Identification of KIT as a Suppressor of BRAFV600E-Mutant Melanoma

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IDENTIFICATION OF KIT AS A SUPPRESSOR OF BRAF^{V600E}-MUTANT MELANOMA

A Dissertation Presented

By

James Victor Neiswender

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
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Program in Molecular, Cell, and Cancer Biology
Dissertation Examination Committee

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A Dissertation Presented By
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This work was undertaken in the Graduate School of Biomedical Sciences Program in Molecular, Cell, and Cancer Biology

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Abstract

Genetic changes acquired in the pigment producing cells of the skin, called melanocytes, can lead to formation of the deadly cancer melanoma. Mutations or amplifications leading to the activation of the RAS/MAPK pathway occur in more than 90% of melanomas. Melanocyte development and survival requires the stimulation of this pathway by the receptor tyrosine kinase (RTK) KIT. In ~2% of melanomas, oncogenic KIT mutations drive tumor formation; however, the majority of melanomas lose wild-type KIT expression, suggesting that KIT could suppress melanoma formation. In human melanoma patients of The Cancer Genome Atlas (TCGA), we found an association between BRAF\textsuperscript{V600E} mutations and low KIT mRNA expression, so we tested whether KIT loss would affect BRAF\textsuperscript{V600E}-driven tumor onset by crossing a kit\textit{(lf)} mutant allele into melanoma-prone \textit{Tg(mitfa:BRAF\textsuperscript{V600E}); p53(lf)} zebrafish. We observed that kit\textit{(lf)}-mutant zebrafish experienced accelerated tumor onset and their tumors had increased RAS/MAPK pathway activation. In BRAF\textsuperscript{V600E}-mutant melanoma cells, KIT activity reduced RAS/MAPK signaling by promoting activation of wild-type BRAF (BRAF\textsuperscript{WT}). Furthermore, we found that overexpression of BRAF\textsuperscript{WT} delayed tumor onset in \textit{Tg(mitfa:BRAF\textsuperscript{V600E}); p53(lf); mitfa(lf)} zebrafish, but had no effect in kit\textit{(lf)}; \textit{Tg(mitfa:BRAF\textsuperscript{V600E}); p53(lf); mitfa(lf)} zebrafish and a cohort of TCGA BRAF\textsuperscript{V600E}-mutant melanoma patients with high KIT expression and high BRAF\textsuperscript{WT} allele ratios experienced a reduced likelihood of metastasis and extended overall survival. These studies indicate that wild-type KIT acts to
suppress melanoma formation through activation of \( \text{BRAF}^{\text{WT}} \), causing reduced signaling output of \( \text{BRAF}^{V600E} \)-mutant cells.
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List of Symbols, Abbreviations, or Nomenclature

TCGA – The Cancer Genome Atlas
RTK – Receptor tyrosine kinase
If – Loss-of-function
EGFP – Enhanced green fluorescence protein
NLS – Nuclear localization sequence
Rluc-CRAF – *Renilla luciferase* tagged CRAF
V-BRAF – Venus tagged BRAF
BRET – Bioluminescence resonance energy transfer
MITF – microphthalmia-associated transcription factor
MAPK – Mitogen activated protein kinase
MEK – Mitogen activated protein kinase kinase
ERK – Extracellular regulated kinase
NTA – N-terminal acidic acid motif
KSR – Kinase suppressor of RAS

Preface

The work presented in this dissertation was done by the following individuals:

Conception and design: James V. Neiswender, Craig J. Ceol

Development of methodology: James V. Neiswender, Robert L. Kortum, Deborah K. Morrison, Craig J. Ceol

Acquisition of data: James V. Neiswender, Caitlin Bourque, Melissa Kasheta, Craig J. Ceol
Analysis and interpretation of data: James V. Neiswender, Deborah K. Morrison, Craig J. Ceol

Study supervision: Craig J. Ceol
Chapter I - Introduction to KIT and RAS/MAPK pathway signaling in melanocytes and melanoma

Genetic aberrations lead to cancer formation

To survive, every multicellular organism must coordinate the growth of its cells. This coordination can be achieved through cellular communication and by following developmental and degenerative programs. If a cell goes rogue by following its own growth program, it may grow and multiply without limit forming masses of tissue and leading to the potentially fatal disease called cancer. Thus, to receive growth stimulatory or inhibitory signals from their environment, cells utilize a variety of mechanisms to communicate, including expression of surface receptors that recognize molecules emitted from other cells in their environment. These receptors transmit the signal into a cell by sparking a cascade of signaling activity that results in altered gene expression, causing a cell to grow, divide, differentiate, or die (2). The transmission of signal is typically achieved by protein enzymes called kinases, which communicate an activation signal by adding a phosphate group to a target protein leading to structural changes and new functions of that target protein. Kinases are among the largest groups of eukaryotic protein families and are well conserved across all of the multi-cellular metazoans (3,4).

Internal programs also help regulate cellular lifecycles where proteins that sense DNA damage or a change in telomere length due to multiple cellular
divisions can also enact genetic expression programs to respond to these issues with slowed growth or death (5,6). As cells successfully grow and divide, genetic changes such as copy number alterations or mutations can accumulate due to genome replication errors and exposure to mutagens such as ultraviolet light (7,8). When these changes affect the structure or expression of the critical regulatory signaling genes, uncontrolled cellular growth can occur leading to formation of tumors (9).

Genes that promote cancer formation due to an increase in function that resulted from a genetic alteration are referred to as oncogenes, whereas genes whose loss of function promotes cancer formation are referred to as tumor suppressors. Oncogenes are often discovered by identification of recurrent, disease-associated mutations or increases in expression of the aforementioned growth regulatory signal transduction genes. These mutations or increases in expression level can cause the proteins from these genes to function constitutively and independently, without upstream pathway stimulation. These proteins will then persistently drive the growth and survival transcriptional programs that normally would have required initiation from an external signal. For example, the product of the ABL gene on chromosome 9 functions as a tyrosine kinase to promote cellular growth and division, but a translocation with the BCR gene on chromosome 22 results in a constitutively active fusion product that acts constitutively and drives the formation of acute lymphocytic leukemia and chronic
myelogenous leukemia (10,11). Tumor suppressors are often negative regulators of growth stimulating pathways, such as NF1 or PTEN (12,13), or proteins that slow down the cell cycle or promote programed cell death such as RB or p53 (5).

Oncogenes and tumor suppressor genes differ in the fate of their corresponding wild-type gene. A gain-of-function mutation in a single copy of an oncogene can drive tumor formation while leaving the wild-type allele intact, such as with the \( \text{BRAF}^{\text{V600E}} \) mutant allele, which maintains heterozygosity in roughly two-thirds of melanomas, though the mutant allele has been shown to be preferentially amplified (14,15). Similarly, several tumor suppressor genes only require the loss of one allele to no longer be capable of inhibiting aberrant proliferation, such as \( \text{PTEN} \), \( p27 \), and \( \text{NF1} \) (16). On the other hand, losses of both copies of a gene have been observed at high frequencies for many tumor suppressor genes including \( \text{RB} \), \( \text{BRCA1/2} \), \( \text{APC} \), and \( \text{CDKN2A} \); such an occurrence is referred to as a loss of heterozygosity (17-21). The requirement for losing one or both copies of a tumor suppressor gene may be context dependent. Indeed, an analysis of tumors from Li-Fraumeni patients showed that slightly less than half had lost the wild-type copy of their \( \text{TP53} \) allele, yet clearly many tumors were able to form with a single germline mutation in \( \text{TP53} \) (22). The form of ocular cancer retinoblastoma that is recessively inherited has been linked to the loss of both copies of the gene for the cell cycle inhibitor RB (23-25). The requirement for a loss of both copies of this gene for disease progression
provides a clear explanation for the observation that children with the inheritable form of this disease are more likely to develop bilateral retinoblastomas, as they would have been born with one defective copy of RB and require only two sporadic genetic mutation events to develop the disease in both eyes. However, children who had sporadic (non-inheritable) mutations are more likely to only develop unilateral disease. These observations led to the formation of the "two-hit hypothesis" stating that cells require multiple genetic “hits” that alter their growth in order to become tumorigenic (26). Since this was proposed, it has been clear that the function and impact of mutations must be interpreted in the context of their genetic background.

Correct classification of the genetic contributions from mutant genes to cancer formation has become essential to understanding how cancer forms and for the development of targeted therapies. The identification of the BCR-ABL translocation allowed development of a small molecule inhibitor, imatinib, which blocks ATP-binding pocket to inhibit the kinase activity of BCR-ABL and other tyrosine kinases to effectively treat leukemia treatment (11). Cancer sequencing studies have revealed a vast number of genetic changes that occur during tumorigenesis. The loci of mutated oncogenes tend to be amplified with multiple copy numbers, while the loci of tumor suppressors are deleted (14,27). Although researchers have been able to identify new “driver genes” of cancer formation by introducing the mutations found in patients into normal cell types and observing a
transformative change (28), there are some genes that are recurrently amplified, deleted, or mutated in cancer, but have no impact on disease progression referred to as “passenger genes” (29). These passengers may have been amplified or deleted due to their proximity to a driver gene, or recurrently mutated due to age or exposure to mutagens. Any gene identified to be frequently altered in cancer must be tested to determine whether its change was a cause or consequence of tumorigenesis. Therefore, determining true cancer-causing genes and searching to understand how they work under various genetic contexts represents a major current challenge in cancer biology.

**Melanoma is a deadly cancer of melanocytes**

One of the most perplexing cancers to sort through its many genetic changes is melanoma, the deadly cancer of the pigment producing cells called melanocytes. Melanocytes reside in the basal layer of the human epidermis, originating from stem cells located in the bulge region of hair follicles (30). These cells produce the pigment melanin in the form of dark brown eumelanin and reddish-colored pheomelanin, which is shipped to neighboring keratinocytes of the skin and establishes skin tone. Eumelanin helps to shield cells from harmful UV radiation (31-35). Radiation from sun exposure has potential to induce C->T mutations, but a degree of this radiation can be shielded from causing serious DNA damage with a sufficient level of melanin pigment (32,36). Indeed, higher amounts of skin pigmentation have been correlated with reduced UV-skin penetration and
reduced risk of developing melanoma (37,38), whereas increased UV exposure through the use of tanning beds has been linked with increased cancer risk (39). Despite a melanocyte’s production of pigment, over time it can still acquire both sun-induced and non-sun-induced mutations that cause uncontrolled cell growth.

Initially, a melanocyte that has acquired a tumorigenic mutation will multiply locally and form benign pigmented spots called nevi or moles. These cells have typically acquired a single mutation, but have become senescent and can be considered benign when they are small, have a smooth defined border and remain the same size over time (40,41). However, if a pigmented patch is asymmetric, has an irregular border, different colors or uneven structural components, and a diameter greater than 6 mm, it is possible the lesion has overcome senescence by acquiring additional genetic changes and would warrant histological testing to determine if it had become a melanoma (42). Although the number of nevi a person has correlates with their likelihood to get melanoma (43), it is unclear whether melanomas necessarily originate from nevi, or rather originate from melanocyte precursor cells or differentiated melanocytes. Once transformed, melanomas have been traditionally classified according to their growth relative to the epidermis where they may remain in place (melanoma in-situ), spread along the epidermis of the skin without invasion (superficial spreading melanoma), invade into subdermal tissues (vertical growth phase), or metastasize to distant locations (44). Melanoma tumor depth has been strongly
correlated with survival outcome. Melanomas with 1 to 2 mm of depth have a 90% or 80% 10-year survival rate, respectively; whereas melanomas with 3 or >4 mm of depth have 63% and 50% 10-year survival rates (45). The presence of regional lymph node or distant metastases further decreases 10-year survivorship rates to 10-20% (45).

An analysis of the Surveillance, Epidemiology, and End Results (SEER) program data that tracked melanoma incidence and survival in the United States from 1982 to 2011 found that melanoma incidence doubled during that time period to a level of 19.7 cases per 100,000 people in 2011 (46). The same study found a steady death rate of 2.7 deaths per 100,000 people, which is expected to remain relatively constant for the next several decades (46). The increasing rate of melanoma incidence has been attributed to increased diagnosis of thin (<1 mm) melanomas, while increased UV exposure may also be a contributing factor (47). Risk of melanoma is also associated with freckling, blonde or red hair, and blue eyes; each of which indicates deficiencies in eumelanin production (38).

Survival rates for surgical removal of low stage (non-metastatic) melanomas are generally high with recurrence rates as low as 2.2% after 10 years (48), but historical treatment options have had minimal survival benefit for metastatic melanoma patients. The standard treatment for advanced stage
melanoma has been with the DNA-alkylating agent dacarbazine, the only standard chemotherapeutic drug with FDA approval for melanoma. Dacarbazine efficacy has been modest. A representative phase III clinical trial of stage IV melanoma patients reported that the cohort treated with dacarbazine had a response rate of 9.8% and median overall survival of 9.1 months (49). Several decades of attempts to improve melanoma outcome with typical chemotherapy regimens yielded little improvement to survival, leaving a great medical need for better treatment options for this disease (49). As described below, hope for improved treatment began to emerge in the 2000s as sequencing studies revealed targetable mutations in melanoma and as scientists improved methods to activate the immune system in cancer treatment (50,51). Additional targeted therapies may be developed as the transcriptional signature of melanoma is further defined. Among the early observations of gene expression changes in melanoma is the observation that many tumors dedifferentiate and re-express neural crest genes (52,53). Many of these genes that play a role during melanocyte differentiation and maintenance also affect melanoma onset and progression; therefore, understanding the complexity of melanocyte development will be essential for describing melanoma biology.
**Melanocyte development and differentiation in relation to melanoma formation**

The developmental history of melanocytes sheds light on the nature of melanoma progression. Melanocytes originate from the embryonic tissue called the neural crest that forms at the boundary between the ectoderm and the neural plate during formation of the neural tube (54). The neural crest cells are characterized by their highly migratory nature as they spread away from the neural tube and migrate throughout the embryo to form the peripheral and enteric neurons, glia, craniofacial cartilage and bone, smooth muscle, and melanocytes (54). The identification of the genes that guide melanocyte development from the neural crest, particularly for the critical factors PAX3, EDN3, EDNRB, SNAI2, SOX10 and MITF, was largely informed through experiments linking mutations in these genes to pigment defects in mice and to the disorder Waardenburg syndrome, a disease characterized by pigment defects, craniofacial defects, and hearing loss due to the absence of cochlear melanocytes (55-58). Prior to the expression of these factors, early BMP signaling is required for establishment of the neural crest during development (Fig. I.1) (59). For melanocytes to develop from the neural crest, BMP signaling must decrease while WNT signaling turns on and neural crest cells become migratory (60,61). Next, melanocyte precursor cells begin to express the transcription factor SOX10 at sufficient levels to promote expression of several critical differentiation factors, including the master regulator of melanocyte differentiation, microphthalmia-associated transcription
factor (MITF) (Fig. I.1) (62-64). MITF is a leucine-zipper transcription factor that promotes expression of genes downstream of E-boxes with the consensus sequence CA[C/T]GTG that play critical roles in defining melanocyte identity such as the pigment production genes TYR and DCT and genes involved in promoting melanocyte survival such as the anti-apoptotic factor BCL2 (65-67). The activity and expression level of MITF in melanocytes can be controlled with upstream activation of the MAPK pathway by stimulation of KIT (Fig. I.2) (68). Loss of KIT activity through mutation or deletion is also associated with a loss of pigment cells, demonstrating that this factor is critical for melanocyte development (69-72). Its role will be discussed more fully below.
Figure I.1 – Genetic regulators of melanocyte and melanoma development

The neural crest is established through activation of BMP and SOX10 signaling. Further melanocyte development requires a loss of BMP activity, but a gain in WNT, KIT, and MITF expression resulting in expression of pigment production genes DCT and TYR. Acquisition of a BRAF or NRAS mutation may lead to
senescence and formation of benign nevi; however, secondary events including strong expression of neural crest genes, loss of tumor suppressors, and loss of \textit{KIT} may lead to cell cycle progression and generation of melanoma. Modulation of MITF expression further influences melanoma growth making it more highly invasive or proliferative with low or high MITF expression, respectively.
Experiments have shown that melanomas regain a transcriptional profile similar to their neural crest origins and less similar to the differentiated state of melanocytes (52,53). This involves high expression of neural crest genes and is thought to contribute to the ability of melanomas to become highly migratory and possess high plasticity, or the ability to switch phenotypes dependent on micro-environmental contexts from highly proliferative to highly invasive forms (73,74). The neural crest identity of melanomas represents a potential target as a therapeutic approach for melanoma patients. Indeed clinical trials have begun evaluating inhibitors of DHODH, a transcription elongation factor that promotes the neural crest transcriptional program in melanoma (52).

A strong, but complicated link exists between specific melanocyte differentiation genes and melanoma progression. The primary facilitator of neural crest induction leading to melanocyte differentiation, SOX10, has been shown to play an oncogenic role in melanomas where it is overexpressed and drives proliferation (75). The link between MITF and melanoma is complex. Although MITF has been shown to be highly expressed in the majority of melanomas and its overexpression can transform a pre-malignant melanocyte cell type, abnormally low MITF activity levels can also be transforming (Fig. I.1) (76,77). This has led to the proposal of a rheostat model of MITF activity in melanoma where high expression levels are associated with a proliferative state with
enhanced survival through upregulation of BCL-2, whereas low expression levels are associated with increased invasiveness (67,73). Intriguingly, the transplantation of cells that had been sorted into groups based on markers for the “proliferative” or “invasive” signatures would grow to produce heterogeneous tumors, demonstrating that melanomas maintain an ability to adapt their transcriptional profiles to suit their environment and growth (74). The control of MITF activity and the activity of other melanocyte specification genes clearly plays an important role in melanoma biology and the determination of the tumorigenic effects of additional melanocyte development genes remains a vital area of research.

**MAPK pathway activation and regulation in melanoma**

The exterior signals that activate MITF to promote melanocyte differentiation are mediated through the RAS/MAPK pathway signaling cascade (Fig. 1.2). This pathway is frequently hyperactivated in cancer and is central to melanoma progression, where activating mutations or other genetic alterations of this pathway are present in greater than 90% of human melanomas (78).

The role of this pathway is to interpret signals from outside the cell and transform that information into action within a cell to instruct it to grow and divide. This can happen when extracellular ligands bind to cell surface receptors on the extracellular side of a cell. This binding of ligand to receptor causes the receptor
to homodimerize and acquire a conformation that favors kinase activity leading to auto-phosphorylation of the tyrosine residues on the tail of the receptor (2). The phosphorylated tyrosine residues will be directly bound by the SH2 domains of GRB2 that will recruit the guanine nucleotide exchange factor SOS, which will switch GDP for GTP binding on the membrane-localized protein RAS, leading to its activation. In a resting state the RAF family members ARAF, BRAF, and CRAF exist as inactive monomers in the cytoplasm, but will relocate to the plasma membrane via an interaction with phosphatidic acid that forms as a result of mitogen-induced phospholipase D activity (79). This positions RAF at the membrane where it will undergo activation if its RAS-binding domain (RBD) is able to bind GTP-RAS (80-82). This binding causes RAF family members to undergo a conformational shift that favors dimerization. Although this dimerization can occur between any two RAF family members, the heterodimerization of BRAF with CRAF is the most predominant and active dimer pair (83). RAF proteins are serine kinases that require dimerization and phosphorylation of both their activation loops and their N-terminal acidic (NTA) domains to become active, in addition to several dephosphorylation events. The NTA of BRAF contains the amino acids SSDD from positions 446-449, imitating a constitutively phosphorylated state. Upon membrane recruitment BRAF will undergo a conformational change, receive several additional activating phosphorylations releasing its activation loop to free its kinase domain, and will proceed to dimerize and transactivate CRAF, which phosphorylates MEK on
serine residues (84). A scaffolding protein named *kinase suppressor of RAS* (KSR), which is similar in shape to the RAF family, helps facilitate the binding between BRAF and CRAF with MEK1 and MEK2 (referred to collectively as MEK in this manuscript) (85). Activated MEK goes on to phosphorylate the threonine and tyrosine residues of the activation loop on ERK1 or ERK2 (Map Kinase, collectively referred to as ERK in this manuscript) that enters the nucleus and phosphorylates and activates multiple substrates resulting in an increase in cellular growth and division (86). Furthermore ERK phosphorylates MITF on S73 leading to its activation (68), though ERK activity also results in downstream activation of the serine/threonine kinase RSK-1 that tags MITF for proteasome-mediated degradation with a phosphorylation at S409 (87). While these temporary bursts of MITF activity may be required for melanocyte development, it has been hypothesized that persistent KIT stimulation would result in reduced MITF expression and therefore inhibit the proliferation and survival of melanocytes (87). Therefore, precise control of the MAPK pathway is required to enable proper melanocyte development (Fig. I.2).

Multiple mechanisms exist within a cell to negatively regulate ERK activity if the extrinsic signal does not cease in a timely fashion. ERK activity leads to an increase in the expression levels of *SPRY* and *DUSP* genes, where the SPRY protein goes on to sequester GRB and SOS to prevent activation of RAS and DUSP will dephosphorylate the threonine and tyrosine residues on ERK (88). An
additional negative feedback loop occurs when ERK directly phosphorylates BRAF on residues S151, T401, S750, and T753, causing BRAF to poorly dimerize with CRAF and thus have reduced capacity to activate the MAPK pathway (89).

Given the critical role BRAF plays in transmitting signals through the MAPK pathway that results in activation of MITF, it is not surprising that multiple sequencing studies have found *BRAF* to be the most mutated gene in cutaneous melanomas (28,78). The landmark study by Davies *et. al* in 2002 found that roughly 2/3rd of the melanoma samples they assayed possessed missense mutations in BRAF, and roughly half of those melanomas contained a valine to glutamic acid mutation at position 600 (*BRAF*V600E)(28). This mutation has been shown to increase the kinase activity of BRAF on its substrate MEK by 500-fold (90) and cause BRAF to have a 138-fold increased transforming capability in NIH-3T3 cells (28). A more recent sequencing study has confirmed the *BRAF*V600E mutation is the most common single mutation in melanoma, affecting roughly 40% of melanomas (78). The kinase domain of BRAF is typically held in an inactive conformation by a hydrophobic interaction within the kinase domain between the valine on position 600 in the activation segment and the p-loop. The V600E mutation disrupts this interaction by replacing that valine with a large, negatively charged glutamic acid residue, thought to mimic activating phosphorylations. This causes BRAF to enter an active conformation that can
phosphorylate MEK in the cytoplasm without upstream RAS activity (90). The high constitutive activity of BRAF^{V600E} has been shown to be insensitive to the aforementioned negative feedback phosphorylations that occur on BRAF as a result of downstream ERK activity (89). BRAF mutations are thought to occur early in tumorigenesis, given that greater than 80% of benign nevi have been found to harbor this mutation (40). This indicates that BRAF^{V600E} alone is insufficient to drive melanoma formation and that other factors must contribute to tumorigenesis. Due to the frequency and clear causative role in melanoma, BRAF^{V600E} has been actively pursued as a therapeutic target. Small molecule inhibitors that can specifically fill the ATP-binding pocket of BRAF^{V600E} to block its activity have been developed (91) and their use led to some degree of tumor regression in 81% of BRAF^{V600E}-mutant melanoma patients; however, resistance to BRAF^{V600E} inhibitors invariably develops over the course of several months (50). Resistance generally occurs through re-activation of the MAPK pathway and may occur intrinsically due to mechanisms such as stromal secretion of the MET-stimulating factor HGF (92), or resistance may be acquired through mechanisms such as BRAF^{V600E} alternative splicing, MAP3K8 upregulation, MEK mutations, upregulation of the RTK PDGFRβ, or acquisition of activating mutations in upstream components such as NRAS or even in alternative pathways such as the PI3K-PTEN-AKT pathway (93-97). RAF inhibitors have also been found to paradoxically promote activation of wild-type BRAF (BRAF^{WT}) due to their promotion of BRAF:CRAF heterodimer formation yielding
transactivation of CRAF to stimulate the MAPK pathway (98). These clinical trials and the pursuant basic research studies on resistance mechanisms underscore the importance of maintenance of MAPK pathway signaling strength to sustain melanoma growth.

Role of KIT in melanocyte development and cancer

The activation of the MAPK pathway plays a critical role in melanocyte differentiation by stimulating the activity of the melanocyte master regulator, MITF, and in melanoma formation to enhance cellular growth and division. Therefore, the role of the RTK KIT in melanoma has been of particular interest to researchers for decades due to its role activating the RAS/MAPK pathway to promote melanocyte development and its role as a proto-oncogene in several cancer types.
Figure I.2

KIT functions as a receptor tyrosine kinase to promote melanocyte development

The binding of KIT to its ligand, stem cell factor, will induce a homodimerization of KIT receptor molecules at the plasma membrane. This dimerization promotes an interface where KIT will auto-phosphorylate its tyrosine residues, which are then recognized as an activation signal by several downstream pathways. Most critical to the function of KIT in melanocytes are its promotion of PI3K/AKT signaling to promote cell survival and activation of NRAS GTPases that go on to promote BRAF/CRAF dimerization leading to MEK and ERK activation, referred
to as the MAP Kinase pathway (MAPK pathway). ERK further phosphorylates MITF, the master regulator of melanocyte development, on S73 leading to increased transcriptional activity, but ERK activity also promotes RSK-1 activity, which phosphorylates MITF on S409 leading to ubiquitin mediated degradation of MITF. Thus, KIT stimulation leads to a temporary burst in MITF activity that is thought to play a critical role in promoting melanocyte development and survival.
In 1986, a gene was cloned from virus purified from a feline fibrosarcoma tumor and designated v-kit (99). The virus transformed feline embryonic fibroblasts and sequencing of the viral DNA revealed a gene containing homology to tyrosine-specific kinase oncogenes, such as v-fms (the viral oncogene orthologue of macrophage growth factor receptor CSF-1) and human epidermal growth factor receptor (99). Southern blotting the v-kit DNA against cat, mouse, or human DNA revealed a likely cellular counterpart for each species, designated c-kit or KIT, later identified to reside on human chromosome 4 and encode a 976 amino acid protein product in humans (99,100). The KIT gene contained sequences encoding an extracellular domain, a transmembrane domain, and a split intracellular tyrosine kinase domain. The immunoprecipitated KIT protein was confirmed to possess intrinsic auto-phosphorylation activity on its own tyrosine residues (100). Shortly after this discovery, various alleles of the W locus on chromosome 5 in mice that are well-known for having hematopoietic defects and lack pigment cells were mapped to the mouse orthologue of KIT (69,101-103). Homozygous W locus mutant mice are almost all embryonic lethal due to severe macrocytic anemia, whereas heterozygous mutants are viable, fertile, and manifest white spots on their bellies, feet, and tail tips (103). As additional mutations and deletions of KIT were linked with pigment defects across many species, it became clear that this gene plays a conserved role in promoting normal melanocyte development, migration and survival (104-109). In humans, the pigmentation disorder piebaldism, which manifests similar
pigmentation spotting phenotypes with patches of skin that lack melanocytes, has also been linked to heterozygous \textit{KIT} deletions and loss-of-function mutations (72,110). Furthermore, a mutation of the steel mutant mouse that has a similar phenotype to \textit{W} locus mutants was also mapped to a gene determined mechanistically to be the \textit{KIT} ligand stem cell factor (SCF) (111).

A pigment defect-linked \textit{KIT} mutation was found to cause a similar amino acid change in mice and humans that occurs near the ATP-binding pocket of the \textit{KIT} kinase domain and abolishes auto-phosphorylation activity, establishing the importance of \textit{KIT} signaling activation in melanocyte formation (69-72). The tyrosine residues that become phosphorylated upon ligand-induced homodimerization of \textit{KIT} become recognized by a variety of signaling intermediary proteins including PI3K, and GRB that, as with a typical RTK described above, leads to the activation of the RAS/MAPK pathway (Fig. I.2). The mechanistic link between \textit{KIT} activity and melanocyte development was realized through work that identified MITF was phosphorylated and activated in response to \textit{KIT} stimulation (68). Importantly, a MEK inhibitor blocked the MITF phosphorylation by \textit{KIT}, suggesting that the signal was mediated through the MAPK pathway (68). It was further demonstrated that the MEK target ERK could directly phosphorylate MITF on serine 73, which increased MITF transcriptional activity on the promoter of tyrosinase, a melanocyte-specific MITF target gene (68). Furthermore, the heightened activity of MITF downstream of \textit{KIT} activation
seems to be tempered by an additional phosphorylation of serine 409 on MITF that results from RSK-1 activity downstream of ERK, which tags MITF for proteasome-mediated degradation (87). This regulatory pattern of dual activation and degradation of this critical transcription factor provides a clear mechanistic connection between KIT and melanocyte development, but the role of KIT in cancer is somewhat more complicated.

In rare cutaneous melanomas that occur on volar surfaces of skin or in mucosa, 15-25% of cases contain mutations or amplifications of KIT that drive tumorigenesis (112-114). Inappropriate activation of KIT leads to ligand-independent, constitutive downstream ERK and AKT signaling (115,116). KIT-mutant melanomas and gastrointestinal stromal tumors (GISTs), which possess similar KIT mutations, have shown clinical responses to imatinib and other tyrosine kinase inhibitors (117,118). Whereas volar and mucosal melanomas represent about 2% of all melanomas, the most common melanomas, representing 90% of all cases, are cutaneous melanomas that occur on intermittently or chronically sun-exposed areas of the skin (119). Paradoxically, KIT expression is either low or undetectable in this latter category of melanomas, which are primarily driven by either BRAF or NRAS activating mutations (120-122). The loss of KIT expression has been attributed to frequent deletion or hypermethylation of the KIT locus in melanoma (27,123). While the loss of KIT in cutaneous melanomas is well documented, it remains unclear whether this loss
is a cause or consequence of tumorigenesis. Although previous work in cultured cells has suggested that KIT activity may impede melanoma progression by promoting apoptosis or restricting migration (124-126), the impact of KIT activity on tumor initiation and oncogenic signaling in melanoma has yet to be explored.

**Zebrafish as a model for melanocyte and melanoma biology**

Manipulating a gene’s expression in an animal model of tumor development can test its contribution to tumor initiation. The zebrafish is an outstanding model to study melanocyte and melanoma biology for several reasons.

First, the development of melanocytes from the neural crest is conserved between zebrafish and humans with many homologous genes determining control of melanocyte development including *SOX10* and *MITF*, named *sox10* and *mitfa* in zebrafish (127,128). *kit* loss-of-function mutants (*kit(lf)*) have been identified among zebrafish and they also present reduced numbers of pigment cells; however, unlike mice, *kit(lf)* homozygous animals are viable and still develop some melanocytes (129,130). The primary *kit(lf)* mutant line in zebrafish was generated by gamma-irradiation and the *sparse(b5)* allele strain was found to contain a frame-shift mutation (T846del) in the gene *kita*, referred to as *kit(lf)* in this text, that results in an early stop allele and a protein product that lacks the transmembrane and kinase domains (130-132). Melanocytes develop poorly in *kit(lf)* zebrafish larvae with reduced numbers and migration failure apparent at 3-
5 days post fertilization (dpf) (129). Melanocytes are then completely absent until the juvenile stage at 3-4 weeks when melanocytes will populate the striped areas of the fish to approximately half the density of wild-type fish, but none form on the dorsal region (129). A further distinction between kit mutant zebrafish and KIT mutant mammals is that kit(lf) zebrafish also maintain normal hematopoiesis (130). This indicates that, although Kit plays an important role in zebrafish melanocyte development, additional compensatory mechanisms are likely to exist in kit homozygous mutants to permit a modest level of melanocyte and hematopoietic development. It is unlikely that kitb, the zebrafish ohnologue (parologue that forms as a result of a whole-genome duplication (133)) of kita, compensates for this loss of melanocyte development due to the lack of kitb expression in the neural crest (134). The homologue of the MITF gene in fish, mitfa, is just as critical for melanocyte development in fish as in other animals. The ENU-induced nacre(w2) allele contains a single base substitution in the mitfa gene that results in an early stop codon and a truncated protein that lacks the basic helix-loop-helix leucine zipper domain required for DNA binding, referred to in this text as mitfa(lf), resulting in animals completely devoid of melanocytes (128).

Second, an autochthonous model of melanoma development has been previously established in zebrafish (135). In this model the human BRAF^{V600E} oncogene is driven to express in melanocytes as a transgene under the mitfa
promoter. On its own in zebrafish, BRAF\textsuperscript{V600E} causes formation of benign hyperpigmented spots with high melanocyte density, referred to as fish-nevi, or f-nevi (135). This is similar to the effect of BRAF\textsuperscript{V600E} causing formation of nevi in mammals. When the BRAF\textsuperscript{V600E} transgene was introduced into a p53 loss-of-function mutation background, p53(\textit{zdf1}), referred to in this text as \textit{p53(lf)}, these zebrafish developed fully-penetrant melanomas with a median onset of 4 months (135). An adaptation of this model was created to facilitate testing of genetic candidates for their contribution to melanoma formation. In this system researchers inject a construct, called \textit{miniCoopR}, that juxtaposes a \textit{mitfa} minigene with a transgene of interest driven by the \textit{mitfa} promoter into a \textit{Tg(mitfa:BRAF\textsuperscript{V600E}); p53(lf); mitfa(lf)} background (136). The \textit{mitfa(lf)} mutation prevents melanocyte formation, thus suppressing melanoma in the \textit{Tg(mitfa:BRAF\textsuperscript{V600E}); p53(lf)} background; however, injection of a \textit{miniCoopR} construct with Tol2 transposase RNA into fertilized single-cell embryos will rescue melanocyte development in adult zebrafish and those melanocytes will go on to form melanomas that overexpress the candidate gene. This system was used to interrogate the overexpressed genes located in the human 1q21 region that is recurrently amplified in human melanoma and found that expression of the histone-methyltransferase SETDB1 significantly accelerated tumor onset compared to expression of EGFP (136). This illustrates several of the major advantages for using fish to study melanoma, namely their relative ease of genetic manipulation, their high fecundity (a single mating pair may produce
several hundred offspring per week, several thousand transgenic animals can be injected per week), and their visible melanoma onset. The use of the miniCoopR system provides a straightforward approach to testing candidate genes for their impact on autochthonous tumor development.

As with any model organism, several drawbacks exist when working with zebrafish that should be considered when planning and interpreting experiments. The zebrafish lineage underwent a whole genome duplication event resulting in many genes having duplicate copies that have diverged in function (137,138). Thus, when identifying a gene to inhibit, it is necessary to justify that the appropriate copy of the gene is being targeted and whether the sister ohnologue will have any compensatory function. Additionally, zebrafish require a generation time of at least three months, thus they require extensive time periods for generation of stable transgenic lines. Melanoma onset also requires 6-12 months to complete analysis for any given test population, therefore experiments must be carefully planned to account for these time-scales.

Additionally, it should be noted that the melanomas from $Tg$($mitfa$:BRAF$^{V600E}$); $p53$($lf$) fish can grow exophytically or become locally invasive, but have not been confirmed to independently form distant metastases as seen in human melanomas (135). Nevertheless, a working model of melanoma metastasis has been established by subcutaneous transplantation of
zebrafish melanoma cell lines into irradiated, transparent casper mutant zebrafish (139) that have been shown to form lesions at sites distant and distinct from their injection sites (140). This quantifiable model of melanoma metastasis has been used to screen zebrafish larvae for modifiers of metastasis and found that the ligand of the G protein-coupled receptor EDNRB, EDN3, which is associated with Waardenburg syndrome and is required for melanocyte development, could induce a proliferation and differentiation of melanoma cells and enhanced metastatic spreading (141). It is anticipated that this method of transplanting zebrafish melanoma cell lines will continue to illuminate the processes involved in late stages of melanoma metastasis. Another contrast between zebrafish melanomas and human melanomas is in their mutation burden. Zebrafish melanomas contain a median of 4 coding mutations per zebrafish tumor, compared to 171 per sun-exposed human melanoma (142). Despite this difference, zebrafish melanomas are riddled with a high number of genomic rearrangements and copy number changes, similar to human melanomas (142). Researchers utilizing zebrafish should also consider the reported high heterogeneity of zebrafish melanomas and plan to use a sufficiently high sample size to give satisfactory statistical power for any tumor phenotype under evaluation (142). Despite these several items that must be considered during experimental planning, the high fecundity and ease of genetic manipulation of the zebrafish make it possible to evaluate a high number of
candidate genes with large sample sizes to test their contribution to genuine melanoma development.

**Rationale and objectives**

Although KIT has been defined as an oncogene in rare acral, mucosal, or in BRAF/NRAS/NF-1 wild-type melanomas, the aim of this work has been to clarify the role of KIT in the more common BRAF-mutant melanomas. Given that this large fraction of human melanomas, roughly 40% of cases, frequently loses KIT expression, it may be expected that the loss of KIT benefits BRAF\(^{V600E}\)-driven tumor formation in some way; however, given the role of KIT in stimulating the RAS/MAPK pathway to promote melanocyte survival and migration, it appears counterintuitive to expect KIT to inhibit melanoma growth. It is possible that the loss of KIT is coincidental to melanoma progression; however, if KIT actively suppresses BRAF\(^{V600E}\)-driven tumor onset, this discovery would help clarify the role of RTKs in RAF mutant cancers and suggest caution against targeting KIT in this class of melanomas. The zebrafish \(Tg(mita:BRAF^{V600E}); p53(lf)\) melanoma model represents a working system to study the role of KIT, given the availability of the \(kit(lf)\) mutant line that may be crossed with \(Tg(mita:BRAF^{V600E}); p53(lf)\) fish to determine the impact of KIT on melanoma development.

The objectives for this project included:

- To determine whether a meaningful association exists between the occurrence of \(BRAF^{V600E}\) mutations and the loss of \(KIT\) expression in
human melanoma. This was carried out through analysis of The Cancer Genome Atlas Database that has published human melanoma RNAseq data for several hundred patient samples where mutation status and expression may be examined.

- To test *KIT* loss in a zebrafish model of melanoma by crossing
  
  $Tg(mitfa:BRAF^{V600E}); p53(\text{lf})$ with $kit(\text{lf})$ mutant zebrafish to generate $kit(\text{lf}); Tg(mitfa:BRAF^{V600E}); p53(\text{lf})$ homozygous lines and compare melanoma onset, invasiveness, and Kit-pathway signaling.

- To test the effect of *KIT* knockdown or overexpression in human melanoma cell lines.

- To analyze the association of *KIT* expression with the clinical outcome of human melanoma patients.
Chapter II - KIT suppresses melanoma formation by attenuating BRAF oncogenic activity

Preface

Note that the contents of Chapter II have been adapted from the Student’s publication Neiswender et al, Cancer Research 2017 (1).

Introduction

Key mutations that drive melanoma progression hyperactivate RAS/MAPK signaling (28,78). Activating mutations in BRAF and NRAS, the well-known drivers of MAPK pathway activity, are the most common oncogenic driver mutations in melanoma, found to occur in in 52% and 28% of the 318 samples published in the TCGA database (78). Loss-of-function mutations in the RAS-GTP-ase activating protein NF1 prevent this protein from inhibiting RAS activity and thus lead to higher MAPK pathway activation in 14% of TCGA samples (78). At the top of this signaling cascade in melanocytes is the receptor tyrosine kinase KIT, known to play a critical role in melanocyte and hematopoietic development (69,101-103). Oncogenic mutations in KIT were found in only 6/318 (2%) of TCGA cutaneous melanoma samples, whereas somewhat higher rates of 15-20% of rare acral or mucosal melanomas were found to have KIT mutations (112-114). Although KIT represents a verified melanoma oncogene in a relatively low percentage of melanomas, the large fraction of sun-exposed cutaneous
melanomas frequently lose KIT expression (120-122), suggesting that KIT may play a suppressive role during melanoma formation.

To investigate the role of KIT in melanoma, we tested whether the absence of Kit affects melanoma formation by introducing a kit loss-of-function mutation into a zebrafish melanoma model that combines melanocyte lineage-expressed $BRAF^{V600E}$ with a $p53$ loss-of-function mutation (135). Loss of kit caused a significant acceleration of tumor initiation. In both zebrafish tumors and human melanoma cell lines, loss of KIT led to an increase in RAS/MAPK pathway signaling. Our mechanistic studies suggest that KIT-mediated activation of wild-type RAF proteins can dampen oncogenic signaling from $BRAF^{V600E}$. These data indicate that, in the context of a $BRAF^{V600E}$ mutation, KIT acts as a tumor suppressor.

Results

As described below, we aimed to test whether the loss of KIT would impact melanoma initiation by using a zebrafish strain that develops fully-penetrant melanomas driven by human $BRAF^{V600E}$ (135). To first confirm that examining the loss of $KIT$ in a $BRAF^{V600E}$-mutant background was appropriate, we investigated whether reduced $KIT$ expression was associated with oncogenic $BRAF$ mutations in human melanoma. Based on normalized transcript amounts from tumor samples in The Cancer Genome Atlas (TCGA) (78), $BRAF^{V600E}$
mutant melanomas generally expressed lower levels of KIT than melanomas with wild-type BRAF (BRAF<sup>WT</sup>) (Fig. II.1A). Contributing to this difference were tumors, in the BRAF<sup>WT</sup> group that contained KIT gain-of-function mutations. As a further indication of the inverse relationship of KIT expression and BRAF<sup>V600E</sup> mutations, gene set enrichment analysis (GSEA) revealed that a set of genes upregulated by BRAF<sup>V600E</sup> (143) was associated with low KIT expression in BRAF<sup>V600E</sup>-mutant melanomas (Fig. II.1B). These data indicate that oncogenic BRAF mutations and activity are correlated with low KIT expression in human melanoma.
Figure II.1 – BRAF<sup>V600E</sup> mutations and gene signature are associated with low KIT expression in human melanoma

(A) KIT mRNA expression was analyzed in TCGA melanoma samples that were sorted according to BRAF and KIT mutation status, *P value < 0.05; Student’s t test. (B) GSEA showing association of a BRAF<sup>V600E</sup>-induced gene set (143) with low KIT expression in TCGA melanoma samples. Nominal P value calculated as described (144), where the observed enrichment score was compared to a set of 1000 randomly generated enrichment scores.
We began investigating the relationship between KIT and BRAF\textsuperscript{V600E} in zebrafish by crossing a \textit{kit(b5)} loss-of-function mutant strain (129), referred to hereafter as \textit{kit(If)}, with a \textit{Tg(mitfa:BRAF\textsuperscript{V600E})} strain, that expressed \textit{BRAF\textsuperscript{V600E}} in the melanocyte lineage (135). This was important because it was unclear whether melanoma could be investigated in a \textit{kit}-mutant background - melanomas in zebrafish predominantly arise from dorsal regions containing scale-associated melanocytes, and \textit{kit(lf)} zebrafish lack these melanocytes. In the resulting \textit{kit(If); Tg(mitfa:BRAF\textsuperscript{V600E})} strain, dorsal scale-associated melanocytes were present in a typical net-like pattern, although at a density somewhat lower than in \textit{Tg(mitfa:BRAF\textsuperscript{V600E})} animals (Fig. II.2A). To determine whether \textit{BRAF\textsuperscript{V600E}} rescued development of dorsal, scale-associated melanocytes that were missing in \textit{kit(If)} fish or whether the oncogene had merely induced mis-migration of otherwise extant stripe-associated melanocytes, we further crossed these strains into an \textit{ednr\textit{b}(lf)} background (129). Whereas, \textit{kit(lf); ednr\textit{b}(lf)} double mutants lacked melanocytes entirely, \textit{kit(lf); ednr\textit{b}(lf); Tg(mitfa:BRAF\textsuperscript{V600E})} fish still developed a population of dorsal scale-associated melanocytes (Fig. II.2B). These results indicate that \textit{BRAF} can act downstream of, or in parallel to, \textit{kit} to promote melanocyte development, which is consistent with KIT receptor signaling through BRAF that is thought to occur in this cell type (68,145).
Figure II.2

(A) Profile images of Tg(mitfa:BRAF<sup>V600E</sup>), kit(lf), and kit(lf); Tg(mitfa:BRAF<sup>V600E</sup>) adult zebrafish showing rescue of dorsal, scale-associated melanocytes in kit(lf); Tg(mitfa:BRAF<sup>V600E</sup>) zebrafish. (B) Profile and dorsal images of kit(lf); ednrb(lf), and kit(lf); ednrb(lf); Tg(mitfa:BRAF<sup>V600E</sup>) adult zebrafish showing melanocyte rescue in kit(lf); ednrb(lf); Tg(mitfa:BRAF<sup>V600E</sup>) zebrafish. The mean density of melanocytes per square millimeter ±SEM on dorsal scales is listed for each genotype.

Figure II.2 – BRAF<sup>V600E</sup> rescues dorsal scale-associated melanocytes absent in kit(lf) zebrafish

(A) Profile images of Tg(mitfa:BRAF<sup>V600E</sup>), kit(lf), and kit(lf); Tg(mitfa:BRAF<sup>V600E</sup>) adult zebrafish showing rescue of dorsal, scale-associated melanocytes in kit(lf); Tg(mitfa:BRAF<sup>V600E</sup>) zebrafish. (B) Profile and dorsal images of kit(lf); ednrb(lf), and kit(lf); ednrb(lf); Tg(mitfa:BRAF<sup>V600E</sup>) adult zebrafish showing melanocyte rescue in kit(lf); ednrb(lf); Tg(mitfa:BRAF<sup>V600E</sup>) zebrafish. The mean density of melanocytes per square millimeter ±SEM on dorsal scales is listed for each genotype.
Rescue of melanocyte development by \textit{BRAF}^{V600E} enabled us to test whether the loss of \textit{kit} would have an effect on \textit{BRAF}^{V600E}-driven melanoma formation. Before doing so, we assessed whether \textit{kit} was expressed in melanomas from \textit{Tg(mitfa:BRAF}^{V600E}); p53(lf) fish. In contrast to most human \textit{BRAF}^{V600E}-mutant melanomas, \textit{kit} was expressed in \textit{Tg(mitfa:BRAF}^{V600E}); p53(lf) zebrafish melanomas (Fig. II.3A), making this model useful for interrogating the effects of the loss of \textit{kit} in melanoma formation. We established a \textit{kit(lf)}; \textit{Tg(mitfa:BRAF}^{V600E}); p53(lf) strain and monitored tumor onset in these animals as compared to \textit{Tg(mitfa:BRAF}^{V600E}); p53(lf) controls. The \textit{kit(lf)}; \textit{Tg(mitfa:BRAF}^{V600E}); p53(lf) fish had markedly accelerated melanoma onset relative to \textit{Tg(mitfa:BRAF}^{V600E}); p53(lf) animals; the median melanoma onset in \textit{kit(lf)}; \textit{Tg(mitfa:BRAF}^{V600E}); p53(lf) fish was 21 weeks, whereas it was 35 weeks in a \textit{Tg(mitfa:BRAF}^{V600E}); p53(lf) background (Fig. II.3B). To confirm that accelerated onset was due to the loss of \textit{kit} rather than background effects, we asked whether a second, independently-derived \textit{kit} loss-of-function allele, \textit{kit(e78)} (146), could also affect onset. Indeed, the \textit{kit(e78)}; \textit{Tg(mitfa:BRAF}^{V600E}); p53(lf) strain experienced accelerated tumor onset similar to \textit{kit(b5)}; \textit{Tg(mitfa:BRAF}^{V600E}); p53(lf) fish (Fig. II.4A), indicating that the loss of \textit{kit} is responsible for the accelerated tumor onset. Melanomas from \textit{kit(lf)}; \textit{Tg(mitfa:BRAF}^{V600E}); p53(lf) (all further use of “\textit{kit(lf)}” refers to the \textit{kit(b5)} strain) were more frequently invasive, with 65% of tumors having penetrated through the musculature and into the spinal column at four weeks post onset, while only 9%
of tumors from control fish displayed such invasion (Fig. II.3C and Table II.1). To investigate whether tumor invasiveness was a cell-intrinsic property, donor blastomeres from \textit{kit}(lf); \textit{Tg}(\textit{mitfa}:\textit{BRAF}^{V600E}); \textit{p53}(lf) embryos were transplanted into \textit{Tg}(\textit{mitfa}:\textit{BRAF}^{V600E}); \textit{p53}(lf); \textit{mitfa}(lf) blastula-stage host embryos or, conversely, donor blastomeres from \textit{Tg}(\textit{mitfa}:\textit{BRAF}^{V600E}); \textit{p53}(lf) embryos were transplanted into \textit{kit}(lf); \textit{Tg}(\textit{mitfa}:\textit{BRAF}^{V600E}); \textit{p53}(lf); \textit{mitfa}(lf) blastula-stage host embryos. Invasiveness was determined to be a cell-autonomous property as melanomas derived from \textit{kit}(lf); \textit{Tg}(\textit{mitfa}:\textit{BRAF}^{V600E}); \textit{p53}(lf) cells were invasive in a \textit{Tg}(\textit{mitfa}:\textit{BRAF}^{V600E}); \textit{p53}(lf); \textit{mitfa}(lf) background, whereas melanomas derived from \textit{Tg}(\textit{mitfa}:\textit{BRAF}^{V600E}); \textit{p53}(lf) cells in a \textit{kit}(lf); \textit{Tg}(\textit{mitfa}:\textit{BRAF}^{V600E}); \textit{p53}(lf); \textit{mitfa}(lf) background were not invasive (Fig. II.4B and II.4C). These results indicate that the loss of \textit{kit} promotes tumor onset and invasiveness, demonstrating that \textit{kit} acts as a tumor suppressor in \textit{BRAF}^{V600E}-mutant melanomas.
Figure II.3

A

Antisense

Antisense (Inset)

Sense

kit

Muscle

Tumor

DAPI

B

\[ \text{Tg(mita.BRAF}\text{ }^{\text{moc}}; \ p53(\text{f}) (n=106)} \]

\[ \text{kit}(\text{f}); \ Tg(mita.BRAF}\text{ }^{\text{moc}}; \ p53(\text{f}) (n=135)} \]

C

\[ \text{Tg(mita.BRAF}\text{ }^{\text{moc}}; \ p53(\text{f})} \]

\[ \text{kit}(\text{f}); \ Tg(mita.BRAF}\text{ }^{\text{moc}}; \ p53(\text{f})} \]
Figure II.3 – *kit* suppresses $BRAF^{V600E}$-driven melanoma formation

(A) In-situ hybridization of a representative $Tg(mitfa:BRAF^{V600E}); p53(If)$ zebrafish melanoma with *kit* antisense and sense probes. Dashed yellow lines indicate the border between normal muscle tissue and tumor. Yellow squares indicate the locations of the inset magnified for the antisense images in the center. Scale bars = 150 µm (B) Tumor onset curves for $Tg(mitfa:BRAF^{V600E}); p53(If)$ and *kit(If); Tg(mitfa:BRAF^{V600E}); p53(If)$ fish. $P$ value = 8.46 x 10^{-12}; log-rank test. (C) Representative adult zebrafish with dorsal melanomas. Lower panels show transverse sections from non-invasive $Tg(mitfa:BRAF^{V600E}); p53(If)$ and invasive *kit(If); Tg(mitfa:BRAF^{V600E}); p53(If)$ tumors. Quantification of invasiveness is shown in Table 1. Scale bars = 0.5 mm.
Figure II.4 – *kit* loss-of-function alleles accelerate melanoma onset and increase invasiveness

(A) Tumor onset curves showing melanoma-free survival for $\text{Tg(mitfa:BRAF}^{\text{V600E}}} ; \text{p53(If)} , \text{kit(b5)} ; \text{Tg(mitfa:BRAF}^{\text{V600E}}} ; \text{p53(If)} , \text{and kit(e78)} ; \text{Tg(mitfa:BRAF}^{\text{V600E}}} ; \text{p53(If)}$ zebrafish. *kit(b5)* is the allele referred to as *kit(If)* elsewhere in the manuscript. $P$ value $= 8.46 \times 10^{-12}$ for *kit(b5)*; $\text{Tg(mitfa:BRAF}^{\text{V600E}}} ; \text{p53(If)}$ vs. $\text{Tg(mitfa:BRAF}^{\text{V600E}}} ; \text{p53(If)}$, $P$ value $= 8.75 \times 10^{-10}$ for *kit(e78); Tg(mitfa:BRAF}^{\text{V600E}}} ; \text{p53(If)}$ vs. $\text{Tg(mitfa:BRAF}^{\text{V600E}}} ; \text{p53(If)}$, and $P$ value $= 0.287$ for *kit(b5); Tg(mitfa:BRAF}^{\text{V600E}}} ; \text{p53(If)}$ vs. *kit(e78); Tg(mitfa:BRAF}^{\text{V600E}}} ; \text{p53(If)}*; log-rank test. (B) Hematoxylin and eosin staining of a transverse section from a $\text{Tg(mitfa:BRAF}^{\text{V600E}}} ; \text{p53(If)} ; \text{mitfa(If)}$ host with a melanoma derived from *kit(If); Tg(mitfa:BRAF}^{\text{V600E}}} ; \text{p53(If)}$ donor cells. 3/3 such animals had melanomas that invaded into the musculature. (C) Transverse zebrafish section from a *kit(If); Tg(mitfa:BRAF}^{\text{V600E}}} ; \text{p53(If)} ; \text{mitfa(If)}$ host with a melanoma derived from $\text{Tg(mitfa:BRAF}^{\text{V600E}}} ; \text{p53(If)}$ donor cells. 0/4 animals had melanomas that invaded into the musculature. Scale bar $= 0.5$ mm.
Table II.1

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Table II.1 – Melanoma invasiveness in kit(lf) mutant zebrafish

Percentages of fish that were positive for melanoma cells at the indicated anatomical depth relating to the representative images in Fig. II.3C.

Tg(mitfa:BRAF<sub>V600E</sub>); p53(lf) versus kit(lf); Tg(mitfa:BRAF<sub>V600E</sub>); p53(lf), P value = 0.0004; chi-squared test.
To explore whether signaling downstream from kit is altered in kit(lf); Tg(mitfa:BRAF$^{V600E}$); p53(lf) tumors, we analyzed levels of phosphorylated Erk (pErk), phosphorylated Akt (pAkt), and Mitfa by western blotting. There was no significant difference in Akt activation or Mitfa expression between kit(lf); Tg(mitfa:BRAF$^{V600E}$); p53(lf) and Tg(mitfa:BRAF$^{V600E}$); p53(lf) tumors (Fig. II.5A-C). However, kit(lf); Tg(mitfa:BRAF$^{V600E}$); p53(lf) tumors did possess 2.6-fold higher levels of pErk (Fig. II.6A and II.6B). Phospho-specific MEK (pMEK) antibodies did not cross react with zebrafish MEK protein, so we could not quantify pMEK/MEK ratios to further measure pathway activity. ERK activation is the major conduit for BRAF$^{V600E}$ signaling, therefore KIT inhibition of melanoma formation could be mediated by inhibition of oncogenic BRAF$^{V600E}$ signaling.
Figure II.5

A

Tg(mita.BRAF<sup>p53<sub>+/&lt;/sup></sub>); p53<sup>+/-</sup>)

kit(/f); Tg(mita.BRAF<sup>p53<sub>+/&lt;/sup></sub>); p53<sup>+/-</sup>)

Mitfa

pAkt

Akt

α-Tubulin

B

C

Box plots showing normalized Mitfa, pAkt, and α-Tubulin levels for different genotypes.
Figure II.5 – Mitfa levels and Akt activation are not significantly altered in

*kit*(*lf*); *Tg(mitfa:BRAF*<sup>V600E</sup>); *p53*(*lf*) zebrafish melanomas

(A) Western blots for Mitfa, pAkt (S473), Akt, and α-Tubulin in seven *Tg(mitfa:BRAF*<sup>V600E</sup>); *p53*(*lf*) and ten *kit*(*lf*); *Tg(mitfa:BRAF*<sup>V600E</sup>); *p53*(*lf*) zebrafish melanoma lysates. (B) Quantification of Mitfa/α-Tubulin. (C) Quantification of pAkt/Akt. Mitfa/α-Tubulin and pAkt/Akt signals were normalized to the mean Mitfa/α-Tubulin and pAkt/Akt signals, respectively, from *Tg(mitfa:BRAF*<sup>V600E</sup>); *p53*(*lf*) tumor samples. Box plot edges represent minimum, 1st quartile, median, 3rd quartile, and maximum of normalized values. *P* values of 0.605 and 0.958 for (B) and (C), respectively. NS, not significant; Students *t* test.
Figure II.6

Figure II.6 – *kit* suppresses *BRAF*<sup>V600E</sup> oncogenic signaling in zebrafish melanomas

(A) Western blots and (B) quantification of pErk/Erk for *Tg*(mitfa:*)BRAF<sup>V600E</sup>*; p53(If) and *kit*(If); *Tg*(mitfa:*)BRAF<sup>V600E</sup>*; p53(If) zebrafish melanomas. pERK/ERK signals were normalized to the mean pERK/ERK signal from *Tg*(mitfa:*)BRAF<sup>V600E</sup>*; p53(If) tumor samples. Box plot edges represent minimum, 1<sup>st</sup> quartile, median, 3<sup>rd</sup> quartile, and maximum normalized values. *P* value < 0.05; Student’s *t* test.
To determine whether KIT also suppressed RAS/MAPK pathway signaling in human melanoma cells, we knocked down KIT in 888MEL cells, a rare melanoma cell line that has a \textit{BRAF^{V600E}} mutation but still expresses KIT (124). 888MEL cells might sustain KIT expression through the transcription factor AP-2, which directly binds and upregulates the \textit{KIT} promoter (147). Although AP-2 expression is frequently absent in melanoma cell lines, it has been previously shown that 888MEL cells have high levels of this factor (147,148). We observed \textit{BRAF^{V600E}} activity by measuring pMEK. MEK is phosphorylated by BRAF and is the most direct measure of pathway activity downstream of BRAF activation. In cells where KIT was knocked down with either of two independent shRNAs, levels of pMEK increased (Fig II.7A). Also, KIT-knockdown cells more readily formed colonies when plated at low density in an anchorage-dependent colony formation assay (Fig. II.7B). Furthermore, stimulation of KIT-expressing 888MEL cells with Stem Cell Factor (SCF), the KIT ligand, reduced both MEK activation and growth of these cells without affecting expression of either KIT or \textit{BRAF^{V600E}} (Fig. II.7C and II.7D). These experiments demonstrate that KIT can function in 888MEL cells to suppress RAS/MAPK pathway activity and reduce their growth potential.
Figure II.7

A

B

C

D
Figure II.7 – KIT suppresses BRAF$^{V600E}$ oncogenic signaling in a human melanoma cell line

(A) Western blots of pMEK and MEK for human melanoma 888MEL cells expressing a control non-silencing shRNA or either of two KIT-targeting shRNAs. (B) Colony formation assay for 888MEL cells expressing a control non-silencing shRNA or either of two KIT-targeting shRNAs. (C) Western blot and quantification of 888MEL cells treated with 200 ng/ml Bovine Serum Albumin (BSA) or Stem Cell Factor (SCF) 30 minutes prior to protein harvest. (D) 888MEL cells were counted during adherent growth with 200 ng/ml BSA or SCF treatment administered on days 1 and 4. Data in B, C, and D are represented as mean ±SEM for experiments done in biological triplicate. *$P$ value < 0.05, **$P$ value < 0.01, NS, not significant; Student’s $t$ test.
We considered different possibilities for how KIT could inhibit BRAF$^{V600E}$-driven signaling. Since KIT activity normally promotes downstream MEK/ERK signaling, it is paradoxical that the loss of KIT would lead to an increase in downstream pathway activity. Excess ERK activation invokes negative feedback loops, so perhaps KIT upregulates signaling to a level where negative feedback is needed to dampen pathway activity. We consider such mechanisms unlikely because A) a major mode of negative feedback is through upregulation of dual-specificity phosphatases, which remove activating phosphoryl groups from ERK (149) – upon loss of KIT in 888MEL cells, we found increased pMEK (Fig. II.7A), arguing that the effect of KIT loss is not centered on ERK, but instead occurs more upstream in the pathway, and B) negative feedback is also accomplished by inhibitory phosphorylation on BRAF by ERK – BRAF$^{V600E}$ is insensitive to such inhibition and thus not subject to this type of negative feedback (89).

For these reasons, we considered mechanisms whereby KIT could more directly interfere with BRAF$^{V600E}$-driven signaling. RTK activation of RAS leads to membrane-localized BRAF:CRAF dimer formation and activation (84,150). The V600E mutation shifts BRAF into a constitutively active conformation capable of phosphorylating MEK in a RAS-independent manner with vastly increased kinase activity (90,151,152). Previously it was reported that BRAF$^{V600E}$ kinase activity was reduced when it heterodimerized with CRAF (153), so we hypothesized that KIT could drive formation of BRAF$^{V600E}$:CRAF complexes, which would have less
activity than uncomplexed BRAF\textsuperscript{V600E}. To test this, we used a bioluminescence resonance energy transfer (BRET) assay to measure the interaction between \textit{Renilla} Luciferase-tagged CRAF (RLuc-CRAF) and Venus-tagged BRAF\textsuperscript{V600E} (V-BRAF\textsuperscript{V600E}). We determined transfection conditions in HEK293T cells where formation of V-BRAF\textsuperscript{V600E}:RLuc-CRAF dimers was induced by co-expression with NRAS\textsuperscript{Q61K} as a constitutively active surrogate for pathway stimulation (STIM). Under stimulating conditions, an increase in dimerization was measured between V-BRAF\textsuperscript{V600E} and RLuc-CRAF as a decrease in BRET\textsuperscript{50}, the acceptor/donor ratio that yields 50 percent of maximal signal of a BRET titration curve (Fig. II.8A and Table II.2). We used site directed mutagenesis of the V-BRAF\textsuperscript{V600E} construct to introduce an R188L mutation, which disrupts the RAS-binding domain of BRAF (82). V-BRAF\textsuperscript{R188L/V600E} was less sensitive to upstream stimulation-induced RLuc-CRAF binding, as indicated by a less dramatic shift in the BRET\textsuperscript{50} for its titration curve (Fig. II.8A and Table II.2). Levels of pMEK increased when cells expressing V-BRAF\textsuperscript{V600E} were stimulated by upstream pathway activity, but did not increase as much for V-BRAF\textsuperscript{R188L/V600E} stimulation (Fig. II.8A and II.8C). These data suggest that, in the context of upstream stimulation, formation of BRAF\textsuperscript{V600E}:CRAF complexes does not diminish the total signaling output of BRAF\textsuperscript{V600E}-mutant cells.
Figure II.8

A

B

C

[Graph showing BRET signal vs. [Acceptor]/[Donor] for different combinations of V-BRAF or V-BRAF<sup>W219C</sup> with STIM, V-BRAF<sup>W219C</sup> with EMPTY, V-BRAF<sup>W219C</sup> with STIM, and V-BRAF<sup>W219C</sup> with EMPTY.]

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[Images of Western blot results showing expression levels of BRAF, pMEK, and MEK under different conditions.]
Figure II.8 – Upstream stimulation drives $\text{BRAF}^{\text{V600E}}$:$\text{CRAF}$ dimer formation and increases oncogenic signaling

(A) BRET titration curves measuring interaction between $\text{V-BRAF}^{\text{V600E}}$ and $\text{RLuc}$-$\text{CRAF}$ in the presence or absence of stimulation with $\text{NRAS}^{Q61K}$ (STIM). The induction of a $\text{VBRAF}^{\text{V600E}}$:$\text{RLuc}$-$\text{CRAF}$ interaction signal was modestly diminished when the RAS binding domain of $\text{V-BRAF}^{\text{V600E}}$ was disrupted with an R188L mutation. Data points denote the mean ±SEM for either the $[\text{Acceptor}]/[\text{Donor}]$ ratio on the x-axis, or the BRET signal on the y-axis for three biological replicates. Quantification of BRET$^{50}$ values is shown in Supplementary Table II.2. (B) Western blots of pMEK and MEK from HEK293T cells expressing $\text{V-BRAF}^{\text{V600E}}$ with or without upstream stimulation. The $\text{BRAF}^{\text{V600E}}$ band in lane 4 runs slightly higher when activated by upstream stimulation. (C) Western blots of pMEK and MEK from HEK293T cells expressing $\text{V-BRAF}^{\text{R188L/V600E}}$ with or without upstream stimulation.
Table II.2

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<td>1.944&lt;sup&gt;**&lt;/sup&gt;</td>
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Table II.2 – BRET<sup>50</sup> values for V-BRAF<sup>V600E</sup> and V-BRAF<sup>R188L/V600E</sup>

BRET<sup>50</sup> and BRET<sup>50</sup> fold changes for Venus-BRAF<sup>V600E</sup> or Venus-BRAF<sup>R188L/V600E</sup> and RLuc-CRAF in HEK293T cells in the presence or absence of stimulation with NRAS<sup>Q61K</sup> (STIM). Associated with the BRET titration curve in Supplementary Fig. II.8A. Data obtained from biological triplicate samples. **P value < 0.01, ***P value < 0.001, ****P value < 0.0001, NS, not significant; F-test.
We therefore examined another hypothesis. Since the $BRAF^{WT}$ allele can continue to be expressed in $BRAF^{V600E}$-mutant cancer cells (15,154,155), we reasoned that upstream activation of this pathway could recruit $BRAF^{WT}$ proteins to form dimers, thus engaging pathway components which possess relatively low kinase activity (Fig. II.15). To determine if $BRAF^{WT}$:CRAF dimers could dampen $BRAF^{V600E}$ activity, we again used BRET assays to determine transfection conditions in HEK293T cells that yielded stimulation-dependent increases in V-$BRAF^{WT}$:RLuc-CRAF heterodimers. Upstream stimulation induced a decrease in BRET, indicating a robust interaction of V-$BRAF^{WT}$ and RLuc-CRAF (Fig. II.9A and Table II.3). Upstream stimulation only modestly induced an interaction between a V-$BRAF^{R188L}$ mutant and RLuc-CRAF. Using transfection conditions that yielded low, medium, and near saturating levels of V-$BRAF^{WT}$:RLuc-CRAF interaction, we measured MEK phosphorylation. With upstream stimulation, pMEK levels increased when any amount of $BRAF^{WT}$ was expressed in cells (Fig. II.9B). A less robust increase was observed when $BRAF^{R188L}$ was expressed. We next asked whether expression of $BRAF^{WT}$ could impact $BRAF^{V600E}$-driven MEK activation. In the absence of pathway stimulation, neither the addition of $BRAF^{WT}$ nor $BRAF^{R188L}$ affected pMEK levels in $BRAF^{V600E}$-expressing cells (Fig. II.9C). However, with upstream stimulation, $BRAF^{WT}$ expression reduced pMEK levels in $BRAF^{V600E}$-expressing cells, whereas $BRAF^{R188L}$ expression had no effect (Fig. II.9D). Quantification of relative pMEK/MEK was calculated in biological triplicate samples of all BRAF titrations for each experimental condition (Fig. II.9E-F).
Figure II.9 – Upstream stimulation of BRAFWT can dampen BRAFV600E oncogenic signaling

(A) BRET titration curves measuring association between RLuc-CRAF and varying levels of V-BRAFWT or V-BRAFV188L in the presence or absence of stimulation from NRASQ61K (STIM). Data points represent the mean ±SEM for either the [Acceptor]/[Donor] ratio on the x-axis, or the BRET signal on the y-axis for three biological replicates. Quantification of BRET50 values is shown in Table 2. (B-D) Western blots of pMEK and MEK from HEK293T cells expressing BRAFWT or BRAFV188L with upstream stimulation alone (B), with BRAFV600E alone (C), or with both stimulation and BRAFV600E (D). The highest bands on the BRAF blots are Venus-tagged BRAFV600E, whereas the lower bands are untagged BRAFWT or BRAFV188L, with BRAFWT running slightly higher in lane 2 of panels B and D when activated by upstream stimulation. (E-G) Quantification of pMEK/MEK levels for cells expressing varying levels of BRAFWT or BRAFV188L with upstream stimulation alone (E), with BRAFV600E alone (F), or with both stimulation and BRAFV600E (G). Fold changes were calculated in comparison to control cells in which EGFP was expressed instead of BRAFV600E or BRAFV188L. Quantification indicates the mean ±SEM of three biological replicate experiments. *P values were calculated comparing each condition of BRAFWT to BRAFV188L expression. *P value < 0.05, **P value < 0.01, ***P value < 0.001, ****P value < 0.0001; Student’s t test.
Table II.3

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Table II.3 – BRET<sup>50</sup> values for V-BRAF<sup>WT</sup> and V-BRAF<sup>R188L</sup>

BRET<sup>50</sup> and BRET<sup>50</sup> fold changes for each of two Venus-BRAF constructs expressed with RLuc-CRAF in HEK293T cells in the presence or absence of stimulation with NRAS<sup>Q61K</sup> (STIM). Associated with the BRET titration curve in Fig. 4A. Data obtained from biological triplicate samples. ***P value < 0.001, ****P value < 0.0001, NS, not significant; F-test.
We repeated these tests using HEK293T cells stably expressing KIT or enhanced green fluorescent protein (EGFP). Upon stimulation with SCF, we detected KIT phosphorylation (Fig. II.10A). In addition, SCF treatment increased levels of pMEK and promoted the interaction of V-BRAF WT with RLuc-CRAF (Fig. II.10A and II.10B and Table II.4), although both were more modest than the increases observed when cells were stimulated by NRAS Q61K. SCF stimulated, KIT or EGFP-expressing cells were transfected with BRAF constructs and, as before, BRAF WT reduced BRAF V600E-driven signaling, but only in the KIT expressing cells (Fig. II.10C and II.10D). These data indicate that BRAF WT can attenuate BRAF V600E-driven MEK activation, and this attenuation is most pronounced in the context of robust upstream stimulation.
Figure II.10

A

Time (min) with SCF: None, 1, 10, 30, 60, 240

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B

BRET signal vs. [Acceptor]/[Donor]

Acceptor: V-BRAF<sup>ΔNT</sup>

Donor: Fluc-CRAF

C

HEK-KIT

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D

HEK-EGFP

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Figure II.10 – Effects of KIT overexpression on RAF dimer formation and downstream signaling in HEK293T cells

(A) Western blots of lysates from HEK293T cells transduced with lentiviral constructs to express either KIT or EGFP. Cells harvested at indicated time points after addition of 200 ng/ml SCF. (B) BRET titration curves measuring association between V-BRAF<sup>WT</sup> and RLuc-CRAF in HEK293T cells expressing KIT, where 400 ng/ml of either BSA or SCF was administered 20 minutes prior to signal assessment. Quantification of BRET<sup>50</sup> values is shown in Supplementary Table II.4. (C) Western blots and quantification of pMEK/MEK from HEK-KIT cells expressing BRAF<sup>V600E</sup> + EGFP or BRAF<sup>V600E</sup> + BRAF<sup>WT</sup> and HEK-GFP cells expressing BRAF<sup>V600E</sup> + EGFP or BRAF<sup>V600E</sup> + BRAF<sup>WT</sup>. Samples harvested 30 minutes after addition of 400 ng/ml SCF. Bar graphs represent the mean ±SEM for biological triplicate samples. *P value < 0.05; Student’s t test.
Table II.4

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Table II.4 – BRET<sup>50</sup> values for V-BRAF<sup>WT</sup> driven by KIT and SCF

BRET<sup>50</sup> and BRET<sup>50</sup> fold changes for Venus-BRAF<sup>WT</sup> with RLuc-CRAF in HEK293T cells expressing KIT and stimulated with SCF. Associated with the BRET titration curve in Fig. II.10B. Data obtained from biological triplicate samples. *P value < 0.05; F-test.
Our studies in HEK293T cell lines suggested that signaling through BRAF^{WT} was important for KIT-mediated tumor suppression. To further explore the relationship between KIT and BRAF^{WT}, we examined signaling in melanoma cells. A375 cells are BRAF^{V600E}-mutant and have no detectable BRAF^{WT} expression, whereas UACC257 cells are BRAF^{V600E}-mutant but retain expression of BRAF^{WT} (156). Neither cell line expressed KIT endogenously (Fig. II.11A). To reconstitute upstream signaling in these cells, we introduced exogenous KIT and grew cells in the presence of SCF. KIT expression promoted growth of A375 cells and increased pMEK levels. The opposite was observed in UACC257 cells, in which KIT expression slowed growth and decreased pMEK levels (Fig. II.11B-D). These results further suggest that the tumor suppressive activity of KIT is related to the expression of BRAF^{WT}. Additionally, it is notable that 888MEL cells, which displayed KIT-mediated growth and signaling inhibition, also expressed BRAF^{WT} (Fig. II.11E and II.11F). Lastly, we examined whether the link between KIT and BRAF^{WT} extended to melanoma clinical data. TCGA melanoma samples were segregated into quintiles based on BRAF^{WT}-to-BRAF^{V600E} ratios, and the upper-most (high BRAF^{WT}:BRAF^{V600E}) and lower-most (low BRAF^{WT}:BRAF^{V600E}) quintiles were examined. Within each group, cohorts with high and low KIT expression were defined. In the high BRAF^{WT}:BRAF^{V600E} group, patients whose tumors expressed high levels of KIT experienced a survival benefit as compared to patients whose tumors had low KIT expression (Fig. II.12A). In the low BRAF^{WT}:BRAF^{V600E} group, there was no correlation between KIT expression and
patient survival (Fig. II.12B). We further observed that high \( KIT \) expression was associated with a decreased likelihood for melanomas to present with regional or distant metastases (tumor stage III/IV) amongst the group of patients with high \( BRAF^{WT}:BRAF^{V600E} \) ratios (Table II.5). \( KIT \) expression had no association with the likelihood of metastases in the group of patients with low \( BRAF^{WT}:BRAF^{V600E} \) ratios (Table II.5). We further analyzed potential relationships between \( KIT \) expression and other common melanoma tumor suppressors. This analysis revealed no significant link between \( KIT \) and the loss of \( PTEN, TP53, \) or \( CDKN2A \), suggesting that there is no interdependence or redundancy of \( KIT \) with these suppressors (Table II.6).
Figure II.11

A

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<tr>
<td>KIT</td>
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<td>![Image](KIT KIT)</td>
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<td>![Image](α-TUBULIN EGFP)</td>
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<tr>
<td>UACC257</td>
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B

A375

![Graph](Cells per well (x10,000))

C

UACC257

![Graph](Cells per well (x10,000))

D

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<td>![Image](MEK EGFP)</td>
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<tr>
<td>UACC257</td>
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E

![Graph](Fluorescence intensity)

F

![Graph](Fluorescence intensity)
Figure II.11 – Synergistic effect of KIT and BRAF<sup>WT</sup> expression on melanoma cell growth

(A) Western blots of KIT overexpression in A375 and UACC257 cells. (B and C) Adherent growth of A375 (B) or UACC257 (C) human melanoma cell lines expressing either EGFP or KIT. Cells were grown in DMEM with 0.5% FBS plus 200 ng/ml SCF added at days 1 and 4. (D). Western blots and quantification of lysate from A375 and UACC257 cells expressing EGFP or KIT. Cells were grown and treated with SCF as above. Data represent the mean ±SEM for samples done in biological triplicate. *P value < 0.05, **P value < 0.01, ***P value < 0.001; Student’s t test. (E and F) Electropherograms from Sanger sequencing of a BRAF PCR product from 888MEL (E) or A375 (F) cDNA. Positions of peaks corresponding to BRAF<sup>V600E</sup> and BRAF<sup>WT</sup> alleles are indicated.
Figure II.12 – Synergistic effect of *KIT* and *BRAF<sup>WT</sup>* expression on melanoma clinical outcome

Overall survival of human melanoma patients over time for high and low *KIT* expression cohorts from (A) the high *BRAF<sup>WT</sup>:BRAF<sup>V600E</sup>* group, *P* value = 0.0024; log-rank test, and (B) the low *BRAF<sup>WT</sup>:BRAF<sup>V600E</sup>* group, *P* value = 0.8927; log-rank test.
### Table II.5

<table>
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<th>Low ( KIT )</th>
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<th>( P ) value</th>
<th>Low ( KIT )</th>
<th>High ( KIT )</th>
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<tr>
<td>(mm)</td>
<td>3.1 ± 0.9 (( n=7 ))</td>
<td>1.4 ± 0.3 (( n=9 ))</td>
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<td>0.08</td>
<td>1.5 ± 0.3 (( n=8 ))</td>
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<td>4/6 (66.7%)</td>
<td>1/7 (14.3%)</td>
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<td>0.10</td>
<td>2/3 (33.3%)</td>
<td>5/7 (28.6%)</td>
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<td>(per mm(^2))</td>
<td>8.0 ± 3.0 (( n=5 ))</td>
<td>3.1 ± 1.0 (( n=8 ))</td>
<td>0.39</td>
<td>0.09</td>
<td>6.0 ± 2.3 (( n=3 ))</td>
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<tr>
<td>Metastasis (Stage III/IV)</td>
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<td>2/9 (22.2%)</td>
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**Table II.5 – Association of \( KIT \) expression with clinical parameters of melanoma progression**

TCGA clinical parameters of patients with melanomas in high or low \( BRAF^{WT}:BRAF^{V600E} \) groups and further subdivided into high and low \( KIT \) expression cohorts. \( P \) values were calculated for Breslow depth and mitotic rate by Student’s \( t \) tests and for ulceration and regional/distant metastasis by Fisher’s exact tests.
Table II.6

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<th>PTEN alterations</th>
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Table II.6 – Lack of association of KIT expression with PTEN, TP53, and CDKN2A alterations in human melanoma

This analysis compared how frequently PTEN, TP53, or CDKN2A tumor suppressor genes (TSGs) were altered in patients with melanomas having high or low KIT mRNA expression. We compared the upper-most 25% of KIT-expressing samples to the remaining 75% of samples to measure enrichment of TSG alterations in melanomas with high KIT expression. Additionally, we compared the lower 25% of KIT-expressing samples to the upper 75% of samples to measure enrichment of TSG alterations in melanomas with low KIT expression. We classified a sample as having a TSG alteration if it contained a mutation or whether the sample was in the lowest (for copy number, mRNA expression, and protein expression) or highest (DNA methylation) quartile within each respective category. For each category, we compared the high or low KIT-
expressing groups for enrichment of the TSG alterations using Fisher's exact tests. These three tables report the $P$ values of these comparisons for each category with each TSG.
To test the link between KIT and BRAF\textsuperscript{WT} in vivo, we employed the established ‘\textit{miniCoopR}’ method for generating transgenic melanomas in zebrafish (136). The \textit{miniCoopR} vector juxtaposes a transgene of interest to a copy of the \textit{mitfa} melanocyte specification gene. When this construct is injected into melanocyte-deficient \textit{mitfa(lf)} zebrafish embryos, melanocytes are rescued cell-autonomously by the \textit{mitfa} gene, and each rescued melanocyte expresses the neighboring transgene of interest (Fig. II.13A). \textit{miniCoopR} vectors containing either BRAF\textsuperscript{WT} or \textit{EGFP} open reading frames under control of the \textit{mitfa} promoter were injected into \textit{Tg(mitfa:BRAF\textsuperscript{V600E}); p53(lf); mitfa(lf)} embryos, and animals with rescued melanocytes were monitored weekly for melanoma onset. Expression of BRAF\textsuperscript{WT} delayed median tumor onset by seven weeks as compared to expression of \textit{EGFP} (Fig. II.13B). This change was not due to altered expression of BRAF\textsuperscript{V600E}, as levels of this oncoprotein were similar in BRAF\textsuperscript{WT} and \textit{EGFP}-expressing cohorts (Fig. II.14). To determine if this delayed onset was dependent on \textit{kit}, the same experiment was performed, except \textit{miniCoopR} constructs were injected into a \textit{kit(lf); Tg(mitfa:BRAF\textsuperscript{V600E}); p53(lf); mitfa(lf)} background. In this background, melanocyte rescue was poor and consequently tumor onset of the \textit{miniCoopR-EGFP} control cohort was slower as compared to \textit{miniCoopR-EGFP} in a \textit{Tg(mitfa:BRAF\textsuperscript{V600E}); p53(lf); mitfa(lf)} background. Within the \textit{kit(lf); Tg(mitfa:BRAF\textsuperscript{V600E}); p53(lf); mitfa(lf)} background, the \textit{miniCoopR-EGFP} control cohort and \textit{miniCoopR-BRAF\textsuperscript{WT}} animals exhibited no difference in melanoma onset (Fig. II.13C). These data indicate that BRAF\textsuperscript{WT}
can inhibit BRAF\textsuperscript{V600E}-driven tumor onset, but this inhibition is dependent upon expression of KIT.
Figure II.13

Figure II.13 – BRAF<sup>WT</sup> delays BRAF<sup>V600E</sup>-driven melanoma formation in a kit-dependent manner

(A) Overview of a miniCoopR tumor onset experiment as described in Materials and Methods. Tumor onset was monitored weekly for fish expressing BRAF<sup>WT</sup> or EGFP. (B) Tumor onset of Tg(mitfa:BRAF<sup>V600E</sup>); p53(If); mitfa(If) zebrafish with melanocytes rescued by miniCoopR-BRAF<sup>WT</sup> or miniCoopR-EGFP, P = 8.05 x 10<sup>-7</sup>; log-rank test. (C) Tumor onset of kit(If); Tg(mitfa:BRAF<sup>V600E</sup>); p53(If);
mitfa(If) zebrafish with melanocytes rescued by miniCoopR-BRAF\(^{WT}\) or miniCoopR-EGFP, \(P\) value = 0.664; log-rank test.
Figure II.14 – BRAF$^{\text{WT}}$ overexpression does not affect BRAF$^{\text{V600E}}$ levels in zebrafish melanomas

Western blots and quantification of lysate taken from $Tg(mitfa: \text{BRAF}^{\text{V600E}}); p53(\text{lf}); \text{mitfa}(\text{lf})$ zebrafish tumors generated with $\text{miniCoopR-EGFP}$ or $\text{miniCoopR-BRAF}^{\text{WT}}$ expression constructs. Data represent the mean of either BRAF$^{\text{V600E}}/\alpha$-Tubulin or total BRAF/\alpha-Tubulin ±SEM normalized to the mean of the $\text{miniCoopR-EGFP}$ group for BRAF$^{\text{V600E}}/\alpha$-Tubulin or total BRAF/\alpha-Tubulin, respectively. The columns represent three $\text{miniCoopR-EGFP}$ and eleven $\text{miniCoopR-BRAF}^{\text{WT}}$ tumors. *$P$ value < 0.05; Student’s $t$ test.
Discussion

Our results show that KIT can suppress BRAF\textsuperscript{V600E}-driven melanoma formation. The loss of KIT resulted in increased BRAF\textsuperscript{V600E}-driven oncogenic signaling in a zebrafish melanoma model and human melanoma cells. Our mechanistic studies suggest that BRAF\textsuperscript{V600E} activity can be reduced by BRAF\textsuperscript{WT}, but only under conditions where BRAF\textsuperscript{WT} receives upstream pathway stimulation. In vivo data support this notion, as expression of BRAF\textsuperscript{WT} suppressed BRAF\textsuperscript{V600E}-driven melanoma initiation in a KIT-dependent manner.

There are several possible ways in which KIT could inhibit BRAF\textsuperscript{V600E}-driven oncogenic signaling. Important negative regulators of oncogenic signaling include the dual-specificity phosphatases (DUSPs), which dephosphorylate and inactivate ERK (149,157). Potentially, KIT could stimulate DUSP activity either by signaling through ERK or by signaling independently, leading to pathway inhibition. However, upon knockdown of KIT in BRAF\textsuperscript{V600E}-mutant melanoma cells, we observed increased pMEK levels. As negative feedback from DUSPs is not expected to have an effect on MEK phosphorylation status (88,149), this led us to further investigate potential mechanisms upstream of ERK. We also considered a mechanism whereby KIT signaling would drive dimerization of BRAF\textsuperscript{V600E} with CRAF, which has been reported to lower the kinase activity of BRAF\textsuperscript{V600E} (153). While this is an attractive model, additional observations suggest that the situation is more complex. In our assay, we did not find
reduction of downstream signaling when BRAF^{V600E} was driven into dimers with CRAF. Our assay differs from the one used by Karreth et al. in that we measured downstream signaling (i.e. MEK phosphorylation) under conditions of upstream pathway stimulation. Stimulation not only facilitates membrane recruitment and dimerization of RAF species, but also promotes their activation through phosphorylation at a series of sites in the negatively-charged region (152). It is possible that BRAF^{V600E}:CRAF dimers, under conditions of upstream stimulation, receive additional activating cues, enhancing the activity of these species.

An alternative model to explain our findings would have the ratio of high activity to low activity BRAF species determine flux through the signaling pathway (Fig. II.15). In this model, under conditions of no upstream stimulation, only high activity BRAF^{V600E} would be functional. By contrast, upon upstream stimulation a mixture of low activity BRAF^{WT}:CRAF and high activity BRAF^{V600E}:CRAF dimers form. Although upstream stimulation could increase the collective number of active BRAF species, overall pathway signaling would be diminished if low activity BRAF^{WT}:CRAF species have a prominent role in determining signaling flux. Such a role could manifest in a variety of ways. For example, BRAF^{WT}:CRAF dimers could compete with BRAF^{V600E}:CRAF dimers for interaction with their shared MEK substrate. Although not considered in our analysis, BRAF^{WT} and BRAF^{V600E} could also potentially compete for interaction
with KSR scaffolding proteins (158). In each of these examples, any interaction of BRAF\textsuperscript{WT} with limiting downstream components could attenuate signaling.
Figure II.15 – Model of pathway stimulation that could reduce signaling of BRAF^{V600E}-mutant cells through activation of BRAF^{WT}

Under normal signaling conditions, KIT will promote activation of NRAS leading to stimulation of active BRAF:CRAF dimers that will phosphorylate MEK with low kinase activity leading to normal activation of the MAPK pathway. Introduction of a BRAF^{V600E} mutation to these cells would result in indiscriminate activation of either high-activity BRAF^{V600E}:CRAF or low-activity BRAF^{WT}:CRAF dimers resulting in moderate pathway activity. Lastly, the BRAF^{WT} expressed in a BRAF^{V600E}-mutant cell that lacks upstream pathway stimulation due to the loss of KIT will remain inactive in the cytoplasm leaving all signaling apparatus to be
used by BRAF$^{V600E}$ monomers or homodimers that possess high intrinsic kinase activity, resulting in overall high pathway activation.
These proposed mechanisms highlight the interplay between wild-type and oncogenic signaling in tumors. During tumorigenesis, oncogenic mutations in genes typically affect one allele, leaving the other unaffected. In tumors driven by oncogenic RAS genes, loss of the corresponding wild-type gene is frequently observed (159). A rationale for this loss comes from elegant genetic studies in the mouse which showed that the wild-type RAS gene has a tumor suppressive effect in certain tumor types (160). By contrast, loss of the $BRAF^{WT}$ allele is rare in tumors driven by oncogenic BRAF, and co-expression of wild-type and oncogenic variants is evident in the majority of tumors (15). In such tumors we propose that signaling through $BRAF^{WT}$ has a suppressive effect, but that the loss of KIT effectively abrogates this effect. More generally, our results reveal that, under certain circumstances, an unexpected increase of oncogenic RAS/MAPK pathway activity could occur upon loss or inhibition of upstream signaling components.

**Materials and Methods**

**Analyses of The Cancer Genome Atlas (TCGA)**

Data from 384 human melanoma RNA-seq samples were downloaded from the Cancer Genomics Hub (CGHub) (https://cghub.ucsc.edu) using GeneTorrent (v 3.8.5a) (161). The RNAseq from the TCGA dataset was comprised of 302 metastatic melanoma samples and 82 primary melanomas (78). We compared RNAseq-derived FPKM values of $KIT$ expression in $BRAF^{WT}$
samples to a $BRAF^{V600E}$ group, where the $BRAF^{WT}$ group excluded $BRAF^{V600E}$ and all other $BRAF$-mutant melanoma samples. Gene Set Enrichment Analysis (GSEA) was performed using GSEA (v 2.2.0) (144,162). A rank-ordered gene list was generated based on Pearson correlation of the expression level of each gene with $KIT$ expression in TCGA melanoma samples. Only genes with reads in $\geq 90\%$ of samples were included. The rank-ordered gene list was analyzed for enrichment of a set of genes at least 3-fold upregulated by overexpression of $BRAF^{V600E}$ in cultured melanocytes (143).

Survival analysis was performed by first sorting patients into groups with high (upper-most 20\%) or low (lower-most 20\%) $BRAF^{WT} : BRAF^{V600E}$ allele ratios, followed by further ranking $KIT$ mRNA expression within each group to define the high and low $KIT$ groups as the upper or lower 50\% of these ranked lists.

**Zebrafish strains and miniCoopR tumor onset assay**

The zebrafish mutant alleles used in this study include $p53(zdf1)$ (163), $Tg(mitfa:BRAF^{V600E})$ (135), $kit(b5)$ (129), $ednrb(b140)$ (129), $kit(e78)$ (146), and $mitfa(w2)$ (128). Strains were housed and cared for as approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Massachusetts Medical School. miniCoopR constructs were cloned to express either enhanced green fluorescent protein (EGFP) or human $BRAF^{WT}$ under the $mitfa$ promoter juxtaposed to a $mitfa$ minigene cassette containing the $mitfa$ promoter, open reading frame, and 3’ UTR. These were injected into embryos at
the one-cell stage along with Tol2 transposase mRNA. Fish with melanocyte rescue were selected as juveniles approximately two months post-fertilization and monitored weekly by visual inspection for exophytic tumor growth (136).

**In-situ hybridization**

*Tg(mitta:BRAF^{V600E}); p53(lf)* zebrafish with dorsal anterior melanomas were euthanized, then stored overnight in 4% paraformaldehyde at 4°C. Samples were incubated in 5%, then 30% sucrose, followed by flash freezing in Tissue-tek O.C.T. compound (VWR) on dry ice. 20 µm transverse sections were placed on Superfrost Plus charged slides (Thermo-Fisher) then desiccated and frozen at -80°C. Digoxigenin-labeled riboprobes were synthesized using a zebrafish *kita* cDNA template transcribed with T3 or T7 polymerases for antisense or sense probes, respectively (MAXIscript Kit; Thermo-Fisher). Tumor samples were rehydrated in a series of decreasing ethanol concentrations and in 2X Saline Sodium Citrate (SSC). They were then incubated in 10 µg/ml Proteinase K for 3-5 minutes and washed with water and triethanolamine (TEA) at pH 8.0. After incubation in an acetic anhydride/TEA solution, samples were dehydrated with increasing ethanol concentrations and dried before beginning an overnight incubation with the riboprobes at 55°C. Washes were performed with 2X SSC, formamide, and then samples were incubated for 30 minutes with RNAse A at 37°C. Samples were incubated in a maleic acid blocking buffer (Roche) for 2 hours followed by an overnight incubation with 1:1000 alkaline phosphatase-
conjugated anti-digoxigenin antibody (Roche) at 4°C. Samples were washed with maleate buffer and fluorescence was detected after incubation with Fast Red (HNPP Fluorescent Detection Set; Roche).

**Mosaic analysis**

Donor blastomeres from *kit(lf); Tg(mitfa:BRAF^{V600E}); p53(lf)* embryos were transplanted into *Tg(mitfa:BRAF^{V600E}); p53(lf); mitfa(lf)* blastula-stage host embryos or, conversely, donor blastomeres from *Tg(mitfa:BRAF^{V600E}); p53(lf)* embryos were transplanted into *kit(lf); Tg(mitfa:BRAF^{V600E}); p53(lf); mitfa(lf)* blastula-stage host embryos. Host animals were raised to adulthood and selected for melanocyte positivity, indicating successful donor cell growth. Fish with melanomas that arose on the dorsal anterior region were euthanized, paraffin-embedded, and stained with hematoxylin and eosin to produce transverse sections that were analyzed for tumor cell invasion.

**Melanocyte scale density assay**

4- to 6-month old fish were treated for 5 minutes with the anesthetic tricaine methanesulfonate and epinephrine, which contracts melanosomes to the central cell body of melanocytes, thereby resolving overlapping cells. Scales were plucked from the dorsal anterior region of fish from the scale rows adjacent to the dorsal midline row. Scales were immediately fixed for ≥30 minutes in 4% paraformaldehyde. After fixation, scales were flat mounted and melanocytes
counted. Area was estimated by multiplying maximal antero-posterior and left-right distances on the scale. Five scales from each of three different animals were used to calculate mean melanocyte density.

**Cell culture**

KIT knockdown experiments were performed in 888MEL cells obtained from the Yale SPORE in Skin Cancer. Cells were grown in F10 (Life Technologies 11550043) media with 5% FBS and 2% penicillin/streptomycin. KIT knockdown was accomplished with the pGIPZ lentiviral shRNA V3LHS_345750 (target sequence 5' - GCATTAAGCAGCGTATC - 3') or the TRC pLKO.1 shRNA TRCN0000000388 (target sequence 5' - AAACCCAGGGCTGCCTTGGAAAAG - 3'). A non-silencing pGIPZ lentiviral shRNA 22-mer that contains at least 3 or more mismatches against any mammalian gene was used as a negative control (sequence 5' - ATCTCGCTTGGGCGAGAGTAAG – 3'). Lentiviral particles were produced over the course of 48 hours in HEK293T cells co-transfected with the lentiviral packaging plasmids psPAX and PMD2.G. After harvesting, viral supernatant was filtered with a 0.45µm filter then mixed 1:1 in F10 media with 5% Fetal Bovine Serum (FBS), 2% penicillin/streptomycin, and 2-8 µg/ml Polybrene (Sigma). Cells were selected using 1 µg/ml puromycin for 2-4 days to establish stable cell lines. An identical approach was used to overexpress KIT and EGFP from *pLENTI-CMV-KIT* or *pLENTI-CMV-EGFP* constructs in HEK293T, A375, and UACC257
cells, but in these cases the viral supernatant was mixed 1:1 with DMEM plus 10% FBS and 2% penicillin/streptomycin. HEK293T, A375, and UACC257 cells were grown in DMEM plus 10% FBS and 2% penicillin/streptomycin. HEK293T transient transfections were performed with Lipofectamine 2000 (Life Technologies) according to the manufacturer’s protocol.

**BRET Assay**

BRET was performed as described by Lavoie et. al. (150). In brief, HEK293T cells were plated at 300,000 cells per well in six-well tissue culture-treated plates. After 48 hours, each well was incubated in 2 ml Opti-MEM, then transfected with 7.5 µl Lipofectamine 2000 plus a specified combination of pLHCX-RLuc-CRAF (100 ng), pLPCS-V-BRAF<sup>V600E</sup>, pLPCS-V-BRAF<sup>R188L/V600E</sup>, pLPCS-V-BRAF<sup>WT</sup>, or pLPCS-V-BRAF<sup>R188L</sup> (0, 50, 100, 200, 300, 400, or 600 ng), and pCDNA3.1-NRAS<sup>Q61K</sup> (250 ng) or pCDNA3.1-EMPTY (250 ng) in 200 µl Opti-MEM. Either KIT stimulation with SCF or NRAS<sup>Q61K</sup> overexpression was used for induction of upstream pathway activity (STIM), as indicated. 48 hours after transfection, cells were resuspended in 500 µl Tyrode’s buffer, then split onto opaque 96-well plates (Perkin Elmer 6005680). An initial reading of total Venus expression was taken on a PE Envision plate reader (excitation 480±30nm, emission at 530±10nm). 10 µl of coelenterazine h (Biotium 10111-1) was then added automatically through the PE Envision robotic pump to a final concentration of 2.5 µM. BRET readings were taken using two filter sets
(emission 485±15nm, emission 530±10nm) 5 minutes after adding coelenterazine. The BRET signal was determined by calculating the 530/485 signal ratio for a given sample and subtracting the 530/485 ratio measured when expressing RLuc-CRAF alone. These values were plotted as a function of the total 530/485 ratio (as a measurement of [Acceptor]/[Donor]) for a range of [Acceptor]/[Donor] concentrations, then fit with a one-site binding hyperbolic equation in GraphPad Prism (v 6.05). Each condition was run in biological triplicate, and each of these replicates was run on a separate plate in immediate succession to one another. Each plate was calculated separately for background subtraction, then all data points from the three biological replicates were combined to calculate Kd, referred to here as BRET<sup>50</sup>.

**Western Blotting**

Protein was harvested from zebrafish melanomas 2-6 weeks post-tumor onset by first euthanizing the fish with tricaine methanesulfonate followed by surgical removal of the tumor. Tumors were tritratured and lysed in ice-cold RIPA buffer containing a cOmplete protease inhibitor tablet (Roche). Protein concentration was measured using the Pierce BCA Protein Assay Kit (Life Technologies). Samples were run on 10% polyacrylamide gels, transferred, and developed using fluorophore-conjugated antibodies (LI-COR). Antibodies against the following proteins were used: Mitfa (136); RAF-1 (c-RAF), pMEK1/2 (S217/221), MEK1/2, p44/42 MAPK (ERK1/2), alpha-Tubulin, pAKT S473, AKT,
pc-KIT, c-KIT (Cell Signaling 9422, 9154, 8727, 4695, 38735, 4060, 4685, 3391, 3308, respectively); pERK (Sigma m8159); BRAF^{V600E} (Spring Bioscience E19290); total BRAF (Millipore 10146); IRDye 800CW Donkey anti-Rabbit and IRDye 680RD Goat anti-Mouse (LI-COR 926-32213 and 926-68070, respectively). All quantitative measurements were calculated based on measurements from a LI-COR Odyssey Imaging System and quantified with Image Studio Lite (v 5.0) software. For background subtraction, a median pixel intensity for regions 3 pixels above and below a selected band was calculated and subtracted from the mean pixel intensity of that band.

Growth Curves

Cells were plated at 20,000-50,000 cells per well in 6-well plates and growth was assayed by resuspending and directly counting the cells on a hemocytometer with samples taken in biological triplicate for 6 days. Cells were grown in their respective media specified above, plus 0.5% FBS with either 200 ng/ml Bovine Serum Albumin or Stem Cell Factor administered on days 1 and 4.

Statistical Analysis

Significance calculations were performed on samples collected in a minimum of biological triplicate. \( P \) values from two-tailed Student’s \( t \) tests were calculated for all comparisons of continuous variables. All further significance tests were performed in Graphpad Prism (v6.05). Fisher’s exact tests were used to calculate
*P* values for 2x2 contingency tables of the association of *KIT* expression with melanoma patient clinical parameters, whereas a *P* value for the 2x3 contingency table of zebrafish melanoma invasiveness was calculated by a chi-squared test. F-tests were used to determine significance of changes upon stimulation of BRET titration curves. Log-rank tests were used to calculate significance of changes in zebrafish tumor onset curves as well as the differences in overall survival of select human melanoma cohorts. A *P* value < 0.05 was considered significant.
Chapter III - Discussion

Impact of our work in the field

Alterations in MAPK pathway signaling in melanoma are critical to disease progression and development of inhibitor resistance. Acquisition of a BRAF(V600E) mutation ramps up MAPK activity and, given sufficient loss of tumor suppressor genes, can lead to disease initiation. Effective targeting of BRAF(V600E) with ATP-competitive inhibitors reduces pathway activity, leading to tumor regression that is eventually reversed as compensatory mutations or gene expression changes reactivate the MAPK pathway (91). The reactivation of the MAPK pathway has led to attempts to target multiple members of the RAS/MAPK pathway to fully inhibit RAF-driven melanoma progression (164). The results of our study with KIT would suggest that inhibition of RTKs upstream of BRAF(V600E) may cause further activation of the MAPK pathway, given sufficient retention of BRAF(WT) expression (Fig. II.15). Although our results suggest caution against targeting RTKs in BRAF(V600E)-driven cancers, the reverse practice of stimulating RTKs would not necessarily be recommended by our results. We have seen that pathway stimulation of BRAF(V600E)-mutant cells that co-express BRAF(WT) reduces MAPK pathway activation by about 20%. The resulting MAPK signaling is still 50% stronger than the signal arising from BRAF(WT) stimulation in our experimental system. Therefore, KIT stimulation would be unlikely to cause melanoma tumor regression, which requires >80% inhibition of pERK levels in BRAF(V600E)-driven melanoma (165). Furthermore, despite their relatively delayed tumor onset,
*Tg(mitfa:BRAF<sup>V600E</sup>); p53(+/−) zebrafish with wild-type *kit* still develop melanomas with 100% penetrance around 60 weeks, suggesting that, although KIT activity may delay tumor onset, it may not change the lifetime risk of developing this disease.

Our studies address the long-standing question regarding what role KIT plays in melanoma. For several decades it has been known that the majority of melanomas lose KIT expression, suggesting that although KIT is required for melanocyte development, it is dispensable for melanoma progression and may indeed be beneficial to tumor growth (120-122). Multiple studies in the 1990s observed that KIT stimulation inhibited the growth of certain melanoma cell lines (124,125), yet greater attention to the role of KIT in melanoma came as activating mutations in KIT were found to drive melanoma formation and could be successfully targeted with tyrosine kinase inhibitors (117,118). Many reviews of melanoma genetics focus on the small fraction of melanomas with KIT activating mutations and simply describe KIT as a melanoma oncogene (166-169) while other reviews do discuss that KIT is absent in most melanomas, but they cannot make conclusions from previous melanoma cell line experiments as to whether KIT acts as a suppressor in melanoma (170,171). Our work balances the discussion to show that the loss of wild-type KIT contributes to the progression of highly common BRAF<sup>V600E</sup>-mutant melanomas. More broadly our work reinforces the concept that melanomas with powerful RAF-activating mutations may be
prone to low RTK and RAS activity. Although previous work has shown that low
RAS-GTP levels in BRAF<sup>V600E</sup>-mutant melanomas may be attributed to the high
SPRY feedback that results from ERK hyperactivity to sequester the GRB-SOS
complex required for RAS activation (88), our work indicates that cancer cells
may further benefit from genetic downregulation of RTKs as this will prevent
induction of wild-type signaling components.

A major technical challenge faced in this work was to determine
physiological conditions of induced BRAF activation as measured by formation of
BRAF:CRAF dimers. Our work had indicated that a key to the KIT-mediated
reduction of BRAF<sup>V600E</sup>-induced MAPK pathway activity would be to understand
the dynamics of RAF dimer formation in response to upstream pathway activity.
Mutations in BRAF that disrupt its dimerization domain dramatically reduce the
signaling capacity of BRAF (172). Additional work has shown that BRAF:CRAF
dimerization induced by upstream stimulation is the most active and effective
form for RAF proteins to transmit the stimulatory signal to activate MEK and
downstream ERK (83,84). The V600E mutation renders BRAF somewhat
immune from this dimerization requirement as it is capable of signaling as a
monomer in the cytoplasm in a RAS-independent manner and dimerization
disrupting mutations only mildly reduce the signaling capacity of BRAF<sup>V600E</sup>
(90,172). While it has been reported that enforced dimerization between
BRAF<sup>V600E</sup> and CRAF reduces the signaling capacity of BRAF<sup>V600E</sup> (153), the
effect of BRAF<sup>WT</sup>:CRAF dimer formation on BRAF<sup>V600E</sup>-expressing cells has not been previously explored. We predicted that signaling through activated BRAF<sup>WT</sup> would result in a lower overall MAPK pathway output due to the mixture of high and low kinase activity RAF species. To investigate this hypothesis, we chose to use a BRET assay that, unlike a more straightforward immunoprecipitation experiment, would allow us to study RAF dimerization dynamics in response to upstream pathway activity in live cells with high sensitivity. This system has been used to study interaction dynamics of G-protein coupled receptors (173) and has been previously applied to BRAF:CRAF dimerization induced by oncogenic KRAS<sup>G12D</sup> (150). We incorporated a similar model for our experiments by titrating a transfection of Venus-tagged BRAF (V-BRAF) with Renilla luciferase-tagged CRAF (Rluc-CRAF) and co-expressing either NRAS<sup>Q61K</sup> or KIT with supplementary SCF. We were able to successfully identify transfection conditions that would result in varying levels of BRAF:CRAF dimer formation. We were also able to confirm that the RAS-GTP-binding defective mutant BRAF<sup>R188L</sup> was significantly less sensitive to upstream pathway stimulation than BRAF<sup>WT</sup>, establishing this as a meaningful negative control. The BRET experiments were critical in demonstrating that the activation of BRAF<sup>WT</sup> though dimerization with CRAF in response to upstream pathway activity was necessary for that activity to cause a reduction of the MAPK pathway signaling in BRAF<sup>V600E</sup>-mutant cells.

While the Lavoie et. al study performed large-scale BRET by using the inexpensive polyethylenamine as a transfection reagent and membrane-localized
RAF-CAAX-fusion constructs that were more prone to dimerization, we used the more effective, but expensive, reagent Lipofectamine with full-length protein constructs that were less sensitive to dimer induction. The high amount of transfection reagent and plasmids used for the methods of our study may represent a burden that would prevent use of our methods for larger scale analyses. More ideal transfection reagents and conditions would need to be found to scale-up these experiments experiments.

**Major issues raised by our findings**

Understanding the role a gene plays in tumor development can be highly context dependent. Contrary to the classic tumorigenic role of oncogenic mutant KIT, wild-type KIT will inhibit tumorigenesis when in the presence of a BRAF\(^{V600E}\) mutation. Furthermore, our analysis of zebrafish miniCoopR tumors and clinical outcome in the TCGA dataset of melanoma patients indicates that KIT will only have a suppressive effect on BRAF\(^{V600E}\)-mutant melanomas when a sufficient amount of BRAF\(^{WT}\) is present to be activated downstream of KIT. Our work highlights the importance of measuring the BRAF\(^{V600E}\) allele ratio, reinforcing previous analysis of TCGA thyroid cancers showing the BRAF\(^{V600E}\) allele ratio correlated directly with tumor size and number of metastatic lymph nodes (174). The BRAF\(^{V600E}\) allele ratio may not be the only deciding factor determining whether KIT should be classified as oncogenic or tumor suppressive, since it is possible that even in a scenario with a high BRAF\(^{WT}\):BRAF\(^{V600E}\) ratio, over-
activation of KIT through locus amplification or acquisition of an oncogenic mutation could still hyperactivate the MAPK pathway to levels similar or even greater than that achieved by monomeric, unstimulated BRAF\textsuperscript{V600E}. While at physiological levels, KIT activation has proven to be tumor suppressive, it remains to be tested whether hyperactivation of KIT would have the same effect in vivo.

Many human melanomas have low KIT expression and our proposed mechanism only accounts for the KIT loss in the tumors that maintain BRAF\textsuperscript{WT} expression in BRAF\textsuperscript{V600E}-mutant melanomas. Based on the TCGA melanoma dataset, we observed that KIT expression correlated with a significant survival benefit for roughly 20% of BRAF\textsuperscript{V600E}-mutant melanomas that possessed BRAF\textsuperscript{WT}:BRAF\textsuperscript{V600E} ratios greater than 70%. KIT-expressing BRAF\textsuperscript{V600E}-mutant melanoma cells may actually have greater sensitivity to low levels of BRAF\textsuperscript{WT} expression since the UACC257 and the 888MEL cells experienced KIT-induced reductions of MAPK pathway signaling and cellular growth with BRAF\textsuperscript{WT}:BRAF\textsuperscript{V600E} ratios of 45% and 30%, respectively. Using this cutoff, we would expect KIT loss to have a suppressive role in 80% of BRAF\textsuperscript{V600E}-mutant melanomas, accounting for roughly 30% of all melanomas. Given that many of the remaining fraction of BRAF\textsuperscript{WT} melanomas have also lost KIT expression more often than expected by coincidence, we anticipate that KIT may either contribute to inhibition of oncogenic signaling of other MAPK pathway activators
such as NRAS by similarly inducing wild-type components (discussed more fully below), or that KIT loss may reduce stimulation of alternative growth-inhibitory pathways, such as the previously reported apoptosis in melanoma cell lines (125).

As our work establishes the loss of KIT expression as an important promoter of melanoma progression, it is important to ask how its expression is regulated in cancer. The transcription of the KIT gene has been shown to be regulated by the transcription factor activator protein 2 (TFAP2) through direct interaction with at least three distinct TFAP2 target sites in the KIT promoter (147). The frequent loss of this transcription factor in melanoma cell lines contributes to the loss of KIT expression in human melanoma (147,148).

Additionally the KIT locus on chromosome 4 has been reported to be recurrently deleted in a series of 123 short term melanoma cultures (27). Another mode of transcriptional regulation of KIT in melanoma was recently discovered as it was identified in a genome-wide promoter methylation screen that the KIT locus was among the few transcription start sites that were hypermethylated in all four of the human melanoma cell lines tested, but in neither of the two cultured epidermal melanocyte cell lines (123). Further investigation revealed that 0/2 benign nevi contained KIT promoter hypermethylation, whereas 57/151 (38%) of melanoma samples comprised of cell lines, primary, and metastatic melanoma contained KIT promoter hypermethylation that correlated positively with KIT
mRNA expression (123). While loss of transcriptional regulatory factors, copy number loss, and promoter hypermethylation provide clues to how KIT expression is lost in human melanomas, it remains unknown why zebrafish melanomas retain expression of Kit. The zebrafish orthologue Tfap2a is known to positively regulate kit expression in zebrafish, but only partially as additional knockdown of its homologue Tfap2e is required to eliminate kit mRNA expression at late stages of embryonic development (175). Given that tfap2a is located on zebrafish chromosome 24 in a region that is not recurrently deleted and tfap2e is located on chromosome 19 of fish, which is the chromosome containing the mitfa:BRAF^{V600E} transgene that has been observed to be recurrently amplified in zebrafish melanomas (unpublished data), it is likely that these factors promote the continued expression of zebrafish kit during tumorigenesis. Additionally the kit locus on chromosome 20 (kita) is adjacent to a recurrently amplified region that should further promote kit expression in zebrafish melanoma (unpublished data).

**Remaining questions and future directions**

The primary observation of our mechanistic experiments reports that pathway stimulation from either KIT stimulated with SCF or from NRAS^{Q61K} will cause reduced MAPK pathway output in BRAF^{V600E}-mutant cells given the presence of RAS-inducible BRAF^{WT}. While an increase in the number of active kinase molecules in a cell would be predicted to raise its signaling output, the fact
that we observe a decrease in overall signaling though MEK suggests that the mixture of low- and high-activity kinase species are likely competing for a limited number of substrates to enact cellular signaling. Among the well characterized components of the MAPK signaling pathway, one could propose that BRAF<sup>WT</sup> and BRAF<sup>V600E</sup> could be competing with each other for access to CRAF, KSR, or MEK (Fig. II.15). CRAF acts as the direct recipient of BRAF kinase activity upon RAS-induced dimerization where the activation loop of CRAF becomes phosphorylated permitting CRAF to further phosphorylate and activate MEK (84). Although BRAF<sup>WT</sup> associates poorly with CRAF in unstimulated cells, BRAF<sup>V600E</sup> has been previously reported to co-IP with CRAF in the absence of stimulation(153), agreeing with our unstimulated BRET titration curves for BRAF<sup>V600E</sup> and BRAF<sup>WT</sup> having a BRET<sup>50</sup> of 3.251 or 4.473, respectively, suggesting a higher latent background association between BRAF<sup>V600E</sup> and CRAF. Nevertheless, if activation of BRAF<sup>WT</sup> were to compete CRAF away from BRAF<sup>V600E</sup>, the V600E isoform can still activate MEK as a monomer or homodimer independently from CRAF (172). A more thorough investigation could be performed overexpressing CRAF to a level where it could no longer act as a limiting component between BRAF isoforms to test whether this abundance of CRAF could eliminate the ability of stimulated BRAF<sup>WT</sup> to inhibit BRAF<sup>V600E</sup> activity.
Alternatively BRAF\textsuperscript{WT} could compete for binding to the MAPK pathway signaling scaffold KSR, which facilitates BRAF:CRAF dimerization and interaction with MEK (176). An absence of KSR expression results in poor signal transduction through BRAF:CRAF dimers induced by oncogenic RAS, while overexpression of KSR can compete with CRAF for binding to BRAF dimerization induced by either RAF inhibitors or RAS oncogenic mutants leading to reduced activity of BRAF and reduced activation of CRAF (158). If we extend these results to our model, we may consider that activated BRAF\textsuperscript{WT} could recruit some combination of KSR and CRAF away from BRAF\textsuperscript{V600E}, reducing its ability to complex with and activate MEK. While the inhibition of the KSR:MEK interaction has been shown to sensitize cells expressing BRAF\textsuperscript{WT} and oncogenic RAS to MEK inhibition, this was shown to have no effect on cells expressing BRAF\textsuperscript{V600E}, suggesting that BRAF\textsuperscript{V600E} oncogenic activity persists regardless of disrupted KSR scaffolding (177).

Finally we may consider the possibility that MEK itself is the limiting factor of this system and an increased proportion of MEK would be activated by low kinase activity BRAF\textsuperscript{WT} under stimulating conditions. This could be investigated by additional BRET experiments testing for the dynamic changes in BRAF\textsuperscript{WT}:MEK and BRAF\textsuperscript{V600E}:MEK interactions as increasing amounts of BRAF\textsuperscript{WT} are expressed in stimulated and unstimulated cells. In such experiments, a limiting role of MEK would be revealed as the increasing amounts
of BRAF<sup>WT</sup> overexpressed in BRAF<sup>V600E</sup>-mutant cells would be associated with a gain of BRAF<sup>WT</sup>:MEK interactions and concomitant loss of BRAF<sup>V600E</sup>:MEK interactions that would correlate with decreased MEK activation. Such experiments could also be expanded to express MEK at levels where it would no longer act as a limited factor to then see whether BRAF<sup>WT</sup> activation would no longer inhibit BRAF<sup>V600E</sup> activity.

We have argued that it is unlikely for the increased MAPK pathway activation seen in BRAF<sup>V600E</sup>-mutant cells with KIT knockdown to be due to negative feedback from ERK-dependent feedback phosphorylations on BRAF or induction of the ERK-phosphatase DUSP family. An additional form of negative feedback from ERK comes from the sprouty (spry) family of RTK inhibitors SPRY1 or SPRY2. It has been previously shown that the spry genes are upregulated in response to the high MAPK activity induced by BRAF<sup>V600E</sup> to cause those cells to have low steady-state RAS-GTP levels (88). It is thus possible that SPRY activity actually helps preserve high MAPK activity by restraining RTK-mediated activation of BRAF<sup>WT</sup> allowing BRAF<sup>V600E</sup> to signal in a RAS-independent manner. SPRY could play an inhibitory role in KIT-positive BRAF<sup>V600E</sup> homozygous mutant cell lines where RTK activity would be predicted to promote BRAF<sup>V600E</sup> signaling.
This concept of upstream pathway activity driving competition between wild-type and oncogenic variants of BRAF provides an additional explanation for the observation that BRAF and NRAS mutations are almost always mutually exclusive in melanoma (78,178). Although it remains possible that this mutual exclusivity is simply due to the epistatic relationship of BRAF and NRAS, it has been proposed that the co-occurrence of their mutations could lead to synthetic lethality through induction of senescence (178,179). Due to the nature of RAF dimer formation in response to RTK and RAS activity (84,150,172), pathway stimulation from either KIT or NRAS could reduce BRAF\textsuperscript{V600E} output by stimulating low-kinase-activity BRAF isoforms to enter into dimers with CRAF and compete for available signaling components. Indeed our data supports the concept that NRAS\textsuperscript{Q61K} negatively affects BRAF\textsuperscript{V600E} signaling through activation of BRAF\textsuperscript{WT} similarly and more robustly than the negative effect from KIT activity (compare Fig II.9d and II.10c). A BRAF\textsuperscript{V600E}-mutant tumor may gain a survival or proliferative advantage by diminishing KIT or NRAS activities so that BRAF\textsuperscript{V600E} could act to freely drive MEK/ERK activation.

In addition to mutual exclusivity of BRAF and KIT or BRAF and NRAS mutations, we also observe in the TCGA melanoma dataset that KIT and NRAS mutations rarely co-occur (2/89) and KIT expression is also generally low in these tumors, similar to the low expression observed in BRAF\textsuperscript{V600E}-mutant melanoma - 9.9 ± 19.9 and 13.6 ± 25.2 KIT mRNA FPKM values ± SEM for
NRAS-mutant and BRAF$^{V600E}$-mutant melanomas, respectively compared to 34.2 ± 6.8 for all BRAF$^{WT}$/NRAS$^{WT}$ melanomas. It remains to be tested in vivo whether the loss of KIT would contribute to NRAS-mutant melanoma as they do to BRAF-mutant melanoma, though one could propose a similar mechanism of action as we have for BRAF where KIT would activate low-activity wild-type NRAS that would lead to reduced activation of the MAPK pathway. Indeed, deletion of a wild-type KRAS allele (KRAS$^{WT}$) has proven to increase the rate of lung tumorigenesis driven by KRAS$^{G12D}$ and overexpression of KRAS$^{WT}$ caused a reduction of pERK levels in KRAS mutant cells (160,180). While it is not clear how wild-type copies of RAS genes inhibit their oncogenic counterparts, it has been proposed that they compete for downstream signaling apparatus, promote differentiation, or stimulate alternative cellular functions that protect against oncogenesis (160,180). Additional zebrafish melanoma models could potentially be generated to directly test whether Kit inhibits NRAS-driven melanomas or whether other RTKs that play a significant role in melanoma, such as EGFR, may also act to suppress BRAF or NRAS-driven melanomas.
Appendix I – KIT promotes adult zebrafish melanocyte regeneration

Introduction

In addition to the important role KIT plays to promote melanocyte development, KIT also plays a critical role in maintaining adult melanocytes during the regenerative process (181). The zebrafish makes an ideal system for studying the contribution of KIT to melanocyte regeneration as several methods to ablate and regenerate melanocytes in adult animals have already been established. Zebrafish will regenerate their melanocyte stripe on their caudal fins after amputation and adult fish will also regenerate the melanocyte stripes that become ablated on the side of the fish after treatment with the copper chelator neocuproine (NEO) (182-184). Kit has been shown to play an important role in melanocyte regeneration after caudal fin resection where the kit(lf) (either the kit(b5) allele used in our work or a kit temperature sensitive allele (kit(ts)) mutant zebrafish will regenerate their melanocytes 20 days after amputation, whereas wild-type fish only require 5 days (182,183). This delay in melanocyte regeneration can be rescued in a background overexpressing RAS from a heat shock promoter, demonstrating the epistatic nature of KIT and RAS is conserved in zebrafish (185). Experiments performing a clonal rescue of melanocytes after 4-hydroxyanisol (4-HA) mediated ablation in zebrafish embryos concluded that kit(ts) animals were deficient in establishment of melanocyte stem cells (MSC) due to the decreased rate of Tg(fTyrp1:GFP) transgene incorporation by Tol2
transposase into *kit(ts)* animals, which may indicate presence of fewer MSCs (186). Given the imprecise nature of using transgene incorporation to estimate the density of MSCs and the recent development of a method by Dr. Sharanya Iyengar in our lab to fluorescently label melanocyte progenitor cells with a nuclear localization sequence-tagged EGFP (NLS-EGFP) to perform lineage tracing after NEO-mediated ablation (187), we aimed to test whether Kit also assisted regeneration of stripe melanocytes in this system, which could lead to a more definitive mechanism of how Kit is affecting regeneration through the use of lineage tracing. Prior to crossing *kit(lf)* mutants into the Tg(mitfa:NLS-EGFP) line, we first sought to determine whether we could reproduce the reported regeneration defect upon fin amputation and determine whether there was a similar regeneration defect post-NEO treatment. We also aimed to determine whether BRAF<sup>V600E</sup> could suppress the KIT regeneration defect similarly to how it suppresses KIT melanocyte developmental defects. We found that although Tg(mitfa:BRAF<sup>V600E</sup>) zebrafish experienced normal melanocyte regeneration, *kit(lf)* zebrafish experienced decreased regeneration and *kit(lf); Tg(mitfa:BRAF<sup>V600E</sup>)* double-mutant zebrafish experienced increased regeneration either post-caudal fin amputation or post-NEO treatment. This suggests that Kit signaling promotes proper functioning of melanocyte stem cells and that the synergistic effect of kit loss in BRAF<sup>V600E</sup>-mutant cells that increased BRAF<sup>V600E</sup> activity observed in tumors could be present within the melanocyte stem cells.
Results

We amputated caudal fins to approximately two-thirds of their original length in AB, *Tg(mita:BRAF\textsuperscript{V600E})*, kit(lf), and kit(lf); *Tg(mita:BRAF\textsuperscript{V600E})* backgrounds. After 17 days we quantified percentage area regeneration and found that while the AB, *Tg(mita:BRAF\textsuperscript{V600E})*, and kit(lf) animals all regenerated 80-90% of the fin area that had been lost, kit(lf); *Tg(mita:BRAF\textsuperscript{V600E})* animals only regenerated approximately 72% of the original fin area and experienced severe ulceration that was unique to the kit(lf); *Tg(mita:BRAF\textsuperscript{V600E})* genotype (App. 1.1A, 1.1B). As anticipated, the AB, and *Tg(mita:BRAF\textsuperscript{V600E})* animals successfully regenerated all of their pre-amputation melanocytes, whereas kit(lf) animals experienced a severe deficit having regenerated only 18% of their melanocytes by day 17 (App 1.1A, 1.1C). The kit(lf); *Tg(mita:BRAF\textsuperscript{V600E})* double mutant animals partially restored the regeneration defect of the kit(lf) animals by regenerating 55% of their original melanocyte total (App 1.1A, 1.1C). These results support the conclusions that KIT and BRAF do act in the same pathway to affect melanocyte regeneration, though full regeneration may be hindered by the poor tissue regeneration of kit(lf); *Tg(mita:BRAF\textsuperscript{V600E})* animals. We performed a similar experiment observing a separate population of stripe melanocytes on the sides of the fish after NEO-mediated ablation of melanocytes.
App. I.1

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Scale bars = 2 mm

B

![Graph showing fin area regeneration](image)

C

![Graph showing melanocyte regeneration](image)

Days post amputation
App. I – The defective melanocyte regeneration after caudal fin amputation in *kit(lf)* animals is partially rescued in *kit(lf); Tg(mitfa:BRAF^{V600E})* animals.

A) Representative images of zebrafish caudal fins before, immediately after, 7, and 19 days after amputation. Magnification provided for the inset on day 19 images. B) Quantification of area regeneration calculated for each fish by comparing the final area to pre-amputation size. C) Quantification of melanocyte regeneration calculated for each fish by comparing the final melanocyte count to pre-amputation counts. *n* = 8 for each group. *P* value < 0.05, **P** value < 0.01, ***P** value < 0.001; Student’s *t* test.
App I.2

A

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**Significance: NS, **p < 0.01, ***p < 0.001**
App. II – The defective melanocyte regeneration after neocuproine treatment in kit(lf) animals is partially rescued in *kit(lf); Tg(mitfa:BRAF<sub>V600E</sub>*) animals.

A) Representative images of zebrafish intermediary stripes immediately ventral to the dorsal fin before, 7, and 22 days after neocuproine treatment. Magnification images provided for the inset on day 19 images. Note that the black and white images have yellow, green, blue, and cyan color values converted to gray for visualization purposes. Original images used for manual quantification of melanocytes. B and C) Quantification of melanocyte regeneration calculated for each fish by comparing the final melanocyte count to pre-treatment count for either DMSO (B) or NEO (C). *P value < 0.05, **P value < 0.01, ***P value < 0.001; Student’s t test.
Upon treating animals from the four genotypes with either DMSO or NEO, we observed a significant clearing of melanocytes in all genotypes within 7 days of NEO treatment, whereas DMSO had no effect on melanocyte number (App. I.2A, I.2B). AB animals regenerated 43% more melanocytes than they had prior to treatment and *Tg(mitfa:BRAF^{V600E})* animals regenerated an equivalent number, though with greater variability as two animals failed to regenerate more than 70% of their original melanocytes (App. I.2C). The *kit(lf)* animals consistently regenerated only 70% of their melanocytes, while the *kit(lf); Tg(mitfa:BRAF^{V600E})* animals hyper-regenerated their melanocytes, growing 140% more melanocytes than they had prior to treatment, but again with high variability (App. I.2C). The *kit(lf)* regeneration pattern produced entire areas of the animal that were devoid of melanocytes (App I.2A) and the regions with melanocytes grew back with lower density than prior to treatment. *kit(lf); Tg(mitfa:BRAF^{V600E})* animals also experienced this patchy melanocyte regeneration pattern (App. I.2A), but the restored patches grew with particularly high melanocyte density. These experiments confirm the role of Kit in melanocyte regeneration and further support the enhanced activity of BRAF^{V600E} that we have proposed to occur in *kit(lf)* mutant animals.
Discussion

This work reaffirms the important role Kit plays in melanocyte regeneration in the fin and confirms that Kit plays a similarly important role during melanocyte regeneration in the stripes on the side of the fish. Furthermore, it is compelling that while BRAF$^{V600E}$ alone had a minimal effect on regeneration after fin amputation or after NEO treatment, the kit(If) allele synergized with the $Tg(mitfa:BRAF^{V600E})$ transgene to promote hyper-melanocytic growth in regenerating zebrafish. Thus, it appears that whatever pathway of melanocyte stem cell development and recruitment is affected by Kit is also affected similarly by BRAF$^{V600E}$, but the impact of BRAF$^{V600E}$ is enhanced in the absence of Kit activity. This result is in line with our findings in chapter II where we identified that MAPK pathway activation downstream of BRAF$^{V600E}$-mutant cells is enhanced in the absence of KIT.

The defective fin regeneration of kit(If); $Tg(mitfa:BRAF^{V600E})$ animals after caudal fin amputation remains a surprise with no obvious mechanism, particularly given the lack of fin regeneration defects among kit(If) or $Tg(mitfa:BRAF^{V600E})$ animals. It has recently been reported that a heightened state of inflammation due to epithelial $il1b$ expression inhibits fin fold regeneration in zebrafish embryos and BRAF$^{V600E}$ has been previously associated with chronic inflammatory states in Erdheim-Chester disease (188,189). It seems possible that BRAF$^{V600E}$-expressing cells may induce a localized inflammatory state that could inhibit fin
regeneration and that *kit*(lf) mutants are either highly susceptible to this inflammation or that BRAF^{V600E} is simply hyperactive in a *kit*(lf) background. A mechanism to explain this phenotype will require additional experimentation.

It remains unclear how KIT and BRAF are affecting melanocyte regeneration. Due to the patchy pigmentation pattern of *kit*(lf) animals after regeneration from NEO treatment, it is possible that a fraction of melanocyte progenitor cells have died or simply fail to be activated during regeneration. The decreased density of melanocytes in the regenerated areas further suggests that even progenitor cells that are stimulated to regenerate are still deficient in the amount of new melanocytes they are able to produce. The *kit*(lf); *Tg*(mitfa:BRAF^{V600E}) animals also displayed patchy melanocyte rescue, but had high density melanocyte growth within rescued patches. It is thus suspected that the defect that occurs in activated *kit*(lf)-mutant progenitor cells is rescued by BRAF^{V600E} and thus distinct from the defect causing the patchy regeneration pigment pattern. To better ascertain what mode of regeneration has been affected by Kit, we can consider recent work from our lab that found a population of unpigmented melanocyte precursor cells that can be labeled by the transgenic *Tg*(mitfa:NLS-EGFP) construct (187). The precursor cells remain dormant until stimulated due to melanocyte ablation, which causes them to enter one of several pathways to reconstitute zebrafish stripe-associated melanocytes. Among the progenitors that respond to regenerate melanocytes, they may 1)
directly differentiate to produce one melanocyte, 2) symmetrically divide to produce two daughter progenitor cells, or 3) asymmetrically divide to produce a progenitor cell and a melanocyte. Wild-type AB fish overwhelmingly utilize the direct differentiation and symmetric division pathways by 45% and 47% of their available progenitor cells upon ablation. Only 7% of progenitors utilized asymmetric division. Use of this system in the future within the kit(If) and kit(If); Tg(mitfa:BRAFV600E) backgrounds should reveal whether these animals have been affected in melanocyte progenitor establishment, or whether one of these three modes of regeneration has been affected.

**Materials and Methods**

**Fin amputation experiments**

Animals were anesthetized in tricaine and melanocyte constriction was promoted by treating fish with 1 mg/ml epinephrine for 5 minutes prior to imaging. Amputation was performed after the first round of imaging by making a vertical incision approximately 1/3rd into the width of the caudal fin posterior to the caudal peduncle. Animals were monitored with additional rounds of anesthetization and epinephrine treatment with imaging on days 7, 17, and 19. Total area was calculated by drawing a region of interest around the fin in ImageJ software and the melanocytes were counted by hand-placing counting pins at each melanocyte, also in ImageJ.
Neocuproine treatment

Anesthetization, epinephrine treatment, and image analysis was performed as in the fin amputation experiments described above, but Photoshop was used instead of ImageJ. Neocuproine treatment was performed as previously described (187) by incubating fish overnight in fish water with 750 nM neocuproine plus 0.0075% DMSO.
Appendix II – Establishment of an accelerated onset zebrafish melanoma model

Introduction

Large-scale chemical or genetic screening in cell lines can reveal novel cancer generating mechanisms and therapeutic targets (190,191). This standard method of screening in tissue culture permits a massive number of compounds or targets to be evaluated that can identify many genuine cancer-associated pathways and therapies, but may fail to find chemicals that are bioavailable and capable of affecting pathways that help initiate tumorigenesis in-vivo, such as a metabolic re-wiring to accommodate to a certain niche or immune-evasion techniques. Autochthonous tumor development in zebrafish represents a promising model system for screening due to the high number of animals that can be produced, their relatively small size, and ease of genetic manipulation. Previous chemical screens in zebrafish have largely focused on phenotypes that can be identified early in development, such as the protection of neuromast hair cells from antibiotic exposure at 5 dpf, or the abnormal oncogene-induced hematopoietic progenitor cell differentiation at 12-16 hours post fertilization (hpf) (192,193). Zebrafish have been successfully used to screen for melanoma modifying compounds by observing the effect of approximately 2000 compounds from the BIOMOL 480 and LOPAC1280 libraries on neural crest development of 5 hpf embryos (52). This screen identified a compound similar to Leflunomide that inhibits the activity of dihydroorotate dyhydrogenase (DHODH) to thus inhibit
transcriptional elongatation of genes essential for neural crest development and melanoma progression. This discovery led to the conduction of a phase I/II clinical trial (NCT01611675) to test for a synergistic effect between Lefluonomide and Vemurafenib in BRAF\textsuperscript{V600E}-mutant melanoma.

Screening for compounds or genes that directly inhibit melanoma formation in zebrafish is challenging due to the fact that \textit{Tg(mitfa:BRAF\textsuperscript{V600E}); p53(lf)} zebrafish do not develop melanomas until adulthood with a median onset around 8 months, making large-scale screening unfeasible. The \textit{miniCoopR} system restores and overexpresses the melanoma oncogene \textit{mitfa}, but only accelerates melanoma onset to a median of 4 to 5 months. One potential factor restraining tumorigenesis in these systems is the weak embryonic melanocyte oncogene expression possible from the 2.1 kb \textit{mitfa} promoter used in this system (135,136). We worked to develop an early-onset zebrafish melanoma model that would develop a larval phenotype or potentially yield tumorigenesis prior to metamorphosis to facilitate large-scale screening experiments.

One possibility to achieve strong, early oncogene expression would be to conditionally activate the ubiquitin promoter by melanocyte-specific CRE expression. A “\textit{ubiswitch}” promoter cassette has been previously developed that contains the highly active \textit{ubiquitin} promoter followed by a \textit{GFP} open reading
frame (ORF) flanked by LoxP sites and an *mCherry* ORF that remains un-transcribed unless the *GFP* ORF and its stop sequence are removed by CRE recombinase activity (194). We reasoned that we could use this system to drive selective oncogene expression, in place of mCherry, with CRE driven to express in the melanocytes by the *mitfa* promoter. Upon establishment of CRE and Ubiswitch lines, they could be maintained as healthy populations and then crossed to yield a high number of offspring with early, strong, melanocyte-specific oncogene expression from the *ubiquitin* promoter (App. II.1).

We established two transgenic lines, *Tg(Ubiswitch:NRAS^{Q61K}); p53(If); mitfa(If)* and *Tg(mitfa:CRE); p53(If)*, that did develop CRE-activated hyper-melanocytic growth in 90% of offspring within 4 days of age. Lethality was observed in 100% of animals after 5 weeks and this was determined to be a melanocyte-independent, off-target effect after lethality was again observed by breeding the transgenic lines in a *mitfa(If)* background. We developed an alternative CRE line using a linearized *miniCoopR-CRE* construct that had improved survival, but no larval phenotype, ultimately leading us to reject either of these models as feasible screening systems. This work demonstrates both the advantages and challenges of using zebrafish for screening experiments of autochthonous tumor development.
Results
To create a model system that would produce a large number of offspring that would rapidly develop melanomas for future screening purposes, we injected single cell $p53(\text{lf})$ embryos with a $Tg(mitfa:CRE)$ construct juxtaposed to a GFP open reading frame driven by the heart tissue-specific cardiac myosin light chain ($cmlc$) promoter (195). We identified heart GFP-positive embryos, raised them to adulthood, then screened F1 animals by outcrossing and searching for offspring that were heart-GFP-positive to identify founder strains that possessed germline transgene integration. We obtained the $ubiswitch:mCherry$ zebrafish line (194) from Dr. Christian Mossiman to test the efficacy of our CRE line (App. II.1). Upon establishment of stable transgene expressing founder lines, we validated the activity of our CRE-switch system by crossing animals heterozygous for the CRE or Ubiswitch transgenes to yield offspring that had no transgenes, were CRE-positive, Ubiswitch-positive, or CRE/Ubiswitch-positive. We initially performed this cross with $Tg(ubiswitch:mCherry)^{+/+}$ zebrafish and observed that $Tg(mitfa:CRE)^{+/+}; Tg(ubiswitch:mCherry)^{+/+}$ double mutant offspring, easily identifiable by their respective combination of heart and full body GFP-positivity, were the only offspring siblings that presented robust mCherry expression (App. II.2A). The mCherry expression did appear in melanocytes, most highly visible after epinephrine treatment to induce constriction of melanosomes to the center of melanocytes, leaving the appearance of an mCherry halo pattern around melanosome pigment clusters (App. II.2Aiv). While no mCherry expression was
detected in embryos lacking the mitfa:CRE transgene, there was apparent off-target effects in $Tg(mitfa:CRE)^+/+$; $Tg(ubiswitch:mCherry)^+/+$ double mutant animals where mCherry expression was visible in non-pigmented cells, possibly xanthophores or other embryonic cell types where the mitfa promoter may have sufficient activity to drive CRE expression (App. II.2A).
App II.1
App. II.1 – Ubiswitch system for driving strong melanocyte-specific transgene expression.

The Tg(ubiswitch:mCherry) line represented here would appear to have whole body GFP-positivity, compared to the Tg(mitfa:CRE) line which expressed heart-specific GFP under a juxtaposed cmlc:GFP cassette. When bred together, the offspring of these lines would have full body-GFP, heart-GFP, and mCherry-melanocytes due to the melanocyte-specific CRE recombination yielding mCherry expression. For tumorigenic assays, the mCherry open reading frame would be replaced with open reading frames for BRAF^{V600E} or NRAS^{Q61K}.

While establishing the functional CRE stable line, we simultaneously developed Tg(ubiswitch:BRAF^{V600E})^{+/+} and Tg(ubiswitch:NRAS^{Q61K})^{+/+} lines and established all CRE and Ubiswitch lines in a p53(lf) homozygous background to facilitate tumor development. BRAF and NRAS are the two most frequently mutated genes in human melanomas and thus their products would make meaningful targets for discovery of genetic cooperating events in melanoma or identification of novel inhibitors (78). We proceeded to cross the Tg(mitfa:CRE) lines with the Tg(ubiswitch:BRAF^{V600E})^{+/+} and Tg(ubiswitch:NRAS^{Q61K})^{+/+} lines. Unfortunately, Tg(mitfa:CRE)\^{+/+}; Tg(ubiswitch:BRAF^{V600E})\^{+/+} embryos showed no phenotype of melanocyte hyper-proliferation and attempts to detect heightened BRAF expression through in-situ hybridization were unsuccessful (data not shown); however, Tg(mitfa:CRE)\^{+/+}; Tg(ubiswitch:NRAS^{Q61K})\^{+/+} animals developed
increased numbers of dorsal head melanocytes by 4 dpf (App. II.2B, II.2C).

These animals also contained melanocytes that had migrated into muscle segments, where this was almost never seen in siblings that contained only a single transgene (App. II.2D).
App. II.2
App. II.2 – Generation of \textit{Tg(mitfa:CRE); Tg(Ubiswitch)} transgenic animals yields functional melanocyte-specific CRE activity.

A) Siblings from a cross of \textit{Tg(mitfa:CRE)/+} (containing heart-GFP) and \textit{Tg(ubiswitch:mCherry)/+} (containing full body-GFP) were sorted according to GFP status. A GFP (A\textsubscript{iii}) or mCherry (A\textsubscript{iv}) magnification of the dorsal head region of a CRE/ubiswitch double heterozygote displays melanocyte-positive mCherry expression. Scale bars = 1 mm (A\textsubscript{i}) and 0.25 mm (A\textsubscript{iv}). B) Dorsal head region images from 4 day old embryo siblings heterozygous for the indicated transgenes. C) Quantification of dorsal head melanocytes comparing wild-type AB fish with \textit{Tg(mitfa:CRE)/+; Tg(ubiswitch:NRAS\textsuperscript{Q61K})/+}, labelled here as “CRE NRAS\textsuperscript{Q61K}”. ****P value < 0.001; Student’s t test. D) Profile images of sibling embryos from panel B illustrating the mis-migration of melanocytes in \textit{Tg(mitfa:CRE)/+; Tg(ubiswitch:NRAS\textsuperscript{Q61K})/+} animals. Scale bar = 1 mm.
We followed the development of the $Tg(mitfa:CRE)^{+/+}$; $Tg(ubiswitch:NRAS^{Q61K})^{+/+}$ double mutant animals and compared to AB animals during the first three weeks of development, prior to metamorphosis while the animals remain a size that could potentially facilitate large-scale screening. We observed that melanocyte populations of $Tg(mitfa:CRE)^{+/+}$; $Tg(ubiswitch:NRAS^{Q61K})^{+/+}; p53(lf)$ animals continued to proliferate and infiltrate tissues during this time (App. II.3A). Furthermore, $Tg(mitfa:CRE)^{+/+}$; $Tg(ubiswitch:NRAS^{Q61K})^{+/+}; p53(lf)$ animals experienced remarkably poor survivorship, where no animals survived juvenile-to-adult morphogenesis stage to live past 5 weeks of age, whereas their siblings with inheritance of individual transgenes had normal survival during this time period (App. II.3B). We considered that if the early-death phenotype was caused by melanocyte hyper-proliferation, this could make a promising model for large-scale screening as the animals were small enough to raise in micro-plates and had a phenotype that would be easy to score by simply observing survival past 5 weeks.

To test whether the death was attributable to melanocyte hyper-proliferation, we crossed our transgenic lines into a $mitfa(lf)$ background to eliminate melanocyte development. To our surprise, although the $Tg(mitfa:CRE)^{+/+}$; $Tg(ubiswitch:NRAS^{Q61K})^{+/+}; p53(lf); mitfa(lf)$ animals appeared normal during embryonic development (App. II.3C), they experienced a similar early-death onset at around 5 weeks of age (App. II.3D). This suggests that CRE
may be activating NRAS\textsuperscript{Q61K} expression in tissues other than melanocytes, making this early-death phenotype unusable for screening melanoma-modifying molecules or genes.
App. II.3

A

4 dpf 14 dpf 21 dpf

AB

Tg(mita:CRE)^+; Tg(Ubiswitch:NRAS^{delK})^+; p53(+/+)
Scale Bars = 1 mm

B

Overall survival

Days post fertilization

P value < 0.0001

C

Tg(mita:CRE)^+; Tg(Ubiswitch:NRAS^{delK})^+; p53(+/+)

D

Overall survival

Days post fertilization

AB (n=73)
mitfa(+/-) (n=75)
CRE; NRAS^{delK} (n=71)
CRE; NRAS^{delK}; mitfa(+/+) (n=61)
App. II.3 – Development of pigmented lesions and early-death apparent in
\(Tg(\text{mitfa}:\text{CRE})^+; Tg(\text{ubiswitch}:\text{NRAS}^{Q61K})^+; p53(\text{lf})\) animals.

A) Dorsal and profile views of AB and \(Tg(\text{mitfa}:\text{CRE})^+; Tg(\text{ubiswitch}:\text{NRAS}^{Q61K})^+\) larvae and juvenile animals. B) Survivorship of siblings generated from a cross of \(p53(\text{lf}), Tg(\text{mitfa}:\text{CRE})^+; p53(\text{lf})\) and \(Tg(\text{ubiswitch}:\text{NRAS}^{Q61K})^+; p53(\text{lf})\), then sorted according to GFP fluorescence as in figure App. II.2 for being \(p53(\text{lf}), Tg(\text{mitfa}:\text{CRE})^+; p53(\text{lf})\) (CRE; \(p53(\text{lf})\), \(Tg(\text{ubiswitch}:\text{NRAS}^{Q61K})^+; p53(\text{lf})\) (CRE; \(\text{NRAS}^{Q61K}; p53(\text{lf})\)), or \(Tg(\text{mitfa}:\text{CRE})^+; Tg(\text{ubiswitch}:\text{NRAS}^{Q61K})^+; p53(\text{lf})\) (CRE; \(\text{NRAS}^{Q61K}; p53(\text{lf})\)). \(P\) value calculated comparing \(\text{NRAS}^{Q61K}; p53(\text{lf})\) and CRE; \(\text{NRAS}^{Q61K}; p53(\text{lf})\) animals; Log rank test. C) Representative profile images of 4 dpf \(Tg(\text{mitfa}:\text{CRE})^+; Tg(\text{ubiswitch}:\text{NRAS}^{Q61K})^+; p53(\text{lf})\) and \(Tg(\text{mitfa}:\text{CRE})^+; Tg(\text{ubiswitch}:\text{NRAS}^{Q61K})^+; p53(\text{lf})\) (CRE; \(\text{NRAS}^{Q61K}\)) and \(Tg(\text{mitfa}:\text{CRE})^+; Tg(\text{ubiswitch}:\text{NRAS}^{Q61K})^+; p53(\text{lf})\) (CRE; \(\text{NRAS}^{Q61K}; \text{mitfa}(\text{lf})\)) animals. D) Survivorship curves for AB, \(\text{mitfa}(\text{lf})\), \(Tg(\text{mitfa}:\text{CRE})^+; Tg(\text{ubiswitch}:\text{NRAS}^{Q61K})^+; p53(\text{lf})\) (CRE; \(\text{NRAS}^{Q61K}\)), and \(Tg(\text{mitfa}:\text{CRE})^+; Tg(\text{ubiswitch}:\text{NRAS}^{Q61K})^+; p53(\text{lf})\) (CRE; \(\text{NRAS}^{Q61K}; \text{mitfa}(\text{lf})\)) animals.
To establish a novel melanoma model that would have alternative CRE expression patterns and increase the copy number of the melanoma oncogene \textit{mitfa}, we prepared a linearized mini\textit{CoopR}-CRE construct, where CRE is still expressed under the \textit{mitfa} promoter, but linearization of the construct should permit for in-vivo concatemerization prior to transgene integration, resulting in animals that have multiple copies of \textit{mitfa} and CRE (App. II.4A). We injected this construct into \textit{p53\textit{lf}}; \textit{mitfa\textit{lf}} embryos, bred F1 animals with melanocyte rescue, and identified several founder animals whose offspring grew melanocytes, indicating successful germline transgene integration. The \textit{Tg(miniCoopR:CRE); p53\textit{lf}}; \textit{mitfa\textit{lf}} animals developed reduced numbers of melanocytes (App. II.4B and compare column 3 of App. II.2C with column 1 of App. II.4C) and the inclusion of a \textit{Tg(ubiswitch:NRAS^{Q61K})} transgene made no difference to melanocyte development in 4 dpf embryos (App. II.4B and II.4C). While the \textit{Tg(miniCoopR:CRE)^+/+; Tg(ubiswitch:NRAS^{Q61K})^+/+; p53\textit{lf}}; \textit{mitfa\textit{lf}} animals did not experience an early death phenotype as severe as that observed in \textit{Tg(mitfa:CRE)} animals, they did have a median survival of 16 weeks and were prone to formation of pigmented melanocytic lesions with a median onset of 7 weeks (App. II.4D). At early stages, these lesions were similar in appearance to the reported f-nevi that form in \textit{Tg(mitfa:BRAF^{V600E})} mutant fish that lack any additional cancer causing mutations (135). The lesions in \textit{Tg(miniCoopR:CRE)^+/+; Tg(ubiswitch:NRAS^{Q61K})^+/+; p53\textit{lf}}; \textit{mitfa\textit{lf}} animals progressed during weeks 4-9 with a spreading growth pattern that did not typically develop the large exophytic
tumor masses of $Tg(mitfa:BRAF^{V600E}); p53(lf)$ zebrafish (App. II.4D) (135). Although the lesions tended not to grow up above the surface of the fish, their effect on animal health was readily apparent as lesion-bearing animals became sclerotic, stunted, and experienced poor survival (App. II.4D). It remains to be tested whether a $mitfa(lf)$ mutation would suppress the poor survival of these animals.
App. II.4

A) Linearized construct
mitfa promoter CRE
mitfa minigene

Inject into
p53(−/−); mitfa(−/−)

Cross chimeric animals
to p53(−/−); mitfa(−/−)
to establish stable line

B) Tg(miniCreR:CRE)69; p53(−/−); mitfa(−/−) "CRE"
Tg(miniCreR:CRE)69; Tg(Ub-switch:NRAS<sup>G12K</sup>)"CRE+NRAS<sup>G12K</sup>"
Scale bar = 0.5 mm

C) Dorsal head melanocytes

D) 4 weeks 5 weeks 6 weeks 7 weeks 8 weeks 9 weeks

Scale bar = 1 mm

Survival percentage
0 10 20 30 40 50 60 70 80 90 100

Weeks

CRE (n=100) overall survival
CRE+NRAS<sup>G12K</sup> (n=100) overall survival
CRE+NRAS<sup>G12K</sup> (n=100) lesion-free survival
App. II.4 – Development of an alternative CRE driver line with a linearized miniCoopR-CRE construct.

A) Scheme for developing a stable melanocyte-restricted CRE line where the miniCoopR vector is linearized and injected into p53(lf); mitfa(lf) animals, raised to adulthood and bred to additional p53(lf); mitfa(lf) animals to identify founders with germline integrations of the linearized vector. B) Dorsal head images of representative 4 dpf Tg(miniCoopR:CRE)/+; p53(lf); mitfa(lf) (“CRE”) and Tg(miniCoopR:CRE)/+; Tg(ubiswitch:NRAS^{Q61K}); p53(lf); mitfa(lf) (“CRE+NRAS^{Q61K}”), scale bars = 1 mm, with accompanying quantification in C. Significance tested by Student’s $t$ test. D) Progression of lesion formation from 4 to 9 weeks of age and associated quantification of lesion-free survival of CRE+NRAS^{Q61K} animals plus overall survival of CRE and CRE+NRAS^{Q61K} animals.

Discussion

The Ubiswitch-NRAS early tumor model represents a potential paradigm shift of what is possible in screening for modifiers of autochthonous tumor development, yet also shows the difficulty in establishing an appropriate model for such a screen. We were able to achieve remarkable early hyper-melanocytic growth in Tg(mitfa:CRE)/+; Tg(ubiswitch:NRAS^{Q61K})/+; p53(lf) animals, where 90% of animals had higher than average numbers of melanocytes by 4 dpf, although we were unable to confirm whether the pigmented lesions that later grew from this hyper-pigmentation represented genuine melanomas prior to the animals death.
Furthermore, we found that the early-death phenotype of these animals was not due to melanocyte formation, as mitfa(If)-mutant animals harboring the CRE and Ubiswitch transgenes still experienced an identical pattern of early, fully-penetrant lethality. We considered the possibility of screening based on the early hyper-proliferation of melanocytes seen in larval zebrafish, but the high variability of this experimental system would make a large-scale screen challenging. We observed that at 4 dpf p53(If) and Tg(mitfa:CRE)^+; Tg(ubiswitch:NRAS^Q61K)^+; p53(If) animals had 25.8 ± 3.8 and 51.9 ± 13.4 mean number of melanocytes/embryo ± standard deviation in the dorsal head region, respectively. This degree of change yields a cohen’s d effect size of 2.65 that would yield a statistical power of 0.89 in a two-tailed test with a standard 0.05 probability level using at least 8 animals per treatment group. This means that, for our known degree of altered melanocyte growth, if we used this model in a large-scale screen where we had at least 8 animals per treatment condition, it would yield a significant result 88% of the time that a real result should have been observed. This power level is above the standard 80% recommended for statistical tests and 8-20 embryos per well of a 48-well assay plate has been previously recommended for screening zebrafish (196). Despite this reasonable level of statistical power possible in our experimental system, it remains to be evaluated how well 8+ zebrafish could be maintained in the ~200 ul allowed in a well of a 48-well plate for the full 4 days required to see our observed phenotype. It is possible that larger volumes would be required once fish begin swimming around
2 dpf and thus 24-well plates with more volume and perhaps a reduced number of fish would be necessary to maintain proper health for 4 days. This represents one factor that would reduce the number of chemicals that could be screened and increase the amount of each chemical required in the screen. An additional difficulty in running this assay would be that it would be necessary to count melanocytes, given that the number of melanocytes of roughly 10-20% of animals in Tg(mitfa:CRE)\(^+/\); Tg(ubiswitch:NRAS\(^{Q61K}\))\(^+/-\); p53(lf) zebrafish overlapped with the number of melanocytes in p53(lf) animals, it would difficult to accurately score animals as simply transformed or not. Quantification of the melanocytes represents a technical hurdle that can be faced since the embryos are easily fixed and stored for later counting. This may represent a challenge due to the time required to potentially count melanocytes on tens of thousands of embryos and automated capillary and image analysis-based techniques could be considered for screens of such size. Although the hyper-melanocytic growth phenotype does present a potentially screenable phenotype, it remains troubling that there is a powerful off-target effect occurring in these fish causing melanocyte-independent lethality that may represent too great of a confounding factor to further consider this model for screening of melanocyte phenotypes that could be affected by the altered physiology of these transgenic animals.

The development of the Tg(miniCoopR:CRE) stable line presented a new option to express CRE and Mitfa at high levels in developing tumors, but these
failed to produce a phenotype in larval melanocytes, with median onset of pigmented lesion formation occurring around 7 weeks, this animal model also did not qualify to be used in a large-scale screening project. Nevertheless, the establishment of these animals demonstrated the full recapitulation of zebrafish melanocytes with Mitfa expression being driven by the 2.1 kb mitfa promoter, which proved to poorly initiate development of larval melanocytes, but adult zebrafish were phenotypically normal. With the increased ease of CRISPR-Cas9 targeting, it may also be possible to develop a CRE-expression line with the CRE ORF inserted at the endogenous mitfa promoter to more perfectly capture true mitfa expression in early melanocytes.

This work provides several principles for consideration if future work attempts to establish transgenic zebrafish with accelerated tumor onset for screening. First, this work could have benefitted from more aggressive development of a variety of founder lines to identify those with the most functional transgenic gene expression patterns. Each founder line for this project only had 2-3 candidate founders that were evaluated, often with 1 being chosen quickly on the basis of fluorescence intensity or melanocyte rescue in offspring. Perhaps functional validation of additional founders could have identified one with better early melanocyte-restricted expression. Next, experiments must carefully plan how to establish a connection between the larval phenotype being screened and the adult phenotype of interest. In our case we identified early...
lesion growth that did not relate to the early-death phenotype we observed in growing animals, but we did demonstrate that growth of these early lesions continuous through development to establish invasive masses of pigment cells. Screening for genes or chemicals that affect formation of such early lesions in larvae would be anticipated to identify modifiers of tumor development.

**Materials and Methods**

**DNA vector preparation**

Human $BRAF^{V600E}$ and $NRAS^{Q61K}$ ORFs were cloned with the “Ubiswitch” 5’ cassette that includes the ubiquitin promoter driving a GFP ORF flanked by LoxP sites and a 5’ polyA sequence using Gateway recombination (ThermoFisher) into the 395 vector from the Tol2 kit (197).

**Statistical analysis**

Cohen’s d effect size and statistical power for the observed differences in melanocyte growth mentioned in the discussion were calculated using the “Effect size” and “Statistical power” calculators available at

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