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Keywords
BRAF, MELK, NF-κB, SILAC, SQSTM1, melanoma

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MELK Promotes Melanoma Growth by Stimulating the NF-κB Pathway

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In Brief
Janostiak et al. find that MELK is overexpressed in melanoma and is necessary for melanoma growth. MELK regulates the NF-κB pathway via SQSTM1, which is necessary partly for its ability to promote melanoma growth.

Data and Software Availability
PXD007872

Highlights
- MELK is upregulated in melanoma by the MAPK pathway via E2F1
- MELK inhibition blocks melanoma growth
- MELK phosphorylates a large number of BRAF and MEK substrates
- MELK partly promotes melanoma by stimulating the NF-κB pathway via SQSTM1
Melanoma is the deadliest form of skin cancer, accounting for
more than 80% of skin cancer-related deaths, and current therapies provide
only short-term benefit to patients. Here, we show in melanoma cells that maternal embryonic leucine zipper kinase (MELK) is transcriptionally upregulated by the MAPK pathway via transcription factor E2F1. MELK knockdown or pharmacological inhibition blocked melanoma growth and enhanced the effectiveness of BRAFV600E inhibitor against melanoma cells. To identify mediators of MELK function, we performed stable isotope labeling with amino acids in cell culture (SILAC) and identified 469 proteins that had downregulated phosphorylation after MELK inhibition. Of these proteins, 139 were previously reported as substrates of BRAF or MEK, demonstrating that MELK is an important downstream mediator of the MAPK pathway. Furthermore, we show that MELK promotes melanoma growth by activating NF-κB pathway activity via Sequestosome 1 (SQSTM1/p62). Altogether, these results underpin an important role for MELK in melanoma growth downstream of the MAPK pathway.

INTRODUCTION

Melanoma is the deadliest form of skin cancer, accounting for ~80% of skin cancer-related deaths (Miller and Mihm, 2006). More than 85% of melanomas are caused by mutations in BRAF or NRAS genes and mutation or deletion of the NF1 gene (Cancer Genome Atlas Network, 2015). These alterations can activate the mitogen-activated protein kinase (MAPK) pathway, which in turn promotes proliferation and facilitates melanoma initiation and progression (Downward, 2003; Karnoub and Weinberg, 2008; Wellbrock et al., 2004a, 2004b).

After the initial discovery of BRAF mutations in a large percentage of melanomas (Davies et al., 2002), specific and highly effective small-molecule inhibitors that target either BRAF or MEK mutants were developed and used to treat BRAF mutant metastatic melanoma in clinics (Chapman et al., 2011; Flaherty et al., 2012). BRAF inhibitors alone or in combination with MEK inhibitors have shown some success; however, within months of treatment, drug resistance emerges and renders these drugs ineffective (Kim et al., 2013; Rizos et al., 2014; Shi et al., 2014). The alternative approach of targeting the MAPK pathway in NRAS mutant and NF1-deficient melanoma has not proved effective (Ascierto et al., 2013; Whittaker et al., 2013). Similarly, new immunotherapeutic approaches, such as anti-CTLA-4 antibody (ipilimumab) and anti-PD1 or anti-PD-L1 antibodies ( pembrolizumab or nivolumab), have benefited only a subset of patients (Hodi et al., 2010; Postow et al., 2015; Robert et al., 2015). Thus, new strategies for treating melanoma and improving patient survival are needed. Maternal embryonic leucine zipper kinase (MELK) is a serine/threonine protein kinase that regulates cell cycle, stem cell renewal, and apoptosis (Badouel et al., 2006; Davezac et al., 2002; Jung et al., 2008; Nakano et al., 2005). MELK knockout mice are viable and display no adverse phenotypes (Wang et al., 2014). This information and the availability of small-molecule inhibitors of MELK with anti-cancer activity in breast and other cancers indicates that MELK might be a druggable target for cancer cell-selective therapy (Gray et al., 2005; Kohler et al., 2017; Nakano et al., 2005).

Here, we show that MELK is necessary for melanoma growth. We found that MELK regulated the phosphorylation of a large number of proteins, many of which were previously identified as substrates of BRAF and/or MEK. We also demonstrate that MELK regulation of the nuclear factor κB (NF-κB) pathway partly mediates the melanoma-promoting activity of MELK. Altogether, our studies identify MELK as an important regulator of melanoma growth downstream of the MAPK pathway.

RESULTS

MELK Is Overexpressed in Melanoma by the MAPK Pathway

MELK is highly overexpressed in several cancer types, and its inhibition has been shown to block the tumor growth of some cancers (Inoue et al., 2016; Joshi et al., 2013; Kato et al., 2016; Wang et al., 2017; Nakano et al., 2005).
Figure 1. MELK Is Upregulated in Melanoma by the MAPK Pathway through the Transcription Factor E2F1

(A and B) Indicated melanoma datasets were analyzed for MELK mRNA expression. Relative expression in patient-derived melanoma samples compared to normal skin (A) and in N1+ versus N0 or primary versus metastatic melanoma (B) is shown.

(C) MELK mRNA expression was measured after treatment with vemurafenib (2 μM) or trametinib (250 nM) for 24 hr. Relative mRNA MELK expression is plotted in reference to DMSO-treated melanoma cell lines.

(D) MELK protein expression was measured by immunoblotting in indicated melanoma cell lines after treatment with DMSO (C0), vemurafenib (V; 2 μM), or trametinib (T; 250 nM) for 24 hr. ACTINB was used as the loading control.

(E) mRNA expression for the indicated genes was measured in A375 cells 24 hr after DMSO, vemurafenib (2 μM), or trametinib (250 nM) treatment. mRNA expression is shown relative to DMSO-treated A375 cells.

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et al., 2014, 2016). MELK knockout mice are viable and do not show specific phenotypes (Wang et al., 2014). Therefore, MELK appears to be a potentially effective and cancer cell-selective target. The role of MELK in melanoma has not been studied, and few MELK substrates have been identified thus far. Therefore, we asked whether MELK plays a role in melanoma growth. We first analyzed the expression of MELK in previously published gene expression datasets of patient-derived melanoma samples. MELK was overexpressed in patient-derived melanoma samples compared to normal skin samples (Figure 1A; Figures S1A–S1C). In addition, MELK expression significantly increased with spreading of melanoma, and metastatic melanoma had higher MELK expression than primary melanoma (Figure 1B; Figures S1B and S1C). A previous study identified increased expression of MELK and other genes as a genetic signature that predicts melanoma progression (Ryu et al., 2007). Altogether, these results suggest an important role for MELK in melanoma.

We aimed to decipher the mechanism of MELK overexpression in melanoma. One of the most altered signaling pathways in melanoma is the MAPK pathway, which is constitutively active in more than 85% of melanomas because of mutations in BRAF/ NRAS genes or inactivation of the NF1 gene (Cancer Genome Atlas Network, 2015). Therefore, we asked whether the MAPK pathway is necessary for transcriptional upregulation of MELK in melanoma. We treated three BRAF mutant melanoma cell lines (A375, M14, and SKMEL-28) with the BRAFV600E inhibitor vemurafenib or the MEK inhibitor trametinib. Treatment of these cells with either inhibitor reduced MELK mRNA (Figure 1C) and protein (Figure 1D) levels. Altogether, these results demonstrate that transcriptional upregulation of MELK in melanoma is primarily mediated by the MAPK pathway.

Transcription Factor E2F1 Is Required for Transcriptional Upregulation of MELK in Melanoma Cells

To determine the mechanism of transcriptional upregulation of MELK, we analyzed the MELK promoter sequence using PROMO and rVista v.2.0. We identified conserved DNA binding sites for E2F1 and MYC transcription factors. We then asked whether any of these transcription factors were upregulated, like MELK, by the action of the MAPK pathway. We treated A375 and M14 cells with vemurafenib or trametinib and analyzed the expression of E2F1-8 and MYC. Only MYC, E2F1, and E2F2 were significantly downregulated after treatment with vemurafenib or trametinib (Figure 1E; Figure S1D). Therefore, we individually knocked down MYC, E2F1, and E2F2 in melanoma cell lines using short hairpin RNAs (shRNAs) and analyzed the effect of these knockdowns on MELK expression. Knockdown of the transcription factor E2F1 significantly reduced MELK expression (Figures 1F and 1G), while E2F2 or MYC knockdown did not (Figures S1E–S1H).

Next, we wanted to determine whether the transcription factor E2F1 directly targets MELK. To this end, we cloned the MELK promoter with an E2F1 DNA binding site upstream of a firefly luciferase reporter gene. This MELK-FLuc construct was tested for responsiveness to the BRAF inhibitor vemurafenib. A375 cells transfected with the MELK-FLuc construct had reduced luciferase activity after vemurafenib treatment (Figure 1H). We also mutated the E2F1 DNA binding site on the MELK promoter and observed a substantial reduction of MELK promoter-driven reporter activity, making this construct non-responsive to vemurafenib treatment (Figure 1H). Finally, to determine whether E2F1 directly associates with the MELK promoter in vivo, we performed chromatin immunoprecipitation (ChIP). We treated A375 cells with vemurafenib, or with DMSO as a control, and performed ChIP for E2F1 for the MELK promoter, or for the GAPDH promoter as a control. E2F1 was significantly enriched at the MELK promoter compared to the negative control GAPDH promoter (Figure 1I). In addition, E2F1 binding of the MELK promoter was inhibited by vemurafenib treatment (Figure 1I). Altogether, these results demonstrate that the MAPK pathway stimulates E2F1 expression, which in turn activates MELK transcription by directly binding to the MELK promoter in melanoma cells.

MELK Inhibition Blocks Melanoma Growth

Because MELK is a kinase that is highly expressed in melanoma cells, we asked whether MELK is a potential target for melanoma therapy. To test whether MELK inhibition would block melanoma growth, we treated melanoma cell lines with the MELK inhibitor OTSSP167 (Chung et al., 2012; Kohler et al., 2017; Wang et al., 2014). OTSSP167 treatment significantly inhibited melanoma cell line proliferation (Figure 2A) and colony formation in a soft-agar assay (Figures 2B and 2C; Figure S2). To confirm that the growth inhibition was due to MELK inhibition and not an off-target effect, we treated cells with a second MELK inhibitor, MELK-8a (Touré et al., 2016). Consistent with our results with OTSSP167, MELK-8a inhibited melanoma cell growth in both the proliferation assay (Figure S3A) and the soft-agar assay (Figures S3B and S3C).

To determine whether the ability of MELK to promote melanoma growth was dependent on its kinase activity, we performed rescue experiments with a wild-type MELK open reading frame or a kinase dead MELK mutant (MELK-D150A). Only wild-type MELK, not the kinase dead mutant, was able to rescue growth in the soft-agar assay, showing that the kinase activity of MELK is required for its ability to promote melanoma growth (Figures 2D and 2E; Figures S3D and S3E).

(F) A375 cells expressing either E2F1 or non-silencing (NS) shRNA were analyzed for E2F1 (left) or MELK (right) mRNA expression using qRT-PCR. mRNA expression in E2F1 shRNA-expressing cells is shown relative to NS shRNA-expressing cells.

(G) Indicated protein levels were monitored in A375 cells expressing either E2F1 or NS shRNAs. ACTINB was used as a loading control.

(H) Relative MELK promoter-driven firefly luciferase (MELK-F-Luc) activity is shown in A375 cells treated with DMSO or vemurafenib and transfected with or without a mutated E2F1 DNA binding site containing the MELK-FLuc construct.

(I) A375 cells treated with DMSO or vemurafenib (2 µM) for 24 hr were analyzed for E2F1 recruitment on either the MELK or GAPDH promoter by chromatin immunoprecipitation assay. Immunoglobulin G (IgG) antibody was used as a negative control. Percentage of enrichment relative to input under indicated conditions is shown.

Data are presented as ± SD for three biological replicates. *p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S1.
MELK Inhibition Blocks the Growth of Vemurafenib-Resistant Cells and Delays the Emergence of Vemurafenib Resistance

Our results showed that MELK is a downstream target of the MAPK pathway and that MELK inhibition blocks melanoma growth. Therefore, we asked whether vemurafenib-resistant melanoma cell lines could also be inhibited by MELK inhibitors. We analyzed A375 and SKMEL-239 parental cell lines and vemurafenib-resistant versions of these two cell lines. To test the effectiveness of MELK inhibitors for blocking growth, we analyzed A375 and SKMEL-239 parental cell lines and vemurafenib-resistant versions of these two cell lines.

Figure 2. MELK Inhibition Blocks Melanoma Cell Growth in Culture

(A) Melanoma cell lines (A375, SKMEL-28, M14, YUGASP, and MeWo) were treated with indicated concentrations of OTSSP167 and analyzed for cell proliferation using the MTT assay. Relative proliferation (%) for each melanoma cell line relative to DMSO-treated cells is shown.

(B) Melanoma cell lines (A375, SKMEL-28, M14, YUGASP, and MeWo) were treated with indicated concentrations of OTSSP167 and analyzed for anchorage-independent growth using the soft-agar assay. Representative images for indicated melanoma cell lines under indicated treatment conditions are shown.

(C) Relative colony size (%) for indicated cell lines at indicated treatment conditions is shown.

(D and E) A375 cells expressing doxycycline-inducible MELK shRNA#1 (D) and MELK shRNA#2 (E) were infected with virus for expression of either MELK wild-type (WT) or MELK knockdown (KD), grown without or with doxycycline, and analyzed for soft-agar colony formation. Representative images are shown. Scale bars, 200 μm. Data are presented as ±SD for three biological replicates. *p < 0.05, **p < 0.01, ***p < 0.001. See also Figures S2 and S3.
vemurafenib-resistant cell lines, we treated parental and vemurafenib-resistant A375 and SKMEL-239 cell lines with vemurafenib alone or with MELK inhibitors (OTSSP167 and MELK-8a). Treatment of parental cell lines (A375 and SKMEL-239) with either vemurafenib or MELK inhibitors (OTSSP167 and MELK-8a) inhibited proliferation and growth in soft agar (Figures 3A–3F; Figure S4). In vemurafenib-resistant cell lines, vemurafenib did not inhibit proliferation or growth in soft agar, but treatment with MELK inhibitors (OTSSP167 and MELK-8a) did inhibit proliferation and growth in soft agar (Figures 3A–3F; Figure S4). Finally, we asked whether MELK inhibition can forestall the emergence of vemurafenib resistance. We treated parental A375 melanoma cells with vemurafenib alone or in combination with OTSSP167 and performed a clonogenic assay to measure the emergence of vemurafenib resistance. After 6 weeks of treatment with these drugs, we visualized and quantified the number of drug-resistant clones. Treating A375 cells with vemurafenib produced several vemurafenib-resistant colonies (Figure 3G). Combined vemurafenib and OTSSP167 treatment did not yield drug-resistant colonies (Figure 3G). Altogether, these results demonstrate that MELK inhibition can inhibit the growth of vemurafenib-resistant melanoma and that the combination of vemurafenib and OTSSP167 can forestall the emergence of vemurafenib resistance.

SILAC Identifies Cellular Substrates of MELK

MELK is a serine/threonine kinase for which few substrates are known. Therefore, to comprehensively identify MELK substrates, we performed a global phosphoproteomic analysis using stable isotope labeling with amino acids in cell culture (SILAC). There were two major goals for this experiment: (1) to characterize the diversity of proteins that are phosphorylated by MELK in melanoma cells and (2) to identify the potential pathway or pathways targeted by MELK to promote melanoma growth. To achieve these goals, we used two melanoma cell lines (A375 and M14) in which cell proliferation is inhibited by MELK inhibition. These cell lines were cultured in light medium, which contains light carbon (\(^{12}\text{C}\)), light nitrogen (\(^{14}\text{N}\)), lysine, and arginine, or in heavy medium, which contains heavy carbon (\(^{13}\text{C}\)), heavy nitrogen (\(^{15}\text{N}\)), lysine, and arginine. After five cell doublings, incorporation of these amino acids exceeded 95% (Table S1), cells in light medium were treated with DMSO, and cells in heavy medium were treated with MELK inhibitor OTSSP167 for 24 hr. SILAC analysis was performed to identify potential MELK targets (Figure 4A). This analysis identified 469 proteins with reduced phosphorylation in both A375 and M14 cells on the same residues (Figure S5; Tables S2, S4, and S5). A comparative analysis showed that 139 substrates identified by our SILAC analysis were previously identified as MAPK pathway substrates (Figure 4B; Tables S2, S4, and S5) (Galan et al., 2014; Stuart et al., 2015). This was not due to reduced MAPK signaling, because OTSSP167 treatment did not inhibit ERK1/2 phosphorylation (Figure S6A). We consider this to be an important observation based on our findings that the MAPK pathway regulates MELK expression and might mediate a large part of the melanoma growth and progression-promoting effect of the MAPK pathway.

We next analyzed the SILAC data to predict the preferred amino acid motif for MELK-induced phosphorylation by a newly developed method. The identified MELK recognition site was broad, and most MELK-mediated phosphorylation of identified substrates occurred at serine (Figures 4C and 4D).

Finally, to identify the key pathways regulated by MELK-mediated phosphorylation, we performed Ingenuity pathway analysis. We identified the NF-\(\kappa\)B pathway as an enriched pathway (Figure 4E). In total, eight proteins involved in NF-\(\kappa\)B pathway regulation, which had decreased phosphorylation as a result of MELK inhibition, were identified by our SILAC experiments in both A375 and M14 cell lines (Figure 4F). We decided to further study MELK-mediated regulation of the NF-\(\kappa\)B pathway because of the previously described role for this pathway in promoting melanoma tumor growth and progression (Dhawan and Richmond, 2002; Madonna et al., 2012; Ueda and Richmond, 2006).

MELK Regulates the NF-\(\kappa\)B Pathway via SQSTM1/p62

Based on our SILAC and Ingenuity pathway analysis results, we asked whether MELK had a role in regulating the NF-\(\kappa\)B pathway. Consistent with our SILAC results, treating A375 and M14 melanoma cell lines with the MELK inhibitor OTSSP167 resulted in attenuated NF-\(\kappa\)B signaling, as assessed by decreased phosphorylation of NF-\(\kappa\)B inhibitor alpha (I\(\kappa\)B\(\alpha\)) (Figure 5A). A similar reduction in NF-\(\kappa\)B signaling was observed in melanoma cells after MELK knockdown using doxycycline-inducible shRNAs (Figure 5B). Furthermore, melanoma cell lines that were treated with MELK inhibitor and cells that expressed MELK-shRNAs both showed reduced luciferase activity when transfected with a NF-\(\kappa\)B-responsive reporter plasmid (pGL4.32[luc2P/NF-\(\kappa\)B-RE/Hygro]) (Figure 5C). Similarly, known NF-\(\kappa\)B transcriptional targets were downregulated after MELK knockdown (Figure 5D) and after OTSSP167 treatment (Figure 5E). We also found that treatment with another MELK inhibitor, MELK-8a, attenuated NF-\(\kappa\)B pathway activity, as determined by decreased phosphorylation of I\(\kappa\)B\(\alpha\) and by decreased expression of NF-\(\kappa\)B-responsive genes (Figures S6B and S6C).

In our SILAC analysis, we identified Sequestosome 1 (SQSTM1) as a protein with decreased phosphorylation after MELK inhibition. SQSTM1 has been shown to be involved in the regulation of NF-\(\kappa\)B signaling (Long et al., 2010; Wooten et al., 2005; Zotti et al., 2014). SQSTM1 has also been shown to be important for NF-\(\kappa\)B-mediated tumorigenesis (Duran et al., 2008). We hypothesized that MELK phosphorylates SQSTM1 to stimulate the NF-\(\kappa\)B pathway. To test this, we performed co-immunoprecipitation (coIP) to detect whether MELK directly phosphorylates SQSTM1 (Figure 5G). To confirm that MELK inhibition reduced SQSTM1 phosphorylation, we performed the in vitro kinase assay using recombinant MELK and SQSTM1 proteins, as described previously (Canman et al., 1998), to test whether MELK directly phosphorylates SQSTM1. Consistent with our SILAC experiments, MELK directly phosphorylated SQSTM1 (Figure 5G).
Figure 3. MELK Inhibition Is Sufficient to Overcome Vemurafenib Resistance

(A–D) Parental and vemurafenib-resistant melanoma cell lines A375 and SKMEL-239 were treated with either DMSO or indicated concentrations of vemurafenib (A and C for A375 and SKMEL-239, respectively) or OTSSP167 (B and D for A375 and SKMEL-239, respectively) and analyzed for proliferation using the MTT assay. Relative proliferation (%) for each cell line relative to DMSO-treated cells is shown.

(E) Parental and vemurafenib-resistant melanoma cell lines A375 and SKMEL-239 were treated with 1 μM vemurafenib or 50 nM OTSSP167 and analyzed for anchorage-independent growth by the soft-agar assay. Representative images for indicated melanoma cell lines under indicated treatment conditions are shown. Scale bar, 200 μm.

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Based on these results, we examined the activity of NF-κB pathway upon SQSTM1 knockdown. Similar to the effect of MELK inhibition, shRNA-induced knockdown of SQSTM1 inhibited the NF-κB signaling pathway and expression of NF-κB target genes (Figures 5I–5K).

**Constitutively Active IKKβ Partially Rescues NF-κB Signaling and Melanoma Growth after MELK Inhibition**

Because inhibitor of NF-κB kinase subunit beta (IKKβ) acts downstream of SQSTM1, we asked whether overexpression of constitutively active IKKβ (IKKβ CA) could rescue the inhibition of NF-κB signaling caused by MELK and SQSTM1 inhibition. Ectopic expression of IKKβ CA partially rescued impaired NF-κB signaling caused by MELK inhibition and by inhibition caused by MELK inhibition, we expressed IKKβ CA in the A375 melanoma cell line and analyzed the growth of melanoma cells in soft-agar assay. Overexpression of IKKβ CA stimulated the growth of A375 cells in soft agar, even in the presence of the MELK inhibitor OTSSP167 (Figures 6E and 6F). Similarly, overexpression of IKKβ CA restored the growth of A375 melanoma cells in the presence of the second MELK inhibitor MELK-8a (Figure S6D). In contrast, expression of an empty vector in the presence of OTSSP167 or MELK-8a did not rescue the growth of A375 cells (Figures 6E and 6F; Figures S6D and S6E). Altogether, these results demonstrate that attenuation of NF-κB signaling is partly responsible for blocking melanoma growth inhibition after MELK inhibition (Figure 7).

(F) Relative colony size (%) for indicated melanoma cell lines at indicated treatment conditions is shown.

(G) A375 melanoma cells were treated with 2 μM vemurafenib alone or in combination with 50 nM OTSSP167 over four weeks. Images of representative plates and surviving colonies or cells are shown.

Data are presented as ±SD for three biological replicates. *p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S4.

**Figure 4. SILAC Analysis Identifies MELK Targets**

(A) Schematic representation of the major steps of SILAC analysis to identify phosphopeptides that are altered after treatment with MELK inhibitor OTSSP167 in melanoma cell lines (A375 and M14).

(B) Venn diagram showing commonly identified proteins that overlap with previously identified BRAFV600E and MEK targets.

(C) Consensus site for the MELK-mediated phosphorylation amino acid recognition motif is shown.

(D) Consensus site for the MELK-mediated phosphorylation recognition motif based on amino acid hydrophobicity is shown.

(E) Ingenuity pathway analysis of the MELK targets identified by SILAC analysis revealed eight NF-κB regulatory proteins that showed down-regulated phosphorylation after treatment with MELK inhibitor OTSSP167.

(F) Site of phosphorylation on NF-κB regulatory proteins for which reduced phosphorylation was observed in SILAC for melanoma cell lines A375 and M14 after treatment with MELK inhibitor OTSSP167.

See also Figure S5 and Tables S1, S2, S4, and S5.
Figure 5. MELK Regulates the NF-κB Pathway via SQSTM1

(A) A375 and M14 cells were treated with OTSSP167 (50 nM) for 24 hr and analyzed for indicated proteins by immunoblot analysis. ACTINB was used as the loading control.

(B) A375 cells expressing doxycycline-inducible MELK shRNAs that were either untreated or treated with doxycycline (2 μg/mL) and analyzed for indicated proteins by immunoblot analysis. ACTINB was used as loading control.

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DISCUSSION

In this study, we show that MELK is important for melanoma growth that functions partly by facilitating NF-κB pathway activity. Our study allows us to draw several important conclusions. First, MELK expression was activated by the MAPK pathway and was necessary for melanoma growth. Second, we unexpectedly found that MELK phosphorylated many proteins that were previously reported to be BRAF or MEK substrates. Third, MELK inhibition blocked the growth of melanoma that was resistant to the BRAF inhibitor vemurafenib. Finally, MELK regulation of the NF-κB pathway occurred via SQSTM1, partly accounting for its role in promoting melanoma growth. These results are important because they describe a role for MELK in melanoma as a survival kinase. This work also demonstrates that pharmacological inhibition of MELK with a highly potent MELK inhibitor can exert strong inhibitory effects on tumor growth in a variety of melanoma types, including NRAS mutant, NF1-deficient, and vemurafenib-resistant melanoma.

MELK Inhibition Blocks Melanoma Growth

Melanoma is an aggressive form of skin cancer, as illustrated by a 5-year survival rate of only 15%–20% for stage IV melanoma (Sandru et al., 2014). Only a fraction of patients experiences long-term benefits from current targeted therapies and immunotherapies (Johnson and Sosman, 2015). Therefore, alternative methods to effectively treat melanoma need to be developed. We found that MELK is a survival kinase for melanoma and that MELK inhibition, by either genetic or pharmacological methods, blocked growth of melanoma cells. Furthermore, MELK inhibition in melanoma cells inhibited tumor growth in a variety of genotypes, including BRAF mutant, NRAS mutant, and NF1-deficient melanoma. In addition, vemurafenib-resistant melanoma cells were sensitive to MELK inhibitors, and we observed that MELK inhibitors forestalled the emergence of vemurafenib resistance in melanoma cells. MELK knockout mice are viable and do not show obvious defects. Altogether, these observations suggest that MELK is an important and broadly applicable therapeutic target in melanoma.

MELK Regulates a Large Number of Previously Reported BRAF-MEK-ERK Substrates

MELK is a serine/threonine protein kinase that regulates the cell cycle, stem cell renewal, and apoptosis (Badouel et al., 2006; Davezac et al., 2002; Jung et al., 2008; Nakano et al., 2005). Previous studies have identified some MELK substrates, including AS1K, ZNF622, BCL2L14, and CDC25B (Davezac et al., 2002; Jung et al., 2008; Lin et al., 2007; Seong et al., 2002). The apoptotic functions of MELK are mediated by ASK1 and BCL2L14 regulation (Jung et al., 2008; Lin et al., 2007), while its cell-cycle regulatory effects are proposed to be mediated by its phosphorylation of CDC25B (Davezac et al., 2002; Mirey et al., 2005). In addition to regulating apoptosis and the cell cycle, MELK regulates other aspects of cell biology. For example, MELK has been shown to inhibit spliceosome assembly during mitosis by phosphorylating ZNF622, thereby contributing to its redirection to the nucleus. Using SILAC, we identified 469 proteins with downregulated phosphorylation after MELK inhibition. In addition, a large number of proteins (139 proteins) were previously identified as potential MEK and BRAF substrates (Galan et al., 2014; Stuart et al., 2015). Because MELK expression is regulated by the MAPK pathway, these findings suggest MELK is a major mediator of MAPK pathway function that promotes melanoma growth.

MELK Is a Regulator of the NF-κB Pathway

The NF-κB pathway is a major tumor promotion pathway in melanoma and several other cancer types (Dhawan and Richmond, 2002; Erstad and Cusack, 2013; Liu et al., 2015; Madonna et al., 2012; Pikarsky et al., 2004). We found that MELK regulates the NF-κB pathway by phosphorylating SQSTM1/p62, which is consistent with a previous study that showed that SQSTM1 is important for NF-κB-mediated tumorigenesis (Duran et al., 2008). In addition, we demonstrated that MELK inhibition decreased the expression of NF-κB transcriptional targets, and we partially rescued diminished melanoma growth after MELK inhibition by expressing IKKβ CA. Altogether, these results identify MELK as a regulator of the NF-κB pathway and show that
Figure 6. Overexpression of IKKβ CA Restores NF-κB Signaling in MELK-Inhibited Melanoma Cells

(A and B) (Left) A375 cells expressing doxycycline-inducible MELK shRNA#1 (A) or MELK shRNA#2 (B) were transfected with constitutively active IKKβ (IKKβ CA) or empty vector. Cells were either untreated or treated with doxycycline (2 μg/mL) for 72 hr and analyzed for indicated proteins by immunoblot analysis. ACTINB was used as a loading control. (Right) A375 cells expressing MELK shRNA#1 (A) or MELK shRNA#2 (B) were transfected with IKKβ CA or empty vector and the NF-κB-responsive F-Luc construct. Cells either remained untreated or were treated with doxycycline and analyzed for firefly luciferase activity. Relative firefly luciferase activity under indicated conditions is shown.

(C) (Left) A375 cells were transfected with constitutively active IKKβ (IKKβ CA) or empty vector, subsequently treated with either DMSO or OTSSP167 (50 nM) for 24 hr, and analyzed for indicated proteins by immunoblot analysis. ACTINB was used as a loading control. (Right) A375 cells were transfected with IKKβ CA or empty vector and the NF-κB-responsive F-Luc construct, treated with OTSSP167 (50 nM) for 24 hr, and analyzed for firefly luciferase activity. Relative firefly luciferase activity for indicated conditions is shown.

(D) A375 cells expressing SQSTM1 shRNA were transfected with constitutively active IKKβ (IKKβ CA) or empty vector and analyzed for indicated proteins by immunoblot analysis 48 hr after transfection. ACTINB was used as a loading control.

(E) A375 melanoma cells were transfected with constitutively active IKKβ (IKKβ CA) or empty vector, treated with OTSSP167 (25 nM), and analyzed for anchorage-independent growth using soft-agar assay. Representative images for soft-agar colonies for indicated melanoma cell lines for the indicated treatment conditions are shown. Scale bar, 200 μm.

(F) Relative colony size (%) for A375 cells expressing empty vector or constitutively active IKKβ were DMSO or OTSSP167 (25 nM) treated. Data are presented as ±SD for three biological replicates. *p < 0.05, **p < 0.01, ***p < 0.001.
MELK at least partly promotes melanoma growth by activating the NF-κB pathway.

EXPERIMENTAL PROCEDURES

Cell Culture
A375, M14, SKMEL28, and MeWo cell lines were obtained from the American Type Culture Collection (ATCC). YUGASP cells were obtained from Yale. A375, M14, MeWo, and YUGASP were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (PenStrep) antibiotics. M14, SKMEL28, and SKMEL239 were grown in RPMI supplemented with 10% FBS and 1% PenStrep antibiotics.

Cell Labeling and SILAC Analysis
Cells were seeded at 15% confluency in the respective complete medium (for A375, DMEM + 10% dialyzed FBS, 1% PenStrep; for M14, RPMI + 10% dia- lyzed FBS, 1% PenStrep). All media was deficient in lysine and arginine and supplemented with light- or heavy-labeled lysine (15N2/15N4) and light- or heavy-labeled arginine (15N6/15N4). Cells were subsequently cultured for at least five doublings in light or heavy medium, which achieved more than 95% labeling efficiency for us in pilot experiments. After labeling, cells were treated for 24 hr with 25 mM (A375) or 50 mM (M14) of OTSSP167. After treat- ment, cells were trypsinized and counted to obtain a cell pellet of 2 x 10^7 cells/condition and subjected to SILAC analysis using mass spectrometry.

Sample Preparation
The heavy and light cell pellets were lysed in RIPA buffer spiked with protease and phosphatase inhibitors using short 15 s sonication bursts. Lysates were centrifuged at 14,000 rpm for 20 min. After centrifugation, the supernatants were collected and the protein concentration was measured using a Hitachi L-8900 Amino Acid Analyzer. From each sample, 200 μg of proteins were aliquoted, combined, and precipitated using a methanol-chloroform precipita- tion method. The protein pellets were resuspended in 8 M urea/0.4 M ammono- nium bicarbonate buffer, reduced with 45 mM DTT for 30 min at 37°C, alkylated with 100 mM iodoacetamide for 30 min in the dark at room temperature, and digested with Lys-C protease (1:20 w/v) by incubating overnight (~16 hr) at 37°C. The Lys-C digest was further diluted and digested with trypsin (1:20 w/v) by incubating for 8 hr at 37°C. The digest was desalted with a MacroSpin column (The Nest Group, Southborough, MA) and dried down in a SpeedVac concentrator. Desalted peptides were then phosphopeptide enriched using titanium dioxide resin imbedded in 10-μL tips (Glygen, Columbia, MD). Flowthroughs were reserved and enriched peptides were eluted using 1:33 ammonium hydroxide/water. The SpeedVac dried flow- through and elution fractions were resuspended in buffer A (0.1% formic acid in water) and subjected to liquid chromatography-tandem mass spec- trometry (LC-MS/MS) analysis.

Mass Spectrometry Data Acquisition and Analysis
The samples were analyzed by LC-MS/MS on an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific, San Jose, CA) interfaced with a nanoAcuity UPLC System (Waters, Milford, MA) at the front end. Samples were loaded into a trapping column (nanoAcuity UPLC Symmetry C18 Trap Col- umn, 180 μm x 20 mm, product 186006527) at a flowrate of 5 μL/min and separated with a C18 column (Peptide BEH C18 nanoAcuity Column, 75 μm x 250 mm, product 186003545). The peptides were eluted with buffer B (0.1% formic acid in acetonitrile) in a gradient from 6% to 35% in 150 min at a flowrate of 300 nL/min. LC-MS/MS data were acquired using 3 s, the topspeed, data-dependent acquisition mode. Details of the instru- ment settings can be found in the Supplemental Information.

Peptides and proteins were identified and quantified with the Sequest HT search engine using Proteome Discoverer v.2.1 software (Thermo Scientific). A standardized SILAC 2plex (Arg10 and Lys8) quantification workflow in the Proteome Discoverer was slightly modified and used for analysis. Briefly, MS/MS data were searched against the SwissProt human database (down- loaded in September 2015; number of protein entries = 20,193). In the Peak Fil- ters node, the signal to noise ratio (S/N) threshold was set to 1.5. The search criteria included 10 ppm precursor mass tolerance, 0.02 Da fragment mass tolerance, and a trypsin mis cleavage setting of 2. Static modification settings included carbamidomethylation (+57.021 Da) on cysteine, while dynamic modifications were set to include oxidation (+15.995 Da) on methionine and phosphorylation (+79.966 Da) on serine, threonine, and tyrosine. Peptide spectrum matches (PSMs) were verified based on q values set to a 1% false discovery rate (FDR) using Percolator. The Precursor Ions Quantifier node was used in the processing step workflow, and the Peptide and Protein Quan- tifier node was selected for the consensus workflow to calculate and quantify peptides, protein abundances, and ratios. The PhosphoRS node (Taus et al., 2011) was used to obtain the localization probability of the phosphorylation sites in the peptides.

SILAC Data Analysis for Identifying the Preferred MELK Amino Acid Context for Phosphorylation
A cutoff of two-fold was used to define downregulation of the phosphorylation level for validation experiments. To identify the MELK phosphorylation consensus site from the SILAC data, we used a prediction algorithm developed in house. The motifs were generated by the R/Bioconductor package dagLogo (v.1.9.2). The background of the motifs was built from the human pro- teome retrieved via the R/Bioconductor package UniProt.ws (v.2.11.9). Lists of all quantified phosphopeptides are presented in Table S4 (for the A375 cell line) and Table S5 (for the M14 cell line). The SILAC proteomics data have been submitted to PRIDE (https://www.ebi.ac.uk/pride/archive/). The accession number for these data is PRIDE: PXD007872.

Melanoma Data Analysis
The Talantov melanoma dataset (Talantov et al., 2005), Riker melanoma da- taset (Riker et al., 2008), and Xu melanoma dataset (Xu et al., 2008) were analyzed for MELK expression using Oncomine (https://www.oncomine. org/resource/login.html), and MELK expression across different samples
was plotted as boxplots. In addition, three previously published melanoma gene expression datasets were analyzed for MELK expression and plotted as boxplots (Eskiocak et al., 2016; Kabbarah et al., 2010; Scatolini et al., 2010).

**Statistical Analysis**

All quantitative data were collected from experiments performed in at least triplicate and expressed as mean ± SD. Differences between groups were assayed with Student’s t test using GraphPad Prism v.6.0h for Macintosh (GraphPad, San Diego, CA) (https://www.graphpad.com/). Significant differences were considered when p ≤ 0.05.

**DATA AND SOFTWARE AVAILABILITY**

The accession number for the SILAC proteomics data reported in this paper is PRIDE: PXD007872.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, six figures, and five tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.11.033.

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**REFERENCES**


