NF1 Loss is a Functional Genomic Event in Melanoma

by

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A Dissertation

Presented to the Faculty of the Louis V. Gerstner, Jr.

Graduate School of Biomedical Sciences,

Memorial Sloan-Kettering Cancer Center

in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

New York, NY

December, 2013

David B. Solit, MD
Dissertation Mentor

12/18/2013
Date

Dedication

"No man can reveal to you aught but that which already lies half asleep in the dawning of your knowledge.

The teacher who walks in the shadow of the temple, among his followers, gives not of his wisdom but rather of his faith and his lovingness.

If he is indeed wise he does not bid you enter the house of his wisdom, but rather leads you to the threshold of your own mind.

The astronomer may speak to you of his understanding of space, but he cannot give you his understanding.

The musician may sing to you of the rhythm which is in all space, but he cannot give you the ear which arrests the rhythm nor the voice that echoes it.

And he who is versed in the science of numbers can tell of the regions of weight and measure, but he cannot conduct you thither.

For the vision of one man lends not its wings to another man.

And even as each one of you stands alone in God's knowledge, so must each one of you be alone in his knowledge of God and in his understanding of the earth."
-Kahlil Gibran, *The Prophet*

For all of my teachers, in gratitude for their unfathomable patience.
For my parents: my first teachers.
For my husband, whose patience conveying the rhythm of space onto occasionally deaf ears deserves distinct laud and recognition.

Abstract

Melanoma is a disease characterized by lesions that activate ERK. Though 70% of cutaneous melanomas harbor activating mutations in the *BRAF* and *NRAS* genes, the alterations that drive tumor progression in the remaining 30% are largely undefined. Vemurafenib, a selective inhibitor of RAF kinases, has clinical utility restricted to BRAF mutant tumors. MEK inhibitors, which have shown clinical activity in NRAS mutant melanoma, may be effective in other ERK pathway-dependent settings. We investigated a panel of melanoma cell lines wild-type for BRAF and NRAS to determine the genetic alteration driving their transformation and their dependence on ERK signaling in order to elucidate a candidate set for MEK inhibitor treatment. From 191 melanoma cell lines, we collected a set of 66 $BRAF^{WT}/NRAS^{WT}$ cell lines for our study. We screened these cell lines for functional alterations that activated RAS, and found a subset of cell lines with elevated RAS-GTP. NF1 negatively regulates RAS and is found to be somatically altered in a growing number of cancers. We examined the NF1 status of these cell lines and identified a cohort of 6 cell lines with high RAS-GTP and loss of NF1 protein expression. Deep sequencing via the IMPACT assay revealed a genomic mechanism for NF1 loss in all of the NF1-null cell lines. Data from the melanoma Cancer Genome Atlas (TCGA) revealed alteration of NF1 via missense mutation, nonsense mutation or deletion in 14% of melanoma tumors, confirming that NF1 loss occurs in human melanomas. In contrast to prior studies in other tissue contexts in which NF1-null cells were shown to be mTOR dependent, we find that NF1-null melanoma cell lines are not dependent on TORC1 but rather on the MAPK pathway for proliferation and cell cycle progression. Inhibition of ERK signaling by some MEK inhibitors was short lived in NF1-null melanoma cell lines due to loss of negative feedback and reactivation of pERK. Enhanced antitumor effects were observed with trametinib, a compound that blocked phosphorylation of MEK by RAF and thus prevented pathway reactivation. NF1 loss in the context of BRAF(V600E) mutation was sufficient to raise RAS-GTP levels and confer resistance

to the RAF inhibitor vemurafenib. However, these cells remained sensitive to the MEK inhibitor trametinib, suggesting a potential therapeutic strategy for patients with NF1 loss and BRAF(V600E) mutation. We questioned whether NF1 loss was sufficient to induce melanoma formation, and modeled somatic NF1 loss in melanocytes using a *Nf1*$^{flox/flox}$*; Tyr::CreER* mouse model. Nf1 loss as a single alteration was not sufficient for melanoma formation, but caused hyperpigmentation and mild hyperproliferation of melanocytes. In summary, NF1 loss occurs in a subset of cutaneous melanomas and results in RAS activation, MEK dependence, and RAF inhibitor resistance.

Vitae

Moriah Nissan (née Heller) was raised in Andover, Massachusetts. She attended the University of Rochester in Rochester, NY with a Bausch and Lomb Honorary Science Scholarship from 2004-2008. There, she was active in the Department of Chemistry as a general chemistry workshop leader under the mentorship of Professor Todd Krauss, and the Department of Biochemistry and Biophysics as a laboratory teaching assistant under the mentorship of Dr. Harold Smith. She joined the laboratory of Dr. Joseph Wedekind in the Department of Biochemistry and Biophysics in 2006 and worked for two years alongside Robert Spitale, a graduate student studying the structure and function of the hairpin ribozyme. Her project was synthesizing modified nucleotides for incorporation into the active site of the hairpin ribozyme to study the cleavage mechanism of this class of ribozymes. Her work yielded two publications with Spitale and Wedekind. Moriah graduated from the University of Rochester in 2008 *magna cum laude* with a BS in biochemistry.

Upon graduation in 2008, Moriah matriculated at the Gerstner Sloan-Kettering Graduate School. There she joined the laboratory of Dr. David Solit in the Human Oncology and Pathogenesis Program to study occult MAPK pathway mutations in melanoma. In addition to this project, Moriah spearheaded an effort to genotype, document and organize a melanoma cell line and matched tumor database with the Wolchok laboratory in order to facilitate use of MSKCC-derived melanoma cell lines. Moriah also worked under the clinical mentorship of Dr. Paul Chapman, initiating an IRB-approved clinical survey of neurofibromatosis patients with melanoma in order to further understand the relationship between the two diseases. She first-authored a review article, a commentary, and a book chapter with Dr. Solit in addition to the first-author peer reviewed article she submitted prior to her defense. Additionally, Moriah presented her work at the 2013 AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

Conference where she was the recipient of a Scholar-in-Training award for “outstanding abstract”.

In addition to her laboratory work, Moriah spent three years actively involved in recruitment for the Gerstner Sloan-Kettering Graduate School, traveling to the 2009, 2010 and 2011 SACNAS and 2011 ABRCMS conferences to meet with potential applicants to the school. She was also co-founder of the GSK Women in Science (GWIS) group, and remained actively involved in the group until her graduation.

Acknowledgements

In kindergarten my parents brought me to Plimouth Plantation, which featured actors dedicated to reenacting life in the year 1627 in the famous harbor-side village. Their commitment to the historical accuracy of the time was, to say the least, convincing. At school the following day I told my teacher I had met the pilgrims, to which she informed me that, actually, the pilgrims are dead. Naturally, what she said conflicted with my experiences and beliefs, so, naturally, I informed her that she was wrong.

Since then, I have come to interpret my stubbornness more as a form of inertia (which, I will have you know, is a property of ALL matter. It's not just me). It is for this reason, among many others, that I am so grateful for the mentorship provided me by David Solit. David spent 5 years nurturing my abilities and interests whilst putting up with my "inertia" on a whole range of topics. In addition to his unwavering patience, he has been a wonderful role model to me in his responsibilities as both scientist and parent. Some of my favorite meetings involved him invoking analogies between lab work and Harry Potter, or between the peer-review process and Legally Blonde. Football analogies, I am sorry to say, were lost on me. However, I will take away from my experience the balance between "getting it done" and "needing to finish early so I can pick up the kids from [insert activity]". David is a living example that success can come to people who work hard but who have lives outside of their careers. For this, I will be eternally grateful.

I have also been extremely fortunate to have the co-mentorship of Neal Rosen. Neal has given me wonderful guidance, input, and, most importantly, criticism over the years; I would worry if Neal didn't have something critical to say, as it meant either that he wasn't paying attention or that he didn't care. I am grateful that the criticism was constructive and frequent. He has made me a better scientist, and continues to do so. I also had the pleasure of having the clinical mentorship of Paul Chapman. Dr. Chapman met with me on a monthly basis to discuss the state of the clinic and the current clinical trials in addition to reviewing data for my project,

and I am extremely appreciative for the time and effort he put into this mentorship. My project was also the result of an outstanding and productive collaboration with Taha Merghoub and the Wolchok lab. Taha was my animal mentor, and helped me plan, execute and analyze my mouse experiments. His guidance and enthusiasm were always welcome and refreshing.

I had help in the lab from a number of talented scientists. Alexis Jones helped me with my mouse work, tissue culture and experiments. Her dedication is inspirational and, for many technical experiments, her skills have come to exceed mine. She is my right-hand-woman, and this body of work would not have been possible without her. Several other people also contributed to my project: Ricardo Ramirez performed the RNA seq experiments; Li Kong initially worked with M308 and found its elevated RAS-GTP; Nikolaus Schultz aided with TCGA data mining and figure composition; and Christine Pratilas initiated the NF1 project, eventually trusting it to my care. I also must thank every member of the Solit lab—current and former—for their friendship and encouragement. Without them there would have been no reason to bake and eat so many treats.

The graduates of Gerstner Sloan-Kettering are blessed with fantastic institutional support staff who are devoted to supporting us through our graduate careers. Ken Marians has led this school by example, and its success can be attributed to his vision and his leadership. Maria Torres, Ivan Gerena and Iwona Abramek devote their lives to the students, and I would not have gotten through graduate school without their constant help and their emotional support. It was calming to know there was always a place at work to which I could escape and find a sympathetic ear. For the record, I only hid in the office to cry three times.

The other unsung heroes of this work are my family members: my siblings, parents, and most importantly, my husband. Keeping it together is a lot easier with someone at home willing to listen to you seesaw alternatively between the despondent “my experiments aren’t working and my life is meaningless” and the manic “SCIENCE IS THE GREATEST AND I AM THE

CONQUEROR OF ALL THE THINGS!" I am lucky to have him as my best friend and my partner in life, and his encouragement is the reason I finished graduate school in one piece.

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4-HT	4-hydroxytamoxifen
aCGH	Array Comparative Genomic Hybridization
APOBEC	Apolipoprotein B mRNA Editing Enzyme, Catalytic Polypeptide-like
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
CD	Common Docking Domain
DNA	Deoxyribonucleic Acid
DUSP	Dual Specificity Phosphatase
DVD	Domain for Versatile Docking
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ERK	Extracellular Signal-Related Kinase
ES cells	Embryonic Stem Cells
EtBr	Ethidium Bromide
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration (of the USA)
FFPE	Formalin-Fixed, Paraffin Embedded
FGF	Fibroblast Growth Factor
GAP	GTPase Activating Protein
GDP	Guanine Diphosphate
GEF	Guanine Exchange Factor
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GRD	GAP-Related Domain
GTP	Guanine Triphosphate

HGF	Human Growth Factor
HVR	Hypervariable Region
IRB	Internal Review Board
IFN	Interferon
IGV	Integrated Genomics Viewer
IL	Interleukin
IMPACT	Integrated Mutational Profiling of Actionable Cancer Targets
IP	Intraperitoneal *or* Immunoprecipitation
kDa	Kilodalton
MAPK	Mitogen Activated Protein Kinase
MEK	Mitogen Activated Protein Kinase Kinase
mM	Millimolar
MPNST	Malignant Peripheral Nerve Sheath Tumor
mRNA	Messenger Ribonucleic Acid
mTOR	Mammalian Target of Rapamycin
TORC1	Mammalian Target of Rapamycin Complex 1
nM	Nanomolar
NF1	Neurofibromin 1 *or* Neurofibromatosis type 1
NGF	Nerve Growth Factor
NPcis	Nf1 +/-; p53 +/- on same murine chromosome
NPtrans	Nf1 +/-; p53 +/- on opposite murine chromosome
ORF	Open Reading Frame
P	Phosphorylated (unless, followed by a number, which means “protein”)
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PI3K	Phosphoinositide 3-kinase

PKC	Protein Kinase C
qPCR	Quantitative Polymerase Chain Reaction
RBD	RAS Binding Domain
RCC	Renal Cell Carcinoma
RNA	Ribonucleic Acid
RNAi	Ribonucleic Acid interference
RTK	Receptor Tyrosine Kinase
SH2	Src-Homology Domain 2
SH3	Src-Homology Domain 3
shRNA	Small Hairpin Ribonucleic Acid
siRNA	Small Interfering Ribonucleic Acid
SOS	Son of Sevenless
SPR	Sprouty-Related Protein
SPRY	Sprouty Protein
TAM	Tumor Associated Macrophage
TCGA	The Cancer Genome Atlas
VEGFR	Vascular Endothelial Growth Factor Receptor
WT	Wild-Type
μM	Micromolar

Chapter One

Introduction

Melanoma is a cancer of the melanocytes: the pigment producing cells of the body. It is the deadliest form of skin cancer. Patients who develop metastatic disease have an average overall survival of 6 months and a 5 year survival rate of about 15% (American Cancer Society 2013). Melanomas are classified based on the site of origin and the type of melanocyte from which the tumor is derived. Cutaneous melanoma, the most common form of melanoma, develops from melanocytes in the epidermal layer of the skin. Non-cutaneous melanomas (acral melanoma, ocular melanoma, mucosal melanoma) arise from the non-cutaneous melanocytes in the eye, gastrointestinal tract, genitourinary tract and meninges (Chin, Garraway et al. 2006). The driving alterations found most commonly vary across subtype. This thesis will focus on cutaneous melanoma.

Traditional chemotherapeutic options for the treatment of metastatic melanoma, and the standard of care, have included the cytotoxic agents dacarbazine and temozolamide and the immunotherapies Interferon-alpha (IFNα) and IL-2 (Sosman 2013). However, these agents have not been shown to prolong the overall survival of patients. Driven by the dearth of treatment options, the field has been focused on developing improved immunotherapies to exploit the innate ability of the immune system to detect and eliminate cancer (Dunn, Bruce et al. 2002). Such drugs have included ipilimumab, an antibody against CTLA-4, which helps the body disable the down-regulation of T-cells and therefore promote T-cell activation and anti-tumor immunity

(Hodi, O'Day et al. 2010), and anti-PD1/PD-L1 antibodies such as nivolumab and lambrolizumab, which promote anti-tumor immunity by preventing T-cell exhaustion and therefore boosting the immune response (Hamid, Robert et al. 2013, Wolchok, Kluger et al. 2013). In addition to the development of immunotherapies, the field has also striven to identify the driving alterations in melanoma tumors and to develop small molecule therapies to specifically target these "driver" alterations, exploiting potential oncogene addiction of melanoma tumors (Weinstein and Joe 2008). These small molecule inhibitors and the alterations they target will be discussed below.

The MAPK pathway

Roughly 90% of cutaneous melanoma tumors have activation of mitogen activated protein kinase (MAPK) (Cohen, Zavala-Pompa et al. 2002), also known as ERK (extracellular signal-regulated kinase). The MAPK pathway is one of several cell-signaling pathways responsible for interpreting mitogenic cues from the extracellular environment and relaying this information to the nucleus to promote growth and proliferation. The MAPK pathway is a three-tiered kinase cascade consisting of the RAF, MEK and ERK kinases that are activated upstream by receptor tyrosine kinases (RTKs) and their small GTPase effector proteins, H-, N- and KRAS.

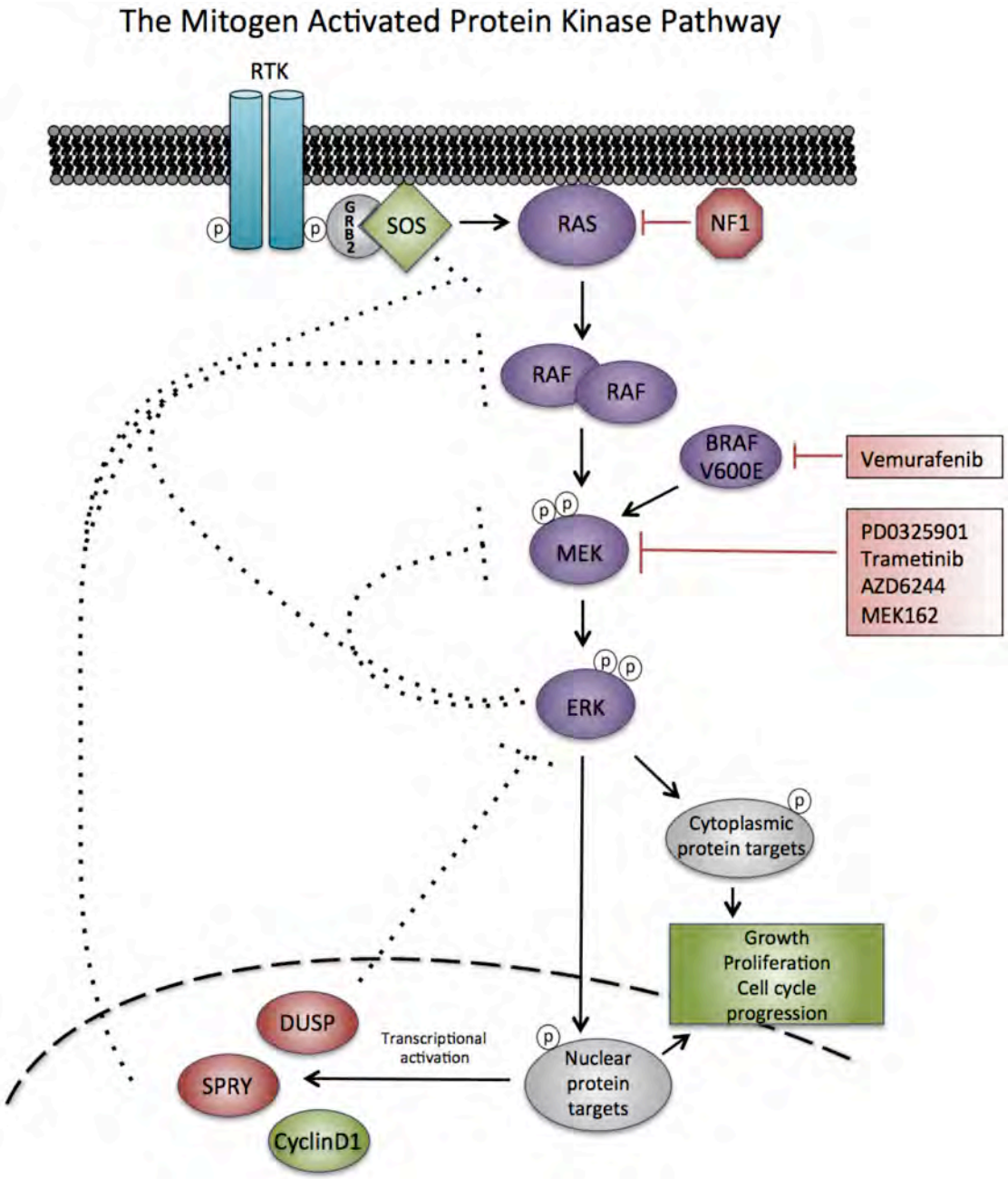

Figure 1.1 The MAPK pathway. Proteins that contribute to positive growth signals are in green, while growth regulatory proteins are in red. Negative feedback is symbolized with dotted hammers. (Adapted from Cancer Discovery, 2013, 3(7), 719-721, Moriah H. Nissan, Neal Rosen, David B. Solit, "ERK pathway inhibitors: how low should we go?", with permission from AACR.)

RTKs, Grb2 and SOS

RTKs are a class of cell surface receptors made up of core extracellular ligand binding domains, transmembrane domains and intracellular kinase domains (Schafer, Marg et al. 2004). The extracellular ligand binding domains in the extracellular matrix bind to mitogens and growth factors: small protein molecules responsible for inducing cell division (mitosis) and growth, proliferation, and differentiation. Structural changes in the receptor induced by ligand-dependent activation results in receptor dimerization, autophosphorylation and kinase domain liberation.

Early landmarks in the RTK field included the discoveries in 1957 of nerve growth factor (NGF) and in 1962 of epidermal growth factor (EGF) (Cohen and Levi-Montalcini 1957, Cohen 1965). NGF and EGF were found to stimulate premature eye opening and tooth eruption in mice, and growth of epithelial cells in culture (Cohen and Levi-Montalcini 1957, Cohen 1965). Using I^{125} labeled EGF in 1975, Carpenter et al. demonstrated that these proteins bind to molecules on the surface of the cell, a discovery that lead directly to the identification of the EGF receptor (EGFR) in 1978 (Carpenter, Lembach et al. 1975, Carpenter, King et al. 1978). In 1984, Kamata and Feramisco showed that RAS switched from its inactive GDP-bound state to its active GTP-bound state as a result of EGF stimulation, which linked growth factors and their receptors to downstream signaling effectors (Kamata and Feramisco 1984).

There are 20 sub-families of RTKs totaling at least 58 members (Gschwind, Fischer et al. 2004). Though some structural and functional differences exist among and within the subfamilies, RTKs share a great deal of similarity. Early work demonstrated this similarity through the formation of a chimeric receptor containing the ligand binding domain of the insulin receptor and the transmembrane and kinase domains of the EGF receptor, the latter of which was activated upon stimulation with insulin (Riedel, Dull et al. 1986). The kinase domain is the most similar and most conserved domain across sub-families.

Receptor dimerization results in autophosphorylation of activation loop tyrosine residues in the receptor's kinase domain, priming the kinase to phosphorylate and activate downstream targets (Schlessinger 1988). The phosphorylated tyrosines on the activation loop also serve as docking sites for proteins that contain SH2 domains, which recognize and bind short peptide motifs containing phosphotyrosines. The specificity of RTK signaling and downstream pathway activation critically depends on the receptor's ability to dock other proteins, as the pattern of RTK phosphorylation may demonstrate fidelity to specific SH2-containing downstream effector proteins and may dictate when one signaling pathway is activated over another (Pawson 1995).

The link between RTKs and RAS is indirect and involves recruitment of GRB2 and SOS (son of sevenless; an activator of RAS) to the activated receptor. GRB2 is an adaptor protein with an SH2 domain flanked by two SH3 domains, which is responsible for recognizing active RTKs and for recruiting SOS. SH2 domains are pockets lined with basic residues that bind negatively charged phosphotyrosine with a Kd of 10-100 nM, and utilize an invariant arginine residue to stabilize the two phosphate oxygens (Pawson 1995). SH3 domains bind proline-rich amino acid stretches with a Kd of 1-100nM (Pawson 1995). The SH2 and SH3 domains of GRB2 are critical for activation of the MAPK pathway, as mutation of either domain in the *c. elegans* GRB2-homologue Sem5 uncouples RTK activation from RAS activation (Pawson 1995). Interestingly, SH3 domains are "pseudo-symmetrical" and therefore may be able to bind to effectors in either orientation (amino- to carboxy-terminal or carboxy- to amino-terminal) suggesting a regulatory mechanism for GRB2 and SOS signaling; binding of an effector in a given orientation may introduce "handedness" to the binding complex and dictate what docking sites are available to further binding partners in the complex (Pawson 1995).

RAS signaling

The 21 kDa RAS proteins (H-, K-, and N-RAS) coordinate the activation of RTKs with the activation of downstream effector proteins. These GTPases lack a catalytic arginine residue common among other GTPase proteins. For example, RAS proteins lack the alpha subunit of G protein coupled receptors, and thus require the coordinate function of RAS GTPase activating proteins (GAPs) and RAS GTPase exchange factors (GEFs) for their activation and regulation.

The three RAS proteins are highly similar in structure. The differences between the proteins mainly lie in their C-terminal regions, also known as the hypervariable regions (HVR). The HVR for each RAS isoform contains a membrane-targeting domain and a CAAX box that together dictate the post-translational lipid modifications and the membrane localization of RAS. HVR differences dictate the specific post-translational modifications made to each protein, and therefore alter the preferred subcellular localization of each protein. KRAS4B, one of two spliced isoforms of *KRAS*, is farnesylated and contains a polylysine stretch that helps it adhere to the disordered plasma membrane via charged interactions (Hancock, Paterson et al. 1990). Therefore, modifications such as phosphorylation or cellular conditions such as high intracellular Ca^{2+} levels affect the ability of KRAS4B to properly localize (Fivaz and Meyer 2005). HRAS, NRAS, and KRAS4A, however, lack this lysine-rich region and are modified with both farnesyl and palmitoyl groups (Hancock, Magee et al. 1989). Though farnesylation is generally required for membrane localization of RAS, N- and KRAS proteins have been shown to utilize geranylgeranyl modifications when farnesylation is inhibited, as in the presence of farnesyl transferase inhibitors (Whyte, Kirschmeier et al. 1997).

Because of the differences in the HVRs of the RAS isoforms, the proteins differ in their intracellular localization. HRAS localizes to disordered membrane as well as both caveolar and non-caveolar lipid rafts; NRAS localizes mostly to lipid rafts in the cell membrane; KRAS is

found mostly in the disordered cell membrane (Hancock and Parton 2005). The different subcellular localizations of the RAS isoforms may contribute to their functional differences, as ERK1/2 shows affinity for different substrates based on its subcellular localization. This suggests that subcellular localization of RAS signaling can control where in the cell ERK is activated and therefore dictate which downstream effectors are ultimately triggered (Casar, Arozarena et al. 2009). Additionally, the subcellular localization of RAS isoforms may affect how frequently they encounter GAPs and GEFS, suggesting that localization may be a mode of regulation (Mor and Philips 2006).

RAS is activated by SOS (son of sevenless), which is found at the cellular membrane in a complex with GRB2 and activated RTKs. SOS is a guanine exchange factor (GEF) responsible for exchanging GDP in the RAS active site for GTP, converting RAS from its inactive state (GDP-bound) to its active state (GTP-bound). RAS has a high affinity for GDP (Cichowski, Shih et al. 1999), which likely prevents the sporadic dissociation of GDP, prevents the subsequent binding of GTP, and thus stabilizes the inactive state of RAS. Because of the high affinity of RAS for GDP, binding with SOS is necessary to facilitate dissociation of GDP from the active site of RAS. This binding is thought to cause a conformational change in both proteins that alters the stability of GDP-RAS binding and facilitates GDP release (Freedman, Sondermann et al. 2006). Driven by the high cytoplasmic concentration of GTP, GTP then occupies the empty nucleotide-binding site. Therefore, the rate-limiting step of RAS activation is release of GDP by SOS, and thus the rate-limiting step is controlled by the extracellular environment through SOS regulation by RTK activation and other upstream signals.

Upon binding or hydrolysis of GTP, RAS undergoes structural changes in two loops, switch I and switch II. Though small changes can be seen across the entire protein, switch regions are literally defined by their structural change upon the GTP-GDP transition (Scheffzek, Ahmadian et al.

1997). Switch I overlaps with the effector binding domain of RAS and contains a negatively charged surface made up of five acidic residues (Asp30, Glu31, Asp33, Glu37, Asp 38). These residues are crucial for binding to both effector proteins and RAS-GAPs, such as NF1. Upon GTP binding, change in this region switches RAS to its "active" conformation and creates a favorable environment for protein binding (Scheffzek, Ahmadian et al. 1997). As the same region of RAS is responsible for binding both effectors as well as negative regulators, downstream RAS signaling competes with and is balanced by RAS inactivation. Switch II overlaps an alpha helix (α2) and a loop region (L4) that, in structures of isolated RAS, are highly mobile. This area is stabilized by binding of RAS-GAPs (Scheffzek, Ahmadian et al. 1997) and is more stable in the GTP-bound form. The Q61 residue, important for hydrolysis of GTP, is proximal to this region, and therefore thought to be unstable and disordered until GTP binds and Switch II gains rigidity (Scheffzek, Ahmadian et al. 1997).

RAF, MEK and ERK

Upon stimulation, RAS can activate the ERK pathway, the PI3K/AKT pathway and the RalGDS pathway, among others. This thesis focuses on the ERK pathway. Active RAS binds to and has affinity for the RAF (A-, B-, and CRAF/RAF1) kinases. Though the kinases can function as monomers, they can and do form homo- and heterodimers (Weber, Slupsky et al. 2001, Wan, Garnett et al. 2004, Rushworth, Hindley et al. 2006). Formation of these dimers depends on active RAS (Weber, Slupsky et al. 2001), and these dimers activate MEK with elevated kinase activity compared to RAF monomers (Rushworth, Hindley et al. 2006). BRAF/CRAF heterodimers have higher kinase activity compared to RAF monomers or homodimers. The increased kinase activity of RAF heterodimers holds true even when one protomer in the dimer is kinase dead, suggesting that the protomers in the dimer synergistically transactivate one another in an allosteric manner.

The dependence of increased kinase activity of the RAF dimers on RAS activation suggests another way RAF activity is regulated by upstream signals.

The three RAF proteins have different patterns of expression and regulation and different roles in the cell. However, they all share similar structures and contain several conserved regions, including the kinase domain and the RAS binding domain (RBD). The RBD has affinity for RAS only when RAS is bound to GTP, likely exploiting affinity to the exposed switch I domain of active RAS. RAS binding to RAF utilizes both the N-terminal regulatory RBD of RAF and the cysteine rich domain of RAF. This latter interaction is responsible for recruiting RAF to the membrane, allowing contact between RAS and the RAF RBD and subsequent RAF activation (Wellbrock, Karasarides et al. 2004). As RAF is recruited to the membrane by RAS, it undergoes a structural change in which the activation loop disengages with the glycine-rich loop and swings into an "open" conformation (Wan, Garnett et al. 2004). The open conformation exposes the regulatory serine and threonine residues of RAF, facilitating phosphorylation of the regulatory residues by their respective kinases. Phosphorylation destabilizes the inactive conformation of the RAF protein and results in its full enzymatic activation (Wan, Garnett et al. 2004).

The extent to which RAS activates each RAF isoform is likely to vary despite the mechanism of activation being similar. Additionally, the activation potentials of each RAF isoform are inherently different from one another (Mason, Springer et al. 1999). All three isoforms contain an "N-region", which must carry a negative charge for kinase activation (Mason, Springer et al. 1999). ARAF and CRAF contain residues in their N-region that must be phosphorylated in order for the protein to be maximally activated. In contrast, though BRAF also has sites that require phosphorylation for activation, several of the conserved sites found in ARAF and CRAF constitutively carry a negative charge in BRAF. For example, the S445 residue of BRAF corresponds to the conserved S338 phosphorylation site in CRAF, and is constitutively

phosphorylated. The Y341 site in CRAF is a negatively charged aspartic acid residue in BRAF (D448) that acts as a phosphomimetic (Mason, Springer et al. 1999). The constitutively negatively charged N-region of BRAF is thought to be the underlying reason why BRAF is found mutated in so many cancers, while CRAF mutations are rare and ARAF mutations are practically nonexistent: it takes fewer steps to maximally activate BRAF, thus it is more likely for a single mutational event to have activating potential (Mason, Springer et al. 1999).

Active RAF kinases bind to and activate their downstream MAPK pathway effectors, the MEK kinases. Three isoforms of MEK (mitogen activated protein kinase kinase), MEK1, the inactive MEK1b and MEK2, are highly homologous, evolutionarily conserved and have only one known physiological substrate, ERK. The MEK proteins are made up of a kinase domain flanked by a regulatory N-terminal domain and a small C-terminal domain. This C-terminal domain contains what is called the domain for versatile docking (DVD) (Takekawa, Tatebayashi et al. 2005) which is responsible for interacting with active MAP kinase kinase kinases, such as RAF or COT. Binding of RAF to the DVD of MEK modulates the phosphorylation of regulatory serine residues (S217/S221) in MEK's activation loop. Upon phosphorylation, these residues activate MEK's kinase activity, which allows binding of MEK to ERK, its only widely accepted effector protein. MEK is a dual-specificity kinase and is uniquely capable of phosphorylating ERK's regulatory tyrosine and threonine residues, whose phosphorylation are required to activate ERK's kinase function.

MEK also has regulatory serines, such as S386, whose phosphorylation can inhibit MEK activity (Matsuda, Gotoh et al. 1993). Interestingly, these sites are generally phosphorylated by ERK, revealing one of the most immediate feedback mechanisms of MAPK pathway activation. MEK may also regulate ERK activity by controlling its subcellular localization: when MEK binds to and activates ERK, ERK translocates to the nucleus where it interacts with its nuclear targets.

MEK enters the nucleus in complex with ERK (Adachi, Fukuda et al. 1999). As MEK, but not ERK, harbors a nuclear export signal, it is likely that MEK is responsible for controlling the movement of ERK back to the cytosol, setting an intrinsic temporal limit on ERK's activity in the nucleus (Adachi, Fukuda et al. 2000).

The ERK proteins (ERK1, ERK2) are the only widely accepted downstream targets of MEK. At ERK's C-terminus its common docking domain (CD) is responsible for interaction with MEK (Tanoue, Adachi et al. 2000). The binding of MEK to this domain exposes ERK's tyrosine and threonine residues to MEK kinase activity. Phosphorylation of these residues (Thr183, Tyr185) then induces a conformational change in ERK that promotes binding of ERK substrates in a manner that facilitates their own phosphorylation by ERK (Zhou, Sun et al. 2006). Activation of ERK leads to the phosphorylation of various cytosolic and nuclear proteins. While still in a complex with MEK, ERK shuttles into the nucleus, however this process is not completely understood and likely involves facilitated transport (Fukuda, Gotoh et al. 1997, Adachi, Fukuda et al. 1999, Adachi, Fukuda et al. 2000). In the nucleus, transcription factors activated by ERK facilitate the transcription of ERK-output genes. Studies aiming to elucidate these genes have used mRNA levels before and after treatment of a MAPK-pathway dependent cell line, generally one with BRAF(V600E) mutation, to determine which genes change significantly following MEK or RAF inhibition (Pratilas, Taylor et al. 2009, Joseph, Pratilas et al. 2010). Several of these ERK output genes are known to promote the transformed state such as cyclin D1, which promotes cell cycle progression from G1 to S phase, while others are transcription factors that promote cell growth and/or survival, such as MYC and the ETS transcription factors (ETV1/4/5). Scaffolding proteins are thought to anchor activated ERK in the cytosol, and may dictate the downstream signaling effects of activated ERK by regulating which proteins are proximal; where in the cell the proteins are located; and the stability of protein-protein interactions (Yu, Fantl et al. 1998).

Feedback

Targets of ERK kinase are genes that affect proliferation and cell cycle progression as described above. However, the other task undertaken by ERK is to shut off the MAPK pathway and therefore regulate its own activation. As was previously described, the first and likely most immediate of these events is the negative regulation of MEK by S386 phosphorylation. Cytosolic ERK also directly regulates other MAPK pathway proteins. Phosphorylation of RAF-1 by ERK on 6 specific serine residues decreases the activity of RAF-1, interferes with the RAS/RAF-1 association, and may target RAF-1 for recycling by attracting phosphatases such as PP2A to dephosphorylate RAF-1 to its inactive state (Dougherty, Muller et al. 2005). ERK phosphorylates four serines in the c-terminal SH3 domain of SOS1, interrupting the SOS1-GRB2 interaction and presumably decoupling RAS activation from RTK activation (Corbalan-Garcia, Yang et al. 1996). ERK also indirectly regulates activation of the MAPK pathway through transcriptional upregulation of genes that negatively regulate MAPK pathway activators, such as the sprouty, DUSP and SPRED genes.

Sprouty proteins (Sprouty1-4) are responsible for inhibiting MAPK pathway activation at the levels of RTKs, RAS and RAF. Their localization at the membrane, and their activation via phosphorylation on a conserved tyrosine residue, play a large role in their function. Membrane localization brings them in proximity to their targets, as RTKs, RAS, and RAF are all located in or near the plasma membrane (Hanafusa, Torii et al. 2002). The conserved tyrosine residue of the sprouty proteins has been shown to interact with SH2 domains, such as in GRB2, suggesting that sprouty binding may sequester GRB2 away from activated RTKs and prevent activation of RAS (Hanafusa, Torii et al. 2002). The conserved cysteine-rich C-terminal domain of the sprouty proteins has also been shown to interact with CRAF/RAF-1 and prevent its phosphorylation by protein kinase C (PKC) (Sasaki, Taketomi et al. 2003). That being said, the roles of sprouty

proteins might differ depending on their cellular context, and other roles for these proteins are still being elucidated.

SPRED proteins (SPRED1-2) were discovered in a yeast two-hybrid screen using an osteoclast cDNA library and the tyrosine kinase domain of c-Kit as bait. As they harbored the C-terminal SPR domain (found in sprouty proteins) they were named SPRED: Sprouty-related protein with EVH-1 domain (Wakioka, Sasaki et al. 2001). These proteins coimmunoprecipitate with RAS and, like their cousins, inhibit the activation of RAF (Wakioka, Sasaki et al. 2001). It was initially thought that these proteins functioned downstream of RAS but upstream of RAF: preventing RAS from activating RAF rather than preventing RAS activation. However, further studies have led to the theory that sprouty proteins inhibit RAS through recruitment of NF1 (Stowe, Mercado et al. 2012). Thus, SPRED proteins may be less effective at inhibiting RAF activation in an NF1-null context. Likewise, there is evidence that NF1 is less effective at controlling RAS activation when SPRED is mutated or deleted, providing the basis for the observation that Legius syndrome, the congenital syndrome resulting from SPRED mutation, is phenotypically similar to neurofibromatosis type 1, which results from germline mutation of the *NF1* gene (Stowe, Mercado et al. 2012).

The DUSP (Dual-specificity phosphatase) genes encode phosphatases that are capable of dephosphorylating ERK on both its tyrosine and threonine residues. Members of the DUSP family demonstrate subcellular localization specificity, with DUSP1, 2, 4, and 5 being nuclear and 6, 7, 8 being cytoplasmic, allowing regulation of ERK in both its subcellular compartments. The number and position of positively charged and hydrophobic residues in a DUSP's kinase interaction motif (KIM) grant specificity for a certain MAPK, such as ERK, JNK or p38 (Tanoue, Yamamoto et al. 2002). Although expression of the DUSPs is controlled by ERK activity, the inherent delay in their expression results in feedback on the MAPK pathway via ERK that is

temporally distinct from immediate feedback events, such as inhibition of SOS1, RAF, and MEK by ERK (Avraham and Yarden 2011). In addition to their role as phosphatases, DUSPs are thought to regulate ERK via sequestration in subcellular compartments, which may regulate ERK activity by enriching or depleting ERK in the presence or absence of its activators and effectors (Rodriguez and Crespo 2011). Most notably, loss of DUSP4 has been implicated as a tumor suppressor in lung cancer (Chitale, Gong et al. 2009), highlighting the important contribution of the DUSPs to negative regulation of mitogenic signaling. It is likely that our understanding of the DUSPs importance in regulating cellular transformation will expand in the future.

Cyclin D1

One of the earliest and arguably most important output effectors of the MAPK pathway is the cell cycle regulator cyclin D1. Transcription of cyclin D1 mRNA is upregulated in a delayed early manner upon mitogenic stimulation of the cell; is stabilized in a mitogenic-dependent fashion; and is translated to a protein whose nuclear translocation helps promote progression of the cell cycle (Sherr, Matsushime et al. 1992, Sherr 1995). Cyclin D1 provides a key link between mitogenic signaling and cell cycle progression. Cyclin D1 is unstable, having a half-life on the scale of 30 minutes, and can be targeted for degradation via phosphorylation by GSK-3β (Diehl, Zindy et al. 1997). Because of the short half life of the protein, as well as the robust induction of mRNA expression by mitogenic stimulation, there is a carefully regulated steady state of cyclin D1 creation and destruction whose balance can be tipped by oncogenes or mitogenic pathway inhibition to affect cell cycle progression (Knudsen, Diehl et al. 2006). This allows cyclin D1 expression to function as a sliding scale regulator rather than a binary on-off switch. However, this may also be the reason why inhibition of cyclin D1 expression requires near complete inhibition of mitogenic signaling pathways responsible for its control to maximally induce growth arrest, as even residual activity of the pathway may be sufficient to maintain cyclin D1 expression

and cell cycle progression (Solit, Garraway et al. 2006, Johannessen, Johnson et al. 2008, Pratilas, Taylor et al. 2009).

The MAPK pathway in melanoma

NRAS

BRAF and NRAS mutations are the most common activating mutations found in cutaneous melanoma (Wellcome Trust Sanger Institute 2013) and were both discovered by direct targeted sequencing efforts. Before NRAS mutations were identified in melanoma, environmental agents had been shown to induce mutations in the RAS genes (Barbacid 1987) and UV exposure was suggested as an important factor in the induction of melanoma formation (Sober 1987). This led van't Veer and colleagues to investigate whether UV exposure could induce RAS mutations in melanoma. They found mutations in the N-isoform of RAS in 19% of tumor and cell line samples (van 't Veer, Burgering et al. 1989). This study and later studies found that NRAS mutations were correlated with UV-induced nucleotide changes, occurred more frequently on continuously sun-exposed skin and were more common in samples representative of later stages of melanoma progression (van 't Veer, Burgering et al. 1989, Ball, Yohn et al. 1994). NRAS mutations, generally at position Q61, are now known to occur in roughly 20% of melanoma tumors (Wellcome Trust Sanger Institute 2013).

Oncogenic mutations of RAS generally alter residues that are critical for RAS GTPase activity. G12 is positioned such that its side chain lies in the protein's active site. G12 mutations, even from glycine to alanine (side chains of –H and $–CH_3$, respectively) have side chains expected to be within Van der Waals radius of both the catalytic arginine of the RAS-GAP as well as the side chain of Q61. This suggests that mutation of glycine at position 12 to any other amino acid would interfere with GTPase function through steric inhibition (Scheffzek, Ahmadian et al. 1997) and

lead to constitutive activation of RAS in either the presence or absence of RAS-GAPs. Mutations at position G13 are expected to have similar steric consequences. Q61 is positioned such that the -NH_2 group of its side chain lies in the active site and is aligned with the phosphate chain of the guanine nucleotide. This interaction is stabilized by the rigidity of the proximal switch II region resulting from RAS-GAP binding, and hydrogen bonding with the catalytic arginine of the RAS-GAP itself (Scheffzek, Ahmadian et al. 1997). Q61 also forms a hydrogen bond with an ordered water molecule in the active site. While prior studies suggested that Q61 stabilized the nucleophilic water in the hydrolysis reaction, data from kinetic isotope effect studies indicate that this theory less likely. Instead, data from kinetic isotope effect studies suggest that the role of Q61 is to stabilize the charge on the leaving group oxygen in the GTP-GDP transition state (Du and Sprang 2009). Thus, mutations at position 61 that disrupt this stabilization or whose side chains occlude the active site lead to constitutive activation of RAS.

BRAF

In 2002, Davies and colleagues at the Sanger Institute set out to sequence genes in pathways frequently mutated in cancers in 923 tumors and cancer cell lines, starting with the RAS-RAF-MEK-ERK genes (Davies, Bignell et al. 2002). By sequencing the exons and intron-exon junctions of BRAF, they found BRAF mutations in roughly 8% of malignant samples tested. Malignant melanoma was the tumor type with the highest percentage of BRAF mutations (66%), with BRAF mutations also identified in a minority of colorectal cancers, gliomas, lung cancers, sarcomas, ovarian carcinomas, breast cancers and liver cancers (Davies, Bignell et al. 2002). The high prevalence of BRAF mutations in melanoma is likely due to the dependence of melanocytes on BRAF for normal physiologic functions, rather than to UV damage, as the T-A mutations seen in *BRAF* are distinct from the CC-TT or C-T changes that commonly result from exposure to ultraviolet light (Davies, Bignell et al. 2002). The melanocyte's dependence on BRAF thus likely

creates an environment that promotes the selection of activating BRAF mutations. BRAF(V600E) mutations are now known to occur in roughly 50% of melanoma tumors, and these mutations are found in a non-overlapping pattern with NRAS mutations (Wellcome Trust Sanger Institute 2013).

The majority of oncogenic BRAF mutations are missense mutations that occur at position V600 of the protein. V600 is adjacent to the T599 and S602 phosphorylation sites, and mutations at this site, for example the most common valine to glutamic acid mutation, mimic the activating phosphorylation described above and thus stabilize the active state of the protein by disrupting the interaction between the activation segment and the glycine-rich loop (Wan, Garnett et al. 2004). The amino acid change resulting from the mutation of a single thymine nucleotide at position 1799 to an adenine nucleotide, suggests that the prevalence of the BRAF(V600E) mutation may be due to the ease with which the mutation is acquired and the mutation's profound functional consequences.

Though activating mutations of BRAF are the most common, several recurrent mutations have been identified that have been labeled as "low activity" mutations. These mutations (G465, G468, N580 among others), cluster in the protein's glycine-rich loop and result in a protein that, as a monomer, has lower kinase activity than wild type RAF. However, these mutants confer elevated kinase activity when present in a dimer with CRAF, suggesting that these mutations promote ERK activation and transformation by allosterically activating RAF heterodimers (Wan, Garnett et al. 2004).

Alteration of cell cycle genes

An activating BRAF or NRAS mutation is not sufficient for melanoma formation. Expression of constitutively active mutant NRAS in a non-transformed cell results in potent oncogene-induced senescence (Dotto, Parada et al. 1985, Denoyelle, Abou-Rjaily et al. 2006). Similar results occur with the expression of oncogenic BRAF into non-transformed cells (Michaloglou, Vredeveld et al. 2005). This data along with the finding that BRAF mutations are present in senescent, non-cancerous melanocytic nevi (Pollock, Harper et al. 2003) suggest that additional mutational events are required to prevent this oncogene-induced senescence in BRAF and NRAS mutant tumors.

Alteration of genes responsible for regulating the cell cycle are common second hits in melanoma and are thought to prevent oncogene-induced senescence (Collado and Serrano 2006). Deletion or alteration at the *CDKN2A* locus is one of the most mutational events in melanoma (Haluska, Tsao et al. 2006). *CDKN2A* encodes two genes important for proper cell cycle regulation: $p16^{INK4A}$ and $p14^{ARF}$ (Quelle, Zindy et al. 1995). $P16^{INK4A}$ is a protein whose expression is activated by cellular stress and whose role is to prevent association of cyclin dependent kinase 4 (CDK4) and cyclin D1 (Serrano, Hannon et al. 1993). When cyclin D1 and CDK4 are associated, these proteins phosphorylate and inactivate retinoblastoma protein 1 (RB1), preventing RB1 from inhibiting transcription of S-phase associated genes. Therefore, loss of $p16^{INK4A}$ can result in uninhibited Cyclin D1-CDK4 association, RB1 inactivation, and thus, uninhibited progression through the G1-S phase of the cell cycle (Lukas, Parry et al. 1995). $p14^{ARF}$ is an inhibitor of MDM2 and therefore a promoter of p53 function (Pomerantz, Schreiber-Agus et al. 1998). p53 is responsible for inhibiting cell cycle progression and proliferation in the presence of cellular stress, such as when there are unresolved DNA double-strand breaks. MDM2 is an inhibitor of p53, and acts by promoting p53 degradation under normal conditions. ARF binds to and can sequester MDM2, leading to uninhibited expression of p53, and therefore, cell cycle and growth arrest. Loss of ARF

therefore results in the uninhibited degradation of p53 and failure to inhibit cell cycle progression under cellular stress (Zhang, Xiong et al. 1998).

Table 1.1 MEK and RAF inhibitors

Target	Inhibitor	International nonproprietary name/generic name
MEK1/2	CI-1040	N/A
MEK1/2	PD0325901	N/A
MEK1/2	AZD6244	Selumetinib
MEK1/2	GSK1120212	Trametinib
MEK1/2	MEK162	N/A
VEGFR, PDGFR, CRAF, BRAF	BAY43-9006	Sorafenib
BRAF(V600E), CRAF, BRAF	PLX4032	Vemurafenib
BRAF(V600E), CRAF, BRAF	GSK2118436	Dabrafenib

Inhibitors of the MAPK pathway and their clinical trials

(Springer and the Current Oncology Reports, 13, 2011, 479-487, "The "SWOT" of BRAF inhibition in melanoma: RAF inhibitors, MEK inhibitors or both?", Nissan, M. H. and D. B. Solit, Figure number 2 and article adaptations, with kind permission from Springer Science and Business Media.)

MEK inhibitors

A series of highly selective, non-ATP competitive, allosteric inhibitors of MEK1 and MEK2 have been in clinical development for over a decade. The class includes CI-1040 (Pfizer), PD0325901 (Pfizer), AZD6244 (AstraZeneca) and GSK1120212 (trametinib; GlaxoSmithKline). These compounds inhibit MEK and ERK activity in all cells, irrespective of their BRAF status, though cell lines with a BRAF mutation are particularly sensitive to MEK inhibition (Solit, Garraway et al. 2006). However, the antitumor effects of MEK inhibitors are not restricted to only BRAF mutant models. Specifically, a subset of RAS mutant cell lines and a small number of cell lines wild type for RAS and BRAF also exhibit MEK-dependence and CI-1040 and PD0325901 sensitivity (Solit, Garraway et al. 2006, Pratilas, Hanrahan et al. 2008, Halilovic, She et al. 2010).

The clinical development of the first-in-class compound CI-1040 was halted due to a lack of clinical activity and in favor of PD0325901, a second-generation compound with greater potency and improved oral bioavailability (Lorusso, Adjei et al. 2005, Brown, Carlson et al. 2007). Clinical trials of CI-1040 were initiated prior to the identification of BRAF mutations by the Sanger group and thus the clinical development of this compound was not directed towards tumor types with a high prevalence of BRAF mutation. The phase 1 trial of PD0325901 was enriched for patients with melanoma and modest anti-tumor activity was observed in three patients, all of whom had melanoma, with all three achieving a partial response (Brown, Carlson et al. 2007). The clinical development of PD0325901 was halted, however, over toxicity concerns, in particular skin rash and the rare occurrence of retinal vein occlusions.

AZD6244 (AstraZeneca) progressed through Phase II testing in several cancer types. Specifically, a Phase II randomization trial of AZD6244 versus temozolomide was completed in patients with melanoma (R. Dummer 2008, Board, Ellison et al. 2009). Modest antitumor activity with AZD6244 was observed, with partial responses documented in six patients, five of whom had V600E BRAF mutant tumors. No significant differences between the treatment arms were noted for the primary endpoint of progression free survival. Similar results were observed in Phase II trials of patients with non–small cell lung and colon cancer in which AZD6244 was compared with pemetrexed and capecitabine, respectively (Lang I 2008, Tzekova V 2008). A summary of the clinical experience with AZD6244 is that the compound has modest clinical activity comparable to, but not superior to, disease-specific, standard chemotherapy in several cancer types. A major weakness in the design of these trials is that they were not enriched for patients whose tumors had activating mutations in the ERK pathway.

The potential advantages of pretreatment stratification by mutation status are highlighted by recent positive results with the MEK inhibitor GSK1120212, or trametinib. Trametinib is a highly potent, non-ATP competitive MEK inhibitor (IC50s for MEK1 and MEK2 of 0.7 and 0.9 nM, respectively) (Infante JR 2010). Preliminary results from the Phase 1 trial of trametinib were reported by Infante et al. at the 2010 American Society of Clinical Oncology annual meeting (Infante JR 2010). As with other MEK inhibitors, skin rash was the most common toxicity. Ocular toxicity proved to be dose limiting with central serous retinopathy reported in three patients. In twenty patients with BRAF mutant melanoma, two achieved complete responses with an additional six patients demonstrating partial responses for a total response rate of 40%. Consistent with the preclinical studies, antitumor activity was most prominent in patients harboring BRAF mutant tumors but was also observed in a small number of patients whose tumors were wild-type for BRAF. Partial responses were observed in 2/19 BRAF wild-type melanomas and 1/22 pancreatic cancers. As the trametinib trial was the only trial of a MEK

inhibitor to have stratified patients based upon BRAF mutational status, it remains unknown whether the greater activity of this compound compared to others in the class was the result of enrichment for patients with BRAF mutations or the compound's specific pharmacologic characteristics. In regards to the latter, the drug has a long half-life (~4.5 days), low peak/trough ratio and low intrapatient variability. The trial investigators speculated that these pharmacologic properties reduced the risk of C_{max}-related toxicities and resulted in sustained drug levels above the threshold required for antitumor activity. Results from the phase 3 trial of trametinib versus chemotherapy in BRAF(V600E/K) malignant melanoma were recently reported and showed a significant increase in progression free survival and overall response rate with trametinib compared to chemotherapy (4.8 vs. 1.4 months and 24% versus 7%, respectively). These data led to the FDA approval of trametinib for the treatment of patients with BRAF(V600E/K) mutant melanoma in 2013 (Robert C 2012).

Although active in patients with BRAF mutant melanoma, the 40% response rate of trametinib is lower than the 60-80% response rates observed with the RAF inhibitors vemurafenib and dabrafenib in BRAF mutant melanoma, which are discussed below. These results have prompted some to suggest that the profound clinical activity of RAF inhibitors obviates the need for further clinical development of MEK inhibitors. However, as RAF inhibitors induce ERK pathway activity in tumors with RAS activation, including those with RAS mutation, MEK (and possibly ERK) inhibitors remain the only therapeutic option in this setting. As RAF is only one of several downstream effectors of RAS, it is not surprising that MEK inhibitors have only modest clinical activity in patients with RAS mutant tumors. The combination of a MEK inhibitor and inhibitors of other RAS pathway effectors may, however, prove to be an efficacious approach (Engelman, Chen et al. 2008, She, Halilovic et al. 2010). Finally, MEK inhibitors may also prove useful in patients who progress on RAF inhibitors or when combined as initial therapy with inhibitors of RAF in BRAF mutant tumors.

RAF kinase inhibitors

Given the high incidence of RAS and BRAF alterations in human tumors, intense efforts have been made to identify potent and selective inhibitors of the ERK pathway for use as anticancer therapies. The first RAF inhibitor to enter broad clinical testing was sorafenib (Nexavar). Sorafenib is a bi-aryl urea identified as a RAF inhibitor on the basis of preclinical studies showing that exogenous RAF constructs expressed in 3T3 cells failed to activate MEK1 when preincubated with increasing concentrations of the drug (Wilhelm, Carter et al. 2004). Sorafenib ultimately demonstrated clinical activity in renal cell carcinoma (RCC) and hepatocellular cancer and was approved for use in these indications (Clark, Eder et al. 2005). Notably, sorafenib lacked meaningful clinical activity in melanoma, the tumor type with the highest prevalence of BRAF mutations. Studies of sorafenib indicate that it lacks selectivity for RAF, and that it is a highly potent inhibitor of VEGFR2, VEGFR3 and several other kinases (Wilhelm, Carter et al. 2004). These findings together with the clinical activity of other VEGF targeted approaches such as sunitinib and bevacizumab in RCC, suggest that the activity of sorafenib in RCC is likely attributable to its anti-angiogenic properties and that inhibition of RAF contributes little if at all to its clinical efficacy in this disease.

In striking contrast to the lack of antitumor activity of sorafenib in patients with BRAF mutant melanoma, remarkable antitumor activity was recently reported with two highly selective RAF inhibitors: PLX4032 (vemurafenib; Plexxikon/Roche) and GSK2118436 (dabrafenib; GlaxoSmithKline). Vemurafenib is an ATP competitive inhibitor of RAF that binds to mutant BRAF(V600E), wild-type CRAF, and wild-type BRAF with Ki_{50} values of 31, 48, and 100 nM, respectively (Bollag, Hirth et al. 2010). In a phase 1/2 trial by Flaherty et al., vemurafenib administered orally on a twice-daily schedule had an 81 percent response rate in patients with V600E BRAF mutant melanomas (Flaherty, Puzanov et al. 2010). These results prompted the initiation of a randomized phase 3 study (BRIM3) comparing vemurafenib to dacarbazine in

previously untreated patients with metastatic melanoma (clinicaltrials.gov identifier: NCT01006980). Eligibility for BRIM3 was restricted to treatment-naïve (no prior systemic anticancer therapy) patients with Stage IIIC and IV melanoma whose tumors were positive for the V600E BRAF mutation. Results from the BRIM3 published in the New England Journal of Medicine showed that vemurafenib was associated with a significant improvement in overall and progression free survival versus dacarbazine (Chapman, Hauschild et al. 2011). Specifically, at 6 months, overall survival was 84% in the vemurafenib treated patients versus 64% in the patients treated with dacarbazine (Chapman, Hauschild et al. 2011).

The question arises as to why vemurafenib succeeded whereas sorafenib failed to demonstrate meaningful clinical activity in melanoma patients with BRAF mutations. One possibility is that vemurafenib's greater selectivity for RAF kinases versus sorafenib allows for more potent inhibition of RAF activation at tolerable doses. Alternatively, a notable property of vemurafenib is that it inhibits ERK pathway activity, as assessed by downregulation of the expression of phosphorylated MEK and ERK, in a BRAF mutant-specific manner (Joseph, Pratilas et al. 2010). In tumor cells expressing the V600E BRAF mutation, treatment with vemurafenib results in downregulation of phosphorylated MEK and ERK expression, coordinate downregulation of cyclin D1 and upregulation of p27 expression. This results in growth arrest in the G1 phase of the cell cycle, and in some cell lines, induction of cell death (Heidorn, Milagre et al. 2010, Joseph, Pratilas et al. 2010, Poulikakos, Zhang et al. 2010). In contrast, in tumors with wild-type RAF including all normal cells, vemurafenib induces a paradoxical increase in the expression of phosphorylated ERK (Hatzivassiliou, Song et al. 2010, Heidorn, Milagre et al. 2010, Poulikakos, Zhang et al. 2010). In some cellular contexts, this increase in ERK activity upon drug exposure is accompanied by an increase in cell proliferation (Halaban, Zhang et al. 2010). Vemurafenib can thus be considered BRAF-selective in regards to ERK pathway inhibition. It should be highlighted that the compound, as noted above, is not BRAF-selective in regards to RAF binding.

As vemurafenib binds to all three RAF isoforms and exhibits only modest selectivity for mutant versus wild-type BRAF, why then does vemurafenib only inhibit RAF activation in BRAF mutant cells? The answer lies in the formation of RAF homo- and heterodimers in BRAF wild-type cells, a process regulated by active RAS (Hatzivassiliou, Song et al. 2010, Heidorn, Milagre et al. 2010, Poulikakos, Zhang et al. 2010). In BRAF wild-type tumor and normal cells, vemurafenib induces ERK signaling by transactivating RAF dimers (Figure 2) (Poulikakos, Zhang et al. 2010). At low concentrations of drug, vemurafenib binds to one protomer within a RAF dimer resulting in transactivation of the other non-drug bound RAF protomer (Poulikakos, Zhang et al. 2010). At higher concentrations, vemurafenib binds to both protomers within such dimers thus inhibiting RAF transactivation and subsequent ERK activation. Biopsies performed as part of the vemurafenib Phase 1 trial suggest that the vemurafenib concentrations necessary to inhibit RAF dimers likely exceed the drug levels achievable at a non-toxic dose (Flaherty, Puzanov et al. 2010). Therefore, in patients, vemurafenib treatment induces RAF and ERK activation in normal tissues and in BRAF wild-type tumor cells. As a result of upstream negative feedback, RAS activity is low in V600E BRAF mutant cells (Poulikakos, Zhang et al. 2010). In such cells, RAS activity and RAF dimer formation are low, and BRAF signals as a monomer. In this context, RAF inhibitors potently suppress BRAF kinase activity and, subsequently, ERK signaling.

Vemurafenib's mutant-selective inhibition of the ERK pathway in BRAF mutant cells is likely the basis for its profound clinical activity and broad therapeutic index. One unknown is whether novel RAF inhibitors capable of inhibiting RAF dimers at non-toxic concentrations can be identified or whether such compounds would suffer from a potentially deleterious paradoxical activation of RAF at low concentrations. If so, such agents could be more efficacious than inhibitors of MEK or ERK as they would have the potential to inhibit non-MEK effectors of

RAF, presuming that such pathways play an important role in RAF-dependent transformation. One implication of the RAF dimerization model is that it suggests that RAS activation through a diversity of mechanisms, including RAS mutation or upstream activation of receptor tyrosine kinases, will result in vemurafenib resistance through induction of RAF dimers, a prediction now born out in studies of vemurafenib-resistance, which are described below.

The mutant-specific effects of vemurafenib on ERK pathway activity also likely accounts for this agent's unique toxicity profile. A common toxicity associated with the use of EGFR and MEK inhibitors is an acneiform skin rash presumed to be the result of ERK pathway inhibition in normal skin (LoRusso, Krishnamurthi et al. 2010). Treatment with vemurafenib on the other hand results in the development of a maculopapular, keratosis pilaris-like skin rash, which is distinct from the rash associated with the use of EGFR and MEK inhibitors. Vemurafenib treatment is also associated with the development of keratoacanthomas and squamous cell carcinomas, the latter toxicity observed in 31% of patients on the Phase 1 trial (Flaherty, Puzanov et al. 2010). It is presumed that these latter adverse effects are the result of ERK pathway activation in normal skin.

Analogous to the promising activity reported with vemurafenib, the RAF inhibitor GSK2118436 (dabrafenib; GlaxoSmithKlein) demonstrated a 63% response rate in a completed Phase I trial (R. Kefford 2010). In contrast to the vemurafenib Phase 1 trial, patients with active brain metastases were eligible for the dabrafenib study, and regression of brain metastases was documented in several patients (R. Kefford 2010). This finding is notable as the development of brain metastases is common and responsible for significant morbidity in patients with advanced melanoma. Finally, dabrafenib showed efficacy in a small number of patients with the V600K/G BRAF alleles but was inactive in patients with the K601E mutation (R. Kefford 2010). These data imply

that a subset of BRAF mutant alleles may demonstrate intrinsic resistance to vemurafenib and dabrafenib.

Mechanisms of RAF and MEK inhibitor resistance

As previously discussed, highly selective inhibitors of RAF and MEK have promising clinical activity in melanoma patients whose tumors express V600E BRAF. However, the rapid onset of drug resistance, similar to the pattern with selective inhibitors of ABL and EGFR signaling, has tempered initial enthusiasm for vemurafenib and dabrafenib. Understanding the mechanisms responsible for clinical progression in patients is critical, as such insights can inform the development of more effective ERK pathway inhibitors or serve as the basis for combination regimens that delay or prevent the onset of resistance. Recently, several groups have reported laboratory and tumor-based studies suggesting possible mechanisms of resistance to RAF and MEK inhibitors. The mechanisms of drug resistance elucidated in these studies can be divided into two general themes: 1) alterations that restore ERK pathway activity despite continued drug treatment and 2) alterations that bypass the requirement for RAF pathway activation ("oncogenic bypass") (Solit and Sawyers 2010).

In regards to the first class, Whittaker et al. have shown using cell culture systems that insertion of a "gatekeeper" mutation analogous to the T315I and T790M mutations in ABL and EGFR respectively can induce RAF inhibitor resistance in BRAF mutant cell lines (Whittaker, Kirk et al. 2010). Although this study confirms that a gatekeeper mutation in BRAF is capable of conferring RAF inhibitor resistance, analysis of tumor samples derived from patients with acquired resistance to vemurafenib has not to date uncovered evidence that secondary mutations in BRAF are responsible for resistance to vemurafenib in melanoma patients (Nazarian, Shi et al. 2010). Nazarian et al. have identified mutations in the NRAS gene in two vemurafenib-resistant tumors (Nazarian, Shi et al. 2010). This latter finding is not surprising as RAS activation was

predicted in preclinical studies to result in vemurafenib-resistance through induction of RAF dimers as discussed previously (Poulikakos, Zhang et al. 2010). Using massively parallel sequencing, Wagle et al. have also identified a downstream mutation in MEK1 (C121S) as the mechanistic basis for treatment failure in a patient with acquired resistance to vemurafenib (Wagle, Emery et al. 2011). Using an ORF (open reading frame) kinase screen, Johannessen et al. identified RAF1, which had been identified earlier as a mechanism of resistance to the RAF inhibitor AZ628 (Montagut, Sharma et al. 2008) and COT/Tpl2, a MAP kinase kinase, as potential mediators of RAF inhibitor resistance (Johannessen, Boehm et al. 2010). Additionally, by culturing sensitive BRAF(V600E) melanoma cells in the presence of vemurafenib over time, Poulikakos et al. identified a 61 kDa splice variant of BRAF that lacked the RAS binding domain and therefore was able to dimerize independently of RAS activity and activate the MAPK pathway in the presence of vemurafenib (Poulikakos, Persaud et al. 2011). In each of the above scenarios (NRAS and MEK1 mutation, RAF1 and COT overexpression), RAF inhibitor resistance was accompanied by failure of the drug to inhibit ERK pathway activity.

Activation of the RTKs PDGFRß and IGF-1R has also been proposed as mechanisms of acquired resistance to RAF inhibitors (Nazarian, Shi et al. 2010, Villanueva, Vultur et al. 2010). Activation of RTKs upstream of RAF is predicted to confer resistance by one of two mechanisms. RTK activation can "bypass" the need for RAF/ERK activation by activating parallel signaling pathways that redundantly regulate downstream mediators of transformation such as cyclin D1, BAD and 4E-BP-1 (She, Halilovic et al. 2010). Further, by activating RAS, RTK activation may attenuate the ability of vemurafenib to inhibit ERK pathway activation by promoting the formation of RAF dimers. In cells with PDGFRß activation, Nazarian et al. did not observe significant reactivation of the ERK pathway and thus they hypothesized that resistance was the result of MEK-independent survival pathway activation (Nazarian, Shi et al. 2010). As Bollag and colleagues have shown that induction of tumor regression by vemurafenib required almost

complete suppression of ERK signaling, even partial restoration of ERK pathway activation in the setting of RTK activation may, however, have contributed to drug resistance (Bollag, Hirth et al. 2010). Straussman et al. have also demonstrated that the tumor microenvironment can play a role in resistance to RAF inhibition, as stromal-produced HGF was shown to reactivate the MAPK pathway through MET activation in vemurafenib resistant cell line/stromal co-cultures (Straussman, Morikawa et al. 2012). Finally, it should be highlighted that very few clinical samples have been analyzed for each of the mechanisms of drug resistance proposed above and therefore additional studies are needed to determine the relative frequency of these events in patients.

One motivation for studying drug resistance mechanisms is that they may suggest combination regimens that delay or prevent its onset. For example, resistance resulting from PDGFRß, IGF-1R or COT activation may be reversed by inhibitors of these kinases. A consistent theme in studies of RAF inhibitor resistance is reactivation of ERK signaling as a result of alterations that promote the formation of RAF dimers. In such cases, MEK or ERK inhibitors may prove useful. Furthermore, combining a RAF and a MEK inhibitor upfront may be a rational strategy as the addition of a MEK inhibitor may attenuate the adverse effects of the RAF inhibitor while simultaneously increasing the magnitude or durability of the ensuing response. This result would be predicted as RAF and MEK inhibitors both downregulate ERK activation in BRAF mutant tumor cells whereas they have antagonistic effects on ERK activation in normal tissues (Joseph, Pratilas et al. 2010).

Differences among tumors regarding the cooperative genetic/epigenetic changes that co-occur with BRAF may also explain the variable degree of tumor regression observed in patients treated with RAF and MEK inhibitors and may account for the small number of patients with BRAF mutant tumors who derive no clinical benefit from vemurafenib and dabrafenib. For example,

loss of PTEN with accompanying AKT activation is common in melanomas with BRAF mutation, and thus the combination of a RAF (or MEK) and a PI3 kinase/AKT inhibitor may prove to be beneficial in this genetically defined subset (Gopal, Deng et al. 2010).

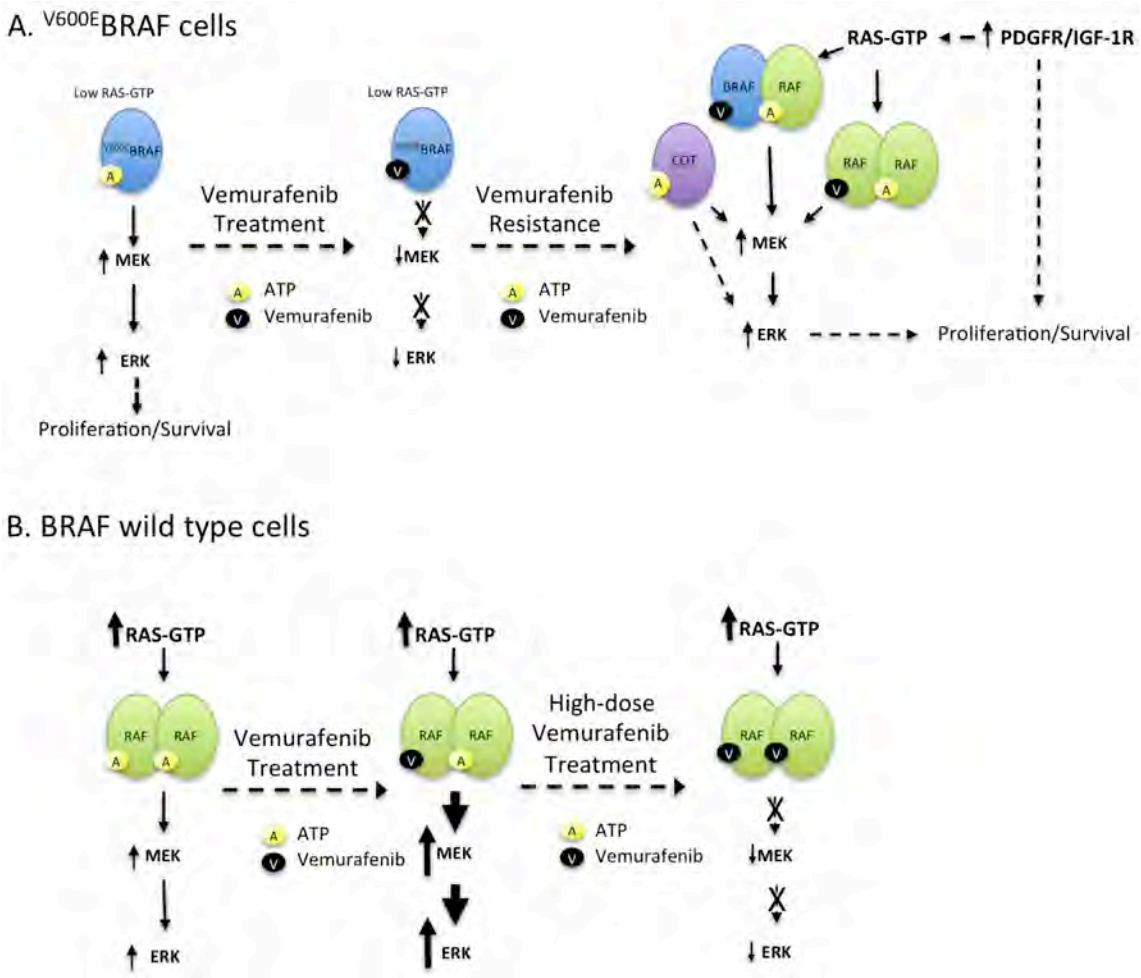

Figure 1.2 Model of vemurafenib resistance. A) In V600E BRAF mutant, vemurafenib-sensitive melanomas, RAS activity is low and RAF dimerization is not required for BRAF activation. In such cells, vemurafenib binds to and inhibits BRAF monomers and thus MEK/ERK activity. RAF inhibitor resistance can result from perturbations that increase the formation of RAF dimers (RAS mutaiton, RAF1 overexpression) or that bypass the requirement for BRAF activation (COT or RTK activation). **B)** In BRAF wild-type tumors and normal cells, RAS activation promotes the formation of RAF homo- and heterodimers. In such cells, binding of drug to RAF results in transactivation of the non-drug bound protomer. This results in a paradoxical hyperactivation of MEK and ERK by the RAF "inhibitor". At higher concentrations of drug that are likely above the maximally tolerated dose of the inhibitor, both protomers with the RAF dimer become drug bound leading to inhibition of RAF activation and thus MEK and ERK activation. (Reproduced from Nissan, M. H. and D. B. Solit (2011). "The "SWOT" of BRAF inhibition in melanoma: RAF inhibitors, MEK inhibitors or both?" Curr Oncol Rep 13(6): 479-487 with permission from Springer)

NF1

Neurofibromatosis Type 1

Neurofibromatosis Type I, or von Recklinghausen's disease, was originally characterized by the German pathologist Freidrich Daniel von Recklinghausen in an 1882 publication entitled "*Über die multiplen Fibrome der Haut und ihre Beziehung zu den multiplen Neuromen*" or "*About multiple fibromas of the skin and its relation to multiple neuromas*". In this work he described the tumors of NF1 as neurofibromas containing interspersed nerve and fibrous tissue and summarized the literature on the subject that had been published. Many publications following that of von Recklinghausen described case studies of patients with the disease, such as one whose "tumours, which were countless, were distributed all over the body and were of two kinds, some being soft, almost fluctuating, subcutaneous masses, others being firmer, projecting from the surface of the skin, and giving a gelatinous sensation to the touch; there were none of the so-called 'seedless raisin' type which have been observed in this disease" (Morris and Fox 1908). These numerous case reports helped shape the clinical understanding of the disease.

NF1 is an autosomal dominant genetic disorder that affects 1:3500 live births. NF1 is commonly characterized by development of benign neurofibromas that develop on the skin and the support cell of the nerves. Pigmented spots on the skin known as café-au-lait spots; plexiform neurofibromas; pigmented nodules in the eye known as Lisch nodules; inguinal and axial freckling; and learning disabilities are all common hallmarks of the disease. Though these symptoms are by no means dismissive, the most serious complication of NF1 is predisposition to cancers such as optic glioma, juvenile myelomonocytic leukemia, gastrointestinal stromal tumors, and malignant peripheral nerve sheath tumors. The clinical symptoms of NF1 tend to affect tissues derived from the neural crest of the embryo, implicating neurofibromatosis as a neurocristopathy, or disease of the neural crest (Bolande 1981). Severity and range of the

symptoms exhibited by patients can vary between family members with the same mutations, and the cause of this variance is not completely understood (Huson, Compston et al. 1989). However, these symptoms can worsen after puberty and pregnancy, suggesting hormonal, or, at least, microenvironmental contribution to disease phenotype (Viskochil, White et al. 1993).

Through the early 20th century, doctors and researchers noticed that NF1 tended to run in families (Barber 1928, Garland 1941, Frank 1947), but the gene responsible for the disease was not identified until the 1980s. Linkage analysis using restriction length polymorphisms and multiple pre-determined markers of families with neurofibromatosis revealed linkage of NF1 with markers around the centromeric region of chromosome 17, which mapped the candidate gene to the long arm of that region (Barker, Wright et al. 1987, Seizinger, Rouleau et al. 1987). However, the large number of genes in the proposed region prevented immediate identification of the candidate gene. Emergence of several NF1 patients with translocations involving the long arm of chromosome 17 helped map the candidate gene's precise location (Cawthon, Weiss et al. 1990, Wallace, Marchuk et al. 1990). The product of this gene was later identified and characterized (Gutmann, Wood et al. 1991, Marchuk, Saulino et al. 1991), thus beginning the molecular study of neurofibromatosis.

Neurofibromin 1

The 250 kDa Neurofibromin 1 protein (or NF1) is encoded by the *NF1* locus on chromosome 17q11.2. The gene is comprised of 350 kilobases of DNA, translating to 2818 amino acids and 60 exons, of which one (23a) can be alternatively spliced. The NF1 gene has a region of sequence similarity to the yeast GAP IRA1 gene, and interacts with and stimulates the GTPase activity of p21 RAS (Martin, Viskochil et al. 1990). Thus, NF1 is a RAS GTPase Activating Protein (GAP) responsible for converting active, GTP-bound RAS to inactive, GDP-bound RAS.

The NF1 transcript is regulated on multiple levels. NF1 mRNA can undergo base-modification editing by ApoB Editing Catalytic Subunit 1 (APOBEC1), which edits the cytidine in an arginine codon at position 2914 to a uridine, creating a stop codon (Skuse, Cappione et al. 1996). Editing of NF1 mRNA was found to be ~8.5 fold higher in tumor tissue of neurofibromatosis patients compared to normal blood leukocytes suggesting a possible mechanism for loss of heterozygosity contributing to malignancy in this setting (Skuse, Cappione et al. 1996). NF1 mRNA can also undergo alternative splicing to exclude exon 23a from the GAP related domain, which is thought to improve RAS-GAP activity roughly 10-fold (Barron and Lou 2012). The translated NF1 protein can be ubiquitinated and targeted for degradation via the proteasome by a Cul3/KBTBD7 complex (Hollstein and Cichowski 2013). Proteasomal degradation is one mechanism of NF1 inactivation contributing to loss of this tumor suppressor in gliomas (McGillicuddy, Fromm et al. 2009) and it is likely that targeted proteasomal degradation occurs in other cancers and contexts. Additionally, there is evidence for mRNA degradation possibly via nonsense mediated decay as a mechanism of NF1 protein loss, as tumors with NF1 point mutations often have significantly lower levels of NF1 mRNA than NF1 wild-type tumors (melanoma TCGA; cbio.mskcc.org).

The protein contains several functional domains. Its catalytic GAP-related domain (GRD) is a conserved domain found in other GTPase activating proteins such as P120RasGAP and the yeast GAPs IRA1 and IRA2. Its leucine-rich domain can be separated into an N-terminal sec14-like domain and a C-terminal pleckstrin homology-like domain, which have been implicated in glycerophospholipid binding (D'Angelo, Welti et al. 2006, Welti, Fraterman et al. 2007). These latter domains are also important for membrane localization and direct or indirect activation of adenylate cyclase (Tong, Hannan et al. 2002). Mutations found in *NF1* are diffuse throughout the whole gene, and do not localize to hotspots. This may be a reflection of the gene's size, but may also suggest that alteration of protein function can occur from changes in more than just the catalytic domain.

The catalytic activity of NF1 lies in the arginine residue at position 1276 of the GRD. This catalytic arginine resides on the arginine finger loop, which is capable of inserting into the ATP binding site of RAS and stabilizing the ATP to ADP transition state by mediating charge buildup on the leaving-group oxygen, in this case the γ-phosphate (Scheffzek, Ahmadian et al. 1997). This stabilization increases the rate-limiting GTPase reaction rate 1000-fold (Ahmadian, Hoffmann et al. 1997, Ahmadian, Stege et al. 1997). However, NF1 also facilitates the GTPase reaction in part by stabilizing the switch II region of RAS upon binding.

NF1 is one of several RAS-GAPs in the cell. The other widely studied RAS-GAP is p120RasGAP, a GTPase activating protein that contains a GRD as well as SH2, SH3 and pleckstrin homology (lipid binding) domains. Many studies have tried to elucidate the overlapping and non-overlapping roles of NF1 and p120RasGAP. The unique detergent sensitivities of the two proteins have facilitated their isolation, characterization and study by allowing selective inhibition of each protein individually (Bollag and McCormick 1991). The most striking difference between the proteins lies in their interaction with RAS, namely in the kinetics and thermodynamics of their interaction (Scheffzek, Ahmadian et al. 1998). NF1 has a high affinity for GTP-bound RAS, which is 50-100 fold higher than that of p120RasGAP. It also has a slower dissociation rate than p120RasGAP. Crystallographic studies of the catalytic domains of NF1 (NF1-GRD) and p120RasGAP (GAP-334) reveal an overall increased flexibility in NF1 (Scheffzek, Ahmadian et al. 1998). Amino acid differences between NF1-GRD and GAP-338, such as a glycine adjacent to the catalytic arginine in NF1, provide flexibility to the arginine finger loop, likely accounting for some of the observed differences in flexibility. These differences could account for the slower binding rate and tighter complex formation between NF1 and RAS compared to GAP-338 and RAS. Besides the kinetic and thermodynamic evidence that NF1 is the preferred RAS-GAP, there is genetic evidence to support this claim. P120RasGAP

mutations and deletions are not commonly found in cancer, while NF1 alterations result in activation of RAS and are found in many cancers. Additionally, p120RasGAP cannot rescue NF1 deficiency, as was seen with NF1-deficient cells transfected with either NF1-GRD, p120GAP-GRD or full-length p120RasGAP. Only the NF1-GRD was able to rescue the hyper-responsivity and hyperproliferation of $NF1^{-/-}$ myeloid cells to GM-CSF (Hiatt, Ingram et al. 2001). Additionally, only the NF1-GRD was able to rescue the hyperproliferation of $NF1^{-/-}$ mouse embryonic fibroblasts through decreased RAS and ERK activation (Hiatt, Ingram et al. 2001). Together, these data suggest that although NF1 is not the only RAS-GAP in the cell, because its loss cannot be rescued by other GAPs, it is the predominant GAP used by RAS.

NF1 loss and cancer

As has been established, NF1 is a crucial regulator of RAS activation, and aberrant RAS activation can have significant consequences on activation of downstream pathways such as the MAPK pathway. Due to the propensity for neurofibromatosis patients to develop cancers, making it a familial cancer syndrome, and the RAS activation that results from NF1 loss, NF1 is widely accepted to be a tumor suppressor. Loss of heterozygosity is found in NF1 patients who progress to pheochromocytomas, leukemias and malignant peripheral nerve sheath tumors (Xu, Mulligan et al. 1992, Legius, Marchuk et al. 1993, Shannon, O'Connell et al. 1994, Cichowski and Jacks 2001). Additionally alterations in *TP53* or deletion of *CDKN2A* commonly co-occur in the invasive tumors of NF1 patients (Brems, Beert et al. 2009). It is therefore not surprising that somatic NF1 loss is implicated in a growing number of cancers. The 2008 glioblastoma project of the Cancer Genome Atlas (TCGA) identified NF1 alterations in 23% of glioma samples tested (mutations, deletions, loss of expression; n=206), with the majority of these alterations predicted to be inactivating (The Cancer Genome Atlas 2008), while NF1 mutations were also identified by the breast project of TCGA, though in fewer samples than in brain (roughly 3%) (The Cancer

Genome Atlas 2012). In addition to these studies, somatic NF1 inactivating events have been found in leukemias and lung cancers (Side, Emanuel et al. 1998, Ding, Getz et al. 2008), and NF1 alterations will likely be identified as important somatic mutational events in other cancer types as methods of detection and deep sequencing platforms continue to improve.

Scope of thesis

We investigated a panel of melanoma cell lines wild-type for BRAF and NRAS to determine the genetic alteration driving their transformation and their dependence on ERK signaling in order to elucidate a candidate set of occult MAPK alterations that may predict for sensitivity to MEK inhibitor treatment. From 191 melanoma cell lines, we collected a set of 66 $BRAF^{WT}/NRAS^{WT}$ cell lines for our study. We screened these cell lines for functional alterations that activated RAS, and found a subset of cell lines with elevated RAS-GTP. NF1 negatively regulates RAS and is found somatically altered in a growing number of cancers. We examined the NF1 status of these cell lines and identified a cohort of 6 cell lines with high RAS-GTP and loss of NF1 protein expression. Deep sequencing via the IMPACT assay revealed a genomic mechanism for NF1 loss in all of the NF1-null cell lines. Data from the melanoma Cancer Genome Atlas (TCGA) revealed alteration of NF1 via missense mutation, nonsense mutation or deletion in 14% of melanoma tumors, suggesting NF1 loss occurs in a subset of human melanomas and was not an artifact of cell line generation. Though NF1-null cells derived from other lineages have been shown to be TORC1-dependent by others, the NF1-null melanoma cell lines were not dependent on TORC1 signaling but rather on the MAPK pathway for proliferation and cell cycle progression. Suppression of ERK activation by the MEK inhibitors PD0325901, AZD6244 and MEK162 was transient in NF1-null melanoma cell lines, but enhanced sensitivity was observed with trametinib, a compound that inhibits MEK phosphorylation by RAF. NF1 loss in the context of BRAF(V600E) mutation was sufficient to increase RAS-GTP levels and confer resistance to the

RAF inhibitor vemurafenib. However, these cells remained sensitive to the MEK inhibitor trametinib, suggesting a potential therapeutic strategy for patients with NF1 loss and BRAF(V600E) mutation. We questioned whether NF1 loss was sufficient to induce invasive melanoma and modeled somatic NF1 loss in melanocytes using an $Nf1^{flox/flox}$; Tyr::CreER mouse model. Nf1 loss as a single alteration was not sufficient to induce melanoma formation, but resulted in hyperpigmentation and mild hyperproliferation of the melanocytes. In summary, NF1 loss is common in cutaneous melanoma and is associated with RAS activation, MEK dependence, and RAF inhibitor resistance.

Chapter Two

Materials and Methods

Cell lines and culture conditions

Cell lines with the prefix "SK-Mel" were generously provided by the laboratory of Jedd Wolchok, and previously Alan Houghton, as well as the Ludwig Collaborative Laboratory under the leadership of Taha Merghoub (MSKCC). MeWo, Malme3M, A375 and SNF96.2 were purchased from the American Type Culture Collection. M308 was a generous gift provided by Antoni Ribas (Jonsson Comprehensive Cancer Center, UCLA, Los Angeles, CA). WM3918 was a generous gift from Katherine Nathanson and Meenhard Herlyn (The Wistar Institute, Philadelphia, PA). All media was purchased pre-sterilized through the Media Core Facility (MSKCC). SNF96.2 was grown in Dulbecco's modified Eagle medium with high glucose (DME HG). A375 was grown in Dulbecco's modified Eagle medium without additional glucose (DME). All remaining cell lines in this study were grown in RPMI 1640 supplemented with 2nM glutamine, 50 units/mL each of penicillin and streptomycin purchased from Gemini Bio Products. SK-Mel cell lines numbered over 300 were grown in 20% fetal bovine serum (FBS) and changed to 10% FBS 24 hours prior to experimentation, while all other cell lines were grown at 10% FBS. FBS was purchased from PAA Laboratories. FBS and pen-strep were filtered through Steritop Filters (Millipore) upon addition to media. All cells were maintained at 37° C in 5% CO_2. PD0325901 was synthesized by the Organic Synthesis Core Facility (MSKCC); Vemurafenib (PLX4032) was obtained from Plexxikon (now Roche); MEK162 was generously provided by Stand Up to Cancer (SU2C); Rapamycin, trametinib (GSK1120212) and AZD6244 were purchased from Selleckchem.com.

Genomic studies

Cellular DNA was extracted from cell pellets using the Qiagen DNAeasy Tissue Kit; concentration (optical density; OD) and purity were measured via NanoDrop 2000 (Thermo Scientific). DNA from all cell lines was analyzed using a mass spectrometry-based fingerprinting assay to validate cell line identity as described previously (Janakiraman, Vakiani et al. 2010). Mutations in NRAS (G12A, G12D, Q61K, Q61R, Q61L), BRAF (V600E, V600K, V600R, K601E), and c-KIT (D816V) were screened for using a mass spectrometry-based assay (Sequenom) performed by the Geoffrey Beene Translational Core Facility (MSKCC) with results validated by Sanger sequencing (Janakiraman, Vakiani et al. 2010). Agilent Comparative Genomic Hybridization was performed by the Genomic Sequencing Core (MSKCC); DNA was hybridized to Agilent 244K or 1M CGH microarrays and compared to pooled control human DNA as has been previously described (Janakiraman, Vakiani et al. 2010).

RNA sequencing (done in collaboration with R. Ramirez)

RNA was extracted from cell pellets using the RNeasy mini kit (Qiagen, Inc.) following the manufacturer's instructions. Quality assessment, poly-A selection, and sequencing with an Illumina HiSeq 2000 were performed by the Genomics Core Laboratory (MSKCC). All samples had a minimum RNA integrity number (RIN) of 7.0 (Schroeder, Mueller et al. 2006). Sequencing produced 40 to 120 million 75bp reads per sample. FASTQ files were produced by the Bioinformatics Core (MSKCC) using the CASAVA 1.8.2 software (Illumina). Low quality bases and adapter sequences were removed with cutadapt. Trimmed reads were aligned to human genome assembly GRCh37 using Tophat 2.0.8 (Flicek, Aken et al. 2010, Kim, Pertea et al. 2013). Gene level quantification and differential expression were calculated using Cufflinks 2.1.1 (Trapnell, Williams et al. 2010). Data visualizations were created with tools from the gplots package for R.

IMPACT

Genomic alterations were profiled in 279 key cancer-associated genes using the IMPACT (Integrated Mutation Profiling of Actionable Cancer Targets) assay in partnership with Michael F. Berger (Dept of Pathology/Human Oncology and Pathogenesis Program, MSKCC) and the Genomics Core Facility (MSKCC). IMPACT utilizes solution phase hybridization-based exon capture and massively parallel DNA sequencing and has been previously described (Iyer, Hanrahan et al. 2012, Wagle, Berger et al. 2012).

Western Blotting

Cells were washed with PBS, scraped and lysed in 1% NP-40 lysis buffer [50 mmol/L Tris (pH 7.4), 1% NP40, 150 mmol/L NaCl, 40 mmol/L NaF, 1 mmol/L, Na_3VO_4, 1 mmol/L phenylmethylsulfonylfluoride, and 10 μg/mL each of leupeptin, aprotinin, and soybean trypsin inhibitor] at 4°C for 1 hour. Protein concentration was measured using the bicinchoninic acid protein assay (Pierce) against a BSA standard curve on a SpectraMAX 190 spectrophotometer (Molecular Devices) using SoftMax Pro5 software for analysis. Samples were resolved using SDS-PAGE run at 100V for 1.5 hours at 25°C and transferred to nitrocellulose (Whatman) at 100V for 1 hour at 4°C. Membranes were blocked in 5% non-fat milk in TBST [0.1% Tween 20, TBS, 10 mmol/L Tris (pH 7.4) and 150 mmol/L NaCl] for 1 hour at 25°C and incubated in primary antibody (1:1000 unless otherwise specified) overnight at 4°C. After 10 minute triple washes with TBST, membranes were incubated with secondary anti-mouse (Amersham), anti-rabbit (Amersham) or anti-goat (Santa Crux Biotechnology) antibodies for 1 hour at 25°C and then triple washed (10 minutes x3) with TBST before developing. Secondary antibodies were detected using Super Signal (Thermo), imaged using a Fuji LAS-4000 (GE Lifesciences) and images cropped and assembled with Adobe Photoshop. Primary antibodies and working concentrations were: Anti-NF1 (1:1000, SC-67), cyclin D1 (1:1000, SC-718), KRAS (1:500, SC-

30), NRAS (1:1000, SC-519), HRAS (1:500, SC-520), actinin (1:1000, SC-17829), SPRY1 (1:200, SC-30048), SPRY2 (1:200, SC-18601), SPRY4 (1:200, SC-30051) all from Santa Cruz Biotechnology. Anti-Ras (1:10,000, #1862335), Thermo Scientific. Anti-pERK (1:1000, #9101), ERK (1:1000, #9102), pMEK (1:1000, #9121), MEK (1:1000, #9122), p-CRAF S338 (1:1000, #9427), DUSP6 (1:1000, #3058), α-tubulin (1:100, #2144) all from Cell Signaling Technology.

Proliferation assays

Cell viability was measured by trypan blue incorporation using a Vi-CELL XR 2.03 (Beckman Coulter). Cells were plated in triplicate at a density of 0.05-0.25 million cells per well in a 6-well plate and allowed to adhere overnight (~18-24h) before either treatment or harvesting the next day. Cells were treated with increasing concentrations of drug (0-500 nM for PD0325901 and GSK1120212; 0-100 nM for Rapamycin; 0-5000 nM for PLX4032;) and counted at days 0 (no drug), 3, and 5. Percent growth = 100*([Day 5 drug]-[Day 0])/([Day 5 DMSO]-[Day 0]). All data graphed and analyzed using Prism 6 software.

FACS analysis

FACS analysis was performed on adherent and floating cells 24 hours after drug treatment unless otherwise indicated using DMSO as a control. Nuclei were collected and stained with ethidium bromide as previously described (Nusse, Beisker et al. 1990). Flow cytometry was performed by the Flow Cytometry Core Facility (MSKCC). Error bars represent S.E.M. from experiments in triplicate. All data graphed and analyzed using Prism 6 software.

RasGTP assays

Active, GTP-bound Ras was isolated via immunoprecipitation using a recombinant Ras binding domain of Raf1 (GST-RAF1-RBD), according to the manufacturer's instructions (Thermo). 500 µg of total cellular protein in 500 µL lysis buffer was precipitated with GST-linked Raf1-RBD and a Glutathione resin in a spin column. After washing, protein was eluted off the resin with 2x reducing sample buffer and separated using SDS-PAGE (15% acrylamide gel, BioRad). The product was probed using total or isoform specific (H-, K-, N-) RAS antibodies as described in "**Western blotting**".

siRNA studies

siRNA studies were accomplished using ON-TARGET plus siNF1 SMARTpool (L-003916) and ON-TARGET plus non-targeting siRNA#2 (D-001810-02) (Thermo Scientific). Cells were transfected with Dharmacon transfection reagent #1 (Dharmacon, Thermo Scientific) in a mixture of Opti-Mem media (Life Technologies) and cell line-specific penicillin/streptomycin-free growth media (i.e. RPMI 1640, DME with 10%FBS) as specified under "**cell lines and culture conditions**". For growth curves and time courses, cell lines were allowed to incubate in transfection suspension for 24 hours prior to addition of drug. In the case of static knockdown, media was changed to complete cell line-specific media (10% FBS, +penicillin/streptomycin) 24 hours after transfection and cells were collected 48 hours after given fresh media (72 hours after transfection).

shRNA studies

shRNA studies were accomplished using GIPZ shNon-Targeting RNA or TRIPZ Inducible shRNA against NF1 (Thermo Scientific) as per manufacturers instructions; shNF1 #2 is CloneID V2THS-260806, shNF1 #4 is CloneID V3THS-380114, shRNA#6 is CloneID V3THS-380110.

Briefly, HEK293T cells were transfected with targeting or non-targeting shRNA vectors and lentiviral packaging mix using calcium chloride ($CaCl_2$) (Thermo Scientific). Collected virus was used to infect target cells (A375). Selection with 0.5 μg/mL puromycin was started 48 hours after transfection and continued for 1 week. shRNAs were induced with 2ug/mL doxycycline daily for 1 week prior to vemurafenib studies.

Mouse models

Nf1$^{flox/flox}$ C57/B6 mice were originally generated by Zhu et al. (Zhu, Romero et al. 2001) and purchased from the Mouse Models of Human Cancer Consortium (MMHCC). Briefly, these mice contain loxP sites flanking exons 31 and 32 of the *Nf1* gene, which are required for Nf1 function in both mice and humans (Brannan, Perkins et al. 1994). Upon cre-mediated recombination, these exons are removed resulting in a null protein. *Tp53*$^{flox/flox}$ mice were generated by Jonkers et al. (Jonkers, Meuwissen et al. 2001). Briefly, these mice contain loxP sites in exons 1 and 10 of the *Tp53* gene such that cre-mediated recombination deletes virtually all coding exons of *Tp53* leaving no functional polypeptide. *Tyr::CreERT2* (referred to as *Tyr::CreER* mice in text) mice were originally generated by Bosenberg et al. (Bosenberg, Muthusamy et al. 2006). Briefly, the *cre* gene is fused to a modified ligand-binding domain of the estrogen receptor (CreERT2) and under the control of the melanocyte-specific tyrosinase promotor (Tyr). Modifications of the ER ligand binding domain (T2) render the domain insensitive to estrogen but exquisitely sensitive to metabolized 4-hydroxytamoxifen (4-HT), such that the fused creERT2 protein is expressed only in melanocytes, localizes to the cytosol, and enters the nucleus only in the presence of 4-HT.

Nf1$^{flox/flox}$*; Tyr:CreER* and *Tp53*$^{flox/flox}$ mice were bred until homologous recombination of chromosome 11 occurred, resulting in a mouse with *Nf1* and *Tp53* floxed alleles on the same chromosome as detected by PCR. Mice carrying this "recombined chromosome" were then bred

until *Nf1*$^{flox/flox}$*; Tp53*$^{flox/flox}$*; Tyr::CreER* mice were born.

Animals were anesthetized with isoforane for administration of topical 4-HT; 2 uL of 2 mg/mL 4-HT dissolved in DMSO was administered to the ear once daily for four consecutive days (QDx4). Animals recovered from anesthesia under direct observation. For administration of tamoxifen via intraperitoneal injection (IP), animals were manually restrained and were not administered anesthesia; 0.1 mL of 40 mg/mL tamoxifen resuspended in sunflower seed oil and sonicated for 15 minutes prior to use was injected into the right peritoneal area once daily for four consecutive days. Animals treated with 4-HT were examined semi-weekly for palpable masses as well as behavioral changes (hunching, changes in grooming habits, lethargy) and photographs of animals were obtained bi-weekly. Mice with suspected tumors were sacrificed and submitted to the animal pathology core facility (MSKCC) for necropsy. All necropsy slides were analyzed by trained pathologists from the animal pathology core facility (MSKCC). Animal care was in strict compliance with guidelines established by Memorial Sloan-Kettering Cancer Center, the Guide for the Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee.

Chapter Three

NF1 loss is a functional genomic event in a subset of melanoma

The MAPK (mitogen-activated protein kinase) signaling pathway is a critical regulator of cell growth and cell cycle progression. Receptor tyrosine kinases (RTKs), activated by extracellular mitogens, stimulate the RAS (H-, N- and KRAS) small GTPase proteins. RAS proteins are activated when guanine exchange factors facilitate binding to GTP and inactivated by GTPase proteins, such as NF1, that facilitate the hydrolysis of GTP to GDP (Downward 1996). Upon activation, RAS facilitates dimerization (Weber, Slupsky et al. 2001) and activation of RAF (A- B- and CRAF/RAF1) kinases, which in turn activate the MEK1/2 (mitogen-activated protein kinase kinase) and ERK1/2 (extracellular signaling-regulated kinase) kinases. Activated, phosphorylated ERK regulates the transcription of proteins such as cyclin D1 that promote cell cycle progression, transcription factors that promote the transformed phenotype and a network of genes that negatively inhibit pathway output by regulating the activity of RTKs, RAS, and RAF (Pratilas, Taylor et al. 2009).

Alterations resulting in aberrant activation of ERK can be found at almost every level of the MAPK pathway and are common in human tumors (Bamford, Dawson et al. 2004). BRAF mutations, particularly at codon 600, are found in several cancers, including 50% of cutaneous melanomas (Davies, Bignell et al. 2002). Selective inhibitors of RAF (vemurafenib, dabrafenib) have unprecedented clinical activity in patients whose melanomas harbor BRAF(V600E)

mutations, and their use results in a prolongation of progression-free and overall survival (Chapman, Hauschild et al. 2011). NRAS mutations, most commonly at codon 61, have been identified in another 15-20% of melanomas and occur in a mutually exclusive pattern with mutations in BRAF(V600E) (Brose, Volpe et al. 2002, Davies, Bignell et al. 2002, Gorden, Osman et al. 2003). Treatment options remain limited for these patients and those whose tumors are wild-type for BRAF, and the prognosis of such patients is particularly grim with a median overall survival of less than one year (Tsao, Atkins et al. 2004).

There have been extensive efforts to understand and distinguish the mutations that drive various tumors in order to identify targetable alterations. Recent technological advances have led to the development of low-cost deep sequencing methods making the in-depth study of a larger population of samples more affordable and feasible (MacConaill 2013). Berger et al. have developed a sequencing and genotyping method that utilizes exon capture followed by massively parallel sequencing of 279 actionable cancer genes (Integrated Mutational Profiling of Actionable Cancer Targets, or IMPACT) (Wagle, Berger et al. 2012). This method sequences all coding exons of the targeted cancer genes and is capable of detecting small insertions/deletions (indels) and larger copy number alterations. It can be performed using DNA derived from formalin-fixed, paraffin-embedded tissues (FFPE), samples to use on other sequencing platforms. It can also easily screen for alterations in cell lines. Thus, IMPACT may be used to screen cell lines for potential driver alterations in cancer genes.

Though there is a clear rationale for high throughput sequencing, one must be able to sift through the glut of information that these methods generate. A study by Pleasance et al. using whole genome sequencing found more than 30,000 mutations and alterations in a single melanoma cell line derived from a patient tumor (Pleasance, Cheetham et al. 2010). It is presumed that the vast majority of these mutations are passenger mutations, defined as mutations that do not provide a

selective advantage to the tumor. Distinguishing the driver and passenger alterations requires testing their functionality.

In light of this problem, we screened melanoma cell lines wild-type for BRAF and NRAS for putative functional alterations that may have resulted in RAS activation. We identified loss of the tumor suppressor protein and RAS GTPase NF1 in a subset of the RAS-active, BRAF/NRAS^{WT} cell lines. Analysis of mutations and copy number alterations in these cell lines using IMPACT revealed focal genomic deletion or nonsense mutation as a mechanism for NF1 loss in all NF1-null cell lines. Additionally, comparison of co-alterations in the NF1-null melanoma cell lines to melanoma tumors analyzed by TCGA revealed an overlapping pattern of NF1 and co-alterations in both sets. These data suggest that loss of NF1 due to inactivating mutations was likely a functional event that occurs in melanoma and that the NF1-null melanoma cell lines are appropriate models to study the biology of melanocytic NF1 loss.

Results

To identify a cohort of $BRAF^{WT}/NRAS^{WT}$ melanoma cell lines for in-depth genomic and biologic characterization, 191 melanoma cell lines were genotyped for BRAF and NRAS mutations using a mass-spectrometry-based (Sequenom) assay (Janakiraman, Vakiani et al. 2010, Xing, Persaud et al. 2012). This screen identified 66 cell lines that lacked hotspot mutations in BRAF or NRAS (Fig. 3.1A, Table 3.1). As this assay was designed to detect only the most common BRAF and NRAS mutations, we further performed pyrosequencing of BRAF exons 11 and 15 and NRAS exons 2 and 3. This analysis identified BRAF mutations not present in the Sequenom assay in two cell lines (D594G in SK-Mel-264 and N581S in SK-Mel-215; Table 3.2). Direct sequencing of KRAS and HRAS further identified activating mutations in KRAS in two and HRAS in one cell line, respectively (Table 3.2). In summary, 61 cell lines were wild-type for RAS and BRAF.

Given the high prevalence of ERK activation in melanoma (Cohen, Zavala-Pompa et al. 2002), we hypothesized that a subset of the $BRAF^{WT}/RAS^{WT}$ cohort likely harbored occult alterations within the RAS-ERK pathway that 1) cause RAS to become refractory to negative feedback and 2) confer constitutive activation of ERK. We thus measured levels of activated, GTP-bound RAS in a subset of the $BRAF^{WT}/RAS^{WT}$ cell lines as a surrogate of upstream pathway activation. RAS-GTP levels are constitutively elevated in RAS-mutant tumors and therefore insensitive to ERK-dependent feedback inhibition of receptor signaling. By contrast, BRAF(V600E) functions as a RAS-independent active monomer (Poulikakos, Zhang et al. 2010, Poulikakos and Solit 2011). Tumors with this mutation therefore typically have very high levels of ERK output, marked feedback inhibition of receptor signaling and very low levels of RAS-GTP (Lito, Pratilas et al. 2012). Similar to human tumors, KRAS- and NRAS-mutant melanoma cancer cell lines exhibit high levels of RAS-GTP whereas BRAF-mutant cell lines have low to undetectable levels of RAS-GTP (Fig. 3.1B and (Pratilas, Taylor et al. 2009, Lito, Pratilas et al. 2012)). RAS was

activated to varying levels in the $BRAF^{WT}/RAS^{WT}$ melanoma cells, with some expressing levels of activated RAS similar to those present in RAS-mutant cells (Figure 3.1B).

The *NF1* gene encodes a protein that functions as the predominant RAS GTPase activating protein (RAS-GAP), which suppresses RAS activity and reduces RAS-GTP levels by promoting endogenous RAS GTPase activity. NF1 is inactivated in diverse human cancers (2008, 2012, Boudry-Labis, Roche-Lestienne et al. 2013) and would be predicted, if lost, to cause RAS to become refractory to negative feedback. We performed western blot analysis to determine whether loss of NF1 protein expression occurred in, and was correlated with, elevated RAS-GTP levels in $BRAF^{WT}/RAS^{WT}$ cell lines. Complete loss of NF1 expression was noted in five of the $BRAF^{WT}/RAS^{WT}$ cell lines, all of which had high levels of RAS-GTP expression (Figure 3.1B, C). Having previously performed high-resolution DNA copy number profiling (array CGH) on 92 melanoma cell lines (Xing, Persaud et al. 2012), we identified a sixth NF1-null cell line that harbored homozygous *NF1* gene deletion and concurrent NRAS (Q61R) mutation (SK-Mel-103) (Figures 3.1C and 3.3C).

NRAS mutations are significantly more prevalent than other RAS mutations in melanoma even though KRAS mutations are the predominant RAS mutations identified in most other cancers (Davies, Bignell et al. 2002). To determine which RAS isoforms were activated in NF1-null melanomas, we assayed activated KRAS, HRAS, and NRAS by performing immunoprecipitation with the RAS binding domain of Raf-1 (Raf1-RBD; see methods) followed by RAS isoform-specific immunoblots. All four NF1-null cell lines examined expressed high levels of total active RAS when compared to a BRAF(V600E) control cell line (Fig. 3.2A). NRAS (Q61K) SK-Mel-30 cells expressed high levels of GTP-bound NRAS, but no detectable levels of activated KRAS, similar to the NRAS (Q61R)/NF1-null SK-Mel-103 line. GTP-bound NRAS was also highly expressed in three other NF1-null cell lines, whereas only a subset had concurrent activation of

KRAS. This included SK-Mel-217, which harbored *KRAS* gene amplification. Elevated levels of GTP-bound KRAS and NRAS were also detected in the KRAS (G12C) mutant SK-Mel-285 cell line. Levels of activated HRAS were low or undetectable in all NF1-null melanoma cell lines examined (Figure 3.2B).

Table 3.1 **Cell lines genotyped by Sequenom**

Genotype	Cell line	Sequenom BRAF	Sequenom NRAS
BRAF (other)	SK-Mel-208	G466E	WT
BRAF (other)	SK-Mel-246	G469A	WT
BRAF V600E	SK-Mel-5	V600E	WT
BRAF V600E	SK-Mel-131	V600E	WT
BRAF V600E	SK-Mel-174	V600E	WT
BRAF V600E	SK-Mel-233	V600E	WT
BRAF V600E	SK-Mel-237	V600E	WT
BRAF V600E	SK-Mel-252	V600E	WT
BRAF V600E	SK-Mel-282	V600E	WT
BRAF V600E	SK-Mel-306	V600E	WT
BRAF V600E	SK-Mel-330	V600E	WT
BRAF V600E	SK-Mel-334	V600E	WT
BRAF V600E	SK-Mel-339	V600E	WT
BRAF V600E	SK-Mel-359	V600E	WT
BRAF V600E	SK-Mel-364	V600E	WT
BRAF V600E	SK-Mel-390	V600E	WT
BRAF V600E	Sk-Mel-413-2	V600E	WT
BRAF V600E	SK-Mel-426	V600E	WT
BRAF V600E	SK-Mel-447	V600E	WT
BRAF V600E	SK-Mel-462	V600E	WT
BRAF V600E	SK-Mel-479	V600E	WT
BRAF V600E	SK-Mel-495	V600E	WT
BRAF V600E	SK-Mel-498	V600E	WT
BRAF V600E	SK-Mel-506	V600E	WT
BRAF V600E	SK-Mel-507	V600E	WT
BRAF V600E	SK-Mel-529	V600E	WT
BRAF V600E	Sk-Mel-457	V600E	WT
BRAF V600E	SK-Mel-483	V600E	WT
BRAF V600E	SK-Mel-513	V600E	WT
BRAF V600E	SK-Mel-1	V600E	WT
BRAF V600E	SK-Mel-7	V600E	WT
BRAF V600E	SK-Mel-11	V600E	WT
BRAF V600E	SK-Mel-12	V600E	WT
BRAF V600E	SK-Mel-13	V600E	WT
BRAF V600E	SK-Mel-19	V600E	WT
BRAF V600E	SK-Mel-22	V600E	WT
BRAF V600E	SK-Mel-26	V600E	WT
BRAF V600E	SK-Mel-27	V600E	WT
BRAF V600E	SK-Mel-28	V600E	WT
BRAF V600E	SK-Mel-29	V600E	WT
BRAF V600E	SK-Mel-32	V600E	WT
BRAF V600E	SK-Mel-37	V600E	WT
BRAF V600E	SK-Mel-39	V600E	WT
BRAF V600E	SK-Mel-40	V600E	WT
BRAF V600E	SK-Mel-41	V600E	WT
BRAF V600E	SK-Mel-73	V600E	WT
BRAF V600E	SK-Mel-100	V600E	WT
BRAF V600E	SK-Mel-105	V600E	WT
BRAF V600E	SK-Mel-133	V600E	WT
BRAF V600E	SK-Mel-170	V600E	WT
BRAF V600E	SK-Mel-178	V600E	WT
BRAF V600E	SK-Mel-181	V600E	WT
BRAF V600E	SK-Mel-188	V600E	WT
BRAF V600E	SK-Mel-190	V600E	WT
BRAF V600E	SK-Mel-192	V600E	WT
BRAF V600E	SK-Mel-196	V600E	WT
BRAF V600E	SK-Mel-197	V600E	WT
BRAF V600E	SK-Mel-200	V600E	WT
BRAF V600E	SK-Mel-202	V600E	WT
BRAF V600E	SK-Mel-207	V600E	WT
BRAF V600E	SK-Mel-222	V600E	WT
BRAF V600E	SK-Mel-227	V600E	WT
BRAF V600E	SK-Mel-228	V600E	WT
BRAF V600E	SK-Mel-230	V600E	WT
BRAF V600E	SK-Mel-232	V600E	WT
BRAF V600E	SK-Mel-238	V600E	WT
BRAF V600E	SK-Mel-239	V600E	WT
BRAF V600E	SK-Mel-243	V600E	WT
BRAF V600E	SK-Mel-244	V600E	WT
BRAF V600E	SK-Mel-256	V600E	WT
BRAF V600E	SK-Mel-267	V600E	WT
BRAF V600E	SK-Mel-268	V600E	WT
BRAF V600E	SK-Mel-269	V600E	WT
BRAF V600E	SK-Mel-271	V600E	WT
BRAF V600E	SK-Mel-272	V600E	WT
BRAF V600E	SK-Mel-275	V600E	WT
BRAF V600E	SK-Mel-276	V600E	WT
BRAF V600E	SK-Mel-279	V600E	WT
BRAF V600E	SK-Mel-283	V600E	WT
BRAF V600E	SK-Mel-284	V600E	WT
BRAF V600E	SK-Mel-441	V600E	WT
BRAF V600E	A2058	V600E	WT
BRAF V600E	A375	V600E	WT
BRAF V600E	Malme3M	V600E	WT
BRAF V600E	WM88	V600E	WT
BRAF V600E	SK-Mel-15	V600E	WT
BRAF V600E	SK-Mel-279	V600E	WT

Table 3.1 Cont.

Genotype	Cell line	Sequenom BRAF	Sequenom NRAS
NRAS Q61K	SK-Mel-307	WT	Q61K
NRAS Q61K	SK-Mel-410	WT	Q61K
NRAS Q61K	SK-Mel-455	WT	Q61K
NRAS Q61K	SK-Mel-21	WT	Q61K
NRAS Q61K	SK-Mel-30	WT	Q61K
NRAS Q61K	SK-Mel-90	WT	Q61K
NRAS Q61K	SK-Mel-109	WT	Q61K
NRAS Q61K	SK-Mel-117	WT	Q61K
NRAS Q61K	SK-Mel-173	WT	Q61K
NRAS Q61K	SK-Mel-186	WT	Q61K
NRAS Q61K	SK-Mel-229	WT	Q61K
NRAS Q61L	SK-Mel-321A	WT	Q61L
NRAS Q61L	SK-Mel-369	WT	Q61L
NRAS Q61L	SK-Mel-127	WT	Q61L
NRAS Q61R	Sk-Mel-301	WT	Q61R
NRAS Q61R	SK-Mel-325	WT	Q61R
NRAS Q61R	SK-Mel-367	WT	Q61R
NRAS Q61R	SK-Mel-394	WT	Q61R
NRAS Q61R	SK-Mel-534A	WT	Q61R
NRAS Q61R	SK-Mel-538	WT	Q61R
NRAS Q61R	SK-Mel-2	WT	Q61R
NRAS Q61R	SK-Mel-103	WT	Q61R
NRAS Q61R	SK-Mel-119	WT	Q61R
NRAS Q61R	SK-Mel-130	WT	Q61R
NRAS Q61R	SK-Mel-147	WT	Q61R
NRAS Q61R	SK-Mel-182	WT	Q61R
NRAS Q61R	SK-Mel-191	WT	Q61R
NRAS Q61R	SK-Mel-199	WT	Q61R
NRAS Q61R	SK-Mel-205	WT	Q61R
NRAS Q61R	SK-Mel-210	WT	Q61R
NRAS Q61R	SK-Mel-234	WT	Q61R
WT	SK-Mel-464b	WT	WT
WT	Sk-Mel-444	WT	WT
WT	SK-Mel-304	WT	WT
WT	SK-Mel-309	WT	WT
WT	SK-Mel-316	WT	WT
WT	SK-Mel-318	WT	WT
WT	SK-Mel-321B	WT	WT
WT	SK-Mel-323	WT	WT
WT	SK-Mel-326	WT	WT
WT	SK-Mel-332	WT	WT
WT	SK-Mel-346	WT	WT
WT	SK-Mel-351	WT	WT
WT	SK-Mel-366	WT	WT
WT	SK-Mel-380	WT	WT
WT	SK-Mel-381	WT	WT
WT	SK-Mel-391	WT	WT
WT	SK-Mel-393	WT	WT
WT	SK-Mel-398	WT	WT
WT	SK-Mel-400	WT	WT
WT	SK-Mel-406	WT	WT
WT	SK-Mel-408	WT	WT
WT	SK-Mel-412Parotid	WT	WT
WT	SK-Mel-423A	WT	WT
WT	SK-Mel-423B	WT	WT
WT	SK-Mel-427	WT	WT
WT	SK-Mel-428	WT	WT
WT	SK-Mel-430	WT	WT
WT	SK-Mel-431	WT	WT
WT	SK-Mel-432	WT	WT
WT	SK-Mel-435	WT	WT
WT	SK-Mel-445	WT	WT
WT	SK-Mel-451	WT	WT
WT	SK-Mel-452	WT	WT
WT	SK-Mel-464a	WT	WT
WT	SK-Mel-481	WT	WT
WT	SK-Mel-509	WT	WT
WT	SK-Mel-524	WT	WT
WT	SK-Mel-24	WT	WT
WT	SK-Mel-31	WT	WT
WT	SK-Mel-35	WT	WT
WT	SK-Mel-36	WT	WT
WT	SK-Mel-75	WT	WT
WT	SK-Mel-110	WT	WT
WT	SK-Mel-113	WT	WT
WT	SK-Mel-118	WT	WT
WT	SK-Mel-146	WT	WT
WT	SK-Mel-161	WT	WT
WT	SK-Mel-176	WT	WT
WT	SK-Mel-187	WT	WT
WT	SK-Mel-215	WT	WT
WT	SK-Mel-217	WT	WT
WT	SK-Mel-264	WT	WT
WT	SK-Mel-265	WT	WT
WT	SK-Mel-266	WT	WT
WT	SK-Mel-285	WT	WT

Table 3.2 **BRAF/NRAS wild type cell lines with alterations found**

Cell line	Sequenom NRAS	Sequenom BRAF	Sanger mutations detected	IMPACT	244k or 1M Agilent CGH
SK-Mel-23	WT	WT	---	BRAF amplification	Broad 7q gain, BRAF amplification, PTEN deletion
SK-Mel-266	WT	WT	NF1 L161F	NF1 L161*, NF1 Q282*	Broad 7q gain
SK-Mel-113	WT	WT	---	NF1 homozygous deletion	Focal NF1 deletion
SK-Mel-217	WT	WT	---	KRAS amplification; focal intragenic NF1 deletion	KRAS amplification; focal NF1 deletion
MeWo	WT	WT	NF1 Q1336*	NF1 Q1336*, heterozygous NF1 deletion, p53 Q317* and E258K	Focal NF1 deletion
SK-Mel-118	WT	WT	KRAS G13R	KRAS amplification; KRAS G13R	KRAS gain
SK-Mel-285	WT	WT	KRAS G12C	---	
SK-Mel-110	WT	WT	KRAS E63K	---	
SK-Mel-146	WT	WT	HRAS Q61K	---	
SK-Mel-215	WT	WT	BRAF N581S	---	Broad 7q gain
SK-Mel-264	WT	WT	BRAF D594G	---	
WM1382	WT	WT	KIT N822K	---	KIT amplification, CDK4 amplification, ERBB3 gain
WM3211	WT	WT	KIT L576P	---	---
WM3918	---	---	---	Focal NF1 deletion	---

Table 3.3 **BRAF/NRAS mutant NF1null cell lines with alterations found**

Cell line	Sequenom NRAS	Sequenom BRAF	Sanger mutations detected	IMPACT	244k or 1M Agilent CGH
SK-Mel-103	Q61R	WT	---	NRAS Q61R, Focal NF1 deletion, TP53 deletion	Focal NF1 deletion
M308	WT	V600E	---	BRAF V600E, NF1 Q1070*	

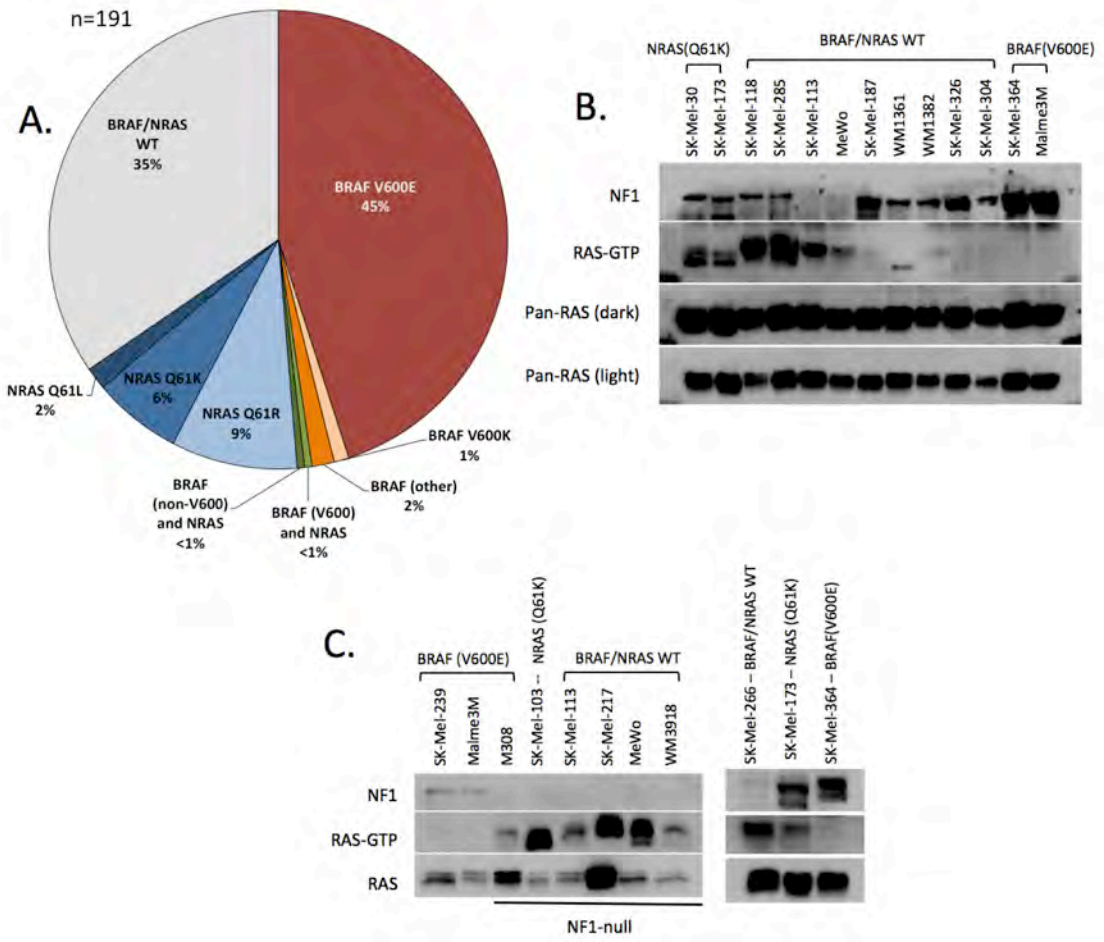

Figure 3.1 NF1-null melanoma cell lines express high levels of activated RAS. A) BRAF and NRAS status of the melanoma cell line panel (n=191). B) Activated RAS protein (RAS-GTP) was quantitated in select melanoma cell lines via immunoprecipitation with the RAS-binding domain of RAF (RAF-1 RBD) followed by immunoblot using pan-RAS antibodies. Expression of NF1 and total RAS (pan-RAS) and actinin were determined by immunoblot from whole cell lysate (WCL). C. Activated RAS protein (RAS-GTP) was quantitated in NF1-null melanoma cell lines as described.

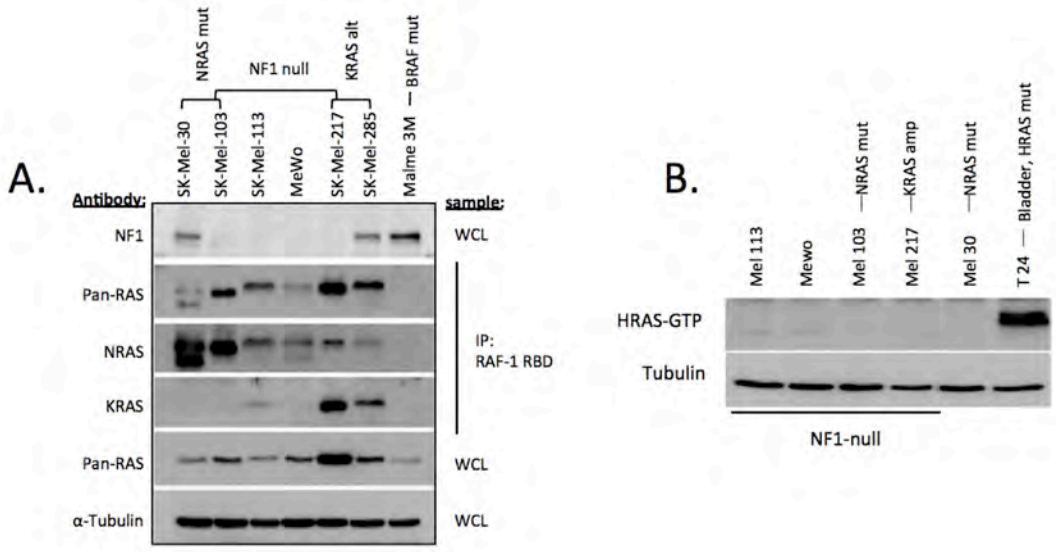

Figure 3.2 NF1-null melanoma cell lines activate the RAS isoforms differently. A) Activated RAS protein (RAS-GTP) was quantitated in select melanoma cell lines as described, followed by immunoblot using RAS isoform specific antibodies. B) Activated HRAS protein (RAS-GTP) was quantitated in select melanoma cell lines as described followed by immunoblot using HRAS antibodies. Alt = alteration, mut = mutant, WCL = whole cell lysate.

To define the mechanistic basis for the loss of NF1 expression and the co-alterations in the melanoma cell lines, we performed next-generation sequencing using an exon capture-based approach (IMPACT assay – see Methods) (Iyer, Hanrahan et al. 2012, Wagle, Berger et al. 2012). Two cell lines were found to harbor nonsense mutations in *NF1* (Fig. 3.3A): MeWo, a hemizygous Q1336* mutation, and Sk-Mel-266, L161* and Q282* mutations. The remaining four cell lines had deletions involving the *NF1* gene locus: SK-Mel-113, focal homozygous loss of the N-terminal domain; SK-Mel-103 and WM3918, focal homozygous loss of the C-terminal domain, and SK-Mel-217, broad monoallelic loss, as well as a focal, intragenic deletion in the second *NF1* allele (Fig. 3.3B, C). In sum, genomic alterations sufficient to account for complete loss of NF1 protein expression were identified in all six NF1-null melanoma cell lines.

Although loss of NF1 was identified in the $BRAF^{WT}/RAS^{WT}$ cohort, it was not mutually exclusive with RAS alterations. Notably, concurrent alterations in the *NF1* and the *RAS* genes have also been noted in two recent whole-exome sequencing studies of melanoma tumors including Hodis et al. (Hodis, Watson et al. 2012) and the melanoma study performed by the Cancer Genome Atlas (TCGA) working group (Fig. 3.4 and (Hodis, Watson et al. 2012)). We analyzed the genes co-altered with NF1 in the melanoma tumors from TCGA. *CDKN2A* and/or *TP53* were among the tumor suppressor genes most commonly co-altered in the NF1-null melanoma cell lines and tumors. However, mutations in *NF1* were also found to co-associate with activating alterations in the RAS/MAP kinase pathway, such as *NRAS* mutation and *BRAF* mutation-- in particular exon 11 "low activity" (Wan, Garnett et al. 2004) *BRAF* mutations.

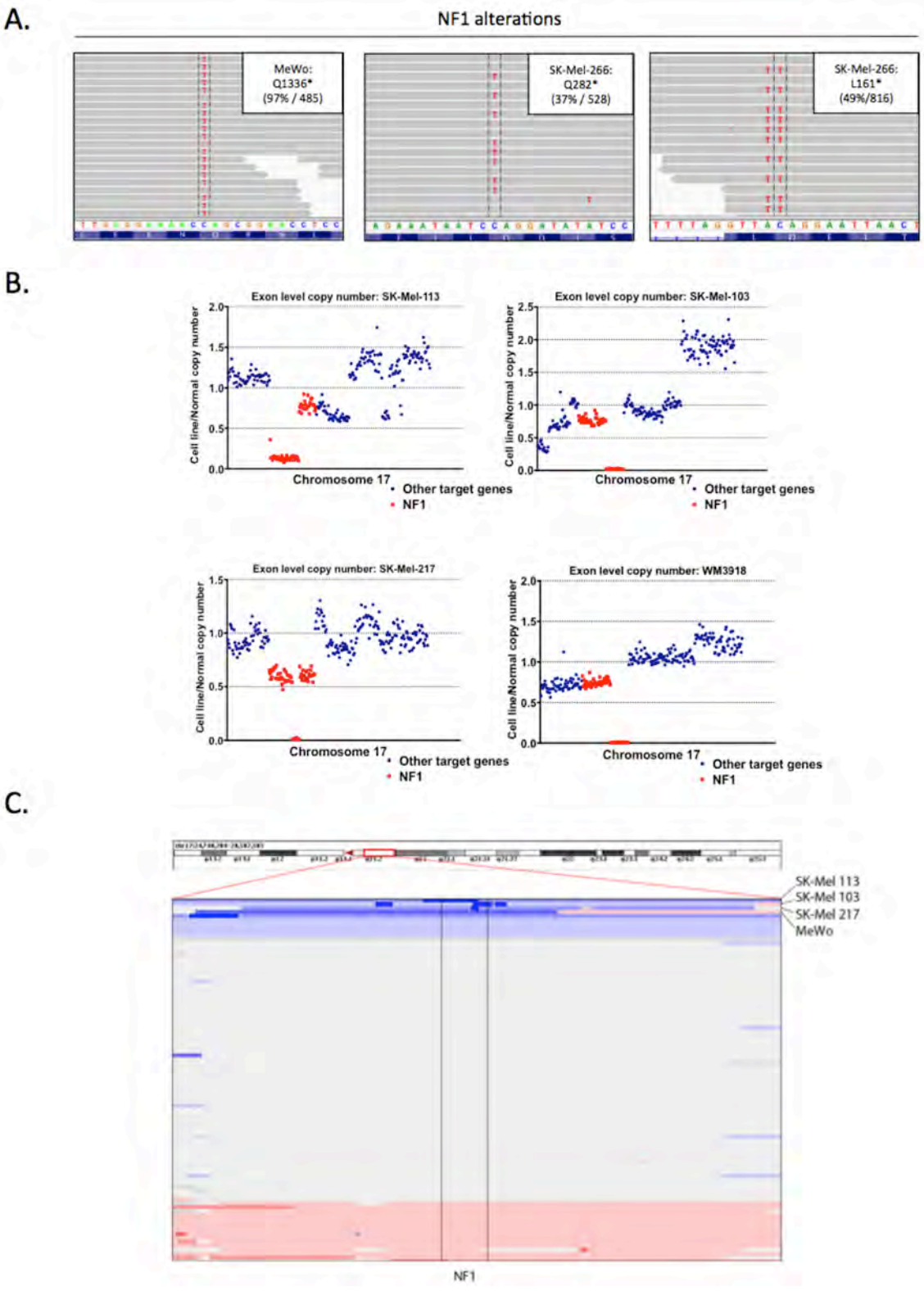

Figure 3.3 The genomic basis of NF1 loss in melanoma cell lines. A) DNA from NF1-null cell lines was analyzed using the IMPACT assay. Shown are aligned sequencing reads highlighting select *NF1* mutations [visualized in the Integrative Genomics Viewer (IGV)] (Robinson, Thorvaldsdottir et al. 2011). Percentages (left) are the ratio of mutant reads over total reads (right). B) Homozygous deletions of NF1 in four melanoma cell lines. Exon-level copy number data is shown for select genes around NF1 on chromosome 17. C) Gains and losses in the NF1 gene across cell lines from Agilent Comparative Genomic Hybridization as visualized in the IGV.

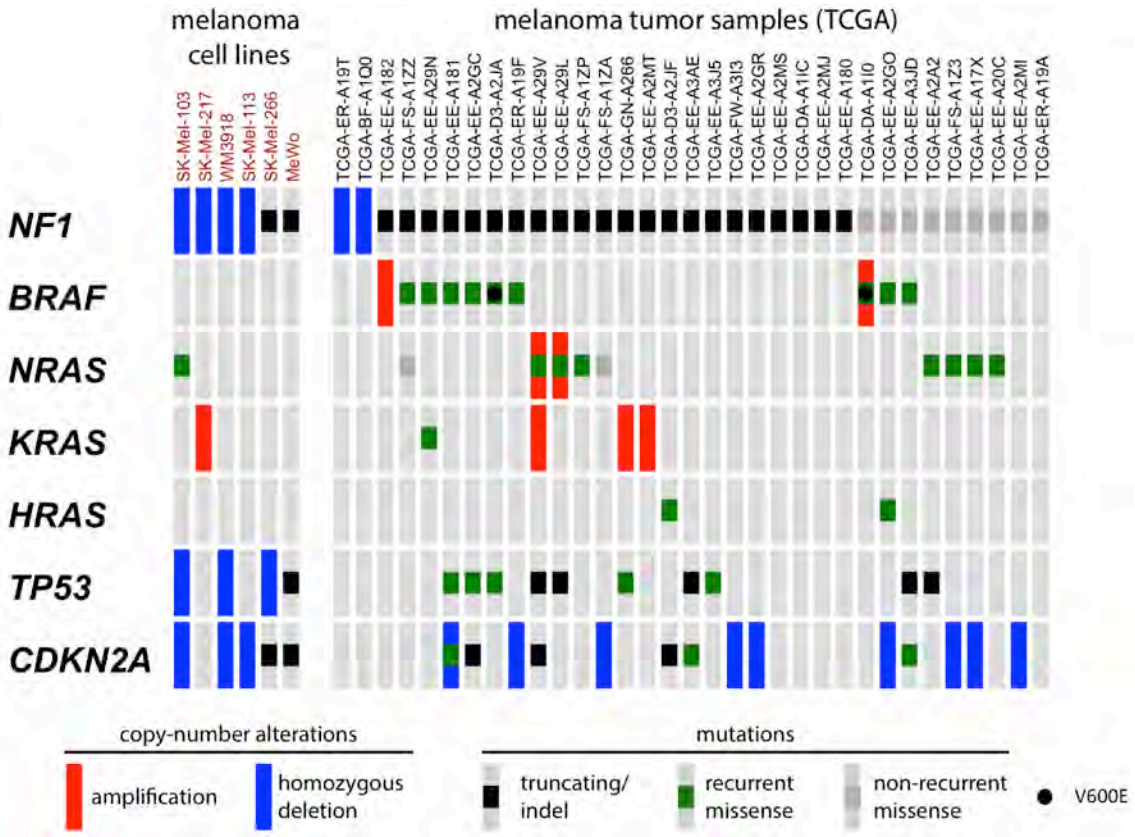

Figure 3.4 NF1 loss in cell lines and tumors. Summary of mutations and copy-number alterations in NF1-null cell lines by IMPACT assay and mutations and copy-number alterations in NF1-null melanoma tumors analyzed by the TCGA.

Discussion

We found that a significant subset of melanoma cell lines, including those wild-type for BRAF and RAS, exhibit total loss of NF1 protein expression. In all cases, a truncating mutation and/or focal deletion of the *NF1* gene, rather than post-transcriptional regulation (McGillicuddy, Fromm et al. 2009) was identified as the basis for NF1 loss. In contrast to prior reports (Andersen, Fountain et al. 1993, Johnson, Look et al. 1993), all the NF1-null melanoma cell lines expressed levels of active GTP-bound RAS comparable to those found in RAS mutant cells. This is the first time that functional *de novo* NF1 loss has been reported in melanoma cell lines, and suggests that NF1 loss is a functional alteration. The preference for activation of NRAS over the other RAS isoforms mirrors the predominance of NRAS alterations in melanoma, and adds evidence to the importance of the N-isoform of RAS in the melanocyte lineage.

Notably, NF1 loss was not mutually exclusive with RAS or BRAF mutations. In both cell lines and tumors, NF1 loss was found concurrently with NRAS and some K- and H-RAS alterations. This is surprising, as activation of the most common RAS mutants was historically thought to be independent of GAP and GEF function (Scheffzek, Ahmadian et al. 1997), and thus would not have been predicted to result in co-selection in the absence of a GAP. In addition to NRAS mutation, NF1 loss was found to commonly co-occur with exon 11 (non-exon 15 or non-V600E) BRAF mutations. These latter mutations in BRAF often exhibit impaired kinase activity and induce ERK signaling by dimerizing with and activating CRAF (Wan, Garnett et al. 2004). As RAS activation through NF1 loss would be predicted to promote the formation of CRAF homo- and heterodimers, NF1 alterations may cooperate with low activity BRAF mutants to induce transformation by enhancing RAF dimer formation.

Overlap of NF1 loss with *CDKN2A* or *TP53* alteration suggests that these latter genes may cooperate with NF1 loss in promoting melanomagenesis, perhaps by preventing the oncogene-

induced senescence resulting from RAS activation. Alterations of these genes have also been reported in other NF1-associated cancer types, such as astrocytomas and malignant peripheral nerve sheath tumors (Brems, Beert et al. 2009).

Though the genomic and biologic data outlined above suggest that NF1 loss is a functional alteration in melanoma, its phenotypic consequences were previously unknown. We thus next examined the dependence of NF1-null melanoma cell lines on downstream effectors of RAS by characterizing their response to small molecule inhibitors of MEK and TORC1.

Chapter Four

NF1-null melanoma cell lines are MAPK pathway dependent

NF1-null cell lines have been widely studied in many contexts to understand the activation state of their signaling networks in order to identify exploitable weaknesses (Bollag, Clapp et al. 1996, Hiatt, Ingram et al. 2001, Johannessen, Reczek et al. 2005, Thomas, Deadwyler et al. 2006, Johannessen, Johnson et al. 2008). Because the NF1-null cells in neurofibromatosis type 1 cause many varied morbidities, a large focus has been to understand the genetic events that cause progression from benign to malignant disease.

Malignant peripheral nerve sheath tumors (MPNSTs) occur commonly in NF1 patients. Work by the Cichowski lab demonstrated that $NF1^{-/-}$ fibroblasts have altered regulation of TSC2 and mTOR (Johannessen, Reczek et al. 2005). Additionally, they found that NF1-null MPNSTs are dependent on TORC1 signaling for proliferation and are exquisitely sensitive to rapamycin, which induces down-regulation of cyclin D1 (Johannessen, Johnson et al. 2008). We asked whether NF1 null melanomas were similarly dependent upon TORC1 for cell growth and/or survival.

Melanocytes originate in the neural crest of the embryo, like the cells most commonly affected in neurofibromatosis type 1 (Bolande 1981, Brannan, Perkins et al. 1994), and therefore may have similar "wiring" to other malignancies that more commonly arise in patients with germline

alterations in NF1. Many melanomas, however, are dependent on the MAPK pathway (Solit, Garraway et al. 2006, Joseph, Pratilas et al. 2010). It was previously unknown whether NF1-null melanomas are dependent on TORC1 or the MAPK pathway for cell cycle progression and proliferation. Additionally, whether NF1-null melanomas, which have activation of RAS, are sensitive to inhibitors of MEK was unknown. While BRAF(V600E) melanoma cells are exquisitely sensitive to MEK inhibition, RAS mutant melanomas are variably sensitive to MEK inhibition suggesting that NF1 null cells may also be variably dependent upon MEK activation for cell growth (Solit, Garraway et al. 2006, Joseph, Pratilas et al. 2010).

Results

The growth of NF1-null cell lines derived from human MPNSTs and MPNSTs that arise in *NF1*$^{-/-}$ mice are dependent on TORC1 signaling and sensitive to the TORC1 inhibitor rapamycin (Johannessen, Reczek et al. 2005, Johannessen, Johnson et al. 2008). To determine whether NF1-null melanoma cells are also TORC1-dependent, we treated the NF1-null melanoma cell lines identified above with rapamycin and compared their sensitivity to that of SNF96.2, a representative human NF1-null MPNST cell line (Fig. 4.1A).

The growth of NF1-null melanomas as a group was significantly less sensitive to rapamycin (IC_{50} ranging from 1 to 286 nM) than SNF96.2 cells (IC_{50} 0.05 nM). The ribosomal protein S6, a component of the 40S ribosomal subunit that is phosphorylated upon translation activation, is downstream of TORC1. Phosphorylated S6 (p-S6) is decreased at one hour following treatment with rapamycin in SNF96.2 but not in the majority of the NF1-null melanoma cell lines (Fig. 4.1B), suggesting that other pathways in these cell lines regulate S6 and translation.

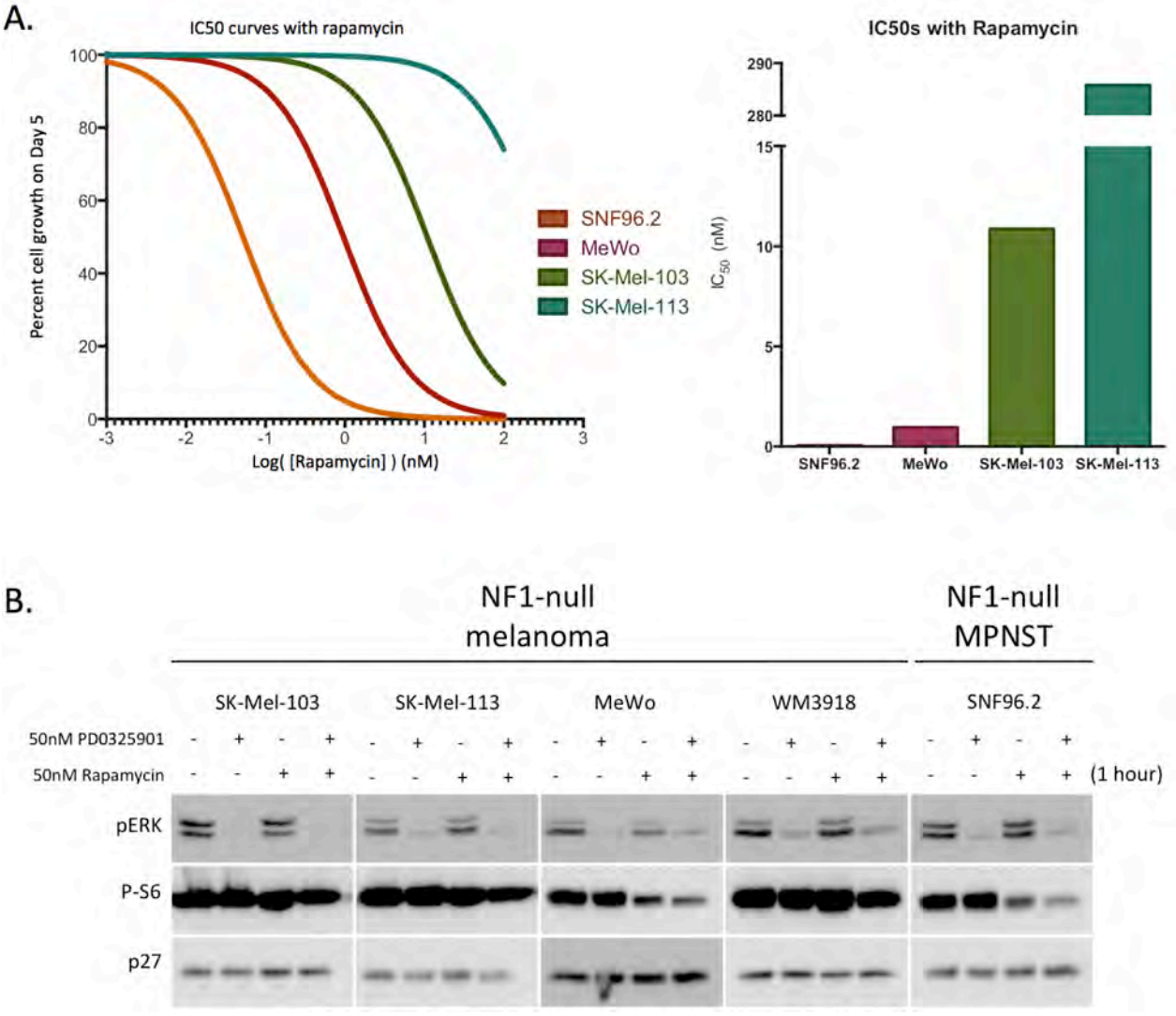

Figure 4.1 NF1-null melanoma cell lines are not mTOR dependent. A) Cells were treated with increasing concentrations of rapamycin for 5 days. Results are percent cell growth as a function of drug concentration (nM). IC50 values plotted to the right. B) Cells were treated with 50nM PD0325901 or 50nM Rapamycin for 1 hour and pathway inhibition was measured by immunoblot.

To probe the MEK dependence of NF1-null melanoma cells, we used PD0325901, a selective allosteric inhibitor of MEK1/2 (K_i^{app} of 1nM for MEK1 and MEK2) (Sebolt-Leopold JS 2004). The effect of PD0325901 on the proliferation and survival of four NF1-null melanoma cell lines was compared to that of SK-Mel-239, a MEK inhibitor-sensitive BRAF(V600E) melanoma cell line and to BT-474, a MEK inhibitor-resistant *ERBB2* amplified breast cancer cell line. The proliferation of all four NF1-null cell lines was inhibited by PD0325901, albeit with IC_{50}s that ranged from 6 to 20 fold greater than that of the BRAF(V600E) SK-Mel-239 cells (Fig. 4.2.1A).

To explore the basis for this differential sensitivity, we assessed the effects of drug exposure on downstream targets of MEK/ERK signaling as a function of concentration and time. Treatment of both BRAF(V600E) and NF1-null cells with 50nM PD0325901 resulted in decreased expression of phosphorylated ERK1/2 (pERK) by 1 hour (Fig. 4.2.1B, C). In BRAF(V600E) cells, suppression of pERK was durable and maintained at 6 and 24 hours and was accompanied by loss of cyclin D1 expression, accumulation of cells in G1 phase, and induction of apoptosis (Fig. 4.2.1B, C and Fig. 4.2.2A). In contrast, a partial rebound in pERK expression was apparent by 6 hours in NF1-null cells and was accompanied by a failure of the drug to potently suppress cyclin D1 expression (Fig. 4.2.1B, C). This rebound in pERK in the NF1-null cells was attenuated with use of a higher concentration of drug (500nM), leading to potent suppression of cyclin D1 expression, maximal accumulation of cells in G1 phase, and inhibition of proliferation (Fig. 4.2.1B,C and Fig 4.2.2A). However, higher concentrations of PD0325901 did not induce cell death in any of the NF1-null cell lines (Fig. 4.2.2A).

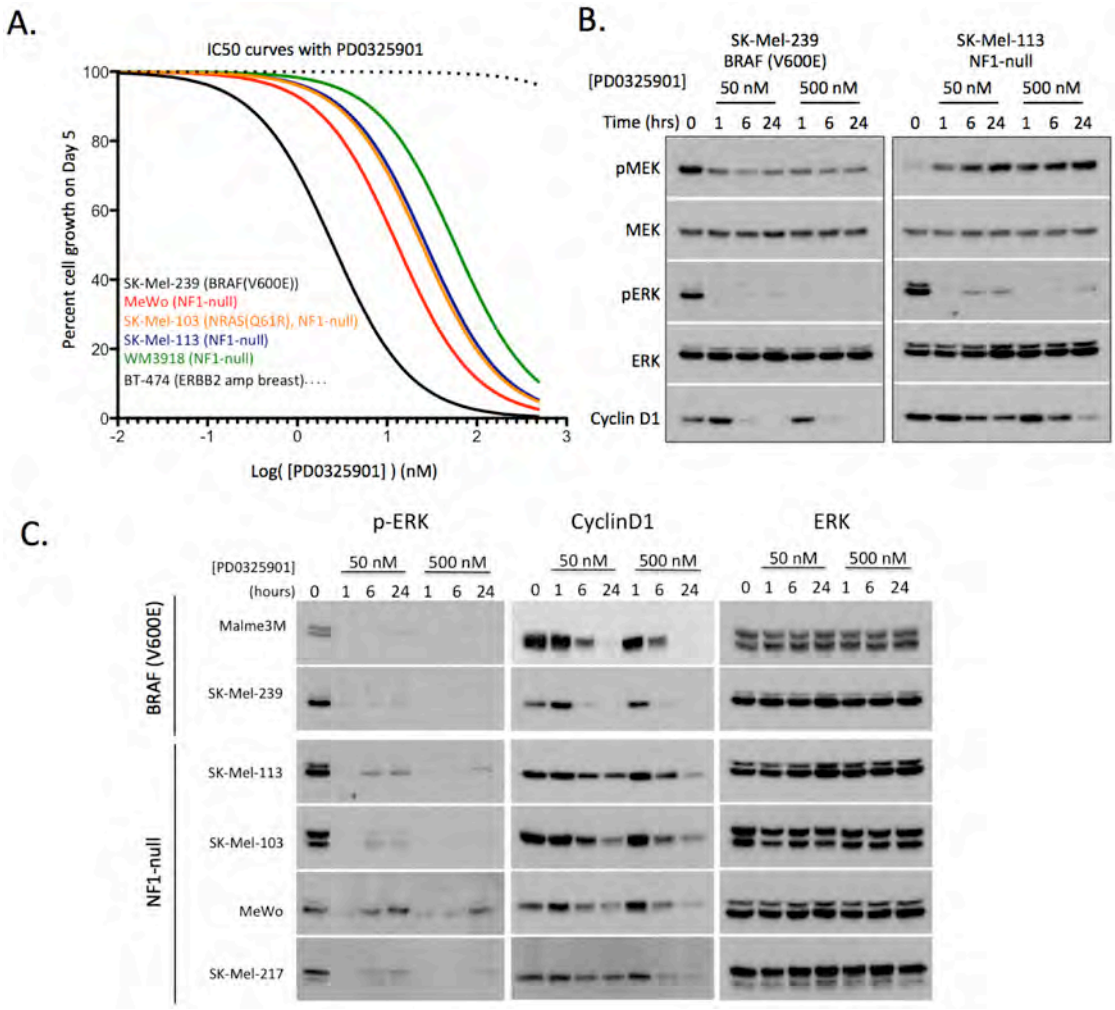

Figure 4.2.1 NF1-null melanoma cell lines are MAPK pathway dependent. A) Cell lines were treated with increasing concentrations of the MEK inhibitor PD0325901 for 5 days. Results are percent cell growth as a function of drug concentration (nM). B) Cells were treated with either 50 or 500 nM PD0325901 for 0, 1, 6 and 24 hours. Phospho- and total levels of MAPK pathway components were determined by immunoblot. C) As in B, pERK and cyclin D1 levels across cell lines treated with 50 or 500 nM PD0325901.

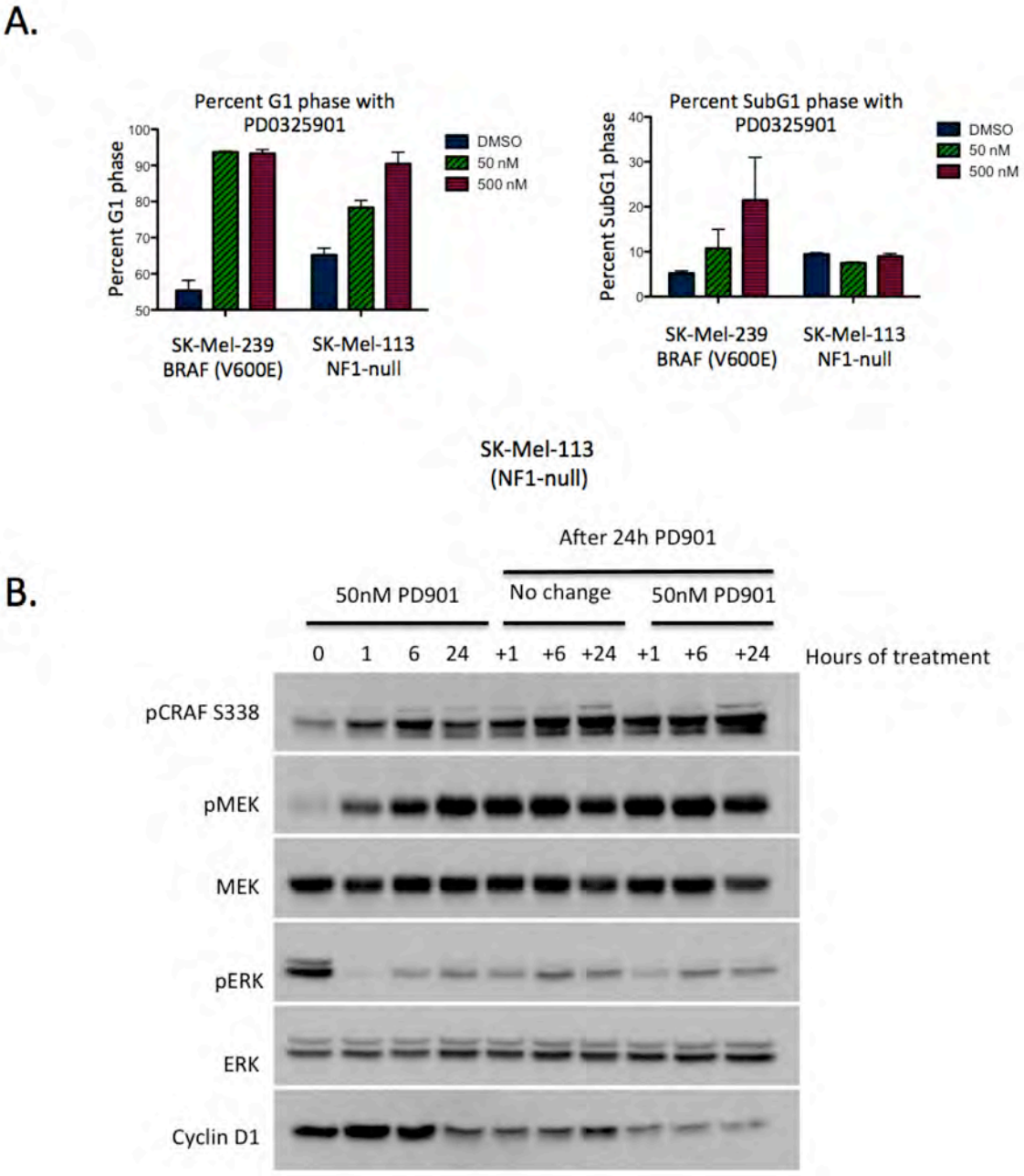

Figure 4.2.2 NF1-null melanoma cell lines are MAPK pathway dependent. A) Cells were treated for 24 hours with 50 or 500nM PD0325901 and stained with Ethidium Bromide before undergoing FACS analysis for cell cycle distribution. Error bars are SEM, n=3. B) NF1-null cells were treated with 50nM PD0325901 for 24 hours, then re-dosed for an additional 24 hours. MAPK pathway effectors were analyzed by immunoblot.

Rebound of pERK was not due to drug turnover, as re-addition of PD0325901 after 24 hours was unable to suppress pERK levels to those seen at 1h in drug-naïve cells (Fig. 4.2.2B). Together, these data suggest that cyclin D1 expression and cell cycle progression are MEK-dependent in NF1-null melanoma cells, but that rapid rebound in ERK activity may account for the lower sensitivity of NF1-null cells to the MEK inhibitor PD0325901.

Resistance to allosteric MEK inhibitors can be induced by upstream pathway hyperactivation (Corcoran, Dias-Santagata et al. 2010, Poulikakos and Solit 2011). We have previously shown that treatment of $BRAF^{WT}$ but not BRAF(V600E) cells with PD0325901 leads to increased expression of phosphorylated MEK (pMEK), which results from relief of upstream ERK-dependent negative feedback (Pratilas, Taylor et al. 2009). Consistent with these prior observations, treatment of NF1-null melanoma cells with PD0325901 resulted in increased expression of phosphorylated MEK (pMEK) (Fig. 4.2.1B). As the induction of pMEK in the NF1-null melanomas paralleled the rebound in pERK expression, we further studied the effects of a second allosteric MEK inhibitor on MEK signaling and cellular proliferation. Trametinib (GSK1120212) has a similar *in vitro* affinity for MEK1 and MEK2 as PD0325901 (IC_{50}s for MEK1 and MEK2 of 0.7 and 0.9nM, respectively), but in contrast to PD0325901, binding of trametinib to MEK blocks its phosphorylation at serine 217 (Gilmartin, Bleam et al. 2011). To compare the relative potencies of PD0325901 and trametinib *in vivo*, we first exposed BRAF(V600E) SK-Mel-239 and NF1-null SK-Mel-113 cells to increasing concentrations of both drugs and assessed the effect of drug treatment on pERK expression at 1 hour. In BRAF(V600E) SK-Mel-239 cells, both drugs were equipotent in their ability to suppress ERK activation at 1 hour (Fig. 4.3A). In contrast, in NF1-null SK-Mel-113 cells, trametinib was considerably more potent in its ability to suppress pERK expression than either PD0325901 or two additional allosteric MEK inhibitors currently in clinical testing (AZD6244 and MEK162) (Ascierto, Schadendorf et al. 2013, Catalanotti, Solit et al. 2013, Patel, Lazar et al. 2013) (Fig. 4.3A).

Additionally, trametinib was the only MEK inhibitor that demonstrated the same potency in pERK inhibition in both BRAF(V600E) and NF1-null cells (Fig. 4.3B).

Treatment of NF1-null melanoma cells with either PD0325901 or trametinib resulted in hyperactivation of RAS and increased expression of activated, phosphorylated CRAF (pCRAF S338), consistent with relief of upstream negative feedback following inhibition of ERK (Fig. 4.4A,B). However, relief of upstream feedback following MEK inhibition was accompanied by a significant increase in the expression of pMEK in PD0325901-treated NF1-null cells, which was attenuated in cells treated with trametinib (Fig. 4.4A). This attenuation of MEK phosphorylation was observed across NF1-null melanoma cell lines (Fig. 4.4B) and was unique to trametinib in comparison to PD0325901, AZD6244, or MEK162 (Fig. 4.4C). Furthermore, the resistance of MEK to upstream hyperactivation by RAS and CRAF in trametinib-treated cells was accompanied by a more durable down-regulation of pERK expression and a more potent inhibition of cyclin D1 as compared to PD0325901 (Fig. 4.4A,E). Consistent with these biological differences among the MEK inhibitors, the anti-proliferative effects of trametinib were similar in BRAF(V600E) and NF1-null cells, whereas BRAF(V600E) cells exhibited greater sensitivity to PD0325901 than did NF1-null cells (Fig. 4.4D).

Differences in the ability of PD0325901 and trametinib to durably suppress the transcriptional output of ERK were also observed in NF1-null SK-Mel-113 cells. RNA-seq was performed on cells treated with PD0325901 or trametinib and changes in genes within a previously defined MAPK pathway gene signature were assessed as a function of time. (Pratilas, Taylor et al. 2009). As shown in Fig 4.5, trametinib more durably suppressed the expression of several ERK dependent genes including SPRY4, ETV1 and MYC as compared to PD0325901 (Fig. 4.5).

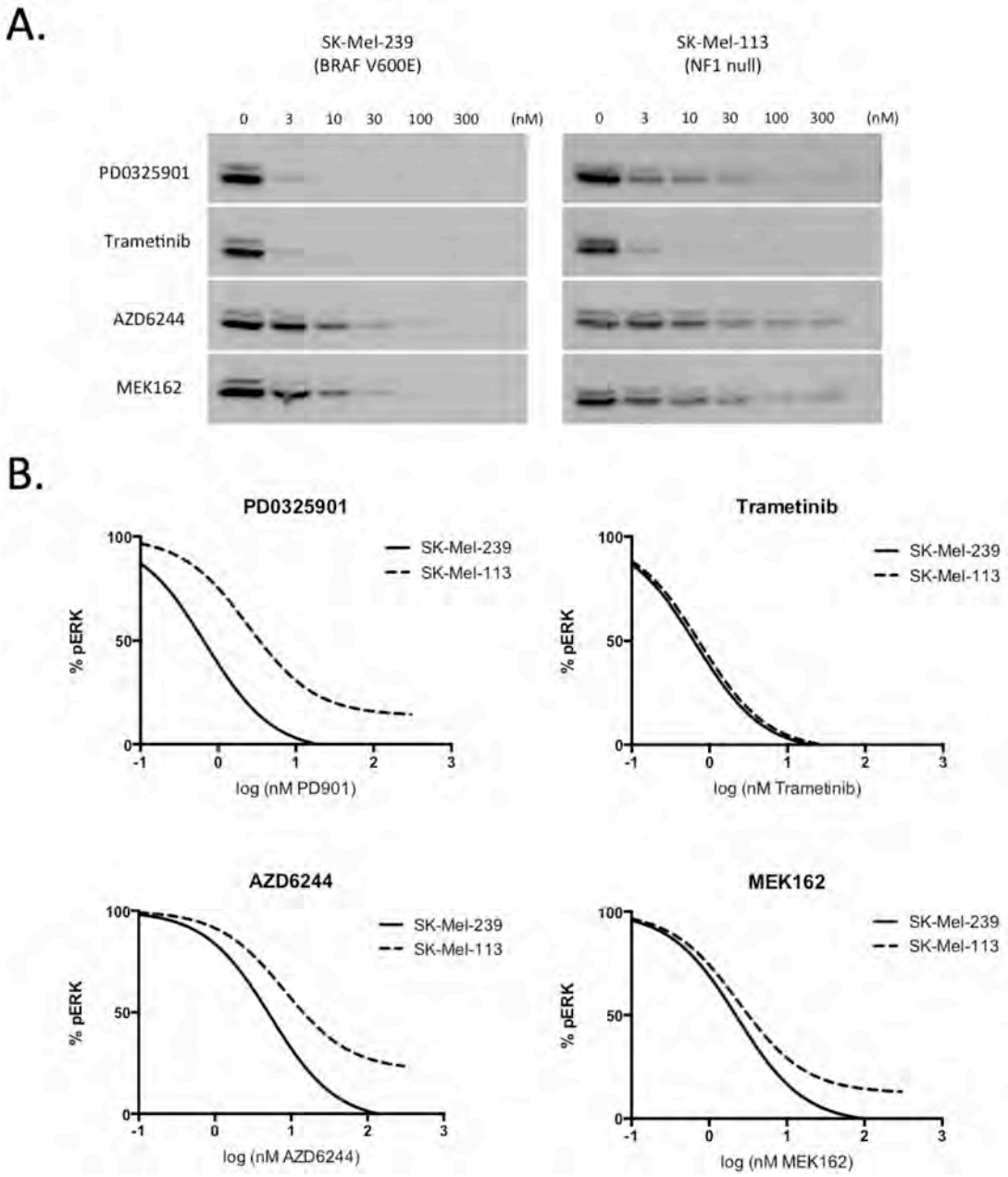

Figure 4.3 Effect of different MEK inhibitors on NF1-null cells. A) pERK levels by immunoblot of BRAF(V600E) or NF1-null cells with increasing doses of PD0325901, trametinib, AZD6244 or MEK162 at 1 hour. B) Quantitation of (A) via densitometry. Percentages calculated with the equation 100*([value of dose]-[value of background])/([value of 0nM]-[value of background]) and graphed with Prism.

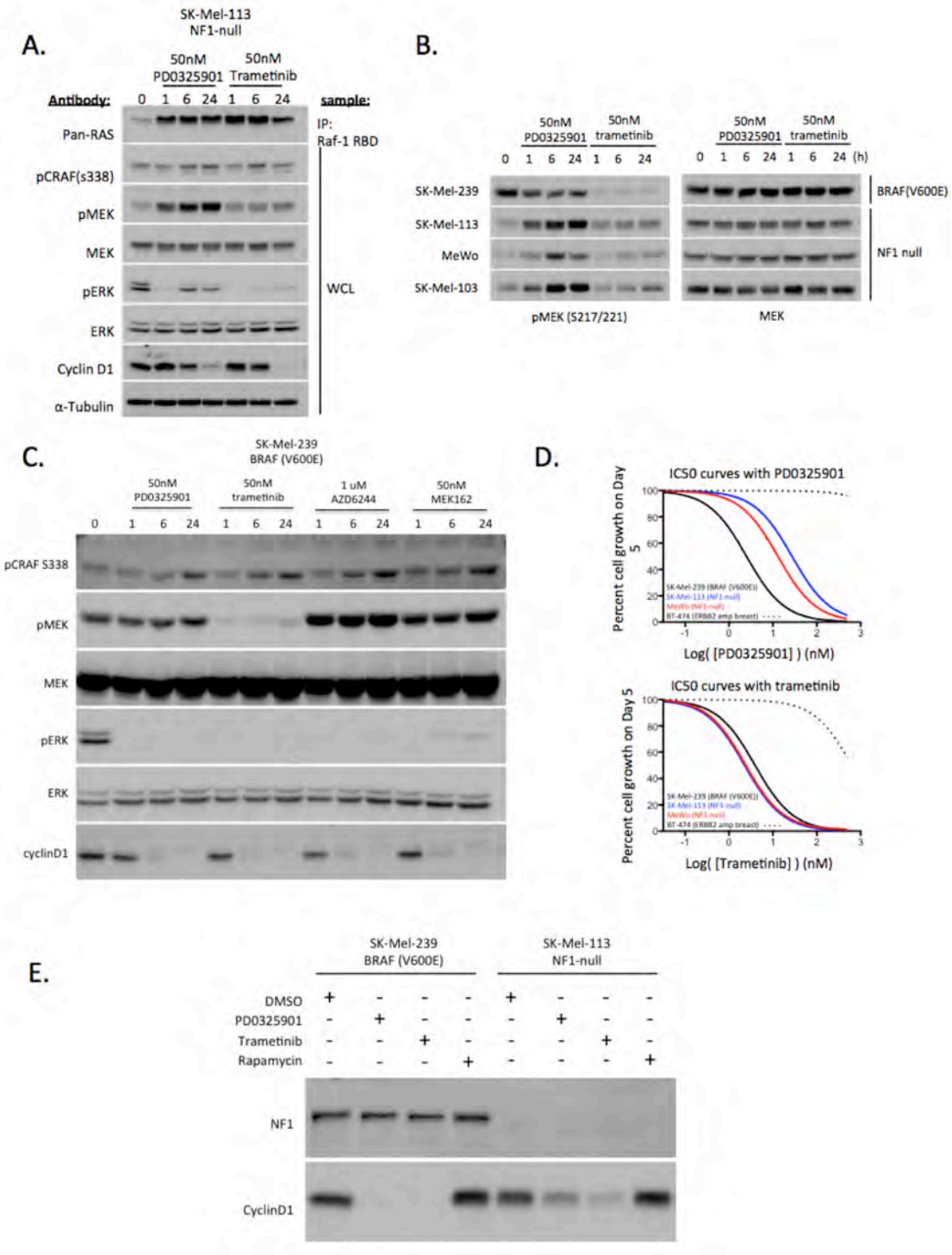

Figure 4.4 Trametinib durably inhibits ERK activation and proliferation in NF1-null melanoma cell lines. A) Cells were treated with 50nM PD0325901 or 50nM trametinib for 0, 1, 6 and 24 hours. Activated RAS protein (RAS-GTP) was quantitated as has been described. Phospho- and total levels of MAPK pathway components were determined by immunoblot. B) pMEK levels with 50nM PD0325901 or 50nM trametinib in NF1-null and BRAF(V600E) cells. C) MAPK pathway effectors in BRAF(V600E) cells treated with different MEK inhibitors for 1, 6, or 24 hours. D) NF1-null cell lines (SK-Mel-113 and MeWo) and BRAF(V600E) cells (SK-Mel-239) were treated with increasing doses of the MEK inhibitors PD0325901 or trametinib for 5 days. Results are percent cell growth as a function of drug concentration (nM). E) Cyclin D1 levels with different drugs at 24h in BRAF(V600E) or NF1-null cells.

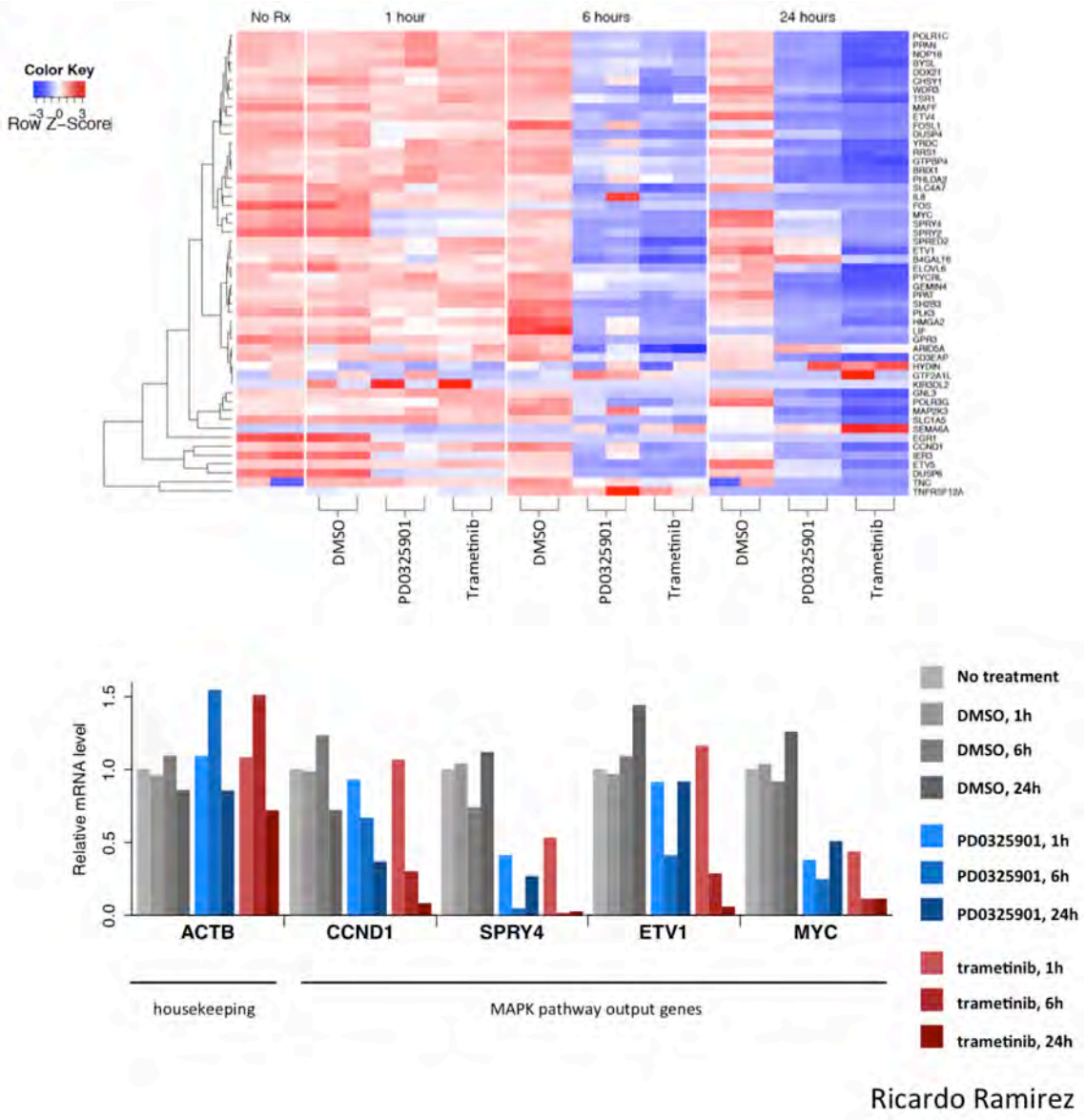

Figure 4.5 Trametinib more durably inhibits MAPK output. A) Z-scores from RNA-seq of the 52 genes considered the MAPK pathway signature output genes with DMSO, 50nM PD0325901 or trametinib for 24 hours. Relative mRNA levels of select genes are highlighted below. Credit to Ricardo Ramirez.

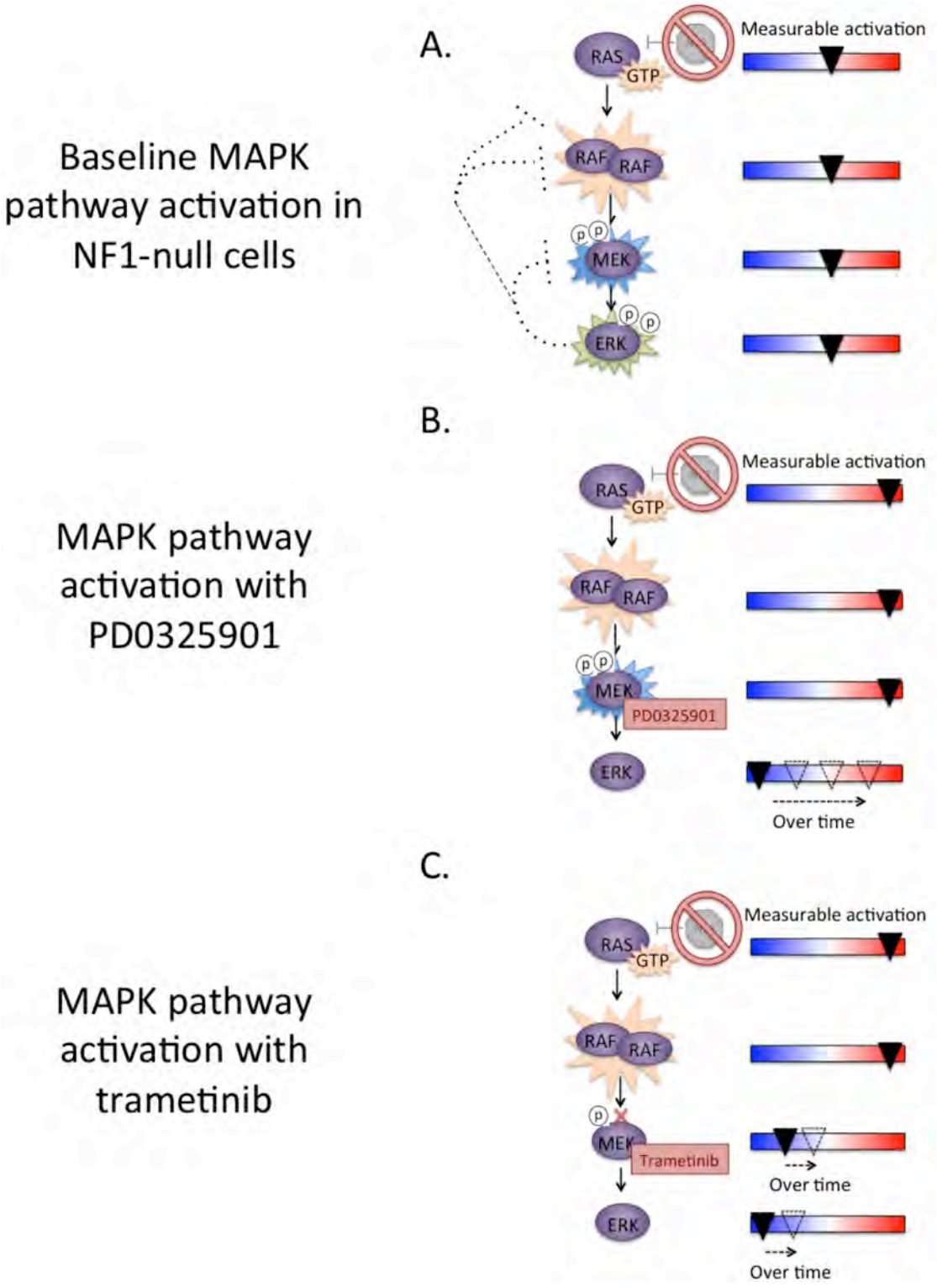

Figure 4.6 Model of measurable MAPK pathway activation with MEK inhibition. PD0325901 treatment results in relief of upstream feedback and activation of RAS and RAF. This results in phosphorylation of MEK by RAF and reactivation of ERK. Trametinib blocks phosphorylation of MEK, abrogating the rebound in pERK levels.

Discussion

Efforts to develop clinically useful direct inhibitors of activated RAS have been unsuccessful to date (Downward 2003). One alternative pharmacologic strategy for the treatment of tumors with constitutive RAS activation, including those with complete loss of NF1 expression, is to target the downstream pathways responsible for maintenance of the transformed phenotype. We observed that in contrast to NF1-null MPNSTs (Johannessen, Reczek et al. 2005, Johannessen, Johnson et al. 2008), NF1-null melanomas were dependent on ERK pathway activation and not TORC1 for cell cycle progression and proliferation. The failure of rapamycin to inhibit S6 phosphorylation at 1 hour in the majority of the NF1-null melanoma cell lines was correlated with the inability to inhibit growth. These data are consistent with the idea that translation is under the influence of multiple signaling pathways, as both the PI3K/mTOR and MEK/ERK pathways control kinases that phosphorylate and activate S6 (p70S6K and p90RSK, respectively) (Roux, Shahbazian et al. 2007). This result indicates that the lineage context within which NF1 is inactivated influences the downstream effector pathways that facilitate RAS-mediated transformation and thus likely dictates the potential utility of targeted pathway inhibitors.

While NF1-null melanomas were dependent upon ERK activation for cell proliferation, we observed significant differences in the relative potency of allosteric, non-ATP-competitive MEK inhibitors in the NF1-null cohort. Specifically, trametinib, which attenuates phosphorylation of MEK by RAF at Serine 217 (Gilmartin, Bleam et al. 2011), had greater antitumor effects than PD0325901. Monophosphorylated MEK has only partial activity (Gilmartin, Bleam et al. 2011) and the ability of trametinib but not PD0325901 to abrogate MEK hyperphosphorylation resulting from relief of upstream negative feedback was associated with more durable pERK inhibition, cyclin D1 suppression and greater anti-proliferative effects. A similar lack of potency was also noted in NF1-null melanoma cells with AZD6244, a second non-ATP-competitive MEK inhibitor incapable of abrogating RAF phosphorylation of MEK. AZD6244's inability to block MEK

phosphorylation likely accounts for the partial resistance to MEK inhibition observed following NF1 knockdown in a prior study (Whittaker, Theurillat et al. 2013). Our data are also consistent with a recent study suggesting that differences in the cellular potency of MEK inhibitors in KRAS-mutant cells can result from differences in the hydrogen bonding strength with S212 of MEK, a critical residue for blocking feedback induced MEK phosphorylation by wild-type RAF (Hatzivassiliou, Haling et al. 2013). The data imply that MEK inhibitors that block phosphorylation by RAF may have greater clinical activity in tumors with activated RAS, including those with loss of NF1 function. Such inhibitors may, however, have a narrower therapeutic index in patients with BRAF-mutant melanomas, as they would be predicted to more potently inhibit RAS-dependent ERK signaling in normal tissues.

In summary, the upstream hyperactivation of RAS and RAF that results from loss of negative feedback following ERK pathway inhibition can lead to an attenuation of the anti-tumor activity of allosteric MEK inhibitors, as modeled in Fig. 4.6. Inhibitors that prevent RAF-mediated phosphorylation of MEK abrogate this adaptive resistance to MEK inhibition and have greater anti-tumor activity in NF1-null cells. With the recent FDA approval of trametinib for the treatment of BRAF-mutant melanomas, these findings have potential therapeutic implications for patients with melanoma and potentially others tumor types with NF1 alterations.

Chapter Five

NF1 loss is associated with resistance to BRAF inhibition

BRAF(V600E) is a powerful oncogene. However, in the absence of additional cooperative alterations, incorporation of BRAF(V600E) into cells results in potent oncogene-induced senescence (Pollock, Harper et al. 2003, Michaloglou, Vredeveld et al. 2005, Dhomen, Reis-Filho et al. 2009, Raabe, Lim et al. 2011). ERK is activated to such an extent in the context of BRAF(V600E) that negative feedback on RAS is highly upregulated. As a result, BRAF(V600E) cells and tumors have extremely low levels of active RAS (Lito, Pratilas et al. 2012). As dimerization of RAF is dependent on active RAS, BRAF(V600E) cells also have very low levels of RAF dimers.

Many mechanisms of resistance to RAF inhibitors have been described. These mechanisms generally fall into two categories: mechanisms that reactivate ERK signaling in the presence of RAF inhibition, or mechanisms that bypass ERK signaling in the presence of RAF inhibition (Nissan and Solit 2011). As RAF inhibitors only effectively inhibit the BRAF(V600E) monomer (Poulikakos, Zhang et al. 2010), alterations that induce RAF dimer formation are associated with RAF inhibitor resistance. These include splice variants that lack the RAS binding domain of RAF, causing the proteins to dimerize in the absence of elevated RAS-GTP (Poulikakos, Persaud et al. 2011), or alterations that increase RAS-GTP levels, thereby facilitating RAF dimer formation (Nazarian, Shi et al. 2010, Villanueva, Vultur et al. 2010).

In analyses of human melanomas, NF1 loss is not mutually exclusive with BRAF(V600E) mutation (see chapter 3). Since BRAF(V600E) cells have high levels of negative feedback regulation on RAS and NF1 loss results in activation of RAS, it was unknown whether NF1 loss in the context of BRAF(V600E) mutation would be sufficient to raise levels of RAS-GTP or whether NF1 loss would be sufficient to confer resistance to RAF inhibitors. We find that NF1 loss results in increased RAS-GTP expression in BRAF(V600E) cells and that loss of NF1 was sufficient to desensitize these cells to RAF inhibitors. Though BRAF(V600E)/NF1-null cells were insensitive to vemurafenib or PD0325901, they remained sensitive to trametinib.

Results

We screened a panel of ten BRAF(V600E) melanoma cell lines for loss of NF1 expression and activation of RAS-GTP. Nine of the ten BRAF(V600E) mutant melanoma cell lines expressed low to undetectable levels of RAS-GTP (Fig. 5.1A). A single BRAF(V600E) cell line (M308) had high basal levels of RAS-GTP expression similar to that of an NRAS(Q61K) cell line. Notably, M308 was also devoid of NF1 expression by immunoblot (Fig. 5.1A). Genomic analysis by IMPACT confirmed the presence of a nonsense mutation in the *NF1* gene (Q1070*) as the basis for the loss of NF1 protein expression in M308 cells (Fig. 5.1B).

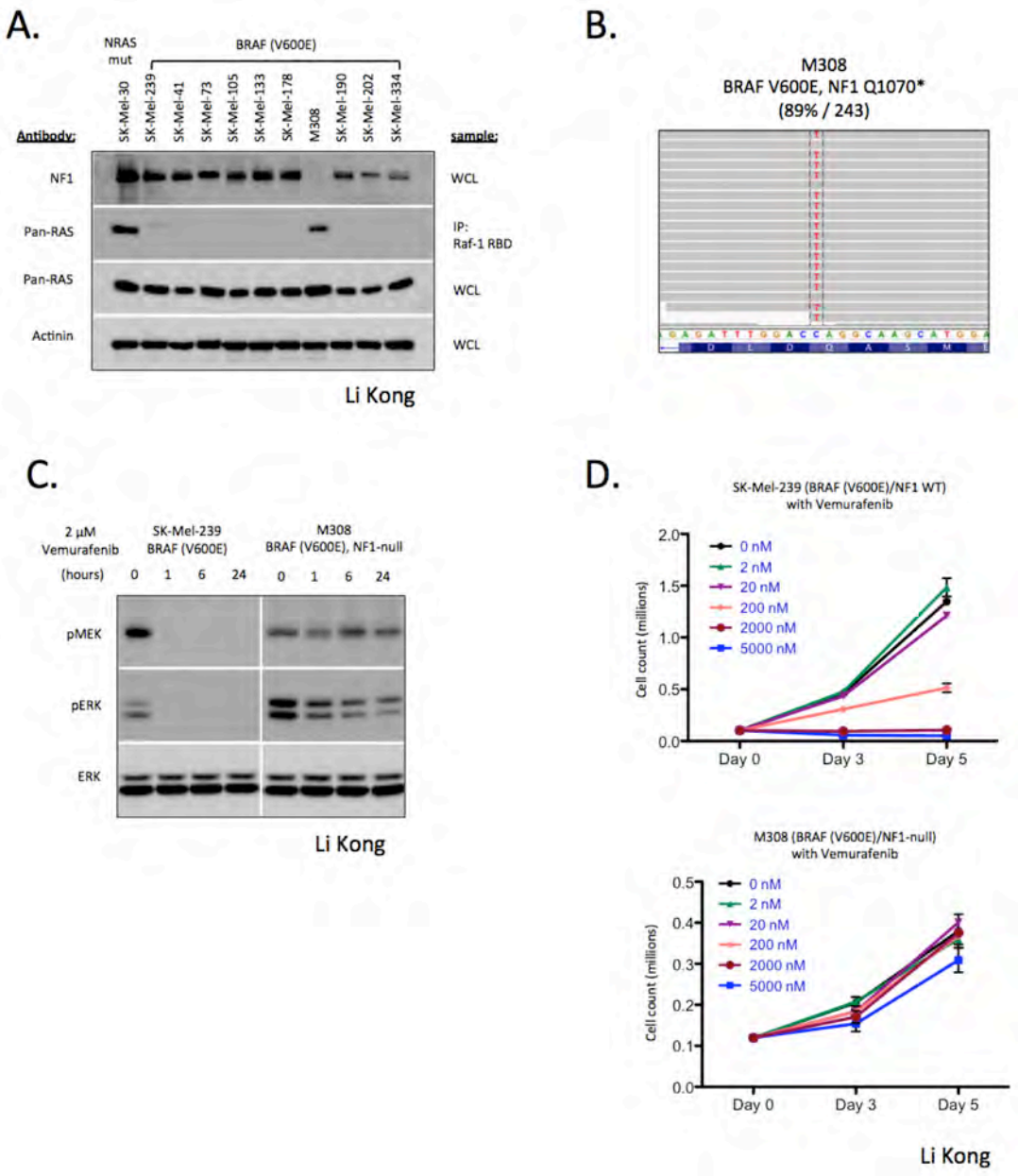

Figure 5.1 NF1 loss in the context of BRAF(V600E) mutation results in elevated RAS-GTP and resistance to RAF inhibition. A) RAS-GTP was quantitated as has been described. NF1, RAS and actinin protein was analyzed via immunoblot. Mut = mutant, WCL=whole cell lysate. Western blot by L. Kong. B) Exon-capture deep sequencing of the NF1 gene in M308 via IMPACT shows a homozygous nonsense Q1070* mutation. C) Cells were treated with 2 μM vemurafenib for 0, 1, 6, and 24 hours. Phospho- and total levels of MAPK pathway components were determined by immunoblot. Western blot by L. Kong. D) Cells were treated with increasing concentrations of the RAF inhibitor vemurafenib for 3 or 5 days. Results are cell count as a function of drug concentration over time. Error bars are SEM, n=3.

To determine whether NF1 loss desensitizes BRAF(V600E) cells to RAF inhibition, we assessed the sensitivity of M308 (BRAF(V600E)/NF1-null) to vemurafenib. Our laboratory and others has previously shown that vemurafenib inhibits ERK signaling only in tumors that express mutated BRAF (Joseph, Pratilas et al. 2010, Poulikakos, Zhang et al. 2010). Consistent with these results, vemurafenib treatment of SK-Mel-239 (BRAF(V600E)/$NF1^{WT}$) cells resulted in potent down-regulation of phosphorylated MEK and ERK and inhibition of cell growth in this cell line (Fig. 5.1C,D). In contrast, vemurafenib had little effect on the expression of phosphorylated MEK and ERK in BRAF(V600E)/NF1-null M308 cells and no effect on cell proliferation (Fig. 5.1C,D).

RAS activation is sufficient to induce vemurafenib resistance in BRAF(V600E) cells (Fig. 5.2A). To determine whether NF1 loss activates RAS sufficiently to overcome ERK-dependent negative feedback and induce vemurafenib resistance, we knocked down NF1 expression in BRAF(V600E) mutant A375 cells and assessed levels of RAS activation in the presence and absence of vemurafenib. si- and shRNA mediated knockdown of NF1 resulted in an induction in RAS-GTP expression (Fig. 5.2B) and decreased sensitivity to vemurafenib (Fig. 5.2C). These data suggest that loss of NF1 function in BRAF(V600E) cells is sufficient to induce RAS-GTP expression and, consequently, vemurafenib resistance.

To assess the MEK-dependence of the BRAF(V600E)/NF1-null M308 melanoma cells, we determined the effects of PD0325901 and trametinib treatment on ERK activation and cellular proliferation. Analogous to the results seen with these inhibitors in the $BRAF^{WT}$/NF1-null melanoma cells, exposure of M308 cells to 50nM PD0325901 was insufficient to durably suppress ERK signaling and cell proliferation (Fig. 5.3A). In contrast, treatment of M308 cells with trametinib resulted in durable suppression of pERK expression, potent downregulation of cyclin D1 expression and potent inhibition of cellular proliferation (Fig. 5.3A, B).

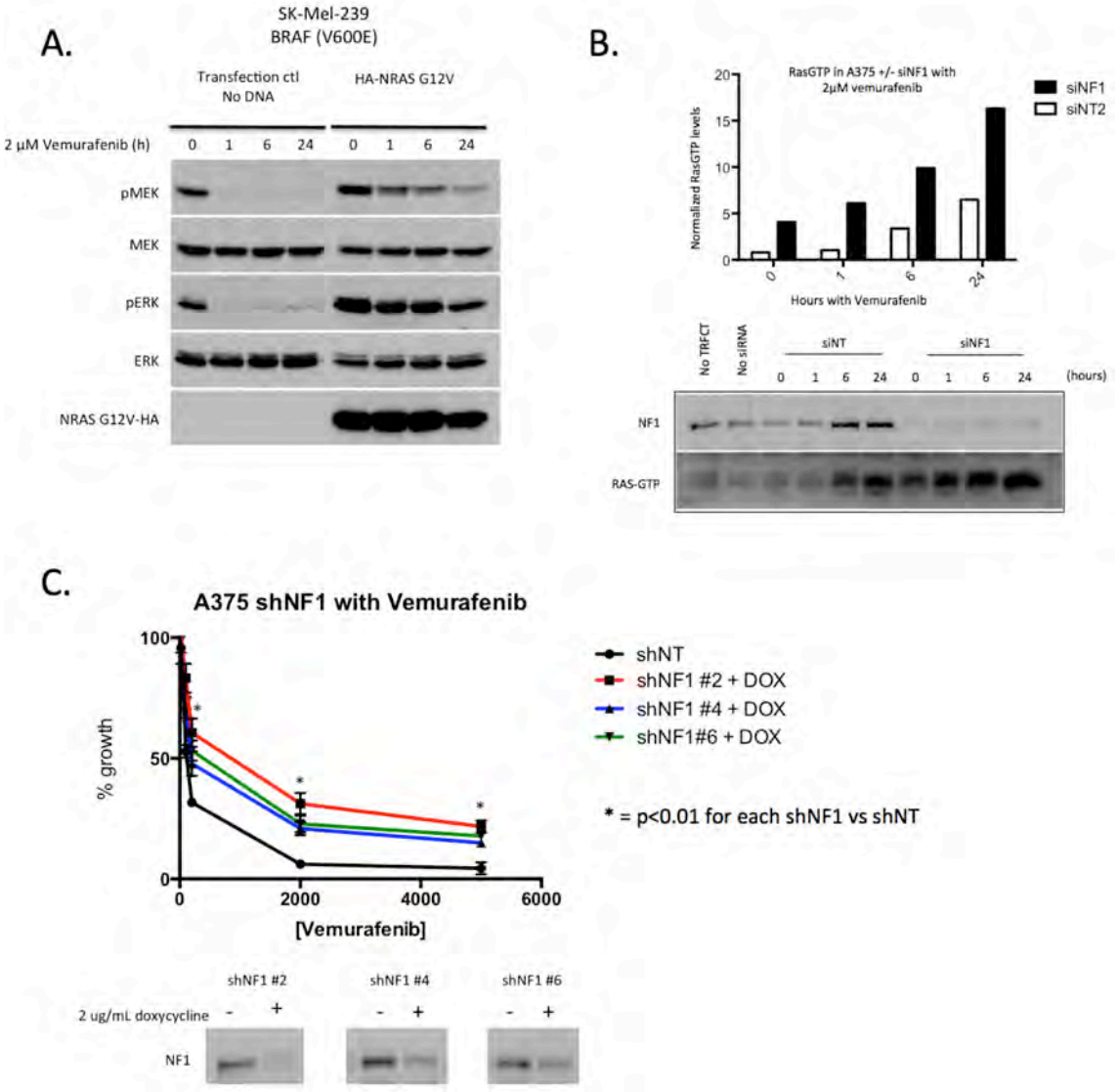

Figure 5.2 Activation of RAS confers resistance of an otherwise sensitive BRAF(V600E) melanoma cell line to vemurafenib. A) SK-Mel-239 cells were treated with 2μM vemurafenib for 0, 1, 6, or 24 hours after transfection of constitutively active NRAS(G12V) or a no-DNA transfection control. MAPK pathway effectors were assessed by immunoblot. B) Quantification of activated RAS protein (RAS-GTP) in a BRAF(V600E) mutant melanoma cell line (A375) with or without knockdown of NF1 by siRNA and treatment with 2μM vemurafenib. RAS-GTP values normalized to no-siRNA transfection control (TRFCT=transfection). C) Cell proliferation on day 4 of treatment with increasing concentrations of vemurafenib (nM) in BRAF mutant A375 after one week of NF1 knockdown with three different shRNAs under a tet-on promoter, or shNon-Targeting (shNT) control. Error bars are SEM, n=3.

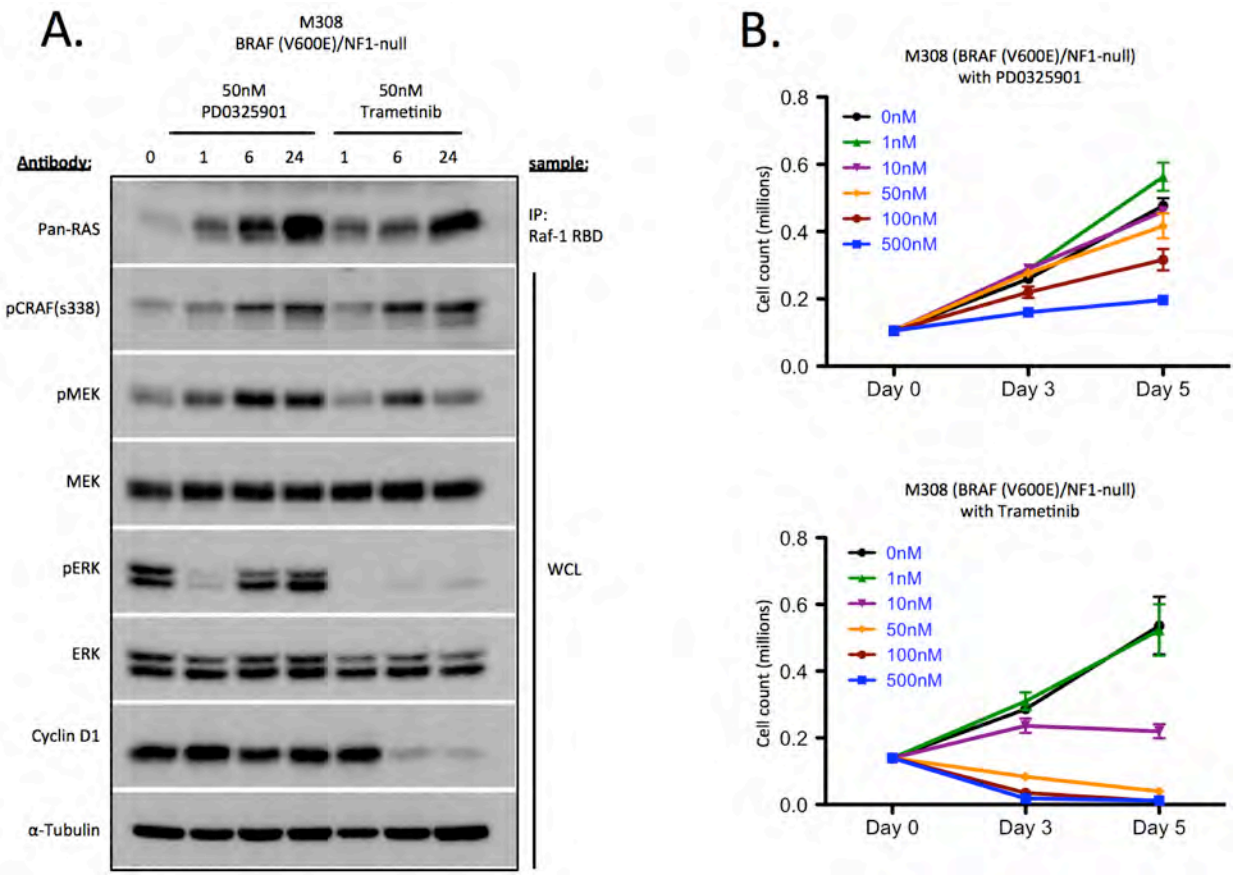

Figure 5.3 NF1 loss in the context of BRAF(V600E) mutation maintains sensitivity to potent MEK inhibition. A) Cells were treated with 50nM of the MEK inhibitors PD0325901 or trametinib for 0, 1, 6, and 24 hours. Activated RAS protein (RAS-GTP) was quantitated as has been described. Phospho- and total levels of MAPK pathway components were analyzed by immunoblot. B) Cells were treated with increasing concentrations of the MEK inhibitors PD0325901 or trametinib for 3 or 5 days. Results are cell count as a function of drug concentration over time. Error bars are SEM, n=3.

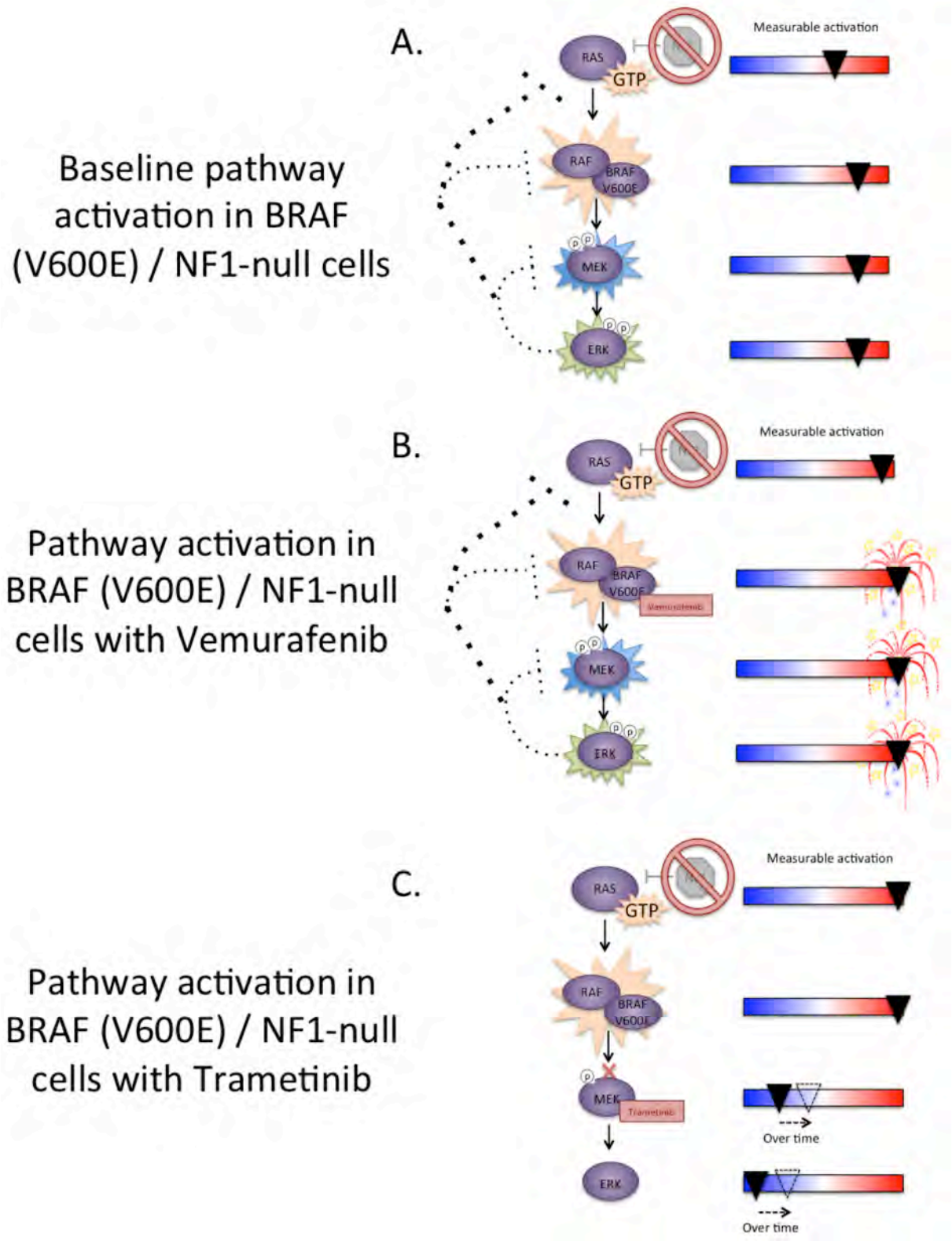

Figure 5.4 Model of measurable MAPK pathway activation with RAF and MEK inhibition in BRAF(V600E)/NF1-null cells. RAF inhibitors hyperactivate the MAPK pathway in BRAF(V600E)/NF1-null cells, while trametinib blocks the activation of the pathway leading to lowered pMEK and pERK levels.

Discussion

NF1 loss in the context of BRAF(V600E) mutation is sufficient to raise RAS-GTP levels despite high levels of negative feedback on RAS (see model in Fig. 5.4). This is a key finding of our study, as 1) data from the TCGA (see chapter 3) confirm overlap of BRAF(V600E) mutations and NF1 loss in human melanomas and 2) it suggests a mechanism of *de novo* and acquired resistance to RAF inhibition with vemurafenib and dabrafenib.

We have shown that M308, a melanoma cell line with NF1 loss and BRAF(V600E) mutation, is resistant to RAF inhibition with vemurafenib, suggesting that melanoma tumors that harbor both alterations will exhibit *de novo* resistance to RAF inhibition. This finding highlights the potential utility of prospective NF1 genotyping before the initiation of RAF inhibitor therapy in BRAF(V600E) patients, as these patients would be predicted to be intrinsically resistant to RAF inhibitors and may also have acceleration of tumor growth based on hyperactivation of the MAPK pathway. Importantly, this finding also applies to BRAF mutant melanoma patients with neurofibromatosis type 1, as RAF inhibitor therapy in such patients may also be intrinsically resistant to RAF inhibitors.

We also have shown that loss of NF1 in an otherwise vemurafenib sensitive BRAF(V600E) cell line can confer resistance to vemurafenib. This finding suggests that NF1 loss may be a mechanism of acquired resistance to RAF inhibitor therapy. Our RNAi studies also suggest that partial loss of NF1 function may result in a more pronounced and rapid restoration of RAS signaling following RAF inhibitor therapy. This could result in an attenuation of drug response sufficient to promote a more rapid emergence of drug resistant clones that harbor additional genetic alterations that decrease RAF inhibitor sensitivity.

Importantly, we have shown that though cells with both NF1 loss and BRAF(V600E) mutation are resistant to vemurafenib, they remain sensitive to MEK inhibition with trametinib. This finding suggests a potential treatment option for patients who have either become resistant to RAF inhibition or whose NF1 status prior to therapy precludes them from treatment with RAF inhibitors.

In summary, NF1 loss is a potential mechanism of resistance to RAF inhibition in BRAF(V600E) melanoma, whereas the MEK inhibitor trametinib may be effective therapeutic option in this patient population, a hypothesis that warrants clinical testing.

Chapter Six

NF1 loss may contribute to, but is insufficient for, melanoma formation

Mouse models are useful experimental tools for studying gene function and their association with human diseases. Various mouse models have been generated to study neurofibromatosis, neurofibromatosis-related malignancies, melanoma and other cancers. These models have aimed to recapitulate human diseases in order to better understand their pathogenesis and to develop novel therapeutic approaches.

The first mouse model of NF1 loss harbored a homozygous genetic deletion of *Nf1* exon 31. Numerous deleterious mutations found in neurofibromatosis patients are located within this exon suggesting it is critical for NF1 function (Brannan, Perkins et al. 1994). This $Nf1^{-/-}$ mouse exhibited embryonic lethality at E13.5 due to a cardiac defect known as double outlet right ventricle resulting from defective cardiac neural crest cells (Brannan, Perkins et al. 1994). The heterozygous $Nf1^{+/-}$ littermates survived to adulthood but were prone to developing with late onset several NF1-associated malignancies such as pheochromocytoma and myeloid leukaemia (Jacks, Shih et al. 1994). However, these mice did not develop neurofibromas, a cardinal NF1 manifestation.

To circumvent the embryonic lethality of the *Nf1*$^{-/-}$ mice, Cichowski et al. generated chimeric mice by transplanting *Nf1*$^{-/-}$ ES cells into *Nf1*$^{+/+}$ embryos (Cichowski, Shih et al. 1999). These mice did develop neurofibromas, but not the dermal neurofibromas that are most typical of the human disease. It remains unclear whether this was due to interspecies differences or whether the *Nf1*$^{-/-}$ ES cells failed to generate the cells of the dermis. Finally, Zhu et al. generated a conditional *Nf1*$^{-/-}$ mouse using the cre/loxP system, permitting tissue-specific knockout of *Nf1* exons 31 and 32 under the control of cre recombinase (Zhu, Romero et al. 2001).

Cichowski et al. later generated mice heterozygous for *Nf1* and *Tp53* in cis (on the same chromosome) or in trans (on opposite chromosomes) to study the progression of benign neurofibromas to malignancies (Cichowski, Shih et al. 1999). NPcis mice were prone to malignancies common in NF1, such as MPNST, while NPtrans mice developed malignancies more common among *Nf1*$^{+/-}$ or *Tp53*$^{+/-}$ mice. Loss of the second chromosome in both mice presumably led to the observed phenotypes, with *Nf1*$^{-/-}$*; Tp53*$^{-/-}$ cells generated in the NPcis mice and a heterozygous allele remaining in cells of the NPtrans mice.

Several mouse models of melanoma have also been developed. Bosenberg et al. generated a mouse with an inducible, melanocyte-specific cre recombinase (*Tyr::CreER*) (Bosenberg, Muthusamy et al. 2006). This mouse was crucial for studying the effects of temporally controlled, melanocyte-specific alterations. The *Tyr::CreER* mouse harbored the cre recombinase gene fused to an altered ligand binding domain of the estrogen receptor (CreERT2; shortened to CreER) (Indra, Warot et al. 1999). The fused protein allows for sensitive and specific binding of 4-hydroxytamoxifen (4-HT), but not estrogen, and subsequent nuclear localization of cre upon 4-HT binding (Indra, Warot et al. 1999, Bosenberg, Muthusamy et al. 2006). In this model, the *CreER* gene is under the control of the tyrosinase promoter, which is selectively activated in melanocytes and some neural crest-derived nerve and neuroepithelial cells (Delmas, Martinozzi et

al. 2003). Thus, this mouse expresses inactive cre in the melanocytes, which is activated only upon administration of 4-HT.

Another mouse that has proven useful in studies of the pathogenesis of cutaneous melanomas is the *BRaf*^{CA} mouse. Dankort et al. initially generated this mouse to study BRAF mutant lung cancer. *BRaf*^{CA} mice express a wild-type *BRAF* allele until exposure to cre recombinase, which then induces excision of the floxed wild-type exons 15-18 and the natural stop codon, allowing transcription of a mutated exon 15 containing the constitutively active (CA) V600E mutation and wild type exons 16-18. (Dankort, Filenova et al. 2007). This mouse allows for expression of BRAF(V600E) under its endogenous promoter only in the target cell of interest when crossed with a tissue-specific Cre.

In 2009, Dankort et al. reported on studies in which the *Tyr::CreER* mouse and the *BRaf*^{CA} mouse were bred to generate a conditional, melanocyte-specific BRaf(V600E) mouse (Dankort, Curley et al. 2009). Induction of BRaf(V600E) in melanocytes with topical 4-HT caused punctate hyperpigmented structures but not melanoma. This data corroborates with BRAF mutation being an early lesion in melanoma and causing oncogene-induced senescence as a single lesion (Pollock, Harper et al. 2003). However, inducing *Pten* deletion in the *BRaf*^{CA} mouse by crossing these mice to mice harboring loxP sites flanking exons 4 and 5 of *Pten* did lead to hyperpigmentation and invasive melanoma requiring euthanasia with a latency of 7 days and 25 days, respectively (Dankort, Curley et al. 2009). The *BRaf*^{CA}*; Pten*$^{flox/flox}$*; Tyr::CreER* mouse was one of the first to develop melanoma within the context of genetic alterations that model the human condition, and was a milestone in the development of more physiologic models of human melanoma.

We crossed several of these mouse models with the goal of generating a conditional, melanocyte-specific Nf1 knockout mouse in order to study the sufficiency of NF1 loss for melanomagenesis. We compared the phenotypes observed in these mice to those of the mouse models of melanoma previously discussed above. We found that Nf1 loss in melanocytes led to hyperpigmentation but not melanoma.

Results

We generated an *Nf1$^{flox/flox}$; Tyr::CreER* mouse to study the effects of somatic NF1 loss in melanocytes. At 8 weeks of age, the ears of *Nf1$^{flox/flox}$; Tyr::CreER* or *Nf1$^{flox/+}$; Tyr::CreER* mice were painted with 4-HT or DMSO and were photographed every other week to monitor for changes in pigmentation or the development of cutaneous tumors (Fig. 6.1A). At 8 weeks post-treatment, the recombined *Nf1* allele could be detected in the experimental mice (Fig. 6.1B). The ears of the *Nf1$^{flox/flox}$; Tyr::CreER* mice painted with 4-HT (experimental mice) were diffusely pigmented, while the ears of the *Nf1$^{flox/+}$; Tyr::CreER* mice and the ears of the mice painted with DMSO (control mice) remained unchanged (Fig. 6.1C). Histology showed an increase in pigmentation in the ears of the experimental mice as well as a small increase in melanocyte proliferation compared to control mice (number of melanocytes per frame). This phenotype emerged at 8 weeks post-treatment and developed in the remaining experimental mice over the course of the following 6 weeks (Fig. 6.1D). These mice were observed for 52 weeks and showed no punctate structures or tumors, and necropsy failed to find abnormalities other than the pigmentation phenotype in the ear.

Loss of p53 is observed in the progression from benign to malignant tumors in neurofibromatosis and is found co-altered with NF1 in melanoma tumors ((Brems, Beert et al. 2009) and Fig. 3.3). We therefore asked whether loss of NF1 with coalteration of p53 could synergize to induce the

development of invasive melanomas. At 8 weeks of age, the ears of *Nf1*$^{flox/flox}$*;Tp53*$^{flox/flox}$*;Tyr::CreER* mice were painted with 4-HT and were photographed every other week to monitor for changes (Fig. 6.2A). Similar to the *Nf1*$^{flox/flox}$*; Tyr::CreER* mice, at 8 weeks post-treatment, the ears of the *Nf1*$^{flox/flox}$*;Tp53*$^{flox/flox}$*;Tyr::CreER* mice and *Nf1*$^{flox/flox}$*;Tp53*$^{flox/+}$*;Tyr::CreER* mice painted with 4-HT were diffusely pigmented, while the ears of *Nf1*$^{flox/+}$*;Tp53*$^{flox/flox}$*;Tyr::CreER* mice painted with 4-HT were not (Fig. 6.2B). Like the *Nf1*$^{flox/flox}$*;Tyr::CreER* mice, histology showed an increase in pigmentation and a mild increase in melanocyte proliferation in the ears of the experimental mice. However, these mice did not show a difference in latency to pigmentation, degree of pigmentation or progression to melanoma when compared to the *Nf1*$^{flox/flox}$*;Tyr::CreER* mice, and thus were phenotypically similar. Latency to pigmentation is summarized in table 6.1.

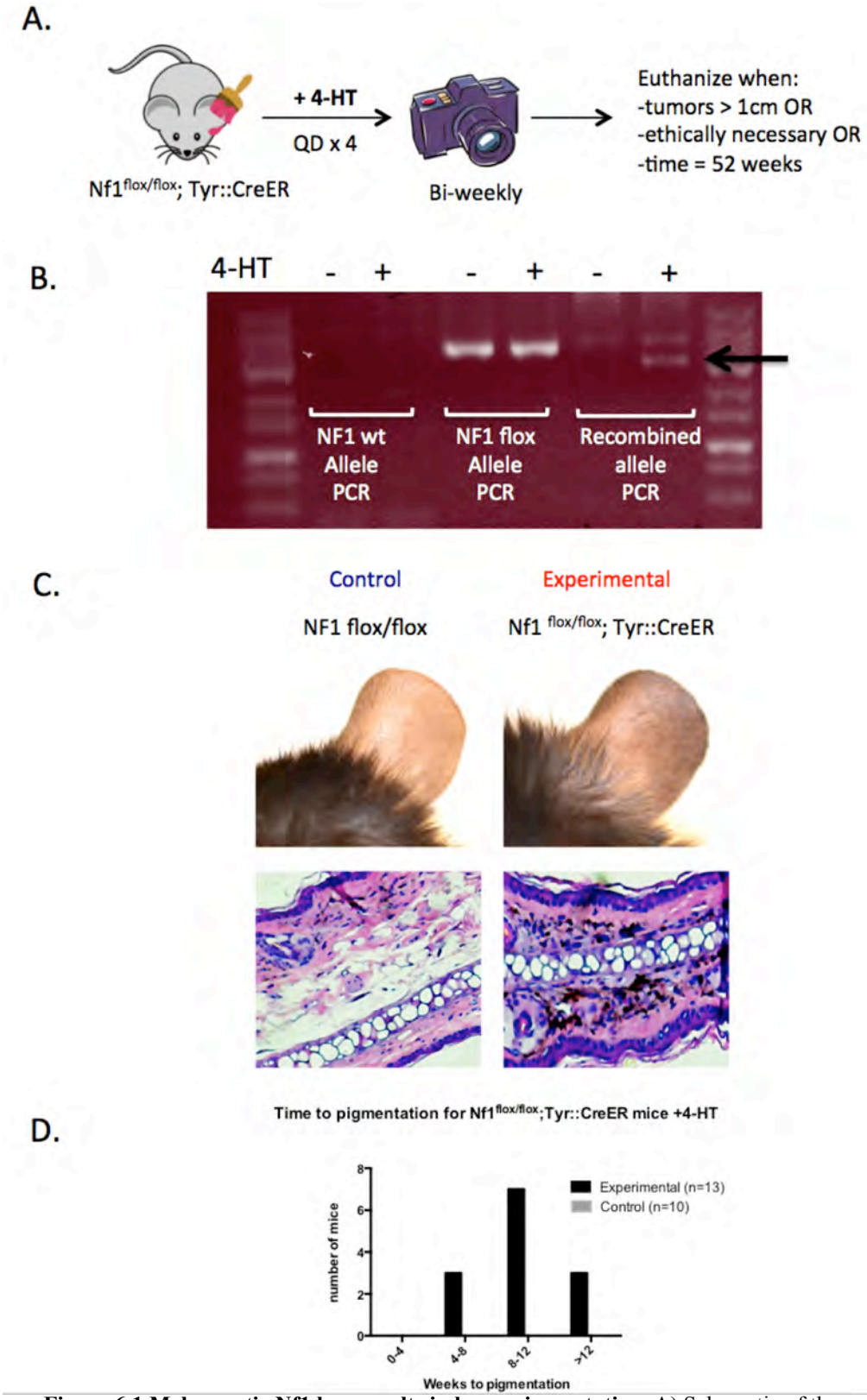

Figure 6.1 Melanocytic Nf1 loss results in hyperpigmentation. A) Schematic of the experimental design. B) PCR reactions specific for the WT, floxed and recombined alleles of NF1 in a mouse before and after 4-HT. C) Pigmentation phenotype in the ears of control and experimental mice (top). H&E staining of control and experimental ears (bottom). D) latency to pigmentation in the control and experimental mice.

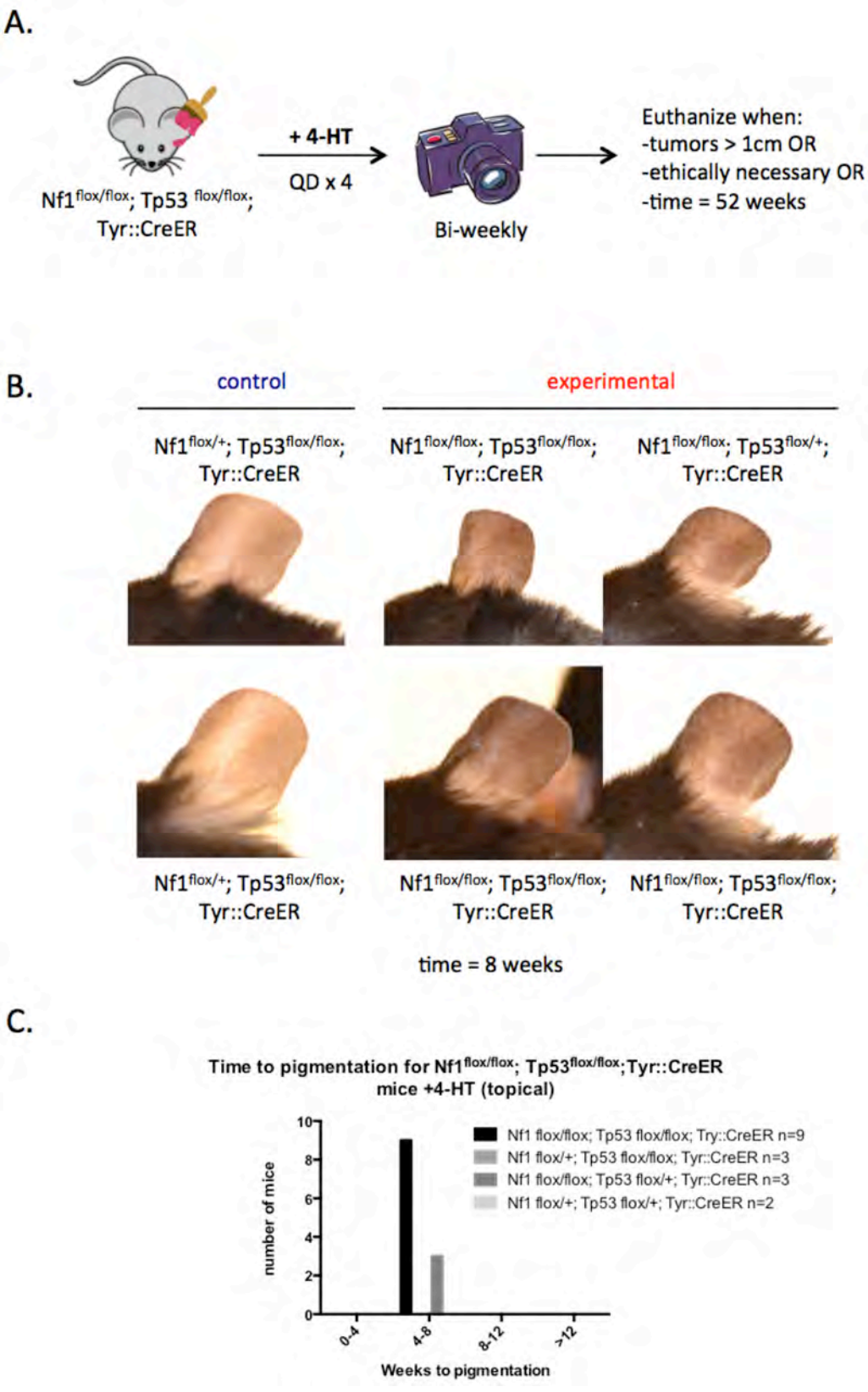

Figure 6.2 Melanocytic Nf1 and p53 loss from topical 4-HT results in hyperpigmentation. A) Schematic of the experimental design. B) Pigmentation phenotype in the ears of control and experimental mice. C) Latency to pigmentation in the control and experimental mice.

Table **6.1**: Time to pigmentation by genotype in 4-HT painted Nf1/p53 mice

Nf1	mouse number	Weeks to pigmentation	p53
Flox/Flox	1	8 weeks	Flox/Flox
Flox/Flox	2	8 weeks	Flox/Flox
Flox/Flox	3	8 weeks	Flox/Flox
Flox/Flox	4	8 weeks	Flox/Flox
Flox/Flox	5	8 weeks	Flox/Flox
Flox/Flox	6	8 weeks	Flox/Flox
Flox/Flox	7	8 weeks	Flox/Flox
Flox/Flox	8	8 weeks	Flox/Flox
Flox/Flox	9	8 weeks	Flox/Flox
Flox/Flox	10	8 weeks	Flox/Flox
Flox/Flox	11	8 weeks	+/Flox
Flox/Flox	12	8 weeks	+/Flox
Flox/Flox	13	8 weeks	+/Flox
+/Flox	14	---	Flox/Flox
+/Flox	15	---	Flox/Flox
+/Flox	16	---	Flox/Flox
+/Flox	17	---	+/Flox
+/Flox	18	---	+/Flox

Discussion

Unlike BRaf(V600E) expression, loss of Nf1 in melanocytes of mice did not lead to a phenotype of punctate melanocytic structures or nevi, but rather resulted in a broad hyperpigmentation and mild melanocyte hyperproliferation. This difference may be due to differences in the functions of these proteins in normal melanocytes. Congenital NF1 loss in patients with neurofibromatosis results in skin manifestations such as inguinal and axial freckling as well as large, pigmented café-au-lait spots, the latter resulting from a modest increase in melanocyte density, large melanosomes and hyperpigmentation (De Schepper, Boucneau et al. 2006). We speculate that loss of NF1 in melanocytes leads to activation of RAS to an amplitude that results in activation of the melanin biosynthetic pathway, such as through activation of MITF by MEK/ERK (Schiaffino 2010), but that MEK/ERK activation that occurs is insufficient to induce significant melanocyte proliferation. In contrast, the pathway activation resulting from BRAF(V600E) mutation may be of sufficient amplitude and/or duration that proliferation is stimulated before oncogene-induced senescence is triggered.

The pigmentation phenotype seen in the *Nf1*$^{flox/flox}$*; Tyr::CreER* mice treated with 4-HT is similar to histological changes seen in human café-au-lait spots and has lead the ears of these mice to be described as "café-au-lait ears" (personal communication, Jedd Wolchok, MSKCC). It is possible that our model of melanocytic NF1 loss is a model of human café-au-lait spots. However, the absence of melanosomes in the pigmented areas of the mice and the differences between melanocyte location in mice and humans (dermal melanocytes versus basal melanocytes, respectively) makes comparing the pigmented phenotype of NF1 loss in the mice with café-au-lait spots in human neurofibromatosis difficult (personal communication, Sebastien Monette, DVM, MVSc; MSKCC) (Reynolds 1954, De Schepper, Boucneau et al. 2006).

We did not see any differences in the phenotypes of *Nf1*$^{flox/flox}$*;Tp53*$^{flox/flox}$*;Tyr::CreER* mice treated with topical 4-HT compared to *Nf1*$^{flox/flox}$*;Tyr::CreER* mice, including severity or latency of the pigmentation phenotype. Notably, homozygous *Nf1* loss seems to be the only relevant factor for the pigmentation phenotype, as *Tp53* status did not change the phenotype, but presence of one copy of *Nf1* (*Nf1*$^{flox/+}$) was sufficient to prevent the phenotype. Though a PCR protocol was developed to detect the recombined *Tp53* allele, we could not confirm the excision of *Tp53* in the *Nf1*$^{flox/flox}$*;Tp53*$^{flox/flox}$*;Tyr::CreER* mice treated with 4-HT, thus cannot make firm conclusions as to the role *Tp53* does or does not play in this context. Inability to detect the recombined *Tp53* allele was likely due to the relatively small amount of tissue that we were able to collect for analysis and the low numbers of melanocytes in the skin, likely masking the recombined allele with DNA from surrounding cells.

We have done preliminary experiments treating the *Nf1*$^{flox/flox}$*;Tp53*$^{flox/flox}$*;Tyr::CreER* mice with systemic 4-HT in order to assess whether systemic administration has any impact on the melanocytes of the mice compared to topical administration. Mice that were *Nf1*$^{flox/flox}$ (n=8), regardless of *Tp53* status, developed pigmentation between 13 and 16 weeks post-4-HT administration, while *Nf1*$^{flox/+}$ mice did not (n=2). Systemic 4-HT treatment led to a more widespread hyperpigmentation phenotype than that observed with topical administration. Systemic 4-HT treatment affected the regions of murine skin in which the melanocytes reside in the basal layer of the surface epidermis, or surfaces where hair is mostly absent (tail, ear, etc) but not regions of the body where hair is abundant and melanocytes reside in the hair follicle (Reynolds 1954). Latency to hyperpigmentation was increased roughly two-fold in the *Nf1*$^{flox/flox}$*;Tp53*$^{flox/flox}$*;Tyr::CreER* mice treated systemically with 4-HT. This may be due to the differences in exposure of a given melanocyte to systemic versus topical 4-HT (time to peak concentration, duration of exposure), or may be due to the differences in how the 4-HT is prepared for intraperitoneal injection versus topical administration (see materials and methods).

Additionally the first two *Nf1*$^{flox/flox}$;*Tp53*$^{flox/flox}$;*Tyr::CreER* mice that were treated systemically with 4-HT developed poorly differentiated spindle cell neoplasms consistent with soft tissue sarcoma or amelanonic melanoma. These tumors arose at sites of injury or chronic inflammation (IP site, ear tag). Inflammation has been known to accelerate tumorigenesis, such as in the case of liver cirrhosis and hepatocellular carcinoma (Altekruse, McGlynn et al. 2009, Capone, Costantini et al. 2010). Additionally, studies of NF1 mouse models have shown that *Nf1*$^{-/-}$ Schwann cells do not lead to neurofibromas in an otherwise *Nf1*$^{+/+}$ mouse, despite being the accepted cell of origin for neurofibromas. Instead, neurofibromas only form when *Nf1*$^{-/-}$ Schwann cells are in *Nf1*$^{+/-}$ mice, suggesting a role for the microenvironment in the formation of these lesions (Zhu, Ghosh et al. 2002). While these results are preliminary and are being validated using a larger cohort of mice, they suggest that inflammation and factors in the microenvironment may cooperate with NF1 loss to induce melanoma.

Chapter Seven

Implications

We have found that NF1 loss occurs in a subset of melanoma tumors and is a functional event in melanoma cell lines. Even in the absence of BRAF or RAS alterations, melanoma cell lines that were NF1-null were dependent on the MAPK pathway for cellular proliferation and were sensitive to selective MEK inhibitors. The feedback-reactivation of the MAPK pathway resulting from MEK inhibition abrogated the antitumor effects of some MEK inhibitors in NF1 null melanoma cell lines. Trametinib, an inhibitor that abrogated this reactivation of MEK, was able to robustly inhibit both the MAPK pathway and cell proliferation in NF1 null cells, which may have clinical importance for the treatment of NF1-null melanoma tumors. When NF1 loss overlapped with BRAF(V600E) mutation, RAS-GTP was elevated and cells exhibited decreased sensitivity to RAF inhibitors. NF1 loss also decreased the sensitivity of BRAF(V600E) cells to some MEK inhibitors through the feedback reactivation of the pathway described above. Though NF1 loss was a functional event in melanoma, it was not sufficient to initiate melanoma development in an *Nf1$^{flox/flox}$;Tyr::CreER* mouse model. It may, however, synergize with other alterations, like loss of p53, for melanomagenesis. Many intriguing questions were raised by our results, providing opportunities for further investigation.

The function-to-alteration versus alteration-to-function approach

As discussed in the introduction, the discovery of other frequent melanoma alterations, such as BRAF and NRAS, were the results of direct sequencing efforts. In contrast, we used a functional approach to identify occult alterations in melanoma cell lines. NF1 is a large gene and thus it is likely that many of the missense mutations that have been reported in this gene are non-functional

passenger events. Additionally, the absence of paired normal DNA for our cell lines makes it impossible to know whether any particular missense mutation is a germline polymorphism. By approaching the $BRAF^{WT}/NRAS^{WT}$ cell lines with a functional screen, we were able to identify a subset of cells with a functional phenotype (RAS activation) then subsequently look for the genetic basis of this phenotype. A caveat of this approach is that RAS activation is qualitative and may vary slightly depending on serum conditions, timing of serum administration, and confluence of the cells, among other factors. However, in conjunction with next generation sequencing approaches, our functional approach may be an effective way to identify functional genomic events using cell line models. The amount of information generated by large screening and deep-sequencing efforts is likely going to expand enormously, and differentiating between driver and passenger alterations will remain an ongoing challenge.

Overlap of RAS and NF1 mutations

An unanswered question posed by this work is what selective benefit arises from NF1 loss in the context of NRAS mutation. Until now, previous work has suggested that the activation of mutant RAS is not dependent on or altered by GAP and GEF expression, suggesting that the cell would derive no additional benefit from having both an NRAS mutation as well as loss of NF1 (Scheffzek, Ahmadian et al. 1997). However, there are several possible explanations as to why these alterations are found concurrently.

Though this work focuses on RAS-related effects of NF1, NF1 may have additional RAS-independent functions in the cell. Evidence for this is abundant. The GRD makes up only 10% of the NF1 protein, suggesting that there may be non-GAP activities regulated by a portion of the remaining 90% (Hsueh 2012). NF1 has also been shown to interact with many other proteins, with putative roles being identified in the cAMP pathway, cell adhesion, and cytoskeletal

dynamics (Hsueh 2012). Thus, it is possible that NF1 loss has non-RAS related effects that provide a selective advantage to an NF1-null/NRAS mutant cell.

Another explanation may be that loss of NF1 activates wild-type RAS proteins in the cells and that this provides a selective advantage. Jeng et al. proposed that mutant RAS can activate wild-type RAS by binding to SOS, causing a conformational change, and leaving SOS in a position to activate wild-type RAS independently of RTK signaling (Jeng, Taylor et al. 2012). In this model, if a selective advantage is conferred to a cell by having both mutant and wild-type RAS activated, there may be further gain of fitness if NF1 was unavailable to downregulate the activity of wild-type RAS proteins. This hypothesis could be tested through knockdown of SOS in NRAS mutant NF1-null cells, or by further general study of SOS in the NF1-null context.

A third reason why NF1 loss may confer advantage to NRAS mutant cells involves the structure of RAS. As mentioned previously, RAS's downstream effectors and its GAPs compete for binding to switch I of RAS. Thus, neither mutant nor wild-type RAS can bind to both its effectors and its negative regulators simultaneously. This sets up a competition between proteins for the same RAS binding site. In normal cells, this competition is likely influenced by the proximity of each protein to RAS as well as RAS subcellular localization. In the case of mutant RAS, even though NF1 does not catalyze the GTPase reaction, the two proteins likely still bind to one another. This suggests that in NRAS mutant cells, NF1 may be competing with the RAF proteins for RAS binding, and may in this way limit the ability of mutant RAS to activate ERK signaling. Loss of NF1 would eliminate competition with RAF for RAS binding. This may maximize the effect of mutant RAS on activation of this key downstream signaling pathway, thus conferring a selective advantage. Studies using isogenic NRAS mutant cell lines with or without NF1 knockdown may help elucidate this question. Additionally, mouse models of concurrent RAS

mutation and NF1 loss may reveal whether the dual alterations have advantage *in vivo*, such as by leading to a more aggressive tumor phenotypes.

Overlap of BRAF and NF1 mutations

In addition to the overlap of NRAS mutation and NF1 loss seen in tumors and cell lines, we also see co-occurrence of BRAF mutations with NF1 loss. We address the effect of NF1 loss in BRAF(V600E) mutant cells in chapter 5. However, the majority of BRAF mutations that co-occur with NF1 loss in tumors are non-V600E mutations that fall in exon 11 of BRAF. In a 2004 study by Wan and colleagues, many of these non-V600E BRAF mutations were biologically characterized and found to be less transforming in *in vitro* transformation assays than wild-type BRAF, leading to their classification as "low-activity" BRAF mutations (Wan, Garnett et al. 2004). However, these mutants hyperactivate the MAPK pathway by dimerizing with and activating CRAF. We know that RAF dimerization is mediated by active RAS (Weber, Slupsky et al. 2001). Therefore, one could speculate that NF1 loss, with resulting RAS activation, would promote dimerization of these low-activity BRAF mutations, and thus ERK activation and transformation. Exploring the relationship between RAF dimers and NF1 loss would help provide evidence for this theory.

MEK phosphorylation and efficacy of MEK inhibitors

We have observed in NF1-null cell lines that increase in pMEK levels after treatment with some MEK inhibitors is associated with a decrease in efficacy of the MEK inhibitor. We hypothesize that the increase in RAF activity resulting from MEK inhibition leads to the increase in MEK phosphorylation, which results in this phosphorylated MEK being primed to activate ERK upon drug dissociation. This hypothesis is supported by data with trametinib, a MEK inhibitor that

abrogates phosphorylation of MEK by blocking the S217 phosphorylation site. Trametinib inhibits activation of ERK in NF1-null cells more potently than three other MEK inhibitors that do not prevent MEK phosphorylation at S217, which results in more potent inhibition of both ERK pathway output and cell proliferation.

However, other factors may account for the difference in potency among the MEK inhibitors over time. It is possible that MEK inhibitors may not bind as well to MEK protein that is phosphorylated on both of its activating serines (S217/S221) as they do to MEK protein that is monophosphorylated or unphosphorylated. Because trametinib prevents dual phosphorylation of MEK, it may bind with great efficiency to MEK, whereas MEK inhibitors that do not prevent dual MEK phosphorylation bind with lower potency over time as the pool of MEK in the cell is increasingly phosphorylated. However, many MEK inhibitors that are less efficacious in NF1-null cells in the face of MEK activation very potently inhibit ERK signaling in BRAF(V600E) cells. As BRAF(V600E) cells have very high levels of pMEK at baseline, this data would suggest that these inhibitors are capable of binding MEK even in the dually phosphorylated state and may suggest that this may not be the reason why the MEK inhibitors differ in their ability to maintain pathway inhibition.

A recent publication by Hatzivassiliou et al. comparing MEK inhibitors in BRAF mutant and KRAS mutant cell lines demonstrated that 1) the strength of hydrogen bond between the MEK inhibitor and S212 of MEK and 2) the effect of the MEK inhibitor on the stability of the RAF-MEK interaction was, at least in part, the basis for differences in the potency of MEK inhibitors in these different genetic contexts (Hatzivassiliou, Haling et al. 2013). These findings may also explain, in part, the differences we observed among the MEK inhibitors in the NF1-null context. Specifically, trametinib may prevent phosphorylation of S217 by stabilizing S212, and may thus act as a dominant negative for RAF by stabilizing the RAF-MEK complex, preventing RAF from

phosphorylating other MEK moieties. Testing the validity of this hypothesis may elucidate why some MEK inhibitors are effective and some ineffective in RAS-active cells, and may aid in the development of newer inhibitors that are more effective treatment options for patients with such alterations.

NF1, pigmentation, and function in melanocytes

As mentioned previously in chapter 6, it is possible that NF1's role in melanocytes is to regulate melanin biosynthesis, as the MAPK pathway can regulate MITF, and MITF can regulate tyrosinase and other melanin-associated genes (Murisier, Guichard et al. 2007). The hyperpigmentation observed with NF1 loss in our mouse model is one piece of evidence to support this hypothesis. The hyperpigmentation phenotype seen in the café-au-lait spots characteristic of neurofibromatosis is another piece of supporting evidence. Additional evidence comes from personal communication with Dr. Kaleb Yohay, head of the neurofibromatosis clinic of Weill Cornell Medical College. Dr. Yohay noted that parents of NF1 patients mention that their children with NF1 tan much easier than other members of their family. This again suggests that NF1 may regulate the melanin biosynthesis pathway in melanocytes.

An additional question of interest is whether or not people with neurofibromatosis type 1 are at an increased predisposition to melanoma. We attempted to conduct an IRB-approved survey of neurofibromatosis type 1 patients to assess the incidence of melanoma in this population. However, the response to the survey was low and we were unable to make conclusions based on the collected data. We also looked for patients on melanoma services at MSKCC and the Abramson Cancer Center at the University of Pennsylvania who also had neurofibromatosis. We identified four patients with metastatic melanoma and a diagnosis of neurofibromatosis type 1. Interestingly, three of these patients were of African American descent, while one of these

patients was of Hispanic descent. Though these numbers are small and may simply reflect the overall patient population seen at these clinics, if neurofibromatosis patients of ethnicities associated with higher levels of pigmentation are more susceptible to melanoma than those with less pigmentation, this might indicate a mutually antagonistic effect of NF1 loss, melanin biosynthesis, and aberrant cell proliferation. To date, no studies have been conducted to investigate this possibility, and the relationship of NF1 and pigmentation remains an outstanding question that would be an interesting area of future investigation.

Deregulation of feedback—NF1/SPRED

As discussed in chapter 4, MAPK pathway feedback plays an important role in NF1-null melanoma cell behavior. In normal growth conditions, the measurable levels of activated MAPK pathway components reflect both the growth signals being sent through the pathway and the negative regulation of these components by feedback elements. In essence, this is the same way a thermostat works. We assume that expression levels of feedback regulators, such as DUSPs, SROUTYs and SPREDs, reflect the actual level of feedback experienced by the pathway. However, an instrumental finding by Stowe et al. suggests that the SPRED proteins negatively regulate the MAPK pathway by recruiting NF1. This provides an explanation why Legius syndrome, or congenital mutation of the SPRED genes, has a similar phenotype to neurofibromatosis type 1 (Stowe, Mercado et al. 2012). Therefore, it is likely that NF1-null cells exhibit some degree of resistance to feedback from the SPREDs. Whether this affects the NF1-null cells' ability to modulate their pathway output or whether it contributes to the activating phenotype of NF1 loss remains to be determined.

Inflammation in NF1-- the microenvironment

An important modulator of tumorigenesis is the microenvironment of the tumor. The microenvironment includes the stromal cells, oxygen conditions and nutrient conditions present at the tumor site (Tlsty and Coussens 2006, Joyce and Pollard 2009). Many studies have demonstrated the contribution of non-tumor cells to the malignant phenotype, whether through transformation, drug resistance or metastasis (Gocheva, Wang et al. 2010, Straussman, Morikawa et al. 2012, Lujambio, Akkari et al. 2013). This includes the contribution of immune cells recruited to sites of inflammation. Thus, it is important to evaluate both the cell-autonomous and cell non-autonomous aspects of tumorigenesis.

An intriguing question that we are currently exploring is whether melanoma formation in *NF1*$^{-/-}$*; Tp53*$^{-/-}$ melanocytes is mediated by or dependent on inflammation, as our early preliminary data discussed above suggests that tumors in *Nf1*$^{flox/flox}$*;Tp53*$^{flox/flox}$*;Tyr::CreER* mice occur at sites of injury or chronic inflammation. Literature supports the possibility that the microenvironment plays a role in NF1-associated disease. For example, neurofibromas in NF1 patients multiply and enlarge during pregnancy and puberty, suggesting a cell non-autonomous contribution to *NF1*$^{-/-}$ cell proliferation (Viskochil, White et al. 1993). In another study, the microenvironment of *Nf1*$^{-/-}$ Schwann cells affected whether or not these cells formed neurofibromas in mice; *Nf1*$^{+/+}$ mice with *Nf1*$^{-/-}$ Schwann cells failed to form neurofibromas, while *Nf1*$^{+/-}$ mice with *Nf1*$^{-/-}$ Schwann cells did form neurofibromas (Zhu, Ghosh et al. 2002). This study suggested a role for a stromal contribution to the initiation of these lesions, which was later identified as mast cell recruitment and activation (Ingram, Yang et al. 2000). Mast cells, cells of the innate immune system, are important for wound healing and the inflammatory process (Prussin and Metcalfe 2003) which suggests that the immune system may play a role in neurofibroma formation in the *NF1*$^{-/-}$ context. A third study which examined cell non-autonomous factors contributing to the *NF1*$^{-/-}$ phenotype showed that injury of *Nf1*$^{-/-}$ nerves in mice resulted in increased pERK signaling and promoted

tumor formation at the injury site (Ribeiro, Napoli et al. 2013). These studies suggest that the microenvironment, particularly the presence of inflammation, may cooperate with loss of NF1 to induce tumor formation.

The possibility that NF1 loss cooperates with the microenvironment, particularly inflammation, to promote tumor initiation suggests a number of potential follow-up studies. Crossing the *Nf1*$^{flox/flox}$*;Tp53*$^{flox/flox}$*;Tyr::CreER* mice with mice harboring various immunodeficiencies may help elucidate which cells or cell systems are contributing to pathogenesis. Co-culturing the NF1-null melanoma cell lines with stromal cell lines, such as was done by Straussman et al., might help elucidate other factors that cooperate with NF1 loss to promote transformation. Alternatively, co-culture of stromal cells with *NF1*$^{-/-}$*; p53*$^{-/-}$ MEFs may help elucidate what factors are needed to promote transformation these cells. Finally, the importance of inflammation and the immune system to NF1-pathogenesis opens up potential treatment options for the NF1 patient population. Prophylactic anti-inflammatory regimens may show benefit in preventing advancement of NF1-pathologies. Additionally, immunotherapies either alone or in combination with MAPK pathway inhibitors may show benefit in NF1-associated malignancies by exploiting the immune cell-rich microenvironment of the tumor.

In conclusion, our work has identified a class of melanomas that are wild-type for BRAF and NRAS but with activation of RAS and the MAPK pathway through loss of NF1. We have shown that these tumors are dependent on the MAPK pathway for cell proliferation, but may be resistant to MEK inhibitors through feedback reactivation upstream of MEK. NF1 loss may be a mechanism of resistance to RAF inhibitors in patients, and may preclude the use of RAF inhibitors in the NF1 patient population. Though NF1 loss causes hyperpigmentation in melanocytes, it is not sufficient for melanomagenesis. NF1 loss likely cooperates with other alterations, such as loss of p53, and possibly cell non-autonomous factors in the

microenvironment to initiate and promote tumorigenesis. We hope this work will be a foundation for future NF1 and melanoma research that will lead to effective treatments for melanoma patients and patients suffering from neurofibromatosis type 1 and its associated malignancies.

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