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MIG6 is MEK-regulated and affects EGF-induced migration in mutant NRAS melanoma

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Abstract

Activating mutations in NRAS are frequent driver events in cutaneous melanoma. NRAS is a GTP-binding protein, whose most well-characterized downstream effector is RAF leading to activation of MEK-ERK1/2 signaling. While there are no FDA-approved targeted therapies for melanoma patients with a primary mutation in NRAS, one form of targeted therapy that has been explored is MEK inhibition. In clinical trials, MEK inhibitors have shown disappointing efficacy in mutant NRAS patients, the reasons for which are unclear. To explore the effects of MEK inhibitors in mutant NRAS melanoma, we utilized a high-throughput reverse-phase protein array (RPPA) platform to identify signaling alterations. RPPA analysis of phospho-proteomic changes in mutant NRAS melanoma in response to trametinib indicated a compensatory increase in AKT signaling and decreased expression of mitogen-inducible gene 6 (MIG6), a negative regulator of EGFR/ERBB receptors. MIG6 expression did not alter the growth or survival properties of mutant NRAS melanoma cells. Rather, we identified a role for MIG6 as a negative regulator of EGF-induced signaling and cell migration and invasion. In MEK inhibited cells, further depletion of MIG6 increased migration and invasion, whereas MIG6 expression decreased these properties. Therefore, a decrease in MIG6 may promote the migration and invasiveness of MEK-inhibited mutant NRAS melanoma especially in response to EGF stimulation.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

INTRODUCTION

Fifteen to twenty percent of melanoma patients harbor an activating mutation in the GTPase, NRAS. Mutant NRAS is a validated target but therapies to directly inactivate forms of RAS have been clinically ineffective (Downward, 2003). One frequently studied RAS effector pathway is the RAF-MEK-ERK1/2 cascade. In melanoma, mutant NRAS activates this pathway utilizing CRAF rather than BRAF (Dumaz et al, 2006, Marquette et al, 2011). In contrast to findings in mutant BRAF V600E/K melanomas (Flaherty et al, 2012), clinical trials of MEK inhibitors in mutant NRAS melanomas have shown limited and inconsistent clinical efficacy. Preclinical and clinical studies of selumetinib (AZD6244) have shown poor anti-tumor responses in cutaneous melanoma (Haass et al, 2008, Gupta et al, 2014). Trametinib (GSK1120212) demonstrated efficacy in mutant BRAF patients (Flaherty et al, 2012) but had weaker responses in mutant NRAS patients (Falchook et al, 2012). While newer MEK inhibitors are showing promise in preclinical models (Micel et al, 2015) and early phase trials (Martinez-Garcia et al, 2012, Zimmer et al, 2014, Ascierto et al, 2013), the underlying reasons for the poor response of mutant NRAS melanoma patients to MEK inhibitors remain unclear.

In vitro studies of mutant NRAS melanoma cell lines have shown a heterogeneous growth arrest response following MEK inhibitor treatment (Solit et al, 2006, Vu and Aplin, 2014). The underlying basis for the varied response is not known. In the mutant BRAF melanoma setting, the adaptive response to both RAF and MEK inhibition has been well-described, with a major mechanism being upregulation of receptor tyrosine kinases (RTK) leading to compensatory PI3K-AKT signaling (Kugel and Aplin, 2014). Our group has shown that ERBB3, a member of the EGFR/ERBB family of RTKs, is rapidly upregulated 4–6 hours following RAF inhibition in mutant BRAF melanoma (Abel et al, 2013). Others have shown upregulation of the RTKs PDGFRβ and EGFR upon MEK-ERK1/2 inhibition in mutant BRAF melanoma (Shi et al, 2014, Sun et al, 2014).

To study altered signaling responses to MEK inhibition in mutant NRAS melanoma, we utilized reverse phase protein arrays (RPPA). In MEK-inhibited mutant NRAS melanoma cells, we detected an increase in AKT activation and a decrease in the adaptor protein, mitogen-inducible gene 6 (MIG6). MIG6 is a non-kinase cytosolic scaffolding protein that binds to ERBB family receptors and inhibits their catalytic activity by blocking the formation of an activating dimer (Zhang et al, 2007). Additionally, MIG6 mediates receptor endocytosis (Frosi et al, 2010, Walsh and Lazzara, 2013) and lysosomal degradation (Ying et al, 2010). We identified a role for MIG6 as a negative regulator of EGF-induced AKT and ERK1/2 signaling and cell migration and invasion in mutant NRAS melanoma. In the presence of MEK inhibition, MIG6 did not modulate growth or apoptosis in mutant NRAS melanoma cells. Rather, MIG6 expression decreased cell migration and invasion; its further depletion in MEK-inhibited cells increased migration and invasion. Therefore, the decrease in MIG6 in the presence of MEK inhibition may be a pro-invasive stimulus in mutant NRAS melanoma.

RESULTS

MEK inhibition decreases MIG6 expression in mutant NRAS melanoma cells

To examine signaling alterations following MEK inhibition in mutant NRAS melanomas, we evaluated the response of mutant NRAS melanoma cells to the MEK inhibitor trametinib by high throughput antibody-based RPPA analysis. Gene set enrichment analysis and hierarchical clustering revealed many proteins that were up- or downregulated, particularly after 72 hours of trametinib treatment (Figure S1). Consistently across all cell lines analyzed, trametinib treatment led to a durable inhibition of phospho-ERK1/2 (Figure 1a). There was a delayed increase in the phosphorylation of RAF1 and MEK1, likely reflecting changes in negative feedback regulators of the ERK1/2 pathway (Pratilas et al, 2009). Increased phosphorylation of AKT (Ser473 and Thr308) and several AKT downstream effectors, including PRAS40, RICTOR, and S6K, were also detected following 72 hours of trametinib treatment (Figure 1A). Paralleling these changes was a decrease in mitogen-inducible gene 6 (MIG6), a negative regulator of ERBB receptors and a corresponding increase in phosphorylation levels of EGFR, ERBB2 and ERBB3. The changes in the phospho-ERK1/2, phospho-AKT, and MIG6 detected by RPPA analysis were validated by Western blot analysis in all four cell lines (Figure 1b).

Additionally, we examined the effects of MEK inhibition in two independent mutant NRAS Q61R melanoma patient biopsy samples, TJUMel-35 and TJUMel-36 (Figure S2a–b). Pieces of biopsy sample were cultured on hemostatic gelatin sponges partially submerged in medium in an ***ex vivo*** explant system. Trametinib treatment of explants showed inhibition of MEK signaling, increased AKT phosphorylation and decreased MIG6 expression through Western blot analysis (Figure 1c). Immunohistochemistry showed inhibition of ERK1/2 phosphorylation and a decrease in MIG6 staining (Figure 1d). Increased AKT phosphorylation, as detected by IHC staining, was variable across the tumor sample perhaps due to differing levels of MEK inhibition.

To further examine the correlation between MIG6 and EGFR/ERBB signaling in a broader set of melanoma patients, we utilized The Cancer Genome Atlas (TCGA) (Cerami et al, 2012, Gao et al, 2013). In 201 cutaneous melanoma samples, MIG6 had a significant negative correlation with AKT pS473 and pT308 (Table 1). In the 55 mutant NRAS samples, the same correlations were observed. MIG6 had a significant positive correlation with total EGFR and a slight negative correlation with phospho-EGFR that was not significant. In summary, MEK inhibition in mutant NRAS melanoma cells and patient samples leads to a decrease in MIG6 expression, which negatively correlates with AKT phosphorylation.

MIG6 has limited effects on growth, proliferation and apoptosis

The role of MIG6 in melanoma is not known. To determine whether alterations in MIG6 expression affected cell growth of mutant NRAS melanomas, we modulated MIG6 by knockdown using tetracycline-inducible shRNA (Figure 2a) and enhanced expression (Figure 2b). Inducible MIG6 depletion in WM1346, WM1361 and WM1366 mutant NRAS melanoma cells had no effect on cell proliferation (Figure 2c) and only a modest increase in anchorage-independent growth in soft agar (Figure 2d). Furthermore, inducible expression

of MIG6 did not inhibit proliferation or anchorage-independent growth in soft agar (Figure 2e–f).

Treatment of mutant NRAS melanoma cells with MEK inhibitors leads to a variable apoptotic response (Solit et al, 2006, Vu and Aplin, 2014). In 3D collagen cultures, which mimic the dermal microenvironment (Smalley et al, 2006), WM1366 cells were relatively insensitive whereas WM1346 and WM1361A were sensitive to trametinib (Figure 2g–h). Depletion of MIG6 did not alter basal levels of apoptosis (Figure 2g). In WM1361A, depletion of MIG6 slightly decreased trametinib-induced apoptosis but this effect was not observed in either WM1346 or WM1366 cells. Additionally, enhancing MIG6 expression did not affect trametinib-mediated apoptosis in 3D collagen (Figure 2h). Overall, these data indicate that altered MIG6 expression does not impact melanoma cell proliferation or the apoptotic response to MEK inhibitors.

MIG6 inhibits EGF signaling to AKT and ERK1/2 in mutant NRAS melanoma cells

MIG6 has been shown to modulate signaling in response to various stimuli, including neuregulin 1 (NRG1) and EGF (Hackel et al, 2001, Ferby et al, 2006). Acute stimulation of WM1366 cells with EGF, HGF, and IGF1 induced AKT activation, whereas serum and NRG1 had little effect on AKT phosphorylation (Figure 3a). EGF and HGF also enhanced ERK1/2 phosphorylation. Inducible expression of MIG6 in WM1366 cells decreased EGF-stimulated phosphorylation of EGFR, AKT and ERK1/2. By contrast, MIG6 expression did not alter HGF- or IGF1-induced signaling. The selective effect of MIG6 expression on EGF-induced AKT phosphorylation was also detected in WM1346 and WM1361 cell lines (Figure S3a–b). Expression of MIG6-FLAG led to a decrease in both the intensity and the duration of EGF-stimulated EGFR, AKT and ERK1/2 phosphorylation (Figure 3b).

Conversely, depletion of MIG6 in WM1366 cells led to an increase in EGF-induced phosphorylation of EGFR, AKT and ERK1/2 that was most evident 30 to 120 min following stimulation (Figure 3c–d). MIG6 depletion also slightly increased baseline ERK1/2 phosphorylation. MIG6 depletion in WM1346 and WM1361A cells enhanced EGF-stimulation of EGFR and ERK1/2 phosphorylation but this effect did not transmit into increased levels of phospho-AKT (Figure S3c–f). Despite the effects on EGFR signaling, the addition of EGF did not modulate growth inhibition or apoptosis induced by trametinib (Figure 3e–f). Together, these data show that MIG6 levels negatively regulate EGF-induced EGFR phosphorylation and downstream signaling in mutant NRAS melanoma.

MIG6 inhibits migration and invasion through AKT in mutant NRAS melanoma cells

Although MIG6 did not affect cell growth or apoptosis, we observed increased scattering of cells at the periphery of colonies in colony growth assays of mutant NRAS cell lines following MIG6 depletion (Figure 4a). Importantly, cell scattering was increased with the addition of EGF and further increased in EGF-stimulated, MIG6-depleted cells. To assess a potential role for MIG6 as a negative regulator of migration, we performed transwell migration assays. Depletion of MIG6 increased migration towards serum-containing medium in all cell lines (Figure 4b). Increased migration following depletion of MIG6 was further enhanced with the addition of EGF (Figure 4b). Conversely, MIG6 expression

decreased EGF-induced scattering (Figure 4c) and migration in the mutant NRAS melanoma cell lines (Figure 4d).

To determine if the effects of MIG6 on migration are mediated, at least in part, by AKT, we utilized the allosteric AKT inhibitor, MK2206. MK2206 effectively inhibited AKT activation at a concentration of 1 μM and above (Figure 4e). In 3D spheroid assays, the invasion of WM1366 cells induced by MIG6 depletion and/or EGF stimulation was reversed with MK2206 treatment (Figure 4f). Accordingly, the increase in migration seen with MIG6 depletion was inhibited with MK2206 both in the absence and presence of EGF (Figure 4g). MK2206 treatment alone did not affect migration. Thus, inhibition of AKT reduced migration and invasion induced by MIG6 depletion in mutant NRAS melanoma cells.

Downregulation of MIG6 increases migration and invasion in MEK-inhibited cells

Given the decrease in MIG6 with MEK inhibition, we expressed MIG6 in MEK-inhibited cells to observe the effects on migration. MEK inhibition did not alter the inducible expression of MIG6 in WM1366, WM1361A, and WM1346 cells (Figure 5a). The increase in AKT activation with trametinib treatment was effectively reversed with MIG6 expression in WM1366 cells but only partially reversed in WM1346 and WM1361 cells. In spheroid and migration assays, treatment of mutant NRAS melanoma cells with trametinib led to a decrease in migration and invasion (Figure 5b–c). The expression of MIG6 in the presence of MEK inhibition led to a further decrease in migration with or without EGF (Figure 5c). As MEK inhibition partially decreases MIG6 expression, we were interested in the effect of a more complete MIG6 reduction. Expression of shMIG6 further decreased MIG6 levels trametinib-treated cells (Figure 5d), which led to an increase in invasion (Figure 5e) and a significant increase in migration (Figure 5f), with or without EGF. These data indicate that the decrease in MIG6 following MEK inhibition in mutant NRAS melanoma cells likely counteracts other negative effects of MEK inhibitors on cell migration and invasion.

DISCUSSION

Targeted therapy treatment options for mutant NRAS-harboring melanoma patients are limited despite recent advances in the treatment of mutant BRAF tumors. MEK inhibitors have long been a logical choice for mutant NRAS melanoma patients yet their performance in clinical trials has been disappointing (Fedorenko et al, 2013). In this study, we utilized a high throughput antibody-based analysis to better understand changes in MEK signal transduction in mutant NRAS melanoma cell lines and identified a decrease in the levels of MIG6. We were able to recapitulate these findings in patient tumor samples. Functionally, we show that MIG6 negatively regulates migration and invasion; thus, its downregulation is likely to promote pro-migratory properties in tumor microenvironments containing high levels of EGF.

Through RPPA analysis, we observed downregulation of MIG6 in MEK-inhibited mutant NRAS melanoma that is associated with increased levels of EGFR and AKT phosphorylation. A previous phospho-proteomic analysis of a MEK-inhibited mutant NRAS melanoma cell line also identified upregulation of EGFR signaling (Fedorenko et al, 2015). Our data in cell lines were supported by findings in an ***ex vivo*** explant system, in which

treatment of two mutant NRAS patient biopsy samples led to decreased levels of MIG6. Additionally, analysis of TCGA melanoma samples showed a negative correlation between MIG6 and levels of phospho-AKT. MIG6 is an immediate early response gene that has been shown to be up-regulated by NRG1 and EGF ***in vitro*** and ***in vivo*** and acts as a negative regulator of ERBB receptors (Hackel et al, 2001, Ferby et al, 2006). Previous studies have shown compensatory upregulation of AKT signaling following MEK inhibition (Gopal et al, 2010, Deuker et al, 2015). In mutant NRAS melanoma, we show that the expression of MIG6 led to a decrease in EGFR phosphorylation and downstream AKT and ERK1/2 signaling. Effects of MIG6 were selective to EGF stimulation compared to other growth factors. Conversely, the depletion of MIG6 enhanced EGFR phosphorylation and AKT and ERK1/2 signaling although some differences were observed between cell lines. MIG6 expression also appears to be regulated by MEK-ERK1/2 signaling in mutant BRAF melanoma lines as treatment of A375 cells with either RAF or MEK inhibitor led to decreased MIG6 expression (Figure S4).

Overexpression of MIG6 has been shown to decrease proliferation and its depletion enhances proliferation in several different cell types (Anastasi et al, 2003, Pante et al, 2005, Ferby et al, 2006). By contrast, the MIG6-regulated changes in signaling in mutant NRAS melanoma cells did not translate to profound effects on cell proliferation. Furthermore, our data show that MIG6 was downregulated regardless of the cell line's sensitivity to MEK inhibition, indicating that MIG6 downregulation is not a biomarker for resistance to MEK inhibition. It is likely that additional pathways, for example the Hippo-YAP1 pathway, may be important in MEK inhibitor resistance (Lin et al, 2015).

Although we did not identify a role for MIG6 in cell growth in mutant NRAS melanoma, we found MIG6 to be a negative regulator of migration and invasion, particularly in the presence EGF stimulation. A previous role for MIG6 in inhibiting migration downstream of HGF/Met in hepatocytes (Pante et al, 2005) and EGF in human liver cancer (Reschke et al, 2010) has been described. Blockade of the MEK-ERK1/2 pathway in melanoma is typically associated with decreased migration and invasion through effects on focal adhesion disassembly (Colo et al, 2012) and expression of the Rho family GTPase, RND3 (Klein et al, 2008, Klein and Aplin, 2009). Utilizing 3D spheroid assays, some groups have shown that MEK inhibition decreases invasion in wild-type NRAS melanoma cells (Haass et al, 2014) while other studies have shown that it increases invasion (Vultur et al, 2014). In the presence of MEK inhibition, we show that the overexpression of MIG6 leads to a further decrease in cell migration. Conversely, the further depletion of MIG6 leads to an increase in the migration of MEK-inhibited cells. Thus, we postulate that MIG6 downregulation following MEK inhibition in mutant NRAS melanoma cells counteracts other negative effects of MEK inhibitors on migration and invasion, especially when EGF is present in the tumor microenvironment.

In mutant NRAS melanoma, MEK inhibition leads to the downregulation of MIG6, which provides a pro-migratory signal. Effects may be mediated by EGFR signaling at least in part through the PI3K-AKT pathway. EGFR is frequently detected in melanoma, with expression in 89% of primary lesions in the vertical growth phase and 80% of metastatic lesions (Rodeck et al, 1991). PI3K-AKT signaling regulates cell migration through phosphorylation

of GSK3 to relieve GSK3-mediated inhibition of migration (Roberts et al, 2004, Kobayashi et al, 2006); girdin to facilitate the formation of stress fibers and lamellipodia (Enomoto et al, 2005), and ACAP to mediate integrin β1 recycling (Li et al, 2005). Consistent with these findings, we were able to reverse the increase in migration upon MIG6 depletion with the AKT inhibitor, MK2206. However, we do not rule out that AKT-independent effects may also be important since MIG6 expression only partially inhibited AKT phosphorylation in trametinib-treated cells (Fig. 5a). The combination of MEK and PI3K inhibitors has been shown to reduce both the growth and invasion of wild-type NRAS melanoma cell lines in 3D spheroid assays (Smalley et al, 2006). In mutant NRAS xenograft models, targeting both MEK and PI3K/mTOR led to tumor regression but the effect on metastasis was not examined (Posch et al, 2013). Our data suggest that it is likely that the cytotoxic effects of MEK inhibition can be combined with EGFR-PI3K-AKT pathway inhibition to prevent the spread and development of mutant NRAS melanoma at new sites.

MATERIALS AND METHODS

Cell culture

WM and SBcl2 melanoma lines were kindly donated by Dr. Meenhard Herlyn (Wistar Institute, Philadelphia, PA). The SKMel2 cell line was purchased from ATCC (Manassas, VA). WM and SBcl2 cell lines were cultured in MCDB 153 medium containing 20% L-15 medium, 2% FBS and 0.2% sodium bicarbonate. SKMel2 cells were cultured in MEM with 10% FBS. Cell lines were validated as being mutant NRAS by Sanger sequencing.

RPPA analysis

Cells lysates were prepared and analyzed by RPPA, as previously described (Tibes et al, 2006), with 216 validated antibodies. Triplicate normalized RPPA data were transformed for use with the Gene Set Enrichment Analysis (GSEA) software (Subramanian et al, 2005) using Gene Ontology gene sets available from MSigDB, as previously described (Kugel et al, 2014). Hierarchical clustering with Pearson correlation was done using MeV 4.9 (Dana-Farber Cancer Institute, Boston, MA).

Western blot analysis

Cells were lysed in sample buffer, separated by SDS-PAGE, and proteins were transferred electrophoretically onto Immobilon P membranes (Millipore Corp., Bedford, MA). Membranes were blocked, incubated with primary antibody, and developed, as previously described (Vu and Aplin, 2014). Antibodies for AKT (9272), phospho-AKT T308 (2965), phospho-ERK1/2 (9101), and phospho-EGFR Y845 (2231) were obtained from Cell Signaling (Danvers, MA); EGFR (sc-03) and ERK1 (sc-94) from Santa Cruz Biotechnology (Santa Cruz, CA); and MIG6 (HPA027206) and actin (A2066) from Sigma-Aldrich (St. Louis, MO).

Ex vivo explants

Tumors from mutant NRAS patients were collected following patient consent at Thomas Jefferson University Hospital under an IRB-approved protocol (#10D.341). Tissue samples were processed into 1 mm^3 pieces and cultured in triplicate on hemostatic gelatin sponge

(Ferrosan, Greensboro, NC) partially submerged in culture media. Cells were treated with DMSO or trametinib for 72 hr, replacing media daily. Full details of this procedure are outlined in Hartsough ***et al.*** (in preparation).

Immunohistochemistry

Tissue from TJUMel-35 and TJUMel-36 explants was fixed in formalin and paraffin embedded. Sections were stained with anti-phospho-ERK1/2 (9101, Cell Signaling), phospho-AKT T308 (38449, Abcam, Cambridge, UK), and MIG6 (HPA027206, Sigma-Aldrich) antibodies.

Cloning and stable cell line generation

Human MIG6 with a FLAG tag was cloned from cDNA using the following primers: forward 5'-CACCATGGATTATAAAGATGATGATGATAAATCAATAGCAGGAGTTGCTGC-3' and reverse 5'-CTAAGGAGAAACCACATAGG-3'. The sh±±±MIG6 construct was cloned using the following primers: forward 5'-CACCGCTGCAGGTTAACCAATTATTCAAGAGATAATTGGTTAACCTGCAGC -3' and reverse 5'-AAAAGCTGCAGGTTAACCAATTATCTCTTGAATAATTGGTTAACCTGCAGC -3'. All DNA constructs were sequence verified. Lentiviral particles and tetracycline repressor (TR)–expressing sublines expressing MIG6-FLAG and shMIG6 were generated, as previously described (Abel and Aplin, 2010). Transgene expression was induced with 0.1 μg/ml doxycycline in the cell culture medium.

Colony formation assay

Cells were plated per 6-well plate in complete medium with −/+ dox and −/+ EGF, with medium being replenished every two days. After 10 days, cells were stained with crystal violet in formalin, 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, cells were isolated, stained with annexin V-APC, and analyzed on a FACSCalibur flow cytometer (BD Biosciences) as previously described (Kaplan et al, 2012). Data were analyzed by FlowJo software (Tree Star Inc., Ashland, OR).

Migration and invasion assay

Serum-starved melanoma cells were placed inside 8.0 μm pore-size cell culture inserts (BD Biosciences, Franklin Lakes, NJ). Cells were allowed to migrate for 24 hours towards an attractant of serum-containing medium, −/+ EGF and −/+ inhibitors. Migrated cells were analyzed, as previously described (Vu and Aplin, 2014)

Spheroid assay

Cells were grown in suspension for 72 hours to form spheroids. Spheroids were harvested and implanted into 3D collagen, as previously described (Smalley et al, 2006). Bright field

images were taken with a Nikon Ti-Eclipse inverted microscope (Nikon, Melville, NY) utilizing the NIS-Elements software package.

Reagents

Trametinib (GSK11202212) and MK2206 were purchased from Selleck Chemicals LLC (Houston, TX).

Statistical analysis

Statistical analyses were performed using SPSS (v22.0). A ***p*** value of <0.05 was considered statistically significant. Two-tailed Student's ***t*** test was used to evaluate apoptosis, colony growth, and migration. Spearman's correlation was used to test the relationships between proteins in the TCGA samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

AKT	v-akt murine thymoma viral oncogene homolog
BRAF	v-raf murine sarcoma viral oncogene homolog
B1 EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERBB	v-erb-b2 erythroblastic leukemia viral oncogene homolog
ERK	extracellular signal-regulated protein kinase
MEK	mitogen-activated protein kinase kinase
MIG6	mitogen-inducible gene 6
NRAS	neuroblastoma RAS viral oncogene homolog

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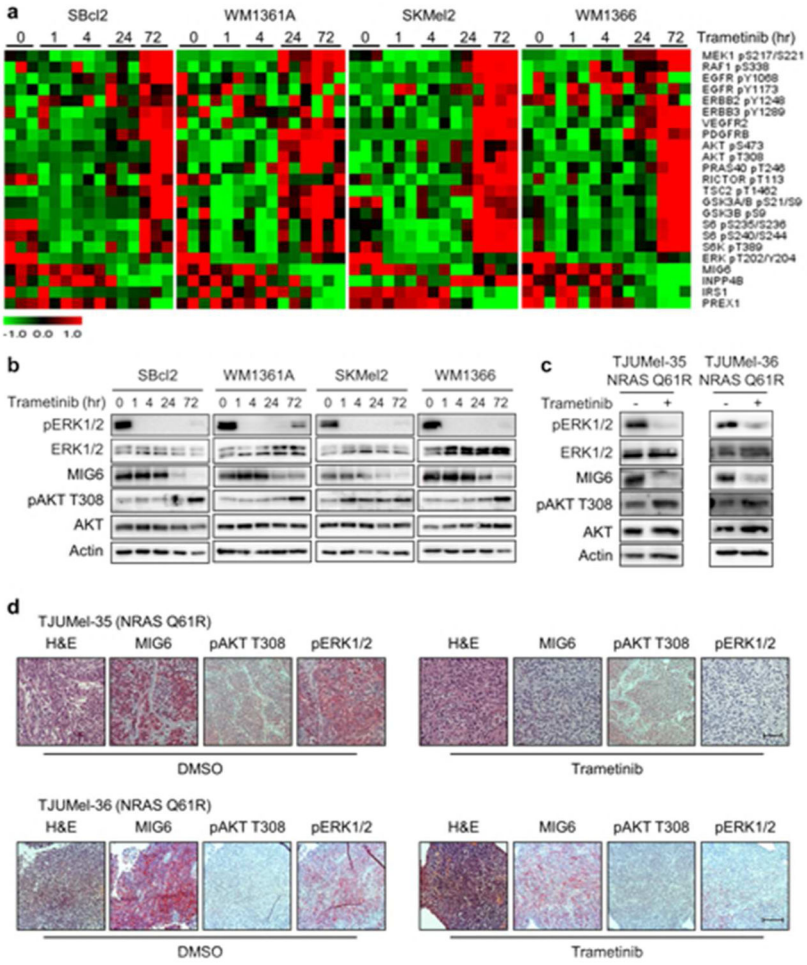

Figure 1. MEK inhibition decreases MIG6 expression in mutant NRAS melanoma cells

(a) RPPA analysis of mutant NRAS melanoma cells treated with trametinib (90 nM) for 0, 1, 4, 24, and 72 hours in triplicate. Proteins levels were normalized to the mean. **(b)** Mutant NRAS cells were treated with trametinib for 0, 1, 4, 24, and 72 hours. Cells were lysed and lysates analyzed by Western blot. **(c)** TJUMel-35 and TJUMel-36 biopsy samples were cultured on hemostatic gelatin sponges and treated with DMSO or trametinib for 72 hours. Samples were lysed and lysates analyzed by Western blot. **(d)** TJUMel-35 and TJUMel-36 samples were cultured as in (c) but were paraffin-embedded for H&E staining and for immunohistochemistry. The scale bar equals 200 μm.

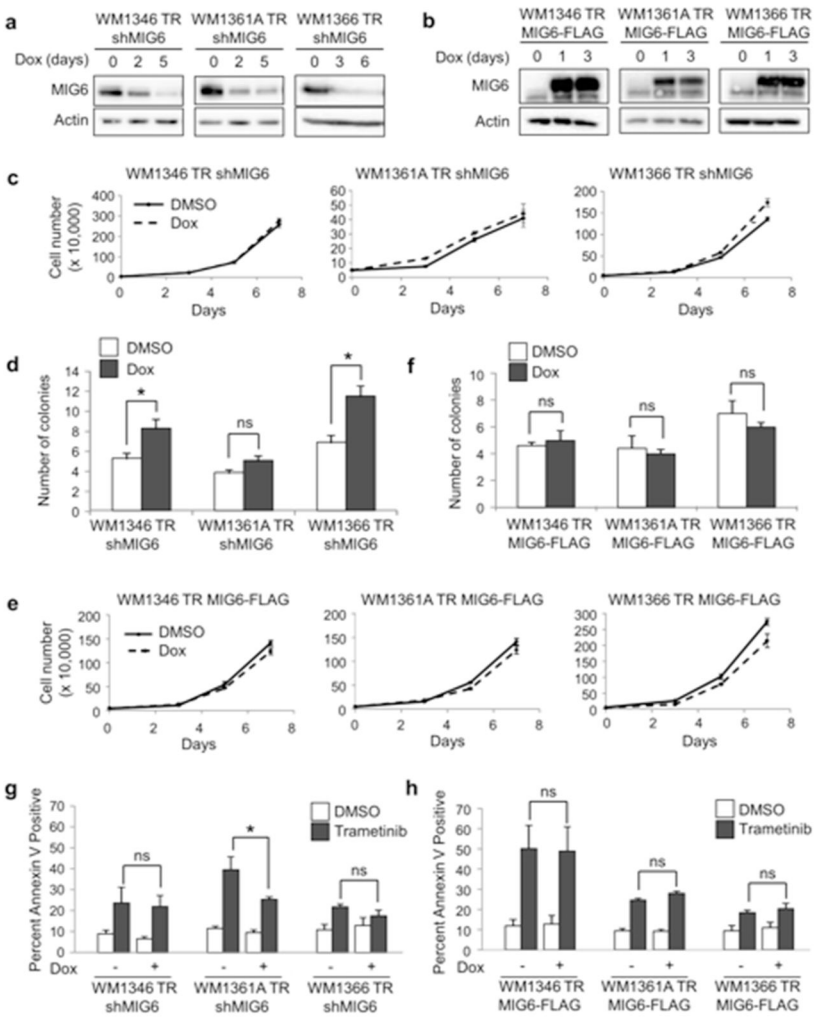

Figure 2. MIG6 has limited effects on growth, proliferation, and apoptosis

Western blot of mutant NRAS cells with inducible shMIG6 **(a)** and inducible MIG6-FLAG **(b)** treated with dox. **(c)** Cell proliferation of mutant NRAS cells with inducible shMIG6 treated with or without dox for 7 days. **(d)** Soft agar assay for mutant NRAS cells with inducible shMIG6 with number of colonies (in triplicate fields of view) determined after 4 weeks. **(e)** and **(f)** NRAS cells with inducible MIG-FLAG were treated as in (c) and (d), respectively. Apoptosis assays of cells with inducible shMIG6 **(g)** and inducible MIG6-FLAG **(h)** in 3D collagen treated with −/+ dox and −/+ trametinib (90 nM) for 48 hours. (n=3; errors bars, S.E; * = $p<0.05$, ns = not significant).

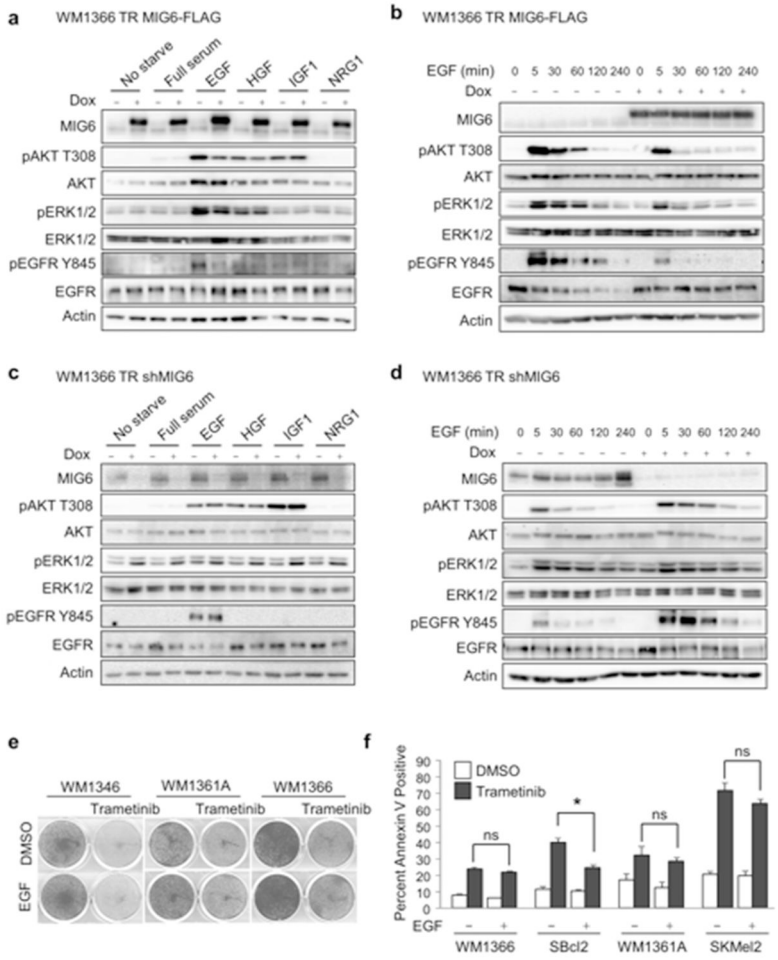

Figure 3. MIG6 affects EGF-stimulated AKT signaling

WM1366 TR MIG6-FLAG cells pre-treated with −/+ dox, serum-starved and treated with serum-free medium, full serum medium, or 10 ng/mL EGF, HGF, IGF1, or neuregulin (NRG1) for 15 min **(a)** or stimulated with EGF for different timepoints **(b). (c)** and **(d)** WM1366 TR shMIG6 cells were treated as in (a) and (b), respectively. **(e)** Mutant NRAS cells were plated at low density and treated with −/+ trametinib (90 nM) and −/+ EGF for 10 days. **(f)** Apoptosis assays for mutant NRAS cells were cultured in 3D collagen and treated with −/+ trametinib and −/+ EGF for 48 hours (n=3; errors bars, S.E; * = $p<0.05$, ns = not significant).

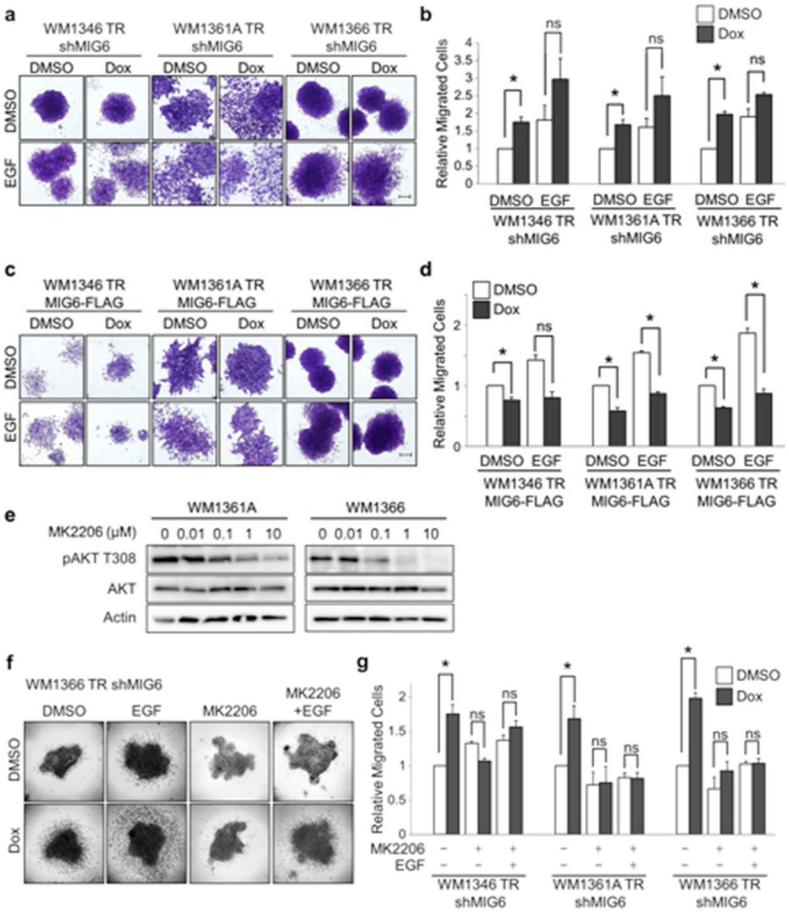

Figure 4. MIG6 inhibits migration and invasion through AKT in mutant NRAS melanoma cells

(a) Colony formation −/+ dox and −/+ EGF (10 ng/mL) for 2 weeks (scale bar = 100 μm) and **(b)** migration toward full serum medium (control migration set at 1.0; n=3; errors bars, S.E.; * = $p<0.05$, ns = not significant) of mutant NRAS cells with inducible shMIG6. **(c)** and **(d)** Mutant NRAS cells with inducible MIG6-FLAG were treated as in (a) and (b), respectively. **(e)** Mutant NRAS cells treated with increasing concentrations of MK2206 for 48 hours. **(f)** WM1366 TR shMIG6 spheroids in 3D collagen treated with −/+ MK2206 (1 μM) for 48 hours. **(g)** Migration of cells with inducible shMIG6 towards medium −/+ EGF and −/+ MK2206.

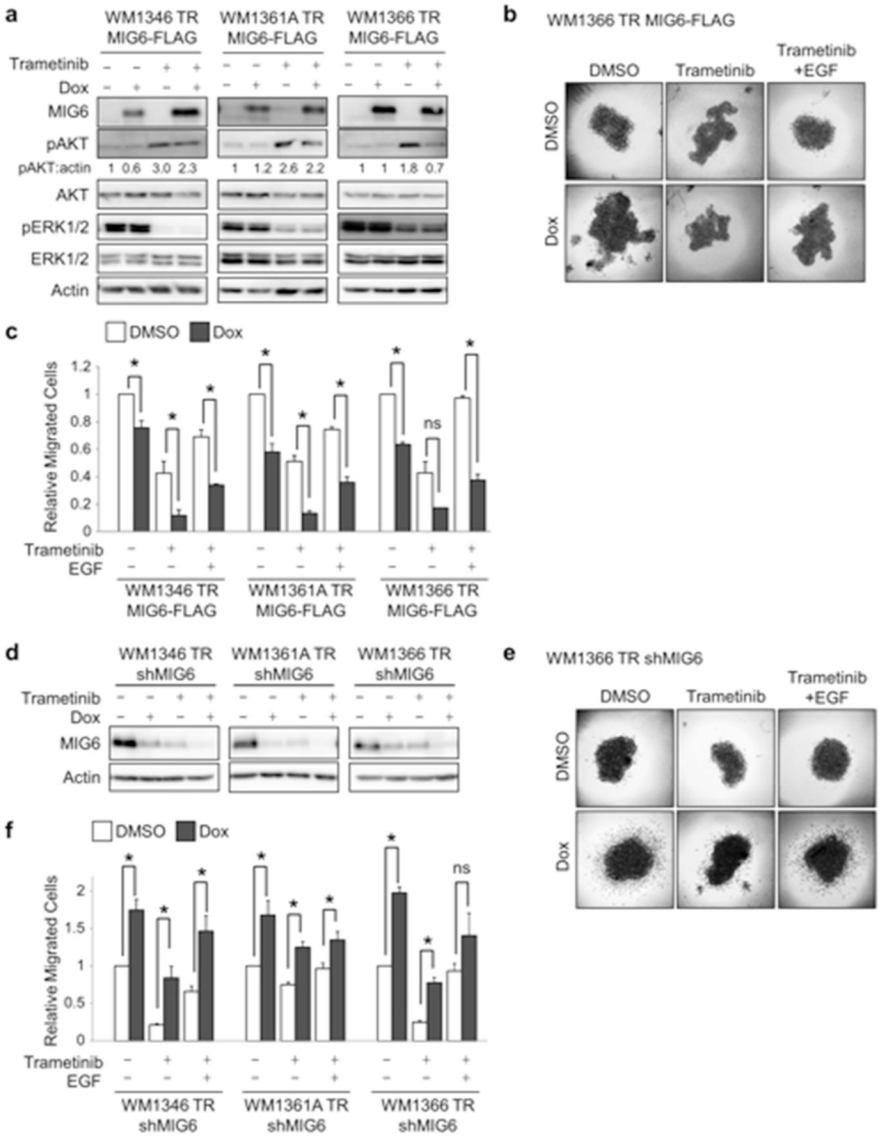

Figure 5. Downregulation of MIG6 increases migration and invasion in MEK-inhibited cells

(a) Mutant NRAS cells with inducible MIG6-FLAG pre-treated with −/+ dox and then −/+ trametinib (90 nM) for 24 hours. **(b)** Spheroids in 3D collagen of inducible MIG6-FLAG cells treated with −/+ dox, −/+ trametinib, and −/+ EGF (10 ng/ml) for 48 hours. **(c)** Migration of inducible MIG6-FLAG cells towards medium with −/+ EGF and −/+ trametinib (control migration set at 1.0; n=3; errors bars, S.E.; * = $p<0.05$, ns = not significant). **(d)**, **(e)**, and **(f)** Mutant NRAS cells with inducible shMIG6 were treated as in (a), (b) and (c), respectively.

Table 1

Protein correlation for cutaneous melanoma samples in TCGA.

Correlation	r_s*	*p*
All Samples, n=201		
MIG6 and AKT pS473	−0.31	<0.001
MIG6 and AKT pT308	−0.29	<0.001
MIG6 and EGFR	0.32	<0.001
MIG6 and EGFR pY1068	−0.10	0.145
Mutant NRAS samples, n=55		
MIG6 and AKT pS473	−0.38	0.004
MIG6 and AKT pT308	−0.32	0.016
MIG6 and EGFR	0.51	<0.001
MIG6 and EGFR_pY1068	−0.07	0.605

*Spearman's correlation