEM with 10% FBS. SKMel30, SKMel173, and SKMel207 were kindly donated by Dr. David Solit (Memorial Sloan-Kettering Cancer Center, New York, NY). WM852 and WM3670 were purchased from Coriell Institute (Camden, NJ). Additional SKMel cell lines were kindly donated by Dr. Meenhard Herlyn (Wistar Institute, Philadelphia, PA). Other WM melanoma lines were purchased from ATCC. WM cell lines, 1205Lu, SKMel28, and SBd2 were cultured in MCDB 153 medium containing 20% Leibovitz L-15 medium, 2% FBS, and 0.2% sodium bicarbonate (WM medium). WM793-Res #5 and WM793-Res #12 were cultured in WM medium in the presence of 5 mmol/L PLX4720 (19). SKMel2 cells were cultured in minimum essential medium (MEM) with 10% FBS. SKMel30, SKMel32, SKMel173, and SKMel207 were cultured in RPMI-1640 with 10% FBS. SKMel3 cells were cultured in McCoy’s 5A medium containing 15% FBS. SKMel5 cells were cultured in MEM with 10% FBS, 10% nonessential amino acids, 1 mmol/L sodium pyruvate, and 0.15% sodium bicarbonate.

Western blot analysis

Cells lysed in sample buffer were separated by SDS-PAGE, and proteins were transferred electrothermally onto Immobilon P membranes (Millipore Corp.). Membranes were blocked with PBS containing 1% BSA and 0.1% Tween 20 for 1 hour and subsequently incubated with primary antibody overnight at 4°C. Antibodies for TBK1/NRK (3504), phospho-TBK1 Ser172 (5483), AKT (9272), phospho-AKT Ser473 (4060), phospho-ERK (9101), IRF3 (4302), and cleaved PARP (9541) were obtained from Cell Signaling Technology; ERK1 (K-23) and NRAS (C-20) from Santa Cruz Biotechnology; actin (A2066) from Sigma-Aldrich; and phospho-IRF3 Ser386 (9076443) from Abcam. Membranes were washed in PBS/Tween and incubated with anti-mouse or anti-rabbit IgG peroxidase conjugates (Calbiochem) for 1 hour at room temperature. Western blots were developed using SuperSignal chemiluminescent substrate (Pierce). Immunoreactivity was detected and quantified using a Fluor-S Multi-Imager and Quantity-One software (Bio-Rad).

Cloning and stable cell line generation

A mutant NRAS<sub>Q61K</sub> construct was generated, as previously described (19). Human TBK1 with a tag for myristoylation was cloned from cDNA using the following primers: forward 5′-CACCATGGGAAGCAGCAAGAGCAAGCC-CAAGGACCCAGCCGCGCCAGACCTTC-TAACATCTGG-3′ and reverse 5′-AAGACAGTCAGCGTTGCGAAG-3′. All DNA constructs were sequence verified. Lentiviral particles and tetracycline repressor–expressing (TR-expressing) sublines WM1361A TR and WM1366 TR expressing doxycycline-inducible TBK1-<i>myr</i> were generated, as previously described (20). Transgene expression was induced with 0.1 μg/mL doxycycline in the cell culture medium.

Immunofluorescence

Melanoma cells growing on glass coverslips were washed with PBS and fixed with 3.7% formaldehyde for 20 minutes. Cells were then permeabilized with 0.2% Triton X-100 for 5 minutes and nonspecific staining blocked with 1% BSA/PBS for 2 hours at room temperature. Coverslips were then incubated with primary antibodies diluted in 1% BSA/PBS overnight at 4°C. After primary antibody, coverslips were washed three times with PBS before incubation with appropriate Alexa Fluor-conjugated secondary antibodies (Invitrogen) for 1 hour at room temperature. In some instances, the coverslips were incubated with 4′, 6-diamidino-2-phenylindole (DAPI) to visualize nuclei. Coverslips were mounted and visualized on an Eclipse Ti inverted microscope with NIS-Elements AR 3.00 software (Nikon).

RNA interference

Mutant NRAS<sub>Q61K</sub> cells were transfected for 4 hours with chemically synthesized siRNA at a final concentration of 25 nmol/L using OligofectAMINE (Invitrogen). Cells were harvested after a further 68 hours. The TBK1 siRNAs (#1: GAACGUAGAUAGCUUAUU; #2: UGACAGAUAGCUUAUA) were purchased from Dharmaco。“

Migration and invasion assay

Subconfluent melanoma cells were cultured overnight in serum-free medium. Cells (4.2 × 10<sup>4</sup>) in serum-free...
medium were placed inside 8.0 μm pore-size cell culture inserts (BD Biosciences). Cells were allowed to migrate for 24 hours towards an attractant of full-serum medium. Chamber filters were fixed in buffered formalin and stained with crystal violet. The cells in the inner chamber were removed. Images in triplicate from each filter were taken with a Nikon Ti-Eclipse inverted microscope and stained cells were counted manually utilizing the NIS-Elements Software package. Images used for quantitation were taken at 10× magnification. The average count from control filters triplicate counts from triplicate independent experiments was normalized to 100% migration. All counts were converted to percent migration in comparison with the control 100% migration. For invasion studies, the migration assay was modified with coating of the inner chamber with 0.75 mg/mL Matrigel (BD Biosciences) for one hour before addition of cells.

**Colony formation assay**

Mutant NRAS cells (4 × 10^5) were plated per 6-well plate in complete medium with or without inhibitors, which were replenished every 2 days. After 7 days, cells were stained with crystal violet in formalin, plates were imaged by scanner, and colonies were imaged on a Nikon Eclipse Ti inverted microscope with NIS-Elements AR 3.00 software.

**3D collagen gels and apoptosis assay**

Collagen gels were cast and cells were isolated and stained with Annexin V-APC as previously described (19). Apoptosis was analyzed by flow cytometry on FACSCalibur flow cytometer (BD Biosciences). Data were analyzed by FlowJo software (Tree Star Inc.).

**Results**

**TBK1 is expressed and activated in a subset of mutant NRAS melanoma cells lines**

TBK1 is an atypical IκB kinase family member that plays an important role in KRAS-mediated oncogenesis through its phosphorylation of AKT at the Thr308 and Ser473 sites (17, 18). Given its role in mutant KRAS-mediated oncogenesis, we examined the potential role of TBK1 in the malignant properties of mutant NRAS melanoma. We initially analyzed the expression level of TBK1 in a panel of mutant NRAS melanoma cell lines from different stages of melanoma progression and NRAS mutation (Supplementary Table S1). By Western blot analysis, TBK1 was ubiquitously expressed in both mutant NRAS and mutant BRAF cell lines examined, albeit to different levels (Fig. 1A). Autophosphorylation of TBK1 at Ser172 is required for its activation (21). Levels of phosphorylated TBK1 at Ser172 were detectable in several cell lines and, in the mutant NRAS panel, correlated with high levels of NRAS. IKKe is another member of the atypical IκB kinase family that shares 67% amino acid identity with TBK1 within the kinase domain (14). As IKKe has been implicated in tumorigenesis, particularly breast, ovarian, and pancreatic cancer (22), we analyzed levels of IKKe and found that its expression level was barely detectable in mutant NRAS melanoma cell lines compared with RAW 264.7 macrophage cells (Supplementary Fig. S1).

Despite previous evidence that KRAS regulates TBK1, it is unknown whether NRAS similarly activates TBK1. To test this, we utilized a lentiviral transduction system to express modest levels of mutant NRAS^{Q61K} in WM3211 cells, which are wild-type for NRAS and BRAF (23). Expression of mutant NRAS led to a marked increase in the phosphorylation of endogenous TBK1 (Fig. 1B). To analyze TBK1 signaling to AKT, we depleted TBK1 in five mutant NRAS melanoma cell lines, and serum-starved cells before stimulation with either serum or insulin. AKT phosphorylation at both Thr308 and Ser473 was low in unstimulated cells (Fig. 1C). TBK1 depletion slightly decreased AKT activation in Sbc12, WM1366, and SKMel2 cells in response to insulin stimulation but no effect was observed in WM1361A and WM1346 cells. Furthermore, TBK1 knockdown had minimal effects on serum-stimulated AKT phosphorylation in the five cell lines analyzed.

Previous efforts to generate active forms of TBK1 by introducing phosphomimetic mutants, such as S172D and S172E, have been unsuccessful and have actually decreased TBK1 activity (24). As subcellular fractionation has revealed that TBK1 is constitutively localized on the membrane (18), we generated a TBK1 construct with an epitope at the N-terminus that can be myristoylated. Myristoylation is an irreversible, cotranslational protein modification that targets proteins to the membrane and therefore enhances signaling (25). The TBK1-myristoylated (myr) construct was expressed and led to a robust increase in the autophosphorylation of TBK1 in WM1366 TR and SKMel2 cells (Fig. 1D), whereas TBK1-myr expression/phosphorylation was low in WM1361A TR cells. Immunofluorescence showed that expression of TBK1-myr leads to increased staining of TBK1 localized at the plasma membrane (Fig. 1E). As with TBK1 depletion, TBK1 activation had a marginal effect on AKT phosphorylation, with a slight increase in WM1366 TR and WM1361A TR but no effect in SKMel2 (Fig. 1D).

**TBK1 affects cell migration and invasion in mutant NRAS melanoma cells**

Through our immunofluorescence studies, we noticed that endogenous TBK1 localized to the edge of the cell and colocalizes, at least in part, with focal adhesion kinase (FAK; Fig. 2A). FAK is a cytoplasmic tyrosine kinase that localizes to focal adhesions, areas where cells interact with the extracellular matrix (26), and is required for migration and invasion of several different cancers, including melanoma (27). In addition, TBK1 is known to be recruited to the exocyst, a hetero-octameric complex that is directly employed for maintenance of epithelial cell polarity, cell motility, and...
cytokinesis (14). Given its colocalization with FAK and its known relationship to the exocyst complex, we tested the role of TBK1 in cell migration and invasion. We utilized siRNA to effectively knock down TBK1 in five mutant NRAS melanoma lines (Fig. 2B). Upon TBK1 depletion in mutant NRAS cells, there was a decrease in Transwell migration towards full serum medium (Fig. 2C). Furthermore, in Matrigel-coated Transwell chamber assays, TBK1 knockdown effectively inhibited invasion of mutant NRAS melanoma cells (Fig. 2D). These findings were recapitulated with a second independent siRNA targeting TBK1 (Supplementary Fig. S3A and S3B). The expression of TBK1-myr showed a trend towards an increase in migration in SKMel2 but this was not statistically significant (Fig. 2E). In both WM1361A TR and SKMel2, the expression of TBK1-myr led to a significant increase in invasion (Fig. 2F). Transduction of WM1366 TR cells with the TBK1-myr lentivirus did not enhance either migration or invasion likely due to less efficient expression (data not shown and Fig. 1D). Overall, these results indicate a novel role for TBK1 in cancer cell migration and invasion.

 Knockdown of TBK1 combined with AZD6244 enhances apoptosis in AZD6244-resistant cell lines in 3D culture

Previous groups have identified a role for TBK1 in promoting the growth and proliferation of KRAS-transformed cancer cells (16, 17). After depleting TBK1 with siRNA, we observed a significant increase in Annexin V staining in some but not all mutant NRAS melanoma cell
Figure 2. TBK1 promotes migration and invasion of melanoma cells. A, immunofluorescence image of WM1361A stained for DAPI (blue), FAK (red), and TBK1 (green). Scale bar = 25 μm. B, WM1366, SBcl2, WM1346, WM1361A, and SKMel2 cells were transfected with nontargeting or TBK1-targeting siRNA for 72 hours and lysates were analyzed by Western blotting. C, mutant NRAS cells were transfected as described in B. Cells were allowed to migrate through Boyden chambers toward an attractant of full serum medium for 24 hours. Counts taken (in triplicate fields of view) from the control siRNA (average set at 100% migration) were used to calculate percent migration (n = 3; error bars, S.E.; **P < 0.05). Representative 10× images of migrated cells are shown. Scale bar = 100 μm. D, mutant NRAS cells were transfected as described in B. Cells were allowed to invade through Boyden chambers coated with Matrigel toward an attractant of full serum medium for 24 hours. Counts taken (in triplicate fields of view) from the control siRNA (average set at 100% invasion) were used to calculate percent invasion (n = 3; error bars, S.E.; **P < 0.05). Representative 10× images of migrated cells are shown. Scale bar = 100 μm. E, WM1366 TR TBK1-myr and WM136A TR TBK1-myr cells were treated with or without doxycycline for 24 hours, and parental SKMel2 and SKMel2 constitutively expressing TBK1-myr were seeded for migration assay as described in C. F, WM1366 TR TBK1-myr and WM136A TR TBK1-myr cells were treated with or without doxycycline for 24 hours, and parental SKMel2 and SKMel2 constitutively expressing TBK1-myr were seeded for invasion assay as described in D.
lines (Fig. 3A). Similarly, TBK1 knockdown inhibited colony growth in 2D assays (Fig. 3B). To further examine the effects of TBK1 on apoptosis, we utilized a 3D collagen culture system that better mimics the dermal microenvironment, where melanomas and cutaneous metastases reside (28), and is thought to be a better medium for the investigation of anticancer drugs (29). In contrast to 2D culture (28), and is thought to be a better medium for the investigation of anticancer drugs (29). In contrast to 2D culture, where melanomas and cutaneous metastases reside in 2 of the 5 mutant NRAS cell lines examined. The combination of TBK1 depletion and AZD6244 significantly increased Annexin V staining in two cell lines that were nonresponsive to either alone: WM1366 and SBC2 (Fig. 3C). These data were supported by the observation of enhanced PARP cleavage in WM1366 cells in 3D culture with the combination of TBK1 depletion and AZD6244 treatment (Fig. 3D).

Given that TBK1 depletion cooperates with MEK inhibition to promote apoptosis, we analyzed the ability of activated TBK1 to protect cells from AZD6244-induced apoptosis. The expression of TBK1-myr in the AZD6244-sensitive SKMel2 cell line reduced AZD6244-induced apoptosis (Fig. 3F). The expression of TBK1-myr

![Figure 3. Knockdown of TBK1 enhances apoptosis in combination with AZD6244 specifically in mutant NRAS cell lines. A, mutant NRAS cells were transfected with non-targeting or TBK1-targeting siRNA #1 and #2, and, after 72 hours in 2D conditions, were analyzed for Annexin V staining by flow cytometry (n = 3; error bars, S.E.; *, P < 0.05). B, mutant NRAS cells were transfected with nontargeting or TBK1-targeting siRNA and plated at low density for a total of 7 days. Full-sized image, top, and 4× magnification, bottom. C, mutant NRAS cells were transfected as described in A and cultured in 3D collagen in the presence or absence of AZD6244 (3.3 μmol/L). After 24 hours, cells were extracted and analyzed for Annexin V staining by flow cytometry (n = 3; error bars, S.E.; *, P < 0.05). D, WM1366 cells were transfected as described in A and cultured in 3D collagen as described in C. After 24 hours, cells were lysed and lysates analyzed by Western blotting. E, WM1361A TR TBK1-myr pretreated with or without doxycycline, SKMel2 and SKMel2 TBK1-myr were cultured in 3D collagen in the presence or absence of AZD6244 (3.3 μmol/L). After 48 hours, cells were released from the gel and analyzed for Annexin V staining by flow cytometry (n = 3; error bars, S.E.; *, P < 0.05). F, WM3211, WM3211 transduced with NRAS<sup>G12V</sup>, and WM983A cells were transfected as described in A and then cultured and treated in 3D collagen as described in C. After 24 hours, cells were extracted and analyzed for Annexin V staining by flow cytometry (n = 3; error bars, S.E.; *, P < 0.05).]
in WM1361A TR did not show a similar response, which may be due to the lower level of activated TBK1 in this cell line (Fig. 1D). To determine whether the effect of TBK1 and MEK inhibition is specific to mutant NRAS melanoma cells, we utilized the WM3211, WM3211-NRAS<sup>G61K</sup>, and WM983A melanoma cell lines. WM983A is a mutant B<sub>R</sub>A<sup>F</sup>/wild-type NRAS melanoma cell line with very high levels of active TBK1 (Fig. 1A). In 3D culture, the depletion of TBK1 cooperated with AZD6244 to enhance apoptosis in WM3211-NRAS<sup>G61K</sup> but not in WM3211 or WM983A (Fig. 3G). In summary, although targeting TBK1 alone has limited effects on melanoma apoptosis in 3D culture, it cooperates with AZD6244 to promote apoptosis in MEK inhibitor–resistant lines.

**BX795 in combination with AZD6244 enhances apoptosis in AZD6244-resistant lines in 3D culture**

To pursue targeting TBK1 by pharmacologic inhibition, we used BX795, an ATP-competitive inhibitor of TBK1 (30, 31). BX795 has significant activity towards both TBK1 and PDK1, with an IC<sub>50</sub> of 6 nmol/L for TBK1 and an IC<sub>50</sub> of 111 nmol/L for PDK1 (30). To determine the ability of BX795 to inhibit TBK1 activity, we analyzed levels of IRF3 Ser386 phosphorylation, a TBK1-specific phosphorylation site (32). In SKMel2 and WM1366 TR expressing TBK1-myr, BX795 efficiently inhibited IRF3 phosphorylation at concentrations of 500 nmol/L and above (Fig. 4A). In contrast, BX795 did not dramatically alter TBK1 autophosphorylation. In 2D cultures, BX795 effectively inhibited
the growth of all mutant \textit{NRAS} cell lines (Fig. 4B). In 3D collagen survival assays, BX795 treatment alone elicited only a minor increase in Annexin V staining (Fig. 4C). As noted before, MEK inhibition alone was more effective at inducing apoptosis in WM1346, SKMEL2 and, to a lesser extent, WM1361A cells compared with WM1366 and SBcl2. Notably, the addition of BX795 with AZD6244 significantly increased apoptosis in WM1366, WM1361A, and SBcl2 cells, the cell lines most resistant to MEK inhibition. This increase in apoptosis was supported by increased PARP cleavage following treatment with the BX795 and AZD6244 combination in WM1366 cells (Fig. 4D).

\textit{NRAS} mutations also mediate acquired resistance to RAF inhibition in mutant \textit{BRAF} melanoma cells (7). Thus, we examined mutant \textit{BRAF} cells that have acquired resistant to the RAF inhibitor, PLX4720, through a secondary mutation in \textit{NRAS} (19). Two PLX4720-resistant lines, WM793-Res \#5 and WM793-Res \#12, both showed an increase in apoptosis in response to AZD6244, that was further enhanced when combined with BX795 (Fig. 4E). These data suggest that in 3D dermal mimetic conditions, BX795 combines with MEK inhibitors to promote apoptosis in mutant \textit{NRAS} melanoma cells.

\textbf{AZ909 combines with AZD6244 to enhance apoptosis in AZD6244-resistant lines in 3D culture}

BX795 is a promiscuous inhibitor and has significant effects on PDK1, and therefore AKT signaling. Because of the effects of BX795 on AKT signaling, we used an ATP-binding site TBK1 inhibitor, AZ13102909 (AZ909, AstraZeneca), with a different selectivity profile. AZ909 has an IC_{50} of 5 nmol/L against TBK1 and an IC_{50} of 100- to 1,000-fold greater for other related kinases (Dr. Claudio Chaqui, personal communication). The structure of AZ909 is very similar to those of previously published azabenzimidazole derivatives (ref. 33; Fig. 5A). Treatment of SKMel2 cells with AZ909 resulted in inhibition of phosphorylation of IRF3 at 100 nmol/L, whereas 1 \textmu mol/L of AZ909 was required to inhibit IRF3 phosphorylation in WM1366 TR cells expressing TBK1-myr (Fig. 5B). In mutant \textit{NRAS} cells, treatment with 1 \textmu mol/L of AZ909 had no effect on PDK1 and IKK\epsilon, and weakly effected inhibited aurora B phosphorylation (Supplementary Fig. S3). In 2D cultures, AZ909 effectively inhibited the growth of all mutant \textit{NRAS} cell lines (Fig. 5C); however, as was noted with BX795, it did not induce significant Annexin V staining in cells in 3D collagen with the exception of SBcl2. Notably, the combination of AZ909 (1 \textmu mol/L) and AZD6244 (3.3 \textmu mol/L) cooperated to significantly enhance apoptosis in several mutant \textit{NRAS} cell lines, namely, WM1366, SBcl2, and WM1346 (Fig. 5D and E). In the PLX4720-resistant WM793-Res\#5 and WM793-Res\#12 cells, AZD6244 and AZ909 alone elicited an increase in apoptosis but the combination of AZ909 and AZD6244 cooperated to significantly enhance apoptosis levels (Fig. 5F).

We examined the levels of the apoptotic proteins Bim-EL, McI-1, and Bmf in cells cultured in 3D conditions treated with TBK1 and MEK inhibitors. The levels of McI-1 did not change and Bim-EL increased with AZD6244 treatment alone and in combination with TBK1 knockdown or AZ909 treatment (Supplementary Fig. S4A and S4B). The levels of Bmf were increased in cells treated with the combination of AZ909 and AZD6244 but this was not seen with the combination of siTBK1 and AZD6244. Because of this difference, we evaluated broader apoptotic pathways with an apoptosis RT\textsuperscript{2} Profile PCR Array (Supplementary Fig. S4C). While the most variation in apoptotic genes was seen between the DMSO and AZD6244-treated groups, some genes were differentially regulated (e.g., nod1, TNFRSF25, and TRAF3) with the combination of AZD6244 and TBK1 knockdown. In sum, these data implicate that targeting TBK1 enhances the susceptibility of AZD6244-resistant mutant \textit{NRAS} melanoma cells to apoptosis.

\section*{Discussion}

\textit{NRAS} is a driver mutation in 15\% to 20\% percent of melanomas. Despite it being the second-most common subset in melanoma, mutant \textit{NRAS} melanomas remain relatively understudied and the treatment options are limited (34). In the past few years, several targeted therapies have been approved for the treatment of patients with mutant \textit{BRAF} melanoma. These include the RAF inhibitors, vemurafenib and dabrafenib, and the RAF/MEK inhibitor combination of dabrafenib plus trametinib. In contrast, no such targeted therapeutic options are FDA-approved for patients with mutant \textit{NRAS} melanoma. To further understand the signaling networks in mutant \textit{NRAS} melanomas, we focused on TBK1, an atypical IkB kinase family member that acts downstream of the Ras effector, RafGEF.

TBK1 has been implicated in several cancers: non–small cell lung cancer (16), pancreatic cancer cells (17), breast cancer (35), and prostate cancer (36). Given this, there has been an interest in developing compounds that target TBK1. To our knowledge, our study represents the first analysis of TBK1 in the malignant traits of melanoma. TBK1 is expressed widely in melanoma cells regardless of genotype but its phosphorylated state was variable. We focused our effort on the subset of melanomas that harbor \textit{NRAS} mutations, as mutant \textit{NRAS} expression was sufficient to promote TBK1 autophosphorylation. Our data indicate that TBK1 has a limited role in AKT signaling in mutant \textit{NRAS} melanoma cells. While one group has found that TBK1 depletion in \textit{KRAS} transformed cells did not change phospho-AKT levels (16), others have found that it leads to a decrease in phosphorylation (17). The mechanism of TBK1 activation of AKT is believed to be through its recruitment to the exocyst complex and subsequent interaction with AKT1 and AKT2, which interact with distinct exocyst subunits (17). In melanoma, reliance on other AKT isoforms, e.g., AKT3 (37), may account for the contrast in findings of AKT regulation by TBK1.

To examine the effects of constitutively active TBK1, we generated a novel \textit{TBK1} construct with a tag for myristoylation (\textit{TBK1-myr}). Unlike previous attempts to generate a phosphomimetic construct of TBK1 (24), the expression of

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TBK1-myr led to a robust increase in levels of phosphorylated TBK1. Recent studies have shown that TBK1 promotes tumorigenesis through its modulation of cytokines, specifically CCL5, interleukin (IL)6, and IL8 (38, 39). While TBK1-myr expression did not dramatically affect signaling through AKT, it did lead to an increase in secretion of CCL5 (data not shown).

Despite its highly metastatic nature, little is known about the regulators of migration and invasion in mutant NRAS melanoma. We observed that TBK1 is localized to areas of focal adhesions. Furthermore, we found that TBK1 depletion decreased migration and invasion. Conversely, the expression of TBK1-myr in some cell lines led to an increase in migratory and invasive properties. Consistent with our findings, other groups have recently implicated TBK1 in glioblastoma and lung carcinoma cell migration, as assessed by wound healing assays (40, 41).

To analyze effects on cell growth, we tested the effects of TBK1 depletion and pharmacologic inhibitors in 2D and 3D collagen. Cells in 3D culture regulate signaling differently from those in 2D conditions (42, 43) and 3D models have been suggested to be a better medium for the investigation of anticancer drugs than 2D monolayers (29). Others have utilized 3D collagen cultures to mimic the dermal microenvironment of invasive primary melanomas and cutaneous metastases (28). In 2D cultures, we found a significant increase in apoptosis with targeting TBK1; in contrast, the effect in 3D cultures was limited. This prompted us to combine TBK1 targeting with MEK inhibitors in 3D to block two major RAS effector pathways.
MEK inhibitors have been noted to elicit highly variable effects in mutant \( \text{NRAS} \) melanoma cells (44). We noted that the combination of TBK1 targeting and MEK inhibition promoted apoptosis, particularly in those mutant \( \text{NRAS} \) cells lines that are resistant to MEK inhibition. This observation was made using molecular knockdowns, as well as pharmacologic inhibitors, underscoring that it is likely a TBK1 selective effect. Notably, we utilized a new inhibitor, AZ909, that has selectivity toward TBK1 versus PDK1. While we sought the use of TBK1 selective inhibitors for our line of investigation, we note that more broad-spectrum TBK1-PDK1 inhibitors may be useful in other aspects. For example, inhibition of PDK1, through both genetic modification and pharmacologic inhibition, attenuated the growth and metastatic potential of \( \text{BRAF}\text{V600E} \) in a \( \text{PTEN}^- \) and \( \text{CDKN2A}^- \) null background melanomas (45).

In summary, melanoma is renowned for its tendency for invasion and its resistance to apoptosis. In the understudied mutant \( \text{NRAS} \) subset of cutaneous melanoma, we identify a role for TBK1 in migration and invasion, and show that targeting TBK1 cooperates with MEK inhibition to promote apoptosis. As the effects of TBK1 in mutant \( \text{NRAS} \) melanomas do not appear to be mediated by AKT, future work should examine the downstream effectors of TBK1 in this context. Furthermore, it may be worth exploring the role of TBK1 in other melanoma backgrounds. In addition to \( \text{BRAF} \) and \( \text{NRAS} \) mutations, the genomic landscape of melanoma has been updated to include the loss of \( \text{NF1} \), which encodes the RAS-GTPase activating protein neurofibromin that is mutated in approximately 10% of melanomas (46). TBK1 may have a role in \( \text{NF1}^- \) mutant melanomas or in those that are triple negative for \( \text{BRAF}, \text{NRAS}, \) and \( \text{NF1} \) mutations.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: H.L. Vu, A.E. Aplin

Development of methodology: H.L. Vu

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H.L. Vu

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H.L. Vu, A.E. Aplin

Writing, review, and/or revision of the manuscript: H.L. Vu, A.E. Aplin

Study supervision: A.E. Aplin

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