ERK3 as a BRAF-Regulated Tumor Suppressor is a New Potential Cancer Target in Melanoma

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

By

MINYI CHEN

B.S., University of Houston-Downtown, 2014

2017
Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Minyi Chen
ENTITLED ERK3 as a BRAF-Regulated Tumor Suppressor is a New Potential Cancer Target in
Melanoma BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
Master of Science.

______________________________
Michael Markey, Ph.D.
Thesis Director

______________________________
Madhavi P. Kadakia, Ph.D.
Chair, Department of Biochemistry
and Molecular Biology
College of Science and Mathematics

Committee on Final Examination

______________________________
Michael Markey, Ph.D.

______________________________
HongMei Ren, Ph.D.

______________________________
Madhavi Kadakia, Ph.D.

______________________________
Robert E.W. Fyffe, Ph.D.
Vice President for Research and
Dean of the Graduate School
ABSTRACT

Chen, Minyi. M.S. Department of Biochemistry and Molecular Biology, Wright State University, 2017. ERK3 as a BRAF-Regulated Tumor Suppressor is a New Potential Cancer Target in Melanoma

Melanoma is the highest mortality rate skin cancer and the sixth most common cancer in the U.S.\textsuperscript{1,2} Arising from melanocytes, melanoma is known for frequent mutation of BRAF to its constitutive activation state \textit{BRAF}\textsuperscript{V600E}, thus over-activating its downstream MAPK targets ERK1/2 and contributing to melanoma progression.\textsuperscript{3,4,5} ERK3, a less studied MAPK family protein which contributes to promoting lung, breast, cervical, head and neck (HNC) cancer metastasis, has also shown a correlation with upregulation of BRAF.\textsuperscript{6,7,8,9} However, the BRAF-ERK3 regulatory mechanism and the function of ERK3 in melanoma are still unclear. Here, we first elucidate the general regulation pattern that \textit{BRAF}\textsuperscript{V600E} and BRAF are able to upregulate ERK3 through two mechanisms: kinase activity-independent protein stabilization and kinase activity-dependent transcriptional regulation. These two mechanisms are not impacted by \textit{BRAF}\textsuperscript{V600E} mutation status, in concordance with our clinical result of \textit{BRAF}\textsuperscript{V600E} mutation decreasing in frequency with melanoma progression. We next proved that unlike in lung cancer and HNC, ERK3 functions as a melanoma tumor suppressor which inhibited both metastasis as well as tumor formation in vitro. Our microarray and immunofluorescence results suggest that ERK3 functions differently in melanoma possibly due to tissue-specific transcriptional and localization differences of ERK3. Meanwhile, microarray elucidates a group of genes clinically relevant to melanoma tumor progression is tightly correlated with ERK3 downregulation, which is found in later stage melanoma.\textsuperscript{10} Clinical patient survival data also supports that ERK3 is a
tumor suppressor in melanoma rather than an oncogene as it is in lung cancer.\textsuperscript{11, 12} Thus, based on these preliminary data we hypothesize that melanoma may evade the usual regulation of ERK3 by BRAF, thus preventing ERK3 suppression metastasis in melanoma. We also revealed several possibilities of how melanoma escapes BRAF-ERK3 regulation from our preliminary data. Further elucidation of the mechanisms by which ERK3 is downregulated in melanoma and to restore these pathways would be a new potential target for treatment of melanoma.
# Table of Contents

I.  **INTRODUCTION** .................................................................................................................................................. 1  
     Melanoma ......................................................................................................................................................... 1  
     BRAF and BRAF<sup>V600E</sup> mutation in melanoma ...................................................................................... 1  
     ERK3 and its known roles in different types of cancer ......................................................................................... 2  
     Hypothesis and specific aims .............................................................................................................................. 3  

II.  **MATERIALS AND METHODS** ........................................................................................................................ 4  
     Cell culture ......................................................................................................................................................... 4  
     Transient siRNA knockdown ............................................................................................................................. 4  
     Mutagenesis of plasmids and transient overexpression .................................................................................... 5  
     Stable expression with lentiviral system ........................................................................................................... 5  
     Western blotting ............................................................................................................................................... 6  
     RT-qPCR (reverse transcription quantitative polymerase chain reaction) ......................................................... 7  
     Protein stability and kinase activity assay ....................................................................................................... 7  
     Co-Immunoprecipitation — ............................................................................................................................... 8  
     Cell migration/invasion assay ............................................................................................................................ 8  
     Wound healing assay ....................................................................................................................................... 9  
     Cell proliferation assay .................................................................................................................................... 9  
     Soft-agar colony formation assay .................................................................................................................... 9  
     Immunohistochemistry (IHC) ........................................................................................................................... 10
III. RESULTS

AIM I. BRAF-ERK3 regulation pathway

BRAF expression levels correlate with ERK3 expression. .......................................................... 13

BRAF regulates ERK3 through both mRNA expression and protein stability .................... 15

BRAF kinase activity is required for control of ERK3 transcription but not protein stability. .... 19

BRAF stabilizes ERK3 protein without requiring BRAF kinase activity .................................. 23

BRAF-ERK3 regulation model ................................................................................................. 26

AIM II. ERK3 functions in melanoma

Knockdown of ERK3 promotes skin cancer cell migration independent of cell type or BRAF\textsuperscript{V600E} mutation status. ......................................................................................................................... 27

ERK3 inhibits melanoma cell invasion ...................................................................................... 32

Reduced migration resulting from knockdown of BRAF is an equilibrium between BRAF promotion and ERK3 inhibition of melanoma metastasis ......................................................... 38

AIM III. The clinical relevance of ERK3 in melanoma

Clinically, BRAF\textsuperscript{V600E} mutation incidence is greater in benign melanocytic lesions than in melanoma and is not co-related with ERK3 expression level ......................................................... 40
In contrast to lung cancer, reduced ERK3 level in melanoma is a positive prognostic............ 42

IV. DISCUSSION .................................................................................................................. 45

V. SUPPLEMENTAL RESULTS AND FUTURE DIRECTIONS ........................................... 48

Reduction of BRAF stabilization of ERK3 might be a key mechanism for melanoma to reduce
ERK3 during tumor progression. .......................................................................................... 48

ERK3 doesn’t feedback to regulate BRAF or its signaling and doesn’t activate another pathway
to induce vemurafenib resistance......................................................................................... 49

BRAF and ERK3 are strongly colocalized in melanoma cells and may affect each other’s cytosol
distribution ................................................................................................................................ 50

Microarray analysis identified several possibilities of the differences between genes affected
by ERK3 regulation in lung cancer versus melanoma. ......................................................... 51

VI. SUPPLEMENTARY DATA .................................................................................................. 53

VII. REFERENCES .................................................................................................................. 61
List of Figures and Tables

Figure 1 BRAF expression levels correlate with ERK3 expression. ........................................ 14

Figure 2 BRAF regulates ERK3 through both mRNA expression and protein stability. .............. 18

Figure 3 BRAF kinase activity is required for control of ERK3 transcription but not protein stability. ........................................................................................................................................ 22

Figure 4 BRAF stabilizes ERK3 protein without requiring BRAF kinase activity. ...................... 25

Figure 5 BRAF-ERK3 regulation model. .......................................................................................... 26

Figure 6 Knock-down of ERK3 promotes melanoma cells migration. ........................................ 29

Figure 7 ERK3 regulation of tumor cell migration depends on cancer types............................... 31

Figure 8 Knock-down of ERK3 in melanoma promotes cell two-chamber transwell invasion independent of BRAFV600E mutation status .................................................................................................................. 33

Figure 9 ERK3 inhibits melanoma cell proliferation independent of BRAF mutation status. .... 35

Figure 10 Knock-down of ERK3 promotes melanoma soft agar colony formation...................... 37

Figure 11 Reduced migration resulting from knockdown of BRAF is an equilibrium between BRAF promotion and ERK3 inhibition of melanoma metastasis................................................................. 39

Figure 12 Clinically, BRAFV600E mutation incidences are reduced during melanoma progression and ERK3 levels do not relate to BRAFV600E mutation status. ...................................................... 41

Figure 13 ERK3 level is reduced with melanoma progression. .................................................. 43

Table S1 BRAFV600E mutation status and background information of different cell lines used in these studies ........................................................................................................................................ 53
Figure S1 BRAF Kinase activity is transcriptionally but not translationally essential for stabilization of ERK3 protein. .......................................................... 54

Figure S2 BRAF may be able to bind rictor-Sin1 in mTORC2 to form a loose complex with ERK3 in 293T........................................................................................................... 55

Figure S3 ERK3 does not feedback regulate BRAF. ........................................................................... 56

Figure S4 Long-term ERK3 knockdown does not induce vemurafenib resistance...................... 57

Figure S5 BRAF co-localizes with ERK3 in the nucleus of BRAFV600E melanoma but crosses over to the cytosol in BRAF WT melanoma............................................................................. 60
Acknowledgment

Foremost, I would like to express my sincere gratitude to my advisor, Dr. Michael Markey. His patient supports and instructions encouraged me to the completion of my Master research project and dissertation. I would also like to give my thanks to my committee members, Dr. Madhavi Kadakia, Dr. Hongmei Ren for all of the guidance. Moreover, I would like to show my great appreciation to other members from cooperated labs, Dr. Long’s lab members in BMB department, and Dr. Michael Kent, Dr. John Moad, Melinda Quattlebaum and other lab technicians in Dermpath Lab of Central States for their supports and instructions in instruments, reagents, technique, clinical samples, experiment works and funding. Our project is also benefited from the normal skin samples from Dr. Mike Kemp (WSU), and cell lines: A375 and OCM3 from Dr. Mitsiades’s Lab (Baylor College of Medicine), WM3211 from Dr. Liu’s Lab (University of California) SK-Mel 239 parental and vemurafenib resistant cell lines from Dr. Solit’s Lab (Memorial Sloan-Kettering Cancer Center)

I would personally special thanks to all of my lab mates and classmates for their support of my research, study, and life: Lobna Elkhadragy, Hitham Abdulrahman, Hadel Mohammed, Amjad Ahmed, Abdullah Alshuduki, Amnah Mahmoud Obidan, Cheng Zhang, Jin Zhang, Marion Morel, Brian James Caprul, et.al. And also say thanks to my college friend Viktor Akhanov.

Last but not the least is, my father, who is far away from the U.S. but never being far away from me. I wouldn’t say thanks to him since I know that he is always being with me.
I. INTRODUCTION

Melanoma

Melanoma is the most dangerous form of skin cancer and the sixth most common cancer in America.\(^2\) It makes up 75% of skin cancer deaths even though only accounts for less than 5% of skin cancer cases.\(^1\) It arises from melanocytes, the melanin pigment producing cells of the skin.

As with other types of cancer, melanoma is caused by genetic alterations. Notably, V-raf murine sarcoma viral oncogene homolog b1 (BRAF) is frequently activated in several cancers and in up to 37-50% of melanomas.\(^4,13,14,15\)

**BRAF and BRAF\(^{V600E}\) mutation in melanoma**

BRAF is the member of Raf serine/threonine protein kinase family, major players in the regulation of the mitogen-activated protein kinase (MAPK) pathway. In response to the upstream kinase Ras activated by extracellular signals, BRAF is the central mediator in the Ras-Raf-MEK-ERK pathway and finally induce many cellular functions including proliferation, differentiation, and transcriptional regulation.\(^4,5,16\)

Structurally, BRAF is composed of three domains. Domains are conserved across all the Raf protein family: the autoinhibitory conserved region 1 (CR1), the serine-rich hinge region CR2, and the kinase catalytic domain CR3. BRAF activation of its catalytic CR3 domain requires two mechanisms: removal of autoinhibition from the regulatory CR1 domain, and phosphorylation of serine and tyrosine residues in the activation loop of the CR3 domain.\(^17\) When BRAF is bound by the activated upstream protein Ras-GTP at the CR1 Ras-binding domain (RBD), BRAF docks at
the cell membrane. This binding will help to release the autoinhibitory CR1 domain of BRAF from its CR3 kinase domain. Further phosphorylation by activated Ras on the BRAF CR3 activation loop will destroy its hydrophobic interactions with the P-loop, the structure which stabilizes non-transferable phosphate groups of ATP in BRAF, to prevent its activation. The destroying of P-loop interaction thus finally causes the change of BRAF DFG (D594, F595, and G596) motif from in- to out-conformation. The out conformation exposes ATP in the CR3 catalytic kinase loop of BRAF to the cytosol for phosphorylation.\textsuperscript{18, 19} Activated BRAF is also able to form a dimer with C-Raf to cause a trans-phosphorylation and thus continue with phosphorylation of downstream kinases at serine/threonine residues to cause a kinase cascade.\textsuperscript{20, 21}

The substitution of glutamic acid for valine at codon 600 (V600E) in the activation loop of the BRAF CR2 domain contributes to approximately 90% of all its kinase activating mutations. This single point mutation in keeps the catalytic C3 domain in the ATP-binding active conformation which can continually phosphorylate and activates downstream targets such as ERK1/2 in the MAPKs (ERK) family to promote cellular function.\textsuperscript{4, 5} BRAF\textsuperscript{V600E} is 500-fold activated to stimulate constitutive MEK–ERK signaling in cells\textsuperscript{22} and is shown able to induce melanoma in mice\textsuperscript{23}. Consequentially, BRAF\textsuperscript{V600E} is an oncogene that is common in multiple types of cancers.\textsuperscript{3}

**ERK3 and its known roles in different types of cancer**

ERK3 (extracellular-signal-regulated kinase 3), an understudied atypical MAPK (mitogen-activated protein kinase) is also known as MAPK6. It is usually an unstable protein but has been shown to promote metastasis in lung cancer\textsuperscript{6}, head and neck cancer (HNC)\textsuperscript{7} and breast cancer\textsuperscript{9}. ERK3 in lung cancer is also shown to promote cell chemoresistance.\textsuperscript{24} Unlike other well-studied
MAPKs such as ERK1 and ERK2, little is known regarding the ERK3 signaling pathway and what functions play in the cell.

In melanoma, BRAF is not only frequently mutated but also is increased in protein expression level compared to nevi. Meanwhile, BRAF is reported to have the ability to up-regulate ERK3. Expression of BRAF or BRAF<sup>V600E</sup> in NIH 3T3 cells identified ERK3 as a novel target of BRAF signaling but the mechanism behind it is unclear. Interestingly, in recent studies, ERK3 upregulation has also been reported in multiple types of human cancer including lung cancer<sup>26</sup>, gastric cancer<sup>27</sup>, and breast cancer<sup>28</sup>.

**Hypothesis and specific aims**

Concluding from all the published data in other cell types, we hypothesize that ERK3 is up-regulated by BRAF overexpression and/or the continuous activity of its mutating form BRAF<sup>V600E</sup>. The result of ERK3 up-regulation further contributes to the promotion of melanoma tumor progression, especially in promoting tumor metastases as in lung cancer.

In order to address our hypothesis, we have **three specific aims:** 1) to elucidate the mechanisms by which BRAF regulates ERK3, 2) to identify ERK3 cellular functions in melanoma, and 3) to investigate the clinical relevance of ERK3 with melanoma progression.
II. MATERIALS AND METHODS

Cell culture -- All cell culture supplements and HERAcell® 150i incubator were purchased from Thermo Fisher Scientific. The human melanoma A375 and ocular melanoma OCM3 were obtained from Dr. Mitsiades’s Lab, Baylor College of Medicine. The tumorigenic vertical growth phase (VGP) primary melanoma WM3211 and human melanoma SE-Mel2 were obtained from Dr. Liu’s Lab, University of California. Human HEK 293T cell line was obtained from Open Biosystems. All the other cell lines squamous cell skin carcinoma A431, mouse melanoma B16F10, cervical cancer HeLa, lung adenocarcinoma NSCLC H23, human alveolar basal epithelial adenocarcinoma A549 were originally purchased from American Type Culture Collection (ATCC) (Table S1).

A375, 293T, A431, B16F10 and A549 cell were maintained in Dulbecco’s modified Eagle medium (DMEM). OCM3, WM3211, HeLa and H23 cell lines were cultured in Roswell Park Memorial Institute medium (RPMI). SK-Mel2 was obtained in Eagle's minimal essential medium (EMDM). All media were supplied with 10% fetal bovine serum (FBS) and 1% antibiotics penicillin-streptomycin (pen-strep). 6% dimethyl sulfoxide (DMSO) (Sigma Aldrich) in complete media was used for cryopreservation. Cells were frozen using the insulated box (Thermo Fisher) in -80°C overnight and then been transfer to liquid nitrogen for long term storage.

The BRAF status of all cell lines can be found in Table S1.

Transient siRNA knockdown -- DharmaFECT Transfection Reagent (Dharmacon) was used to transiently knock down of mRNA following the manufacturer’s instructions. siRNA used were:
siBRAF (Dharmacon siGENOME SMART Plus BRAF, and Qiagen Hs_BRAF_1 Functionally verified Flexi tube siRNA); siERK3 (Ambion Silencer® Select Pre-Designed siRNA); and Negative control siRNA pool (Dharmacon SMART pool: siGENOME siRNA, Qiagen All Stars Negative Control Flexi tube siRNA, and Ambion® Silencer® Select Negative Control siRNA).

**Mutagenesis of plasmids and transient overexpression** – The plasmids pcDNA3-BRAF (Addgene 40775) was used to generate a single point mutation in the kinase domain of BRAF. This kinase dead (KD) BRAF construct contained the mutation D594A. It was synthesized following Quick Change II XL site-directed mutagenesis kit (Agilent Technologies) protocol with designed point mutation primers (Invitrogen): 5’-CTCACAGTAAAAATAGGTGCTTTTGGTCTAGCTACAGTG-3’ and 3’-CACTGTAGCTAGACCAAAAGCACCTATTTTTACTGTGAGG-5’. The synthesized plasmid was amplified using MAX Efficiency® DH5α™ Competent Cells (Invitrogen) followed by purification using GeneJET Plasmid Midiprep Kids (Thermo Scientific) according to the manufacturer’s protocol. The construct was verified by DNA sequencing (Retrogen). Plasmids were transiently overexpressed in cells using FuGENE® HD transfection reagent (Active Motif) following the manufacturer’s protocol. 25% of glycerol in Lennox L Broth (Invitrogen) was used to store bacteria with the generated plasmid in -80°C.

**Stable expression with lentiviral system** — pcDNA3-Myc6-ERK3 lentiviral construct for ERK3 stable overexpression (with 6 Myc tags at the N-terminus) was pre-generated in Dr. Long’s lab as described in the previous publication. Lentiviral expression systems include pGIPZ lentiviral shRNA-mir constructs of shGIPZ-shERK3 (Open Biosystems V3LHS_344615) or shGIPZ-
shCtrl (Open Biosystems RHS4346) \textsuperscript{24}, and pCDH-Myc6-ERK3 or its empty vector control construct pCDH-CMV-MCS-EF1-Puro (System Bioscience). Following the manufacturer’s protocol, constructs were next packed into the packing vectors PAX2, PMD2G lentiviral expressing system (System Bioscience) and transfected using Lipofectamine\textsuperscript{®} 3000 Reagent and P3000\textsuperscript{TM} Reagent (Invitrogen) into HEK 293T cells (Open Biosystems). Lentivirus was collected after 48 hours and concentrated by PEG-it Virus Precipitation Solution (System Biosciences). shRNAs or cDNAs were expressed in A375 or WM3211 cells using polybrene (5 μg/ml hexadimethrine bromide, Sigma-Aldrich). The stable cell lines were generated after 1.5μg/ml puromycin (Sigma Aldrich) selection for 10 days.

**Western blotting** -- EBC lysis buffer was prepared to lyse cell as follows: 50 mM Tris [pH 7.5], 150 mM NaCl, 0.5% NP-40, 1 mM Complete Protease Inhibitors (Roche Diagnostics) and 1 mM Phosphatase Inhibitor Cocktail III [Sigma-Aldrich]). The cell lysate was mixed with Laemmli sample buffer then used to perform Western blotting following the procedures as described previously. \textsuperscript{6} The following primary antibodies were used: anti-BRAF (sc-5284, Santa Cruz Biotechnology), anti-ERK3 (ab53277, Abcam), anti-pERK1/2 (4370, Cell Signaling), anti-Rictor (A300-459A-M, Bethyl), anti-sin 1 (A300-910A-T, Bethyl), anti-β-Actin (3700, Cell Signaling), anti-GAPDH (ab181602, Abcam). β-Actin and GAPDH were used as loading control. Western blot imaging was performed and analyzed by Amersham Imager 600 and ImageQuantTL software (GE Healthcare Life Sciences).
RT-qPCR (reverse transcription quantitative polymerase chain reaction) -- Total RNA of each cell line was extracted using Trizol® reagent (Ambion) and then used as templates for reverse transcription to generate cDNA using SuperScript® VILO™ MasterMix (Invitrogen) on a SimpliAmp™ Thermal Cycler (Applied Biosystems) according to the manufacturers’ protocols. Quantitative polymerase chain reaction (qPCR) was performed using TaqMan® Universal Master Mix (Applied Biosystems), designed Roche Universal primers and Universal Probe (Roche Diagnostics) on the 7900HT Fast Real-Time PCR Systems (Applied Biosystems). Relative expression level was expressed using the delta-delta CT method with internal control of GAPDH.

Protein stability and kinase activity assay -- Stock protein translation inhibitor cycloheximide (Sigma Aldrich, 100mg/ml) was dissolved in 100% ethanol and BRAFV600E kinase inhibitor vemurafenib (Selleckchem, 10mM) was dissolved in dimethyl sulfoxide (DMSO) (Sigma Aldrich). Ethanol and DMSO were used as vehicle controls respectively. Pre-transfected or plated cells were treated with the decided dose of drugs (100µg/ml CHX and/or 2µM Vemurafenib) for different time periods (as described in each figure) by adding directly to the plated cells. Protein or total RNA were collected for analyzing by Western blotting and RT-qPCR as described previously. The protein level of each time point was normalized with β-Actin and then normalized to their first-time point level as 100%. Protein half-life and degradation speeds were calculated from densitometry analysis of the western blots in IQTL as described. Trend lines and standard errors were calculated from multiple replicates of each experiment.
**Co-Immunoprecipitation** – Cells were lysed with EBC lysis buffer. 1mg total protein lysate was used for each immunoprecipitation using either a specific antibody (Ab) or the corresponding IgG control. The supernatant was precleared with 40 μl Pierce® Protein A/G Agarose (Thermo) for 1 hour at 4°C with constant rotation. 5% of the protein was retained as input and the remaining sample was then incubated with 3μg/ml specific Ab for 1.5 hours following by an additional 1 hour binding to 50μl of protein A/G agarose beads. The beads were washed 3 times (10 minutes per wash) with lysis buffer before elution by boiling with 2× Laemmli sample buffer and further used for Western analysis.

**Cell migration/invasion assay** – A modified 2-chamber transwell system (8.0 μm pore, BD Biosciences Falcon™) was used to analyze cell migration and invasion following the manufacturer’s instructions and the Long lab’s protocol as follows. For invasion assay, inserts were pre-coated with DPBS-diluted 1mg/ml of Growth Factor-Reduced Matrigel Matrix (Corning) following the manufacturer’s protocol. Trypsin-EDTA detached cells were resuspended in 0.2ml serum-free medium. $5 \times 10^4$ to $1 \times 10^5$ cells were plated for migration and $1.5 \times 10^5$ for invasion. Cells were cultured in inserts and placed in wells containing 0.5ml of complete culture media following with 8-12 hours migration or 20 hours invasion in a 37°C cell incubator. Cells that failed to migrate were removed using cotton swabs. Then, cells that migrated to the outer side of the insert were fixed with 4% paraformaldehyde for 10 minutes. Migrated were stained with water diluted 0.5% crystal violet solution for 5 mins and captured to count with a 20x primovert microscope (Zeiss) with a Motic camera/software system.
**Wound healing assay** – Cells were grown in complete media on the 24-well plate overnight until they formed a monolayer. Then, pipette tips were used to scratch the center of the well. Images were captured using the microscope and camera system described above at time points described in each figure. Healing distances were measured using Motic camera software following the manufacturer’s instructions.

**Cell proliferation assay** -- Cell proliferation was determined by MTS [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega), following the manufacturer’s instructions. Absorbance was measured on a Synergy H1 microplate reader (BioTek) at 490nm.

**Soft-agar colony formation assay** – 3-dimensional in vitro colony formation was performed using the EMD Millipore Transformation assay kits (including agarose, Cell Staining Solution, and Cell Quantification Solution) following the manufacturer’s protocol. 1500 cells per well of a 24-well plate were grown in 0.25ml of 0.15% agarose in complete media. 0.25ml complete media was replenished every 3 days for a total of 2 weeks culture in a 37°C cell incubator. Colonies’ morphology was captured using a microscope as described above. Then cells in agarose were either stained with 0.5ml of Cell Stain Solution overnight for counting colonies or 0.08ml of Cell Quantification Solution for quantification by measuring absorbance at 490nm using Synergy H1 microplate reader (BioTek).
**Immunohistochemistry (IHC)** – IHC staining was performed at Dermpathology Lab of Central State (DLCS, Dayton, OH) following the universal protocol applied in that lab. Clinical samples were formaldehyde fixated in hospitals and collected at DLCS from all over the United States. Samples ID were assigned at DLCS. Fixed tissues were then paraffin-embedded, sectioning on a rotary microtome, and stained. Staining was performed on BenchMark ULTRA platform (Roche) using an Automated IHC/ISH slide staining Module with UltraView Red (v1.02.0018) procedure for hematoxylin & eosin staining (H&E, Roche VENTANA HE 600), or desired first Ab and ultraView Universal Alkaline Phosphatase Red Detection Kit staining (Roche VENTANA). Images were captured in the lab using a Leica microscope and software system following the manufacturer’s protocol.

**Genotyping** – 8 to 10 5µm sections were taken from clinical tissue specimens at DLCS. Paraffin was removed by rinsing briefly with 1ml of xylene. Xylene was removed by rinsing with 100% ethanol (Fisher Chemical). Samples were used for DNA extraction by QIAamp DNA FFPE Tissue Kit (Qiagen) following the manufacturer’s protocol. BRAFV600E mutation detection of extracted DNA was performed on the QuantStudio 7 Flex Real-Time PCR System platform using TaqMan® Mutation Detection Assays and TaqMan® Gene Expression Master Mix (Applied Biosystems) following the manufacturer’s protocols and software for analysis.

**Immunofluorescence (IF)** – Cells were cultured on coverslips in 24-well plate and were fixed with 4% paraformaldehyde for 10 -15 min and permeabilized with 0.15-0.3% PBST (phosphate buffered saline with Tween-20). 10% NGS (normal goat serum) was used for blocking samples
and dilution of Ab before an overnight primary Ab binding at 4°C. Goat anti-rabbit IgG (H+L) Alexa Fluor® 555 conjugated and anti-mouse 488 conjugated secondary Ab (A-11034, A28180, Invitrogen) were incubated for 30 min at room temperature. 1µl/ml DAPI (Invitrogen) diluted in dH₂O was used for nuclear staining and then mounted with antifade reagent (D1306, Invitrogen). Coverslips were sealed on slides and observed under Leica DMi8 Fluorescence Imaging microscope following the manufacturer’s software and protocol. All solutions are purchased from Thermo Fisher.

**Drug tolerance assay** – Cells were cultured overnight in 96-well plate in complete medium before drug treatment. Desired gradient concentrations of vemurafenib (Selleckchem, 10mM) were mixed with 0.1ml/well complete media and this replaced the old media. Cell viability was determined after 3 days culture in a 37°C incubator using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega), following the manufacturer’s instructions. Absorbance was measured by Synergy H1 microplate reader (BioTek) at 490nm. IC₅₀ was calculated from the absorbance trend line and used for determining tolerance differences between conditions.

**Microarray** – Total RNA was extracted using Trizol® reagent (Ambion) three days post-siRNA-transfection. RNA quality was assessed on an Agilent 2100 Bioanalyzer following the manufacturer’s protocol. Samples with RNA integrity numbers (RIN) >6 were used for further analysis. RNA concentration was measured by NanoDrop™ One Spectrophotometer (Thermo Fisher Scientific). Affymetrix microarray system was used for gene expression analysis using Clariom™ S Pico Assay (human) platform with WT PLUS Kit and GeneChip® Hybridization, Wash,
and Stain Kit following the manufacturer’s protocols. Data were analyzed using the manufacturer’s software Transcriptome Analysis Console (TAC) 3.0 and its protocol.

**Statistics** – All quantified data were expressed as a mean plus with standard deviation (S.D.). Significance was quantified by the Two-tailed Student’s t-test where P value less than 0.05 was considered as statistically significant. Asterisks are used here to signify p-values as follows:

P<0.05 (*), P<0.001 (**), P<0.0001 (***), and P<0.00001 (****)
III. RESULTS

AIM I. BRAF-ERK3 regulation pathway

BRAF expression levels correlate with ERK3 expression.

We first confirmed that ERK3 upregulated by BRAF and that this is a general phenomenon across cancer types. The embryonic kidney human 293T cell line represents the non-cancer cells, while A375 (with mutant BRAF^{V600E}) and WM3211 (BRAF WT) are melanoma cell lines, and HeLa is cervical cancer (data not shown). Overexpression of the BRAF construct by transient transfection in different cell lines was able to upregulate ERK3 protein expression (Fig 1A). Similarly, the same phenomenon was observed when BRAF^{V600E} was overexpressed. However, BRAF^{V600E} doesn’t induce dramatically greater expression of ERK3 than wild type BRAF does (Fig 1A, left A375 cell line). In contrast, ERK3 upregulation is correlated more closely to cell line the BRAF total protein expression level. Comparing BRAF and BRAF^{V600E} overexpression levels (Fig 1A), higher total BRAF protein level appears to show more upregulation of ERK3. This suggests that ERK3 regulation by BRAF is independent of BRAF^{V600E} mutation status.

Conversely, the BRAF-ERK3 regulation was further confirmed in different types of melanoma cell lines by BRAF knockdown. Cutaneous melanoma cell line A375 (BRAF^{V600E}), uveal melanoma OCM3 (BRAF^{V600E}), and BRAF WT primary melanoma WM3211 were treated with siRNA against BRAF. This was able to reduce ERK3 level (Fig 1B). The reduction level of ERK3 was also correlated to BRAF total protein knockdown efficiency but not mutation status or cell types.
Figure 1 BRAF expression levels correlate with ERK3 expression.

(A) Expression plasmids for wild-type BRAF or constitutively active BRAF<sup>V600E</sup> were transiently transfected into melanoma cell lines A375 (BRAF<sup>V600E</sup>) and WM3211 (BRAF Wild Type) or embryonic kidney non-cancer cell line 293T (BRAF WT). The Western blotting analysis reveals an increase in ERK3 protein concomitant with increased BRAF. (B) Western blot analysis of ERK3 reduction correlating with knockdown of BRAF by siRNA in three different melanoma cell lines A375, WM3211, and uveal BRAF<sup>V600E</sup> melanoma OCM3. Beta actin serves as a loading control.
BRAF regulates ERK3 through both mRNA expression and protein stability

Given that ERK3 expression was more affected by total BRAF expression than by BRAF mutation status, we next moved to further define the regulatory mechanism. RT-qPCR indicated that ERK3 mRNA expression level was significantly reduced (P<0.05) after BRAF knockdown in different melanoma cell lines independent of BRAF\textsuperscript{V600E} mutation status (Fig 2A).

Meanwhile, previous studies have shown that ERK3 is an unstable protein which is rapidly targeted for ubiquitin-mediated degradation.\textsuperscript{33} We thereby performed protein stability assays using the protein synthesis inhibitor cycloheximide (CHX) in three different melanoma cell lines (Fig 2B, 2C, 2D, upper panels). All cell lines indicated a similar regulatory effect of BRAF on ERK3 protein stability. In response to BRAF knockdown, the dramatic decrease of ERK3 protein half-life indicated that BRAF stabilizes the ERK3 protein. In all melanoma cell lines, ERK3 protein half-life dropped from 1-2 hours to less than 0.5 hour as calculated by protein degradation trend lines (Fig 2B, 2C, 2D, lower panels). Therefore, we are confident that besides transcriptional regulation, BRAF stabilizes ERK3 protein by an unclear mechanism, also contributing to upregulation of ERK3. Notably, ERK3 half-life in melanoma cell lines A375, OCM3, and WM3211 are dramatically less than 293T independent of BRAF\textsuperscript{V600E} mutation status.
Figure 2 BRAF regulates ERK3 through both mRNA expression and protein stability.

(A) RT-qPCR was used to measure ERK3 mRNA levels in three melanoma cell lines A375, OCM3, and WM3211. BRAF was knocked down by siRNA for 2 days. Reduced ERK3 mRNA expression corresponds to the reduction in BRAF. Significance (p-values) were determined by Student’s t-test and expressed as mean ± SD (n=3). (B) Cycloheximide (CHX) protein stability assay indicates ERK3 protein stability reduction in A375, OCM3, and WM3211 in response to BRAF reduction. Western blots indicate ERK3 protein degradation after 100ug/ml protein translation inhibitor CHX treatment for 0, 0.5, 1, 1.5 and 2 hours. For densitometry analysis in IQTL software, the ERK3 level at each time point was first normalized to beta actin in each lane, then to ERK3 levels at 0 hour of siBRAF or siCtrl. These normalizing controls were set as 100% representing the initial protein levels respectively. Western blots and degradation trend-lines are representative of multiple experiments and expressed as mean ± SD (n>3). Protein half-life was calculated from the corresponding exponential trendline equation.
**BRAF kinase activity is required for control of ERK3 transcription but not protein stability.**

BRAF is a serine/threonine kinase. Following the discovery of two possible BRAF-ERK3 regulation mechanisms, we further explored whether these two mechanisms depend on BRAF kinase activity or not. Vemurafenib is a BRAF\textsuperscript{V600E} mutation-specific targeting drug. We used vemurafenib to inhibit BRAF\textsuperscript{V600E} kinase activity which was verified by monitoring phosphorylation of the BRAF downstream target ERK1/2 (pERK1/2).\textsuperscript{18} 2\,\mu M of vemurafenib is able to remove BRAF kinase activity up to 36h post-treatment (Fig 3A). We observed a significant reduction of ERK3 protein as well as mRNA (P<0.05) (Figure 3A and B). Interestingly, an increase in ERK3 protein is observed in the first several hours’ post DMSO treatment. It is apparent that DMSO, the solvent used for vemurafenib and our vehicle control, induces ERK3 expression by an unknown mechanism (Fig 3A, Fig S1). Over a detailed time course, a similar pattern of ERK3 expression was observed in the vemurafenib treatment group (Fig S1). We thus conclude that this phenomenon is due to the same stimulation by the solvent DMSO. Although the mechanism of ERK3 increasing by this stimulation is unknown, the side effect of DMSO indicated that ERK3 translation was not impacted by the removal of BRAF kinase activity. ERK3 was still able to be translated from the remaining mRNA left in the cell prior to kinase inhibition. However, inhibited BRAF kinase activity prevented further ERK3 mRNA transcription. Thus, combined with the degradation of the remaining mRNA by 24 hours post-inhibition, ERK3 drops in levels of both protein and mRNA.

For further analysis, A375 cells were used for protein stability assays in the presence or absence of vemurafenib (Fig 3C). BRAF kinase activity is able to be removed immediately, within 5 minutes post-treatment (data not shown). Accordingly, co-treatment of CHX with vemurafenib was monitored over 2 hours. ERK3 protein stability was not affected by the inactivation of BRAF.
kinase activity, demonstrating that BRAF kinase activity is not critically required for the stabilization of ERK3. Notably, the previous study showed that BAY 43-9006 (Sorafenib, a RAF kinase activity inhibitor) was able to reduce ERK3 protein half-life as CHX did. Their study thus concluded that BRAF kinase activity is required for ERK3 stabilization in A375 cell. However, BAY 43-9006 is a less-specific type II kinase inhibitor (inactive conformation binder) for B-RAF (V600E or WT), C-RAF and other several kinases. Vemurafenib, which we used, is a type I kinase inhibitor which binds to the active DFG motif to specifically prevent ATP binding to BRAF\textsuperscript{V600E}. Our vemurafenib study suggested that BRAF kinase activity is not required for ERK3 stabilization. Comparing to their results, we consider that ERK3 protein stability might be impacted by other RAF kinase activity or kinase pathways. We don’t exclude the possibility of ERK3 regulation by other kinases but can conclude that BRAF kinase activity is not critical for regulation of ERK3. Further study is warranted.

However, BRAF kinase activity does seem to be required for phosphorylation of ERK3. We observed a reduction of pERK3 in BRAF-KD overexpressing cells compared even to the pCDNA3 control group (data not shown). Further examination needs to be done to confirm or refute this finding.
A375

**A**

<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>Vemurafenib</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERK3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRAF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pERK1/2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(T202, Y204)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**B**

Gene expression fold change

- BRAF: DMSO 1.00, Vemurafenib 1.00
- ERK3: DMSO 0.44, Vemurafenib 0.44

**C**

Time (hours) | DMSO | Vemurafenib | CHX |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0, 0.5, 1, 1.5, 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERK3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRAF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pERK1/2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(T202, Y204)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- DMSO
- Vemurafenib

Remaining Protein Level

- DMSO
- Vemurafenib
**Figure 3** BRAF kinase activity is required for control of ERK3 transcription but not protein stability.

(A) Western blot of ERK3 regulation by kinase inhibited BRAF. A375 cells were treated with 2µM vemurafenib (Vemu) to inhibit BRAF activity and compared to control cells treated with the vehicle DMSO. (B) RT-qPCR analysis of ERK3 mRNA level changes in response to BRAF kinase activity inhibition. ERK3 or BRAF mRNA levels of Vemu or DMSO treated A375 cells were normalize to DMSO controls. Significance levels (p-values) were determined by Student’s t-test and expressed as a mean ± SD (n=3). (C) ERK3 protein stability is not impacted by BRAF kinase activity inhibition. Cycloheximide (CHX) treatment was performed in A375 cell lines simultaneously in vemurafenib or DMSO groups at 0, 0.5, 1, 1.5 and 2 hours. Western blots and degradation trend-line are representatives of three experiments and expressed as a mean ± SD (n=3). Beta actin serves as a loading control.
**BRAF stabilizes ERK3 protein without requiring BRAF kinase activity**

To further confirm the impact of BRAF regulation on ERK3, we generated a single point mutation BRAF kinase dead (KD) construct with the residue 594 mutated from aspartic acid (D) to alanine (A) in the activation loop of BRAF C2 domain.\(^{32}\) This mutation was identified from cancer patient\(^{35}\) and its BRAF kinase dead phenotype was verified by overexpression in 293T, A375 (Fig 4A, B), WM3211 and HeLa cells (data not shown) without the phosphorylation of its downstream target ERK1/2 at T202 and Y204 residues. A375 cell line didn’t induce a dramatic upregulation of pERK1/2. This may due to the highly phosphorylated endogenous ERK1/2 in BRAF\(^{V600E}\) cell line. In concordance with our previous results, BRAF-KD overexpression was able to up-regulate ERK3 the same as BRAF WT and BRAF\(^{V600E}\) did in all tested cell lines. However, all of the overexpression BRAF constructs in 293T (Fig 4C) and A375 (data not shown) were not able to induce significant upregulation in ERK3 mRNA level. We would expect this phenotype is due to the high endogenous BRAF level in these cells. As a result, overexpression of BRAF was not able to significantly affect ERK3 through its mRNA expression level. In contrast, the increased BRAF level in all three overexpression condition was able to directly increase ERK3 protein stability through a potential protein-protein interaction independent of its kinase activity. CHX protein stability assays demonstrate the fact that BRAF-KD overexpression is able to dramatically extend ERK3 half-life from around 2 hours up to 3.6 hours (Fig 4D). We thus further confirmed that ERK3 is an unstable protein that is up-regulated by BRAF through a kinase-independent effect on of its protein stability probably depending on a protein-protein interaction mechanism. Immunofluorescence stain confirmed a close co-localization of BRAF and ERK3 in all melanoma cells (further discussion in the Discussion section below) (Fig S5).
Figure 4 BRAF stabilizes ERK3 protein without requiring BRAF kinase activity.

(A, B) Western blotting analysis of ERK3 level by BRAF (WT), BRAF<sup>V600E</sup> and BRAF kinase-dead (KD) plasmids transiently overexpression for 2 days in (A) non-melanoma cell 293T or (B) A375. All three BRAF constructs are all able to induce ERK3 levels compared to pcDNA3 control in both cell lines. (C) RT-qPCR analysis of ERK3 mRNA level in 293T cells with BRAF (WT), BRAF<sup>V600E</sup> and BRAFKD overexpression at 1.5 days post-transfection indicates that ERK3 mRNA up-regulation levels are not affected by BRAF kinase mutations status. Each overexpression condition was normalized to the pcDNA3 empty vector control expressed as a mean ± SD (n=3). Significance levels (p-values) were determined by Student’s t-test. (D) BRAF-KD overexpression in 293T results in ERK3 protein stabilization. CHX treatment was performed at 2 days post-transfection of pcDNA3 control or BRAF-KD. Western blots and degradation trend-line are representatives of multiple experiments expressed as a mean ± SD (n=3). Beta actin or GAPDH serves as a loading control.
BRAF-ERK3 regulation model

In conclusion, we established the general BRAF-ERK3 regulation model that (Fig 5): 1) BRAF transcriptionally promotes ERK3 mRNA expression through its kinase activity; 2) BRAF stabilizes ERK3 protein in a kinase-independent manner; 3) Both mechanisms are not melanoma specific; 4) Kinase activity of BRAF is likely not required for ERK3 translation; 4) BRAF stabilization of ERK3 is a major mechanism for upregulation ERK3.

![BRAF-ERK3 regulation model diagram](image)

*Figure 5 BRAF-ERK3 regulation model.*

*BRAF kinase activity is required for BRAF to transcriptionally upregulate ERK3. At the same time, BRAF protein is able to stabilize ERK3 protein by an unknown mechanism, independent of its kinase activity. Both mechanisms are common in all analyzed cell types.*
AIM II. ERK3 functions in melanoma

Knockdown of ERK3 promotes skin cancer cell migration independent of cell type or BRAF<sup>V600E</sup> mutation status.

ERK3 knockdown promotes skin cancer cell line migration in our study, including BRAF<sup>V600E</sup> positive melanomas A375 and OCM3 (Fig 6A), BRAF WT melanomas WM3211, B16F10 (Fig 6B), and A431 (Fig 7). The ability of ERK3 to inhibit migration across cell lines with active and wild type BRAF indicates this effect is not dependent on BRAF status, which is consistent with Aim I.

Meanwhile, A375 and WM3211 are human cutaneous melanomas, whereas OCM3 is a uveal melanoma. B16F10 is a murine cutaneous melanoma, and A431 is a human squamous cell carcinoma. The consistency across these cancer lines suggests that the effect of ERK3 on migration is a more general phenomenon, not limited to human cutaneous melanomas. This phenotype is not impacted by whether it is transient knockdown by siRNA or long-term by lentivirus shRNA knockdown of ERK3 (Fig 6C), or by targeting different regions of ERK3 by siRNA/shRNA (data not shown).

Considering that the trans-well migration system assays require a serum starvation condition by which cells such that cells are placed in serum-free media in the upper insert and attracted by outer insert complete media with 10% serum chemoattraction in the outer insert. For the sake of remove nutrition effect. To address concerns that the serum-free condition could interfere with the normal migration of cells, we therefore further performed scratch healing assays simultaneously in which a lawn of cells is allowed to refill a gap left by scraping away cells from the plate, all in the presence of complete medium (Fig 6D). Under the complete media condition,
ERK3 knockdown consistently promoted migration in this assay, consistent with transwell migration findings.
Figure 6 Knock-down of ERK3 promotes melanoma cells migration.

(A, B, C) Transwell migration assays were performed 2 days post-transfection with siERK3 in (A) BRAF\(^{V600E}\) human melanoma cell lines A375 and OCM3, and (B) human WM3211 and murine B16F10 BRAF WT melanoma cell lines, or using (C) A375 by shRNA stably depleted using a lentiviral system targeting the ERK3 coding region with shGIPZ as empty vehicle control. Transwell migration was consistent with transient knockdowns. Knockdown efficiencies were verified by western blots. Migration was quantified by two-chamber transwell migration assays. Migrated cell numbers were quantified from an average of 6 captured fields (20x magnification) per insert for two technical replicates per biological replicate (n>3) after 8 to 12h depending on the cell line. Presented captures were from the replicate with cell number closest to the average. Significance levels (p-values) were determined by Student’s t-test and expressed as mean ± SD. (D) Stable shERK3 A375 were used for scratch healing assays. Healing distances were measured under a 4x magnification microscope field with 6 wells per cell line after 24h. Presented captures were from the one with cell number most close to the average. Significance levels (p-values) were determined by Student’s t-test and expressed as mean ± SD (n=6).
**ERK3 regulation of tumor cell migration depends on cancer types**

To further verify if this regulation of migration by ERK3 is specific to the skin cancers or broadly applied to epithelial cancers, we performed the same trans-well migration assay in non-small cell lung adenocarcinoma H23. As was found in early publications in other lung cancer cell lines H1299 and A549, ERK3 also promotes migration in lung adenocarcinoma H23 cells (Fig 7). However, based on the skin cancer cell line examined in our experiment, we conclude that ERK3 inhibits skin cancer migration in general.
Figure 7 ERK3 regulation of tumor cell migration depends on cancer types.

Transwell migration assays were performed in squamous cell carcinoma A431 and non-small cell lung adenocarcinoma H23. ERK3 was transiently knocked down by siRNA for 2 days. Knockdown efficiencies were verified by western blots and migration was measured by transwell migration assays as described previously. Migrated cell numbers were quantified from an average of 6 captured fields (20x magnification) per insert for two technical replicates per biological replicate (n=2) after 12h. Presented captures were from the replicate with cell number closest to the average. Significance levels (p-values) were determined by Student’s t-test and expressed as mean ± SD.
ERK3 inhibits melanoma cell invasion

These melanoma studies have yielded results that conflict with previous findings of the role of ERK3 in lung cancer. We are also interesting to know whether ERK3 functions in other aspects of melanoma progression. Transwell invasion assays (Fig 8) were used to mimic in vitro cell invasion. Matrigel was coated on transwell insert membrane to mimic the extracellular matrix condition. Besides increased migratory potential, cancer cells develop an increased ability to digest the extracellular matrix. This assay model that ability by requiring cells to digest matrigel in order to migrate across the transwell insert. Knockdown of ERK3 in A375, OCM3, and WM3211 promote cell invasion (Fig 8A), while stable overexpression of ERK3 in WM3211 decreased invasion compared to its CDH lentiviral backbone control (Fig 8B). These results consistently suggest that ERK3 functions as a suppressor of cancer invasion in vitro.
Figure 8 Knock-down of ERK3 in melanoma promotes cell two-chamber transwell invasion independent of BRAFV600E mutation status.

Transwell invasion assays in (A) BRAF\textsuperscript{V600E} cell lines A375 and OCM3 and (B, left) BRAF WT cell line WM3211. ERK3 was transiently knocked down by siRNA for 2 days in A375, OCM3 and WM3211 cells or stably overexpressed ERK3 in WM3211 prior to transwell invasion assays. ERK3 knockdown or overexpression efficiency was verified by western blot. The lentiviral ERK3 overexpression construct expresses ERK3 with a Myc tag, which was indicated by the larger molecular size compared to CDH lentiviral backbone only expressing endogenous ERK3. Migrated cell numbers were quantified from an average of 6 captured fields (20x magnification) per insert for two technical replicates per biological replicate (n>2) after 16-24h depending on cell lines. Presented captures were from the replicate with cell number closest to the average. Significance levels (p-values) were determined by Student’s t-test and expressed as mean ± SD.
**ERK3 inhibits melanoma cell proliferation independent of BRAF mutation status.**

In order to determine whether the ERK3 expression has an effect on the proliferation of melanoma cell lines, a BRAF\textsuperscript{V600E} cell line (A375) and WT cell line (WM3211) were used for MTT assays. A stable expression of shERK3 was able to efficiently reduce ERK3 expression (Figure 9A, bottom panel). Over the six-day course of the assay, cells depleted of ERK3 proliferated significantly more than the shGIPZ control cells (Figure 9A, top panel). Conversely, when exogenous ERK3 was overexpressed in WM3211, there was a dramatic inhibition of proliferation (Figure 9B).
Figure 9 ERK3 inhibits melanoma cell proliferation independent of BRAF mutation status.

MTT cell proliferation assays were performed in (A) A375 cells with ERK3 depleted by a lentiviral shRNA system with the empty lentiviral construct shGIPZ serving as a control, and (B) WM3211 cells stably overexpressing ERK3. Plasmids expression efficiencies were verified by western blots. The ERK3 overexpression lentiviral system expresses ERK3 with a Myc tag which was indicated by the upper band and larger molecular size compared to the empty expression construct CDH.

Significance levels were determined by Student's T-test of the average of 4 times experiments and expressed as mean ± SD. Asterisks indicate P<0.05 (*), P<0.001 (**), P<0.0001 (***), and P<0.00001 (****)
Knock-down of ERK3 in melanoma promotes soft agar colony formation.

To further verify whether ERK3 is able to suppress melanoma tumor formation, soft-agar colony formation assays were used to mimic vitro tumor formation condition (Fig 10). Soft agar colony formation clearly showed ERK3 knockdown in A375 tended to push the cells toward an aggressive phenotype. Under the 20x microscope (Fig 10A), colony morphology tended to be more irregular, spreading and diffusing throughout the agar instead of forming a smooth tumor surface, underlining the invasive tumor stage. Although the knockdown of ERK3 only slightly increased cell proliferation in 2-dimensional surface culture conditions (Fig 9A), colonies increased in size and number dramatically in 3-dimensional agar condition. This indicates more clinical relevance to real tumor progression.
Figure 10 Knock-down of ERK3 promotes melanoma soft agar colony formation.

A375 ERK3 stable knock-down using a lentiviral system and its shGIPZ control cell lines were grown in soft agar to mimic in vivo growth for 2 weeks. (A) Tumor morphology captured under a 4x microscope (left) and proliferation measured after staining with Cell Quantification solution (provided with kids) (right) were affected by ERK3 depletion. ERK3 expression was verified by western blot. (B) Colonies were quantified after staining with Cell Staining Solution (provided with kids) (left), and colony size distribution was measured using IQTL software (right). Colony formation ability was determined by the average of 5 experiments with 4 technical replicates each time. Presented captures were from the replicate with cell number, morphology, and colony size closest to the average. Significance levels (p-values) were determined by Student’s t-test and expressed as mean ± SD.
Reduced migration resulting from knockdown of BRAF is an equilibrium between BRAF promotion and ERK3 inhibition of melanoma metastasis.

We hypothesized that the moderate decrease of cell migration under BRAF inhibition represents an equilibrium with the migration promoting effect by ERK3. To verify this hypothesis, we removed ERK3 using a stable shERK3 A375 cell line (Figure 11). As we expected, shERK3 by itself is able to dramatically increase migration ($P=2*10^{-11}$). Knockdown of BRAF by siRNA has a moderate impact on migration, decreasing it 1.3-fold ($P=5.4*10^{-4}$). However, when ERK3 is removed and BRAF knocked down, the decrease in migration is even more dramatic (2-fold, $P=2.8*10^{-9}$). These results suggest that removal of the anti-migration effect of ERK3 is synergistic with the inhibition of BRAF kinase activity. More experiments are needed to confirm this conclusion.
Figure 11 Reduced migration resulting from knockdown of BRAF is an equilibrium between BRAF promotion and ERK3 inhibition of melanoma metastasis.

(A) Transwell migration assays were performed in A375 stably expressing lentiviral shRNA against ERK3 or the control expressing only the lentiviral construct shGIPZ. At the same time, these cells were transiently transfected with siRNA against BRAF or a control siRNA. Transwell assays were otherwise performed and quantified as previously described. Migrated cell numbers were quantified from an average of 6 captured fields (20x magnification) per insert for two technical replicates per biological replicate (n=2) after 8h. Presented captures were from the replicate with cell number closest to the average. Significance levels (p-values) were determined by Student’s t-test and expressed as mean ± SD. (B) ERK3 or BRAF knockdown efficiency was verified by western blotting.
AIM III. The clinical relevance of ERK3 in melanoma

Clinically, BRAF<sub>V600E</sub> mutation incidence is greater in benign melanocytic lesions than in melanoma and is not co-related with ERK3 expression level.

To identify the clinical relevance of ERK3 with melanoma progression and BRAF<sub>V600E</sub> mutation status, we retrieved the clinical specimens from DLCS. The BRAF<sub>V600E</sub> mutation was detected in 19 out of total 21 benign samples but only 14 out of 57 melanomas (Fig 12C). BRAF<sub>V600E</sub> mutation incidence is lower in advanced melanomas, which has been observed previously. We hypothesize that this may due to the senescence induced by when BRAF is activated by mutation without additional oncogenic mutations. This hypothesis fits into our finding that ERK3 acts as a tumor suppressor which will be upregulated by overexpressed or mutated BRAF. BRAF protein is clinically overexpressed in melanoma compared to benign. We therefore further hypothesize that only the tumor which develops a mechanism to limit ERK3 can overcome this senescence to undergo progression. We believe the induction and stabilization of ERK3 by active BRAF in melanomas is somehow bypassed, such that it no longer has a controlling role in inhibiting metastasis. Indeed, we only sometimes observed slightly increased expression of ERK3 in metastatic melanomas (Fig 12, upper IHC panel) but the level is not correlated with BRAF mutation status, tumor size, and Clark’s level (Fig 12, lower table).
Figure 12Clinically, BRAFV600E mutation incidences are reduced during melanoma progression and ERK3 levels do not relate to BRAFV600E mutation status.

(A and B) IHC staining of ERK3 in clinical (A) benign and (B) melanoma samples. Pink staining indicates expression of ERK3 (up panel). Each specimen medical record was obtained from DLCS and BRAFV600E mutation status was detected from the extracted DNA of each sample (lower panel). (C) Genotyping of results BRAFV600E mutation analysis in a total 21 benign and 57 melanoma clinical specimens (DLCS).
In contrast to lung cancer, reduced ERK3 level in melanoma is a positive prognostic

Because we hypothesize a flipped role for ERK3 in melanoma compared to what has been observed in lung adenocarcinoma, we compared clinical data for these two cancers. Survival data for patients in The Cancer Genome Atlas LUAD (lung adenocarcinoma) and SKCM (cutaneous melanoma) datasets are consistent with this difference. High expression of ERK3 correlated to worse prognosis in lung cancer, but was a positive indicator in melanoma (Fig 13 A and B). Further evidence of reduced ERK3 with melanoma progression is seen in the microarray data produced by Talantov et al., (Fig 13C). ERK3 is significantly reduced in melanomas as compared to normal skin or benign melanocytic lesions, consistent with our IHC results. These data support our assertion that ERK3 functions as a tumor suppressor in melanoma despite acting as an oncogene in lung cancer.
Figure 13 ERK3 level is reduced with melanoma progression.

Kaplan plot for MAPK6 in (A) LUAD (lung adenocarcinoma) and (B) SKCM (skin cutaneous melanoma). Lung cancers with the lowest 10% of ERK3 expression vs. those with the highest ERK3 expression indicate higher expression of ERK3 is significantly worse for survival (Log-rank p-value=0.0203, n=49). Melanomas with lowest 10% of ERK3 expression vs. those with highest ERK3 expression indicates that higher expression is better for survival (p-value=0.167, n=45).

Plots were made in OncoLnc from TCGA survival data. http://www.oncolnc.org/. (C) MAPK6 expression in the Talantov Melanoma data set was measured by human genome 133A
microarrays (Affymetrix). Normal skin (n=7) is compared to benign melanocytic nevi (n=18) and cutaneous melanoma (n=45). Overall average genes expression level was set as 0 and ERK3 expression level was normalized to the average level. Melanomas had a significantly lower ERK3 expression (p=2.56*10^-8) but the difference between benign nevi and normal skin was insignificant (p=0.781). The gene expression reporter is Affymetrix probe 207121_s_at. Plots indicate the 25th-75th percentiles (boxes), minimum and maximum values (whiskers), and the median (horizontal line) of gene expression.
IV. DISCUSSION

Because ERK3 functions as an oncogene in many other types of cancer, we were also concerned with ERK3 cellular functions in melanoma. We surprisingly found that ERK3 functions entirely opposite to its known roll in all the other studied cancer types. ERK3 knockdown promotes skin cancer cell line migration and other cellular functions of melanoma in our study.

BRAF is expressed in many melanomas, and we have proved that ERK3 is able to be upregulated by BRAF. However, BRAF\textsuperscript{V600E} kinase activity inhibition by vemurafenib is able to dramatically reduce cell migration as well as other cellular functions such as proliferation. Therefore vemurafenib is widely used as a BRAF\textsuperscript{V600E} targeted therapy clinically. Thus clinical, as well as cellular study results, seem to conflict with the tumor suppressive effect we observe for ERK3 in melanoma.

In order to explain that, we have shown that ERK3 upregulation by BRAF is partly through a kinase-independent stabilization effect. As a result, simple inhibition of BRAF kinase activity is not able to dramatically decrease level as we demonstrate in Fig 3 and 4. In vitro, complete knockdown of BRAF results in cell death so we are unable to establish stable shBRAF cell lines (data not shown). It is thus hard to achieve strong downregulation of ERK3 when BRAF is still present (even if inactive). As a result, the moderate decrease of cell migration under BRAF inhibition represents an equilibrium with the migration promoting effect by ERK3. Only after we remove the ERK3 effect in melanoma, we then are able to observe the full BRAF effect on cell migration. We thus used stable ERK3 knockdown cell line A375 shERK3 (Fig 11) for further migration study. As we expected, BRAF inhibition by siRNA was able to induce a more dramatic
decrease of cell mobility without ERK3 present. The result perfectly supports our hypothesis and all findings of BRAF-ERK3 regulation and ERK3 cellular functions in melanoma.

Meanwhile, in lung cancer, ERK3 plays a major role in cell migration but less effect on proliferation (data not shown). In order to determine whether the ERK3 expression has an effect on the proliferation of melanoma cell lines, the BRAF^{V600E} cell line (A375) and WT cell line (WM3211) were used for MTT assays. A stable expression of shERK3 was able to efficiently reduce ERK3 expression (Figure 9A, bottom panel). Over the six-day course of the assay, cells depleted of ERK3 proliferated significantly more than the shGIPZ control cells (Figure 9A, top panel). Conversely, when exogenous ERK3 was overexpressed in WM3211, there was a dramatic inhibition of proliferation (Figure 9B).

In conclusion, the results of this thesis are summarized:

- ERK3 is regulated at the transcriptional level by BRAF in a kinase-dependent manner.
- ERK3 protein is stabilized by BRAF in a kinase-independent manner.
- ERK3 functions as a suppressor of cell migration, invasion, proliferation, and soft agar colony formation. This was consistent across all tested melanoma cell lines (BRAF mutated and wild type, human and mouse), and observed in non-transformed cells and a squamous cell carcinoma. However, it is not the case in lung cancer.
- Clinically, patients with the highest expression of ERK3 have a better prognosis than patients with the lowest ERK3. This is counter to findings in lung cancer.
• Clinical melanoma specimens express lower levels of ERK3 than normal skin or benign melanocytic nevi.

• Finally, restoring ERK3 level is a potential therapeutic goal for treatment of melanoma.
V. SUPPLEMENTAL RESULTS AND FUTURE DIRECTIONS

Reduction of BRAF stabilization of ERK3 might be a key mechanism for melanoma to reduce ERK3 during tumor progression.

A recent report suggested that BRAF\textsuperscript{V600E} is able to bind with Rictor in the mTOR complex\textsubscript{2} (mTORC2) to negatively regulate AKT.\textsuperscript{41} Accordingly, our preliminary data found a BRAF kinase-independent protein stabilization regulation of ERK3 and our co-IP data suggested that ERK3-BRAF might also directly interact with the mTORC2 complex. Additionally, ERK3 transient knockdown in melanoma seems correlated with pAKT up-regulation (data not shown). We examined the possibility that BRAF-ERK3-mTORC2 is able to form a complex by co-IP. In 293T cells, BRAF was able to be pulled down as part of a Rictor-sin 1 complex in mROTC2 (Fig S2A left panel) while ERK3 was hard to identify due to the non-specific band in the similar position of IgG control loading. However, ERK3 conversely was able to pull down Rictor and BRAF in 293T (Fig S2A left panel). The direct ERK3-BRAF pull down was weak compared to BRAF binding to Rictor. It suggests that Rictor might be a mediator between ERK3 and BRAF in the same complex, which could play a role in the BRAF stabilization of ERK3. However, this BRAF-ERK3 binding interaction was only confirmed in non-melanoma 293T cells with BRAF WT. The strength of co-IP in BRAF\textsuperscript{V600E} A375 cells indicates that melanoma might decrease the BRAF-ERK3 protein-protein interaction either due to the BRAF mutation or in a cell type-specific manner (Fig S2B). Notably, the half-life of ERK3 in A375 is the shortest observed in all tested cell lines (less than 1 hour), while other melanomas demonstrated ERK half-lives of 1 to 2 hours (Fig 2B, C, D), and non-melanomas 293T cells 2 to 4 hours (Fig 4D). Taken together, we consider that melanoma may actually have a reduced BRAF stabilization effect on ERK3, and this could be one of the possible
mechanism for melanoma to suppress the ERK3 overexpression caused by BRAF hyperactivation. To further address our hypothesis, CHX protein stability assays could be used for testing additional cell lines of different cancer, especially expanding the comparison of lung cancer and melanoma. Moreover, co-IP experiments in A375 (BRAFV600E) and WM3211 (BRAF WT) would be a good model to confirm whether the mutation of BRAF is disrupting the interaction between BRAF and ERK3. More experiments could be designed for driving our hypothesis if the phenomenon can be verified by co-IP.

**ERK3 doesn't feedback to regulate BRAF or its signaling and doesn't activate another pathway to induce vemurafenib resistance**

In the clinic, BRAFV600E melanoma patients soon develop acquired resistance to the drug. Recent reports reveal one mechanism where the drug treatment forces melanoma cells to reactivate ERK signaling despite ongoing inhibition of mutant BRAF.42, 43 We performed simple tests to exclude the possibility that decreased ERK3 expression along with BRAF inhibition by vemurafenib could represent a means to reactivate MAP kinase signaling. However, stable knockdown of ERK3 in A375 cell did not show effects on BRAF protein level, mRNA expression or its downstream ERK1/2 signaling (Fig S3). Similarly, vemurafenib remains able to impact the viability of shERK3 cells at the same IC50 (Fig S4). Therefore we conclude that, although BRAF has a strong impact on ERK3 expression, ERK3 level does not, in turn, affect BRAF levels. BRAF activity and vemurafenib sensitivity remain unchanged. Decreasing ERK3 level is not the mechanism for ERK1/2 reactivation leading to vemurafenib resistance.
In the future, we could perform migration and proliferation assays as before to monitor the effect of BRAF knockdown in A375shERK3 cells. Vemurafenib could also be used for BRAF kinase inhibition in A375shERK3 cells. The cell proliferation and vemurafenib IC\textsubscript{50} tests can be used for deciding whether the effects of BRAF kinase inhibition are a combined result of ERK3 and BRAF effects.

**BRAF and ERK3 are strongly colocalized in melanoma cells and may affect each other’s cytosol distribution**

Given that kinase-independent stabilization of ERK3 by BRAF is likely through protein-protein interactions (Fig S2), we performed immunofluorescence for visualizing of BRAF and ERK3 localization in A375, OCM3, SK-Mel2, and WM3211 cell lines. In BRAF\textsuperscript{V600E} cell lines (Fig S5 A and B), BRAF and ERK3 are strongly co-localized around the nucleus while BRAF WT cell lines WM3211 (Fig S5C) and SK-Mel2 (Fig S5D) exhibited colocalization of ERK3 and BRAF throughout the cytosol. Although we can’t conclude that BRAF\textsuperscript{V600E} has different protein-interactions impacting ERK3 cellular localization, both BRAF and BRAF\textsuperscript{V600E} exhibited strong co-localization with ERK3 in melanoma cell lines.

Interestingly, after knockdown of BRAF, we observed a strange ERK3 movement to the cell membrane’s leading edge in WM3211 (data not shown), which exhibited a similar distribution pattern of β-catenin and F-actin in melanoma. This indicates strong adhesion with reduced metastasis ability.\textsuperscript{44} Notably, a similar ERK3 re-localization pattern was observed in the less metastatic A375shGIPZ cell line compared to A375shERK3 (data not shown). We hypothesize
that besides the ERK3 reduction, ERK3 is also prevented from localizing at the leading edge of melanoma cells by some mechanism. This could be another mechanism to promote melanoma progression to a highly metastatic stage.

These results remain preliminary. To further explore ERK3 localization and its effects on cell migration, we would like to determine whether ERK3 localization at the cell membrane is related to differences in gene expression between cell lines. We have performed microarray analysis of gene expression in response to ERK3 knockdown both melanoma and lung cancer (data not shown). This indicates a group of cell adhesion genes affected differently by ERK3 in the two cell types. For further verification, more co-IF can be performed to monitor the interactions between ERK3 and the proteins encoded by these genes.

**Microarray analysis identified several possibilities of the differences between genes affected by ERK3 regulation in lung cancer versus melanoma.**

In order to explore the differences between lung cancer and melanoma in response to ERK3 regulation, we further performed microarray analysis in lung cancer A549 and melanoma A375 cell lines. We observed a series of dramatically changed genes affected by the ERK3 knockdown (data not shown). There were many genes uniquely regulated in each cell line. Firstly, a group of 8 genes involved in focal adhesion (p=0.002) and adhesion junctions (p=0.007) were dramatically increased only in the lung after the ERK3 knockdown, which indicates the reduction of migration ability. Secondly, 1 gene falls into the pathways of reducing migration including ECM-receptor interaction (p=0.005) and focal adhesion (p=0.01) is inhibited by ERK3 in the lung.
but promoted by ERK3 in melanoma (fold change > 2). This is a likely target for further exploration. Both of these finding consistent with the known role of ERK3 in promoting migration in lung. Oppositely, 2 genes involved in the inhibiting migration pathway of complement and coagulation cascades are dramatically inhibited by ERK3 only in melanoma (fold change = 2.5 and 4.5, p=0.002). It is thus reasonable to hypothesize that the functional differentiations caused by ERK3 in these two types of cancer are primarily due to tissue-specific differences in the suite of genes found downstream of ERK3.

Microarray also supported the possibilities of resolving the regulatory differences by ERK3 in lung and melanoma. On one hand, the top upregulated or downregulated (fold change > 3) genes in melanoma have significant endogenous expression level differences between melanoma and lung. We thus further hypothesize that these genes might be suppressed by other genes in lung so that ERK3 is no longer able to regulate them. On the other hand, further analysis of potential tissue-specific transcriptional factors (TFs) regulated by ERK3 will also help determine the mechanisms behind the different functional effects of ERK3.

Another notable finding from the microarray analysis is that the top expression changes induced by siERK3 are in the same positive or negative direction during melanoma progression in the GEO database. We thus reasonable to believe that the reduction of ERK3 is the key mechanism to promote melanoma progression along with the modification of these genes expression.
VI. SUPPLEMENTARY DATA

<table>
<thead>
<tr>
<th>CELL</th>
<th>TISSUE</th>
<th>ORGANISM</th>
<th>BRAF</th>
<th>RESOURCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>A375</td>
<td>Metastasis of cutaneous melanoma</td>
<td>Human</td>
<td>V600E</td>
<td>Dr. Mitsiades’s Lab</td>
</tr>
<tr>
<td>OCM3</td>
<td>Uveal melanoma</td>
<td>Human</td>
<td>V600E</td>
<td>Dr. Mitsiades’s Lab</td>
</tr>
<tr>
<td>WM3211</td>
<td>Primary cutaneous melanoma</td>
<td>Human</td>
<td>WT</td>
<td>Dr. Feng Liu-Smith</td>
</tr>
<tr>
<td>SK-MEL2</td>
<td>Metastasis of cutaneous melanoma</td>
<td>Human</td>
<td>WT</td>
<td>Dr. Feng Liu-Smith</td>
</tr>
<tr>
<td>B16F10</td>
<td>Metastasis of cutaneous melanoma</td>
<td>Mice</td>
<td>WT</td>
<td>ATCC® Number: CRL-6475</td>
</tr>
<tr>
<td>A431</td>
<td>Skin squamous cell carcinoma</td>
<td>Human</td>
<td>WT</td>
<td>ATCC® Number: CRL-1555</td>
</tr>
<tr>
<td>H23</td>
<td>Non-small cell lung cancer</td>
<td>Human</td>
<td>WT</td>
<td>ATCC® Number: CRL-5800</td>
</tr>
<tr>
<td>293T</td>
<td>Immortalized embryonic kidney</td>
<td>Human</td>
<td>WT</td>
<td>Dr. Long’s Lab</td>
</tr>
</tbody>
</table>

Table S1 BRAF V600E mutation status and background information of different cell lines used in these studies.
Figure S1 BRAF Kinase activity is transcriptionally but not translationally essential for stabilization of ERK3 protein.

Western blot of ERK3 regulation by kinase inhibited BRAF. A375 cells were treated with 2μM vemurafenib or DMSO control. Protein was collected at 0, 1, 2, 4, 8, 24 and 36 hours.
Figure S2 BRAF may be able to bind rictor-Sin1 in mTORC2 to form a loose complex with ERK3 in 293T.

Rictor or ERK3 was immunoprecipitated from (A) 293T and (B) A375 cell lysates, then western blotting was performed to identify proteins co-precipitating proteins. Rabbit Immunoglobulin G (IgG) was used as a negative control when using Rictor or ERK3 anti-Rabbit antibody (Ab) for co-precipitating. A protein-protein pull down assay was performed by incubating total cell lysate immobilized on Ab pre-bound agarose beads.
**Figure S3 ERK3 does not feedback regulate BRAF.**

BRAF protein level was measured by western blotting (A) and mRNA level was measured by RT-qPCR (B). ERK3 was depleted by a lentiviral system targeting the ERK3 coding region in A375 cells. A375 shGIPZ was used as a control and mRNA level is normalized to this control, then expressed as the mean ± SD (n=3). Significance levels (p-values) were determined by Student’s t-test.
Figure S4 Long-term ERK3 knockdown does not induce vemurafenib resistance.

A375 stable ERK3 knockdown and its control shGIPZ cell lines were treated with gradient doses of BRAF<sub>V600E</sub> kinase inhibitor vemurafenib for 3 days and cell viability was analyzed by MTT assay. Results were representative of multiple experiments (left panel) and knockdown efficiency was verified by western (right panel). Results are expressed as a mean ± SD (n=5). Beta actin serves as a loading control.
Figure S5 BRAF co-localizes with ERK3 in the nucleus of BRAFV600E melanoma but crosses over to the cytosol in BRAF WT melanoma.

Co-immunofluorescence staining of BRAF and ERK3 in melanoma BRAF\textsuperscript{V600E} cell lines (A) A375 and (B) OCM3, and BRAF WT melanoma cell lines (C) WM3211 and (D) SK-Mel2. Images were taken under 100x magnification. BRAF (red) and ERK3 (green) staining were merged with DAPI (blue) nuclear staining (left panels) where yellow indicates the colocalization of two proteins (right panels).
VII. REFERENCES


