RAS SIGNALING AND THERAPEUTIC RESISTANCE IN MELANOMA

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ABSTRACT

BINGYING ZHOU: RAS signaling and therapeutic resistance in melanoma
(Under the direction of Adrienne D. Cox)

Increasing appreciation of the complexity of RAS signaling in cancer has led to a renewed wave of RAS research. I have focused on two key areas: the role of wild-type RAS isoforms in RAS-mutant cancers, and mechanisms of resistance to molecularly targeted therapies directed against RAS effector pathways. Melanoma, the most aggressive form of skin cancer, presents an excellent model to study RAS signaling; ~90% of melanomas have a driver mutation in NRAS (26%) or BRAF (63%), thus hyper-activating the canonical RAS-RAF-MEK-ERK effector signaling pathway. An army of small molecule inhibitors has emerged to target this pathway, and several are FDA-approved for melanoma treatment. However, all targeted therapies face the challenge of resistance. Most validated mechanisms of resistance to these inhibitors involve either reactivation of ERK signaling or other bypass routes that result in cancer cell survival.

In my investigation of resistance mechanisms in BRAF-mutant melanoma. I found that CK2α was sufficient to drive resistance to inhibitors of BRAF (BRAFi) and of MEK (MEKi). CK2α facilitated rebound of ERK phosphorylation in the presence of BRAFi, and maintained ERK phosphorylation upon treatment with MEKi. Surprisingly, by using a kinase-inactive mutant of CK2α, I showed that RAF-MEK inhibitor resistance did not rely on CK2α kinase catalytic function. That both wild-type and kinase-inactive CK2α bound equally well to the RAF-MEK-ERK scaffold KSR1 suggested that CK2α increases KSR facilitation of ERK phosphorylation. Accordingly, CK2α did not cause resistance to direct inhibition of ERK by the ERK1/2-selective

inhibitor SCH772984. These findings support a new mechanism whereby a kinase-independent scaffolding function of CK2α promotes resistance to RAF- and MEK-targeted therapies.

Another pressing issue is understanding the biological activities of wild-type RAS isoforms in RAS-mutant tumors. Most studies have investigated KRAS-mutant cancers, but little is known about NRAS-mutant cancers. NRAS-mutant melanomas comprise the second largest subgroup of melanoma patients, and no targeted therapy is approved for these patients. Exploring the roles of wild-type RAS isoforms in NRAS-mutant melanoma cells, I found that WT KRAS is essential for their proliferation and survival. Interestingly, depletion of KRAS resulted in a unique cellular morphology and in signaling outcomes distinct from those due to silencing of NRAS or HRAS. Moreover, KRAS knockdown stabilized p53 protein, which was accompanied by an increase in p53 target genes. Intriguingly, by using reverse phase protein array analysis, I found that KRAS knockdown severely impaired phosphorylation of ribosomal protein S6, dependent on S6K1 (p70 S6K) activity, but independent of Akt-mTOR, or ERK-p90RSK activity. These results may shed light on the potential efficacy of pan-RAS inhibition in NRAS-mutant cancers.

Together, my findings uncover a novel kinase-independent scaffolding function of CK2α in promoting resistance to inhibitors of BRAF and MEK in BRAF-mutant melanoma; highlight the critical importance of WT KRAS in NRAS-mutant melanoma; and describe the unique role of WT KRAS in maintaining phosphorylation of ribosomal protein S6. How CK2α engages in scaffolding to maintain ERK signaling, and how KRAS circumvents Akt-mTOR to regulate ribosomal protein S6, are issues to be addressed in future studies of RAS signaling.

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LIST OF ABBREVIATIONS AND SYMBOLS

2D	two-dimensional
3D	three-dimensional
4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1
AKT	protein kinase B
AML	acute myeloid leukemia
Arf	ADP ribosylation factor
ATCC	American Type Culture Collection
C-terminus	carboxyl-terminus
CAAX	cysteine-aliphatic-aliphatic-unconserved amino acid
Cdc42	cell division cycle 42 small GTPase
cDNA	complementary deoxyribonucleic acid
Chk1	checkpoint kinase 1
CK1	casein kinase 1
CK2	casein kinase 2
COSMIC	catalogue of somatic mutations in cancer
CRD	cysteine-rich domain
DMEM-H	high glucose Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DSS	dextran sulfate sodium
DUSP	dual-specificity phosphatase
DUSP5	dual-specificity phosphatase 5
DUSP6	dual-specificity phosphatase 6
EGFR	epidermal growth factor receptor

eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinase
Ets	E-twenty six transcription factor
FBS	fetal bovine serum
FDA	Food and Drug Administration
FGFR	fibroblast growth factor receptor
FLAG	an octapeptide fusion tag consisting of eight amino acids, DYKDDDDK
Flt3	Fms-Related tyrosine kinase 3
FRS2	fibroblast growth factor receptor substrate 2
FTI	farnesyltransferase inhibitor
GAP	GTPase-activating protein
GDP	guanine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GI_{50}	growth inhibitory 50, concentration of drug to cause 50% reduction in proliferation
GIST	gastrointestinal stromal tumor
Grb2	growth factor receptor-bound protein 2
GSK-3	glycogen synthase kinase 3
GTP	guanine triphosphate
GTPase	guanosine triphosphatases
h	hour
HA	hemagglutinin
HRAS	Harvey rat sarcoma viral oncogene homolog
HVR	hypervariable region
IQGAP1	IQ motif-containing GTPase activating protein 1
JNK	c-Jun N-terminal kinase

kDa	kilodalton
KRAS	Kirsten rat sarcoma viral oncogene homolog
KSR1/2	kinase suppressor of RAS 1/2
LCCC	Lineberger Comprehensive Cancer Center
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
min	minute
MKK4	MAP kinase kinase 4
ml	milliliter
MP1	MEK partner 1
mRNA	messenger ribonucleic acid
Mst1	macrophage stimulating 1
mTOR	mechanistic target of rapamycin or mammalian target of rapamycin
mTORC	mTOR complex
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
Myc	v-Myc avian myelocytomatosis viral oncogene homolog
NF1	neurofibromin 1
NHM	normal human melanocytes
NRAS	neuroblastoma rat sarcoma viral oncogene homolog
NSCLC	non-small cell lung cancer
N-terminus	amino-terminus
NT	non-targeting
p90RSK	p90 ribosomal kinase
PAK	p21-activated kinase
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor

PDGFR	platelet-derived growth factor receptor
PDK1	phosphoinositide-dependent protein kinase1
PI3K	phosphatidylinositol 3-kinase
PI	propidium iodide
PIP2	phosphatidylinositol-4,5-bisphosphate
PIP3	phosphatidylinositol-3,4,5-trisphosphate
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PLCε	Phospholipase C epsilon
PTEN	phosphatase and tensin homolog
PVDF	polyvinylidene difluoride
Rab	rat brain small GTPase
Rac	Ras-related C3 botulinum toxin substrate
Raf	rapidly accelerated fibrosarcoma kinase
Ral	RAS-like
RalA	RAS-like A
RalB	RAS-like B
RalBP1	Ral-binding protein 1
RalGDS	Ral guanine nucleotide dissociation stimulator
RalGEF	Ral guanine nucleotide exchange factor
Ran	RAS-related nuclear protein
RAS	rat sarcoma viral oncogene homolog
RASSF	RAS-association domain family
RB1	retinoblastoma 1
RBD	RAS binding domain
RECIST	Response Evaluation Criteria In Solid Tumors

Rho	Ras homology
RhoA	Ras homology A
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
RPPA	reverse phase protein array
rpS6	ribosomal protein S6
RTK	receptor tyrosine kinase
qPCR	quantitative polymerase chain reaction
S6K1	p70 ribosomal S6 kinase 1
S6K2	p70 ribosomal S6 kinase 2
SEER	Surveillance, Epidemiology, and End Results
SEM	standard error of the mean
SDS	sodium dodecyl sulfate
SH2	Src homology 2
SHP2	SH2-domain-containing protein tyrosine phosphatase-2
shRNA	short hairpin ribonucleic acid
SOS	son of sevenless
SPRY	Sprouty
STAT3	signal transducer and activator of transcription-3
SV40	simian vacuolating virus 40
$t_{1/2}$	half-life
TBK1	TANK-binding kinase 1
TBST	Tris buffered saline with Tween-20
TCF	Tissue Culture Facility
TCGA	The Cancer Genome Atlas
TEY motif	threonine-glutamic acid-tyrosine motif

Tiam1	T-cell invasion and metastasis gene 1
TPA	12-O-tetradecanoylphorbol-13-acetate
TRC	The RNAi Consortium
VEGFR	vascular endothelial growth factor receptor
WT	wild-type
γH2AX	H2AX phosphorylated on Ser139
μm	micron
μM	micromolar
μl	microliter

CHAPTER I: INTRODUCTION

RAS is a small GTPase

RAS (rat sarcoma viral oncogene homolog) genes were initially identified in acute transforming retroviruses as genetic sequences responsible for their oncogenic properties (Barbacid, 1987). The identification of mutationally activated human RAS genes as potently transforming oncogenes in several contemporary studies (Der et al., 1982; Goldfarb et al., 1982; Parada et al., 1982; Pulciani et al., 1982; Santos et al., 1982; Shih and Weinberg, 1982) has spawned decades of extensive research into RAS structure, biochemistry, biology, and pathobiology (Cox and Der, 2010; Malumbres and Barbacid, 2003).

RAS proteins are the founding members of the Ras superfamily of small guanosine triphosphatases (GTPases) that comprises over 150 members, which are divided into five major subfamilies, namely Rab, Ras, Arf, Rho, and Ran (Wennerberg et al., 2005). They are a conserved class of low molecular weight (~ 21 kDa), monomeric G-proteins that serve as molecular switches in coupling extracellular cues to a variety of intracellular signaling events, to regulate a plethora of fundamental cellular processes, including differentiation, proliferation, survival, adhesion, migration, vesicular trafficking, nuclear-cytoplasmic transport, *etc* (Cox and Der, 2010). Small GTPases cycle between a GTP-bound, active state, and a GDP-bound, inactive state. RAS proteins possess low intrinsic GTP hydrolyzing and guanine nucleotide exchange activities. In cells, these processes are accelerated by GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs), respectively. In resting cells, RAS is predominantly GDP-bound. Extracellular stimuli, such as growth factor stimulation, activate

GEFs, thereby promoting nucleotide (GDP) release from RAS. Due to the ten-fold higher intracellular concentrations of GTP than GDP, RAS transiently binds to GTP, after which GAPs quickly return RAS to its resting state (Figure 1.1). Structural studies have uncovered two critical stretches of RAS that undergo significant conformational changes when RAS cycles between GTP- and GDP-bound states. Termed switch I (amino acids 30-38) and switch II (amino acids 60-76), these regions are also crucial to RAS interaction with its regulators and effectors. Oncogenically activating mutations in RAS, predominantly occurring at residues G12, G13, and Q61, impair both intrinsic and GAP-mediated GTP hydrolysis, rendering mutant RAS constitutively active (Cox et al., 2014). Analysis of the COSMIC (catalogue of somatic mutations in cancer) database reveals that somatic mutations in the three RAS genes are the most frequent mutations of oncogenes in human cancers (Cox et al., 2014). In the following section, the specific mutational profiles of the RAS isoforms will be introduced.

RAS isoforms are structurally similar but functionally distinct

The human genome harbors three RAS genes (*HRAS*, *KRAS*, and *NRAS*) at entirely different chromosomal locations. *HRAS*, located on chromosome 11, was the first characterized isoform, and dominated RAS research in the early years. The underlying assumption was that RAS proteins are functionally redundant and thus interchangeable, which turned out to be an oversimplification (Malumbres and Barbacid, 2003; Reddy et al., 1982; Tabin et al., 1982; Taparowsky et al., 1982). *KRAS*, residing on chromosome 12, encodes two splice variants, KRAS4A and KRAS4B, the latter of which had been regarded the major isoform expressed in human cells. A recent study by Tsai *et al*. showed that KRAS4B can be expressed at comparable levels as KRAS4B (Tsai et al., 2015). A third RAS isoform, previously not identified in any retrovirus studies, came to light a year after *KRAS*, and was designated *NRAS* (chromosome 1), because it was discovered in human neuroblastoma-derived DNA (Hall et al., 1983; Shimizu et al., 1983; Taparowsky et al., 1983). RAS proteins share 82-90% sequence

identity, differing only at their C-termini (hypervariable regions), which are critical for their respective lipid modifications, and hence for their membrane targeting and subcellular localizations (Cox et al., 2014) (Figure 1.2).

Although highly similar in structure, the RAS isoforms have been shown to carry out overlapping but still very distinct functions (Castellano and Santos, 2011; Newlaczyl et al., 2014). Genetic knockout studies in mice suggested that KRAS is the most important isoform during development, since *Kras*-ablated mice die during embryogenesis (Johnson et al., 1997; Koera et al., 1997). In contrast, *Nras* gene function was shown to be dispensable for normal mouse development, growth, and fertility (Umanoff et al., 1995), yet was later found to be important for antiviral immune response and T-cell function in mice (Perez de Castro et al., 2003). Neither did abrogration of *Hras* result in any developmental defects (Esteban et al., 2001). However, it reduced the numbers of papillomas formed compared with wild-type littermates after 20 weeks of 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment (Ise et al., 2000). Strikingly, $Hras^{(-/-)}/Nras^{(-/-)}$ double knockout mice were viable, and displayed normal development, growth, fertility, and neuronal development (Esteban et al., 2001). Both wild-type (WT) NRAS and KRAS were found to be required for SV40 TAg-induced transformation in mouse embryonic fibroblasts, during which they performed unique functions by engaging different signaling pathways (Fotiadou et al., 2007). Specifically, wild-type NRAS regulated adhesion through RAF and RhoA, whereas KRAS coordinated motility through AKT and Cdc42.

In addition to the divergence of their physiological roles, there is also a huge body of evidence to suggest different oncogenic potentials and signaling preferences of the mutant RAS isoforms, which seem highly context-dependent. For example, in Rat-2 and NIH 3T3 fibroblasts, transformation assays showed that a G12 mutant of HRAS was more transforming than the corresponding NRAS or KRAS mutants. By contrast, in the human multipotent haematopoietic cell line, TF-1, $NRAS^{G12mut}$ exhibited greater biological activity (Maher et al., 1995). Importantly, these observations were not due to differences in protein expression or

stability, suggesting tissue-specific regulatory components as major players in shaping signaling outcomes. Another study compared the effects of the effector loop mutation P24G in RAS isoforms in the context of the G12V activating mutation. As expected, P24G diminished both RAF-MEK-ERK and PI3K-AKT pathway activity. However, whereas $HRAS^{G12V/P34G}$ retained its ability to induce transformation of NIH 3T3 cells and to activate RalGDS-RalA and Rac signaling, both $NRAS^{G12V/P34G}$ and $KRAS^{G12V/P34G}$ did not (Oliva et al., 2004). A comparison of leukemogenic potentials between $NRAS^{G12D}$, $KRAS^{G12D}$ and $HRAS^{G12V}$ in a bone marrow transplantation mouse model revealed different strengths and distinct phenotypes induced by different RAS mutants (Parikh et al., 2007). It was suggested that differential PI3K-AKT pathway activation underlies the phenotypic differences among the RAS isoforms. In mice conditionally expressing G12D mutants of KRAS or NRAS, $KRAS^{G12D}$ led to hyperproliferation of the colon epithelium in a MEK-dependent manner. In comparison, $NRAS^{G12D}$ did not affect growth of the epithelium, but conferred resistance to dextran sodium sulfate (DSS)-induced apoptosis (Haigis et al., 2008). Further analysis showed that NRAS formed a signaling complex with RAF-1 and STAT3, which activates STAT3 signaling independent of ERK activity (Wang et al., 2013c). In immortalized, non-transformed Ink4a/Arf deficient melanocytes, $NRAS^{G12V}$ exhibited superior tumorigenicity compared to $KRAS^{G12V}$, which was attributed to AKT activation that prevents GSK-3-mediated phosphorylation of Myc at T58 (Whitwam et al., 2007), a site commonly associated with proteasomal degradation of Myc protein. A retrospective analysis to assess the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer in >1,000 human tumor DNA samples concluded that, while $KRAS^{mut}$ has a negative effect on outcome, NRAS mutations are significantly associated with a low response rate (De Roock et al., 2010).

Another piece of compelling evidence that the three RAS genes are unique is the biased frequency and distributions of RAS mutations in human cancers. Analysis of the current COSMIC database (http://cancer.sanger.ac.uk/cosmic) (Cox et al., 2014) revealed that HRAS,

the isoform that had attracted most attention in the early RAS years, is in fact the least frequently mutated isoform (3%) in human cancers. KRAS, on the contrary, is the most commonly mutated isoform (86%), and is almost the exclusive isoform mutated in some of the most deadliest cancers, such as pancreatic ductal adenocarcinoma and lung adenocarcinoma. NRAS is the second most frequently mutated isoform in human cancer (11%), and has been, for both historical reasons and its low occurrence in the most dreaded cancers, the "neglected" isoform in most RAS studies to date. In addition to the overall mutation frequencies of RAS isoforms, there are two other mutational biases. One is the preferential mutation of a specific isoform in a given disease. For example, although rare in all cancers, HRAS is the predominantly mutated isoform in bladder cancer (57%) and in head and neck squamous cell carcinoma (86%). NRAS, which is rarely found in pancreatic ductal adenocarcinoma or lung adenocarcinoma, is the major oncogenic isoform in cutaneous melanoma (94%) and acute myeloid leukemia (59%). The other bias is a codon-specific mutation signature. As alluded to earlier, most RAS mutations (98%) occur at codons G12, G13, or Q61. While 83% of KRAS mutations occur at G12, 62% of NRAS mutations occur at Q61. Yet in cutaneous melanoma, Q61 mutations account for 87.5% of all NRAS mutant cases (Burd et al., 2014). These observations are strong evidence for distinct functionalities of RAS isoforms.

The roles of wild-type RAS in RAS-mutant cancers remain controversial

While the major focus of RAS research is still on oncogenic RAS, emerging studies are casting more emphasis on the interplay between oncogenic and wild-RAS isoforms in tumor development and maintenance. In general, wild-type RAS studies are divided into two categories: 1) studies of the wild-type counterpart of the oncogenic RAS isoforms, and 2) studies of the remaining two wild-type RAS isoforms in the presence of the oncogenic isoform. The latter will be a focus of this dissertation.

Studies of the functions of wild-type RAS began not long after the characterizations of oncogenic RAS. Those early studies primarily focused on the wild-type counterpart of the mutant isoform, and most concluded tumor-suppressive roles for wild-type RAS. For example, Spandidos and colleagues reported that transfection of the normal human H-ras1 (HRAS) gene suppressed the transformed morphology and tumorigenic phenotypes of rat 208F fibroblast transformed with the human T24 H-ras1 (HRAS) oncogene. Under these circumstances, the expression level of the normal HRAS gene predominated. In rare instances, where transformed fibroblasts escaped suppression by normal HRAS, and eventually formed tumors in nude mice, expression of normal HRAS was markedly reduced. These findings suggest that HRAS can act as a tumor suppressor in mutant-HRAS transformed cells (Spandidos and Wilkie, 1988; Spandidos et al., 1990). Likewise, wild-type *Kras2*, the murine *KRAS* gene, has been shown to suppress the formation of chemically induced lung tumors that harbored Kras2 (Zhang et al., 2001). Of note, this study utilized a *Kras2*$^{+/-}$ mouse model that has a heterozygous loss of wild-type KRAS, which was compared to wild-type mice in all readouts. A similar conclusion was drawn for NRAS, which, however, was not studied in the presence of oncogenic NRAS. Instead, the study used a carcinogen-induced thymic lymphoma mouse model, and found tumor-suppressive effects of wild-type NRAS both in the presence of mutant KRAS, and in the absence of any mutant RAS (Diaz et al., 2002). More recently, Balmain and colleagues also reported tumor suppressive functions of HRAS^{WT} and KRAS^{WT} in experimental models of HRAS-driven non-melanoma skin cancer and KRAS-driven lung cancer, respectively (To et al., 2013). Interestingly, wild-type *Kras4A* was identified as the main mediator of both the oncogenic activity of mutant KRAS and the suppressor activity of wild-type *Kras* (To et al., 2008). An interesting role of wild-type *Kras* gene dosage was uncovered in selecting the specific mutation induced by urethane. Specifically, wild-type mice carried mostly (94%) Q61R *Kras* mutations, while those from *Kras* heterozygous animals carried mostly (92%) Q61L mutations (Westcott et al., 2015).

Loss of the wild-type RAS allele in RAS-mutant cancers is frequently observed, and suggests a tumor suppressor role of the wild-type counterpart of oncogenic RAS. When using the Kras2LSLMx1-Cre (KM) mouse model to study $Kras^{G12D}$-induced leukemia, Bergo and co-workers found that all T-cell acute lymphoblastic leukemia (T-ALL) tumors in bone marrow-transplanted mice showed loss of the wild-type *Kras2* allele, indicating a tumor suppressive role of wild-type *Kras* (Staffas et al., 2015). Zhang et al. showed that, in a fraction of endogenous oncogenic KRAS-induced hematopoietic malignancies including acute T-cell lymphoblastic leulemia/lymphoma (T-ALL) and myeloproliferative neoplasm (MPN), wild-type *Kras* expression is lost by epigenetic or genetic mechanisms (Kong et al., 2016). Loss of endogenous wild-type *Hras* has also been found at high frequencies in carcinogen-induced skin tumors (Bremner and Balmain, 1990). Thymic lymphomas have also been demonstrated to lack the normal allele of *Nras* in carcinogen-induced mouse models (Guerrero et al., 1985).

However, there are also studies that have demonstrated tumor-promoting roles of wild-type RAS (Diaz et al., 2004; Maruyama et al., 2001; Matallanas et al., 2011; Tsunematsu et al., 1994). By comparing signaling events in an isogenic colorectal cancer cell line pair HCT-116 ($KRAS^{G13D/WT}$) and Hke3 ($KRAS^{-/WT}$) with or without selective siRNA-based silencing selectively targeting $KRAS^{WT}$, Matallanas et al. demonstrated that $KRAS^{G13D}$ activates the proapoptotic MST2 pathway. whereas $KRAS^{WT}$ antagonizes this activation (Matallanas et al., 2011). This indicates that, in these colorectal cancer cells, wild-type KRAS supports mutant *KRAS*-induced transformation. Collectively, while the vast majority of studies demonstrate an oncosuppressive role of the wild-type RAS allele in tumors driven by oncogenic activation of the same RAS isoform, it is also true that tissue and/or cellular specificity can influence the role that wild-type RAS plays in tumors.

Most of the other subset of RAS studies that focused on wild-type RAS isoforms other than the oncogenically mutated isoform have, in contrast, revealed tumor-promoting functions. For example, in a study aimed at understanding the mechanism of FTI (farnesyl transferase

inhibitor)-mediated radiosensitization in cell lines that express oncogenic KRAS, the authors found that wild-type HRAS, although not wild-type NRAS, contributes to radiation survival in most pancreatic and colorectal carcinoma cell lines tested (Cengel et al., 2007). Similarly, Panayotou et al. reported that ectopic expression of *KRAS*^{G12V} in the Caco-2 colorectal cancer cell line increased expression and activity of endogenous HRAS, and that oncogenic KRAS partly exerted its effects, such as enhanced invasiveness, through wild-type HRAS (Ikonomou et al., 2012). Likewise, Keller and co-workers demonstrated that the presence of an oncogenic *KRAS* allele resulted in elevated levels of GTP-bound NRAS in two human colorectal cancer cell lines, HCT 116 and DLD-1, compared to their isogenic counterparts in which the mutant *KRAS* allele was disrupted by homologous recombination (Keller et al., 2007). Detailed mechanistic insight was first provided by Lim *et al.*, who showed that activation of eNOS (endothelial nitric oxide synthase) by phosphorylation of S1177 promotes C118 *S*-nitrosylation and activation of endogenous wild-type RAS proteins, suggesting that an oncogenic RAS^{mut}-PI3K-AKT-eNOS-RAS^{WT} pathway is required for tumor initiation and maintenance (Lim et al., 2008). They pointed out in particular that the activation of the *other* wild-type RAS family members by eNOS may serve as an important means to diversify RAS signaling beyond that of oncogenic RAS. In agreement with their speculation, they found that loss of wild-type HRAS does not inhibit oncogenic *HRAS*^{G12V}-mediated oncogenesis in TtH cells expressing either scramble or *HRAS* shRNA in addition to RNAi-resistant oncogenic *HRAS*^{G12V}. In fact, the wild-type counterparts of the oncogenic RAS proteins are often deleted in cancers (Li et al., 2003; Wan et al., 2006), suggesting that, in contrast to the non-counterpart wild-type isoforms, these play a tumor-suppressive role.

It seems that the interaction between oncogenic and wild-type RAS proteins is the net result of a multitude of diverse mechanisms, as the following studies have presented ways of crosstalk. Oncogenic KRAS has been demonstrated to promote allosteric stimulation of SOS, a well-known RAS GEF, and leads to activation of wild-type HRAS and NRAS (Jeng et al., 2012).

A later study by the same group provided additional insights into RAS protein interactions (Grabocka et al., 2014), where wild-type HRAS or NRAS depletion in mutant *KRAS* cancer cells resulted in hyperactivation of both ERK-p90 RSK and PI3K-AKT pathways, thereby phosphorylating Chk1 at an inhibitory site, S280. Consequently, the G2 DNA damage checkpoint was inhibited, leading to increased sensitivity of *KRAS*-mutant cells to DNA damaging agents, such as irinotecan, a topoisomerase I inhibitor that is FDA-approved for the treatment of colorectal cancer. Furthermore, oncogenic and wild-type RAS isoforms have been reported to be responsible for regulating different aspects of signal transduction, with oncogenic RAS modulating basal mitogen-activated protein kinase (MAPK) pathway signaling, and wild-type isoforms controlling response to growth factor signaling (Young et al., 2013). However, surprisingly little is known about whether NRAS-mutant cancers require $KRAS^{WT}$ or $HRAS^{WT}$ for tumor initiation and/or maintenance. It is therefore an aim of my thesis work to understand the roles of wild-type RAS isoforms in a NRAS mutant setting.

RAS effector pathway signaling is frequently deregulated in cancer

There are at least 11 identified direct effectors of RAS, including RAF, PI3K, RalGDS, Tiam1, PLCε, etc. (Figure 1.3). Among all downstream pathways, the RAF-MEK-ERK and the PI3K-AKT-mTOR pathway are by far the best studied. The RAF-MEK-ERK pathway, the first RAS effector pathway delineated, has been shown in various contexts to contribute significantly to tumor development (Blasco et al., 2011; Collisson et al., 2012; De Luca et al., 2012; Khosravi-Far et al., 1995; Stang et al., 1997), and thus has been a major focus of targeted therapies (Roberts and Der, 2007; Samatar and Poulikakos, 2014; Santarpia et al., 2012). A detailed discussion of this crucial pathway is provided in the following section.

The PI3K-AKT-mTOR pathway is the second-best studied pathway identified downstream of RAS (Cantley, 2002), and is involved in many cellular processes relevant to cancer, including metabolism, inflammation, cell survival, motility, and overall cancer

progression (Vanhaesebroeck et al., 2010). In 1994, the p110 catalytic subunits of class I PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase) were identified as direct downstream targets of RAS (Kodaki et al., 1994; Rodriguez-Viciana et al., 1994). PI3K is a lipid kinase that phosphorylates the 3'-hydroxyl group of PIP_2 (phosphatidylinositol-4,5-bisphosphate), converting the latter into PIP_3 (phosphatidylinositol-3,4,5-trisphosphate), which then recruits AKT to the plasma membrane to further transmit signaling. On the other hand, PIP_3 is negatively regulated by dephosphorylation by the tumor suppressor, PTEN (phosphatase and tensin homolog). Upon translocation to the plasma membrane, AKT can be phosphorylated by PDK1 on its activation loop (T308) (Wick et al., 2000), which is sufficient to activate the mammalian target of rapamycin complex 1 (mTORC1) by direct phosphorylation on Ser2448 (Nave et al., 1999). Activation of mTORC1 results in increased protein synthesis and cell survival upon phosphorylation of two key effectors. Phosphorylation of 4E-BPs (eIF4E-binding proteins) terminates their binding to eIF4E and relieves the block on translation initiation (Aoki et al., 2001; Nave et al., 1999), whereas phosphorylation of p70 ribosomal S6 kinases (S6K1 and S6K2) (Dufner and Thomas, 1999; Saitoh et al., 2002) activates their major target, ribosomal protein S6 (rpS6). While mTORC1 signals downstream of PI3K-AKT, another mTOR complex, mTORC2, contributes to the full activation of AKT by phosphorylating it on Ser473 (Zhang et al., 2012) (Figure 1.4). PI3K-AKT-mTOR signaling has long been known to be deregulated in cancer (Sheridan and Downward, 2013; Yuan and Cantley, 2008), which can occur either through upstream activation by oncogenic RAS, or through genetic alterations of pathway-specific components, such as mutations in p110α (Gustin et al., 2008; Ligresti et al., 2009; Samuels and Waldman, 2010; Samuels et al., 2004), inactivation of PTEN (Li et al., 1997; Parsons, 2004; Steck et al., 1997), and amplifications in AKT (Cheng et al., 1992; Cheng et al., 1996; Staal, 1987), etc. Notably, many tumors depend on pathway signaling for tumor initiation and/or maintenance (Castellano et al., 2013; Gupta et al., 2007; Lim et al., 2008; Lim and

Counter, 2005), resulting in epic efforts to develop small molecule inhibitors against components of this pathway. To date, at least 53 inhibitors of PI3K-AKT-mTOR inhibitors have reached clinical evaluation (Cox et al., 2014). While showing minimal promise as monotherapeutics, they potentially yield powerful synergism when combined with inhibitors of the RAF-MEK-ERK pathway (Engelman et al., 2008; Ewald et al., 2014; Jin et al., 2011; Lasithiotakis et al., 2008; Meier et al., 2007; Pitts et al., 2014).

Another major RAS downstream effector pathway is the RalGEF-RalA/RalB axis. RalA and RalB are two highly related (82% overall amino acid sequence identity) small GTPases of the RAS subfamily (Gentry et al., 2014). The importance of Ral activity has been established in many human cancers, including non-small cell lung cancer (NSCLC), melanoma, ovarian cancer, colorectal cancer, bladder cancer, pancreatic cancer, etc. (Coltart et al., 1990; Guin and Theodorescu, 2015; Lim et al., 2006; Mishra et al., 2010; Smith et al., 2007; Wang et al., 2010; Wang et al., 2013a; Zipfel et al., 2010). In contrast to the RAF-MEK-ERK pathway or the PI3K-AKT-mTOR pathway, Ral downstream signaling is less well characterized, and components of this pathway are not mutated in cancer. The first effector identified for Ral was RalBP1 (Cantor et al., 1995; Jullien-Flores et al., 1995; Park and Weinberg, 1995), which is also the most studied. Xenograft studies have demonstrated that RalBP1 plays an important role in several tumor types, such as pancreatic (Leake et al., 2012), prostate (Wu et al., 2010), colorectal (Mollberg et al., 2012), bladder (Wu et al., 2010), and glioblastoma (Wang et al., 2013b). Other well-established effectors of Ral include Sec5 and Exo84 (Moskalenko et al., 2002), which are subunits of the octomeric exocyst complex, and have been implicated in fostering oncogenic RAS-mediated tumorigenesis (Issaq et al., 2010).

Although the aforementioned pathways remain the top three most influential RAS effector pathways studied to date, significant contributions of other pathways to RAS-mediated oncogenesis cannot be excluded, owing to a lesser understanding of them. Indeed, some of these pathways have started to emerge as important players in cancer, such as Tiam1-RAC-

PAK signaling (Baker et al., 2014; Dummler et al., 2009), RASSF-Mst1/2 signaling (Chao et al., 2015; Maruyama et al., 2008; Mezzanotte et al., 2014; Zhou et al., 2014), and PLCε-PKC signaling (Dowling et al., 2016; Leonard et al., 2015; Martins et al., 2014).

The RAS-RAF-MEK-ERK "pathway" is really a "web"

The RAS-RAF-MEK-ERK cascade is without doubt one of the best-characterized signal transduction pathways in science history. The initial illustration of this pathway as a linear and unidirectional flow was later extensively revised based on studies that continued to add complexities to it, rendering the pathway now the core of a complex signaling network (Figure 1.5). In 1993, four groups reported the direct interaction of the RAF serine/threonine kinase (specifically RAF-1 or CRAF) with RAS, making it the first bona fide mammalian RAS effector to be identified (Moodie et al., 1993; Vojtek et al., 1993; Warne et al., 1993; Zhang et al., 1993). In fact, the human RAF family comprises three evolutionarily conserved cytosolic RAF kinases, ARAF, BRAF and CRAF (Desideri et al., 2015). When GTP-bound, whether through growth factor stimulation or mutation, the affinity of RAS towards RAF increases due to conformational change, leading to RAS-RAF binding via the RAS-binding domain (RBD) (Nassar et al., 1995; Vojtek et al., 1993) and the cysteine-rich domain (CRD) (Mott et al., 1996) on RAF (Roskoski, 2010). This physical interaction tethers cytoplasmic RAF to the plasma membrane, and is considered the first step in its activation (Morrison and Cutler, 1997). Once bound to RAS, the conformation of RAF changes, rendering it accessible for additional modifications, such as phosphorylation at Y340/341 by Src family kinases, which had been shown to enhance the catalytic activity of RAF (Fabian et al., 1993; Marais et al., 1995). In addition, oligomerization, which can be induced by the enrichment of RAF at the plasma membrane when bound to RAS, is also a means of RAF activation (Farrar et al., 1996; Luo et al., 1996). Indeed, it is well established that RAF dimerization is necessary for its kinase activity (Rajakulendran et al., 2009; Rushworth et al., 2006). Upon full activation of RAF, it can further transmit the signal by

phosphorylating and activating MEK (or MKK, mitogen-activated protein kinase kinase). Two highly-related MEK1 and MEK2 proteins share 80% sequence identity, and are the only well-validated RAF substrates. Activated MEK then propagates the signal to its downstream effectors ERK1 and ERK 2 (extracellular signal-regulated kinase), which have 86% sequence identity, and are the only well-validated MEK substrates. ERK1/2, however, has over 200 substrates, distributed throughout the cell (Roskoski, 2012). The most thoroughly studied nuclear substrate of ERK1/2 is Elk1 (Yoon and Seger, 2006), a member of the ternary complex factor subfamily of Ets (E-twenty six)-domain transcription factors, which play a major role in inducing the expression of immediate early genes. The best-known cytoplasmic substrates of ERK1/2 are p90 RSK family kinases, which are known to regulate cell growth, motility, and survival (Anjum and Blenis, 2008).

In addition to forward propagation of signal, the pathway is also regulated by feedback phosphorylation to restrict signaling output and provide a sensitive and temporal modulation of RAS-RAF-MEK-ERK signaling. Morrison and colleagues reported that a series of ERK-catalyzed phosphorylation sites (Ser151, Thr401, Ser750, and Thr753) are associated with feedback inhibition of BRAF (Ritt et al., 2010). Specifically, phosphorylation of these sites inhibits BRAF binding to activated RAS and disrupts heterodimerization with CRAF, whereas mutation of each feedback site increased transformation and correlated with enhanced heterodimerization and activation of CRAF. Similarly, ERK1/2 phosphorylation of HA-BRAF at Thr753 exerts negative feedback on the persistence of the HA-BRAF/FLAG-CRAF heterodimer and decreases BRAF kinase activity (Rushworth et al., 2006). The phosphorylation of six serine residues (29, 43, 289, 296, 301, and 642) on CRAF/RAF-1 has been reported to be catalyzed by ERK1/2, hyperphosphorylation of which inhibited RAS-CRAF interaction and desensitized RAF to additional stimuli (Dougherty et al., 2005). Moreover, ERK1/2 also catalyzes inhibitory feedback phosphorylation of MEK1 on Thr292 (Brunet et al., 1994; Catalanotti et al., 2009) and Thr398 (Brunet et al., 1994; Matsuda et al., 1993). Catalanotti et al.

showed that the MEK1/MEK2 heterodimer is negatively regulated by ERK-mediated phosphorylation of MEK1 on Thr292, a residue not present in the proline-rich sequence of MEK2 (Catalanotti et al., 2009). This phosphorylation blocks the ability of PAK to phosphorylate S298 and of Rac-PAK signaling to enhance MEK1-ERK complex formation (Eblen et al., 2004). The mechanism by which the other phosphorylation site, Thr398, attenuates MEK-ERK signaling, however, is less well understood. ERK, and its immediate substrate p90 RSK-2, are both capable of phosphorylating son of sevenless 1 (SOS1) at several residues, thereby interfering with SOS1 binding to Grb2, an adaptor protein that couples RTKs to RAS, leading to the ultimate downregulation of RAS signaling (Corbalan-Garcia et al., 1996; Douville and Downward, 1997). Further downstream, activation of ERK1/2 signaling induces the transcriptional upregulation of some negative regulators of the pathway, including dual-specificity MAP kinase (MAPK) phosphatases (MKPs or DUSPs) and Sprouty (SPRY) proteins. ERK1/2 signaling drives the expression of a variety of DUSP proteins, including the ERK-specific phosphatases DUSP5 (nuclear) and DUSP6/MKP3 (cytoplasmic) (Kidger and Keyse, 2016; Zhang et al., 2010), thus providing a straightforward means of controlling its own activity. The importance of DUSPs in controlling RAF-MEK-ERK signaling is exemplified by the frequent loss of DUSP6 in EGFR- and KRAS-driven non-small cell lung cancers (Zhang et al., 2010) and by the demonstration that the loss of DUSP5 accelerates HRAS-driven skin cancer in mice (Rushworth et al., 2014).

SPRY proteins have also been identified to act as negative regulators of RTK-RAS-RAF-MEK-ERK signaling (Hanafusa et al., 2002; Kim and Bar-Sagi, 2004). Growth factor stimulation leads to the tyrosine phosphorylation and plasma membrane translocation of SPRY1 and SPRY2, where they bind the adaptor protein Grb2 to prevent recruitment of the Grb2-SOS complex either to the fibroblast growth factor receptor (FGFR) docking adaptor protein FRS2 or to SH2-domain-containing protein tyrosine phosphatase-2 (SHP2), thereby blocking the necessary step in coupling FGFR stimulation to RAS activation (Hanafusa et al., 2002).

Understanding such negative feedback regulation is important for predicting the consequences of pharmacologically inhibiting elements of this pathway for cancer treatment, as well as for unraveling the consequences of signaling from wild-type RAS isoforms in the presence of oncogenic RAS.

Scaffolding proteins, such as kinase suppressor of RAS (KSR), also play an important role in shaping RAS-RAF-MEK-ERK signaling (Kolch, 2005). A variety of scaffold proteins including KSR1/2, IQGAP1, MP1, β-Arrestin1/2 participate in regulation of the RAF-MEK-ERK kinase cascade (Morrison and Davis, 2003). The human genome encodes two KSRs, KSR1 and KSR2. Sequence analysis indicates that KSR1/2 belong to the protein-serine/threonine kinase family. These proteins were considered catalytically inactive owing to the absence of critical conserved amino acid residues. Emerging studies have shown otherwise, but the bulk of evidence still supports a kinase-independent function of KSR. KSR1 binds all modules of the RAF-MEK-ERK pathway, but, whereas MEK is associated constitutively, RAF and ERK might bind in a stimulus-dependent manner (Morrison, 2001). $KSR1^{-/-}$ mice were found to be less susceptible to oncogene-induced tumors than their wild-type counterparts (Kortum and Lewis, 2004; Lozano et al., 2003; Nguyen et al., 2002), supporting a role for KSR1 in promoting the proliferative and transforming functions of the RAF-MEK-ERK pathway. As discussed in Chapter 2, I have found a previously unrecognized role for a KSR-interacting protein in modulating responses to pharmacological inhibition of the RAF-MEK-ERK kinase cascade.

Finally, there is extensive crosstalk among the different RAS effector pathways. For example, there are multiple levels of crosstalk among the RAF-MEK-ERK, PI3K-AKT-mTOR, and RAC-PAK pathways (Aksamitiene et al., 2012; Ebi et al., 2013; Eblen et al., 2002; Wang et al., 2013d). Of note, cross-talk is context-dependent and pathways can activate or inhibit each other.

CK2 modulates RAF-MEK-ERK pathway activity and vice versa

Amongst all the molecules that can modulate RAF-MEK-ERK activity, protein kinase CK2 is not one that is commonly thought of. However, there have been multiple reports of CK2 affecting the signaling output of the RAF-MEK-ERK pathway. Protein kinase CK2, formerly known as "casein kinase 2", is a widely expressed, constitutively active kinase that phosphorylates nearly 300 protein substrates, and plays important roles in cellular survival, proliferation and differentiation (Pinna, 2002; Pinna and Allende, 2009; Trembley et al., 2009). The CK2 tetrameric holoenzyme is composed of two regulatory (β) and two catalytic (α or α') subunits. There is substantial evidence to suggest that the subunits can also function independently of the tetramer (Hanif et al., 2010).

The modulatory effect of CK2 on RAF-MEK-ERK signaling can happen at various levels. The Goldberg laboratory reported CK2α binding to the core dimer of protein phosphatase 2A (PP2A) (Heriche et al., 1997), a phosphatase that is known to be principally responsible for the down-regulatory dephosphorylation of kinases belonging to the RAF-MEK-ERK pathway (Hunter, 1995). Overexpression of wild type CK2α inhibited serum-stimulated activation of co-transfected MEK1 and suppressed cell growth. CK2α also dramatically reduced (by 60%) the focus forming capability induced by *Ras*^{G12V}, but not by constitutively active MEK1, in NIH 3T3 cells. These data suggest that CK2α binding to PP2A may enhance PP2A activity toward MEK1. Later, the same group showed that CK2α could also inhibit serum-stimulated activation of ERK2, and that expression of activated viral Raf disrupted CK2α-PP2A association (Lebrin et al., 1999). While these studies identified a negative regulatory role of CK2α on the RAF-MEK-ERK kinase cascade, others found positive roles. For example, CK2α has also been shown to interact with and phosphorylate the ERK-specific dual specificity phosphatase 6 (DUSP6), also known as MAP kinase phosphatase 3 (MKP3). This resulted in a slight increase in EGF-stimulated ERK phosphorylation in COS-7 cells (Castelli et al., 2004). *In vitro* studies have

demonstrated that, upon EGFR activation, ERK2 directly binds CK2α via the ERK2 docking groove and phosphorylates CK2α at Thr360 and Ser362, thereby enhancing CK2α activity toward α-catenin phosphorylation (Ji et al., 2009). Furthermore, CK2 has been characterized as an integral component of the KSR1 scaffolding complex, and has been shown to be critical to the maximal activation of this pathway (Ritt et al., 2007). Aside from fine-tuning the signaling amplitude of the RAF-MEK-ERK pathway, CK2 has been reported to regulate ERK nuclear translocation and translation of nuclear targets of ERK, which, in essence, can also affect the signaling efficiency of the pathway (Plotnikov et al., 2011). Mechanistically, CK2 can phosphorylate Ser244 and Ser246 in the nuclear translocation signal (NTS) of ERK, allowing ERK binding to Imp7 (importin 7), a protein that is responsible for the nuclear import of many proteins.

Although the interactions between CK2 and the RAF-MEK-ERK pathway are sophisticated and relatively poorly defined, the following can be concluded from the above studies related to CK2 and RAF-MEK-ERK signaling: 1) CK2 is both a target and a regulator of RAF-MEK-ERK signaling, and 2) there are multiple ways by which CK2 and components of the RAF-MEK-ERK pathway can interact with and modulate each other's activity.

RAS-RAF-MEK-ERK signaling is critical to RAS- and RAF-driven cancers

Hyperactivation of the RAS-RAF-MEK-ERK pathway occurs very frequently in solid tumors, and is often the result of activating mutations in upstream receptor tyrosine kinases, RAS, or BRAF. For example, it is known that EGFR-mutant non-small cell lung cancers constitutively express active ERK (Balko et al., 2009; Britson et al., 2009). Activating RAS mutations or loss of the RasGAP NF1 can cause activation of the pathway at the level of RAS. *RAS*, especially *KRAS*, gain-of-function mutations occur at extremely high frequencies in pancreatic (Almoguera et al., 1988), colorectal (Cancer Genome Atlas, 2012), lung (Cancer

Genome Atlas Research, 2014a), and endometrial adenocarcinomas (Cancer Genome Atlas Research et al., 2013), whereas NF1 loss-of-function mutations are more commonly seen in melanomas (Cancer Genome Atlas, 2015), glioblastomas (Verhaak et al., 2010), and lung squamous cell carcinomas (Cancer Genome Atlas Research, 2014a). The next module in the tier, *RAF*, specifically *BRAF*, is frequently mutated in melanomas (Cancer Genome Atlas, 2015) and in papillary thyroid carcinomas (Cancer Genome Atlas Research, 2014b). In sharp contrast, MEK and ERK mutations are very rarely found in human cancers.

Given the frequency of mutational activation of the pathway and its importance in tumor maintenance, it is therefore not surprising that considerable efforts have been made to develop targeted therapies against RAS-driven cancers that are directed against the RAF-MEK-ERK kinase cascade (Samatar and Poulikakos, 2014). Attempts to target active RAS in the early days focused on targeting RAS membrane association, using FTIs to block RAS obligate lipid modification by farnesylation. However, despite their preclinical efficacy in tumor models, FTIs proved unsuccessful in the clinic for a variety of reasons including their failure to block alternative prenylation of KRAS and NRAS (Berndt et al., 2011). The next big step was a leap towards designing inhibitors that target RAS downstream signaling, in particular the RAF-MEK-ERK and PI3K-AKT-mTOR cascades. Here, I will focus on inhibitors of the RAF-MEK-ERK pathway.

Mutant BRAF-specific inhibitors vemurafenib and dabrafenib, which specifically inhibit $BRAF^{V600E}$, the predominant oncogene in melanoma patients, cause >80% inhibition of phosphorylated in ERK in tumors, and thus have shown significant clinical efficacy (Bollag et al., 2010; Chapman et al., 2011; Hauschild et al., 2012). However, using these first-generation mutant-specific inhibitors in non-BRAF-mutant settings, such as for RAS-mutant cancers, can lead to the paradoxical activation of MEK-ERK signaling resulting from increased BRAF-CRAF dimerization. Drug binding to the heterodimer inhibits BRAF, but transactivates CRAF, thereby hyperactivating the pathway (Poulikakos et al., 2010). This paradoxical activation of RAS-MEK-

ERK signaling can be manifested by the appearance of an array of secondary lesions, including papillomas, squamous cell carcinomas, keratoacanthomas, and basal cell carcinomas, in those parts of the skin without BRAF mutations (Holderfield et al., 2014a; Su et al., 2012).

A huge number of studies has reported the use of various MEK inhibitors to inhibit tumor cell proliferation in culture and to block tumor growth in animal models (Sebolt-Leopold and Herrera, 2004), underscoring the wide range of MEK dependency in different tumor systems. However, the dependence on MEK appears highest in tumors that harbor a *BRAF* mutation (Solit et al., 2006), perhaps because mutated BRAF transmits signal only through the RAF-MEK-ERK pathway, whereas mutated RAS, for instance, can utilize several effector pathways. Interestingly, structural and functional studies demonstrated that MEK inhibitors with superior efficacy in *KRAS*-mutant cancers, such as GDC-0623 and G-573, form a strong hydrogen bond with Ser212 in MEK and thus block MEK feedback phosphorylation by wild-type RAF (Hatzivassiliou et al., 2013). In comparison, MEK inhibition in *BRAF*-mutant cancers requires potent inhibition of active, phosphorylated MEK, which is achieved by inhibitors such as GDC-0973 (cobimetinib). Another study agrees with this finding and revealed that MEK activated by CRAF was less susceptible to MEK inhibitors than when activated by $BRAF^{V600E}$, and that MEK inhibitors induced RAF-MEK complexes in *KRAS*-mutant models, explaining the marginal efficacies of MEK inhibitor in RAS-driven cancers (Lito et al., 2014). Therefore, the mechanisms by which MEK inhibitors function can determine their efficacies in *BRAF*- versus *KRAS*-mutant tumors. Generally, MEK inhibitors are classified into ATP-competitive and non-ATP-competitive (allosteric) inhibitors, but most MEK inhibitors belong to the latter category. At present, at least 15 MEK inhibitors have entered clinical evaluation (Cox et al., 2014). Among them, trametinib and cobimetinib have been approved by the FDA for use in BRAF-mutant metastatic melanomas, whereas selumetinib is approved for treatment of uveal melanoma, a rare eye cancer driven largely by mutations in GNAQ and GNA11 rather than in BRAF or RAS.

ERK inhibitors are also emerging, prompted by the observation that ERK reactivation is a major cause for resistance to RAF or MEK inhibition (Lito et al., 2013). Three ERK inhibitors (BVD-523, GDC-0994, MK-8353/SCH-900353) have reached clinical evaluation (Cox et al., 2014), and SCH772984, an analog of MK-8353, has been well characterized in preclinical models (Morris et al., 2013). Whether ERK inhibitors will be superior to RAF or MEK inhibitors remains to be determined.

Resistance to targeted therapies remains a major challenge in treating RAS- or RAF-driven cancers

A common theme of targeted therapies is the emergence of drug resistance, despite remarkable initial tumor responses. The study of resistance mechanisms is necessary for uncovering new therapeutic targets or rationalizing drug combinations that forestall or delay tumor relapse (Zhou and Cox, 2015). Among all classes of inhibitors, the resistance mechanisms for BRAF inhibition are best characterized (Hartsough et al., 2014; Lito et al., 2013; Spagnolo et al., 2015) (Figure 1.6). Resistance is broadly divided into two not mutually exclusive categories: intrinsic and acquired resistance. Approximately 50% of BRAF-mutated melanoma patients show no response (~15% of them) or little response (~35% of them), as defined by a degree of tumor shrinkage sufficient to meet the RECIST criteria for a partial response to BRAF inhibition (intrinsic resistance) (Chapman et al., 2011; Flaherty et al., 2010; Sosman et al., 2012). The other half of treated patients initially display response to therapy (>30% tumor shrinkage), but eventually develop secondary tumors that display acquired resistance to these inhibitors.

Compared to intrinsic resistance, acquired resistance is more clearly defined and investigated. Mechanism-wise, acquired resistance largely fits into two subcategories: mechanisms that ultimately result in ERK reactivation (major), and mechanisms that reduce the dependence of tumors on ERK signaling (minor). Based on the above discussion regarding

RAS-RAF-MEK-ERK signaling, it can be predicted that factors that enhance RAF dimerization/signaling will eventually reactivate ERK. Accordingly, Nazarian *et al*. (Nazarian et al., 2010) reported that acquired resistance to PLX4032 (vemurafenib) develops by mutually exclusive upregulation of PDGFRβ or mutations in *NRAS*, mechanisms that have also been found in patient tumor samples. In one subset, PDGFRβ-upregulated tumor cells have low levels of activated RAS, and, when treated with vemurafenib, do not reactivate the MAPK pathway significantly. In contrast, in another subset, high levels of activated NRAS resulting from NRAS mutations lead to significant MAPK pathway reactivation upon vemurafenib treatment. The occurrence of secondary *NRAS* mutations in previously regressed tumors was independently validated in a separate study, where they were found in 4 out of 19 tumor progression samples (Poulikakos et al., 2011). Loss of NF1, a RAS GAP and tumor suppressor, has also been shown to promote resistance to RAF inhibition by enhancing ERK signaling, and has been validated as a potential mechanism of intrinsic resistance in cell culture, as well as of acquired resistance in patient tumor samples (Maertens et al., 2013; Whittaker et al., 2013). These alterations upstream of RAF can promote signal transmission to wild-type RAF, particularly CRAF, the active protomer of the inhibitor-bound BRAF-CRAF heterodimer. At the level of RAF, amplifications in *BRAF* (Shi et al., 2012b) or *CRAF* (Johannessen et al., 2010; Montagut et al., 2008), alternative splicing of mutant *BRAF* (Poulikakos et al., 2011), as well as increased expression of an alternative MAPKKK, COT (also known as Tpl-2) (Johannessen et al., 2010), have been documented as resistance mechanisms. A 61 kDa alternatively spliced form of $BRAF^{V600E}$ lacking exons 4-8 (encoding the RAS binding domain of BRAF) has been reported to dimerize independently of RAS, thus conferring resistance to vemurafenib, consistent with the model that only $BRAF^{V600E}$ monomers are sensitive to inhibition. Importantly, this splice variant has been identified in both resistant cell lines generated *in vitro* and vemurafenib-resistant patient tissue. Downstream of RAF, mutations such as C121S (Wagle et al., 2011), P124L (Emery et al., 2009) and Q56P (Emery et al., 2009) in *MAP2K1*, which

encodes MEK1, have also been shown to confer RAF inhibitor resistance. However, the significance of other *MEK* mutations needs further interrogation, because although P124S and I111S mutations in MEK1 have been detected in pre-treatment samples, they do not provide resistance to RAF inhibitors (Shi et al., 2012a).

Mechanisms of acquired resistance that attenuate the dependence of tumor cells on ERK usually involve the activation of other parallel pathways that bypass the need for ERK signaling. For example, increased AKT pathway signaling, resulting from the concurrent loss of the *PTEN* and *RB1* tumor suppressors, is capable of diminishing RAF-MEK-ERK dependence in *BRAF*-mutant melanomas (Xing et al., 2012). Upregulation of RTK signaling is another means by which tumor cells become less dependent on ERK signaling. Using a panel of kinase-'addicted' human cancer cell lines, Settleman and co-workers found that most cells can be rescued from drug sensitivity by simply exposing them to one or more RTK ligands (Wilson et al., 2012). Girotti *et al*. demonstrated that BRAF inhibitor-mediated activation of EGFR-SFK (Src family kinase)-STAT3 signaling mediated drug resistance in patients with *BRAF*-mutant melanoma (Girotti et al., 2013). Villanueva, Herlyn and colleagues reported an IGF-1R/PI3K-dependent survival mechanism in the development of resistance to BRAF inhibitors (Villanueva et al., 2010).

Resistance mechanisms to MEK-targeted agents (Caunt et al., 2015; Poulikakos and Solit, 2011; Sale and Cook, 2014) are often distinct from those to RAF-targeted therapies and have largely been characterized preclinically in colon cancer cell lines. In sharp contrast to RAF inhibition, where no secondary BRAF gatekeeper mutation has been identified (Whittaker et al., 2010), resistance to MEK inhibition can arise through mutations in MEK. For example, *MEK1*^{V211D} and *MEK1*$^{G128D/L215P}$ mutations were identified in RKO (*BRAF*^{V600E}) cells and in HCT-116 (*KRAS*^{G13D}) colon cancer cells, respectively, that displayed acquired resistance to the MEK1/2 inhibitor trametinib (Morris et al., 2013). Likewise, a *MEK1*^{F129L} mutation was found in HT-29 (BRAF-mutant, KRAS-WT) colon cancer cells as a molecular mechanism responsible for

resistance to MEK inhibitor RO4927350 (Wang et al., 2011). The clinical relevance of *MEK1* mutations as a mechanism of acquired resistance to MEK1/2 inhibitors was highlighted by the identification of a *MEK1*^{P124L} mutation in a metastastic focus of a patient post-selumetinib relapse that was undetectable in pre-treatment samples (Emery et al., 2009). Additionally, concurrent *MEK2*^{Q60P} mutation and *BRAF*^{V600E} amplification was found to promote resistance to trametinib (Villanueva et al., 2013). Another type of MEK inhibitor resistance mechanism involves amplification of the driving oncogene. For example, resistance to MEK inhibitor selumetinib was driven by analogous mechanisms in two separate colon cancer cell lines (Little et al., 2011). In drug-resistant COLO-205 (*BRAF*^{V600E}) clones, increased abundance of BRAF was the result of *BRAF* amplification. Similarly, in drug-resistant HCT-116 (*KRAS*^{G13D}) clones, elevated KRAS abundance was due to *KRAS* amplification (Little et al., 2011). In the same vein, BRAF amplifications were found in two additional colorectal cancer cell lines (COLO201 and COLO206F) with *BRAF*^{V600E} mutations when chronically exposed to selumetinib (Corcoran et al., 2010). Increased abundance of activated KRAS confers resistance to MEK inhibitor CI-1040 was demonstrated in C26 murine colon cancer cells, which harbor *KRAS*^{G12V} (Wang et al., 2005).

Since ERK inhibitors have only recently reached the clinic, there is limited data on the mechanisms of resistance to ERK inhibition. A study by Jha et al. sought to preemptively define modes of resistance to ERK inhibition (Jha et al., 2016). Chronic exposure of the HCT-116 (*KRAS*^{G13D}) cell line to ERK inhibitor SCH772984 led to emergence of a G186D mutation in ERK1, which impairs binding to the ERK inhibitor.

In sum, a multitude of resistance mechanisms have been identified for the inhibition of each signaling module in the RAF-MEK-ERK pathway. Further validation of each of these mechanisms in clinical samples is warranted to justify rational drug combination design to prevent or delay tumor recurrence.

Melanoma is an excellent disease model for studying RAS-RAF-MEK-ERK signaling and therapeutic resistance

Melanoma is a malignancy that arises from melanocytes, the melanin-producing cells that reside in a number of different anatomic sites including skin, mucosal epithelia, and meninges (Sullivan et al., 2015). For historical reasons, unless otherwise unspecified, melanoma normally refers to “skin cutaneous melanoma”, which is the most common form of the disease, and also a focus of this dissertation.

The incidence rate of melanoma has been steadily rising over past few decades (Siegel et al., 2015). Well-known as a very aggressive disease, it accounts for 75% of all skin cancer deaths. According to the SEER (Surveillance, Epidemiology, and End Results) statistics of the National Cancer Institute, melanoma is currently the sixth most common cancer in the United States, with an estimated 73,870 new cases and 9,940 deaths in 2015. Although the 5-year survival rate for localized melanoma is 98.3%, once metastasized, it plummets to 16.6%.

Advances in deep-sequencing technologies have contributed dramatically to the in-depth understanding of melanoma. Since the publication of the first melanoma genome in 2010 (Pleasance et al., 2010), subsequent large-scale sequencing studies have discovered numerous previously unknown melanoma-associateed genes involved in the regulation of the RAS-RAF-MEK-ERK and other signaling pathways (Cancer Genome Atlas, 2015; Hodis et al., 2012; Krauthammer et al., 2012; Stark et al., 2012). It is now widely accepted that, based on genomic classification, melanomas are divided into four major subtypes: mutant *BRAF*, mutant *RAS*, mutant *NF1*, and Triple-WT (wild-type) (Cancer Genome Atlas, 2015) (Figure 1.6). Focal amplifications of *BRAF* were observed at significant frequencies in the *BRAF* mutant subtype, whereas *NRAS* amplifications co-occurred in tumors with *NRAS* mutations (Cancer Genome Atlas, 2015). Among a collection of 318 tumor samples analyzed, the mutation frequencies of BRAF, RAS and NF1 were 52%, 30%, and 14%, respectively (Cancer Genome Atlas, 2015),

indicating that the vast majority of melanomas have hyperactivation of the RAS-RAF-MEK-ERK pathway.

Melanoma is distinct in its "choice" of *RAS* mutation. As introduced earlier, while most cancers preferentially harbor mutations in *KRAS*, melanomas exhibit a strong bias towards *NRAS* mutations (94%), compared to *KRAS* (2%) or *HRAS* (4%) (Cox et al., 2014). Furthermore, in contrast to acute myeloid leukemia (AML), which also favors NRAS mutations, melanomas preferentially harbor mutations at codon Q61, rather than at G12 or G13 (Burd et al., 2014; Johnson et al., 2014). Most studies on the roles of wild-type RAS isoforms in RAS-mutant cancers to date have focused on KRAS mutant disease, likely due to the prevalence of mutations in this isoform (Grabocka et al., 2014; Jeng et al., 2012; Lim et al., 2008; Weyandt et al., 2015). Owing to their distinct biological functions in cancers (Castellano and Santos, 2011), observations from KRAS-mutant cancers may not apply to cancers with another mutated RAS isoform. Therefore, interrogating the contributions of wild-type RAS in different mutant-isoform system, such as NRAS-mutant diseases, is desired. Hence, NRAS-mutant melanoma is a naturally occurring disease model that is highly suitable for the study of RAS isoform interactions in a new mutant-isoform setting. Moreover, NRAS mutations are an independent prognostic indicator for worse clinical outcomes (Devitt et al., 2011; Jakob et al., 2012; Mann et al., 2013), and this subgroup does not have access to any FDA-approved targeted therapy. There is thus a strong urge to better understand this subpopulation of melanoma patients so as to improve their prognosis.

BRAF-mutant melanoma patients, on the contrary, are currently treated with 4 out of all 7 FDA-approved inhibitors of RAS effector signaling: BRAF inhibitors vemurafenib or dabrafenib, and MEK inhibitors trametinib and cobimetinib, sometimes in combination (Bollag et al., 2010; Chapman et al., 2011; Flaherty et al., 2012a; Flaherty et al., 2012b; Hauschild et al., 2012; Larkin et al., 2014). Even so, they face one of the greatest challenges in targeted therapy – resistance. As discussed in detail above, both intrinsic and acquired resistance can account

for tumor relapse, and there are many possibilities as to how a secondary tumor develops. Therefore, because of the extensive use of targeted agents in this genetic subtype, and due to the inevitable occurrence of tumor relapse, *BRAF*-mutant melanoma presents a suitable disease model to study therapeutic resistance to inhibitors of the RAS-RAF-MEK-ERK pathway.

Rationale and objectives for this project

Define a novel mode of resistance to RAF-MEK-ERK pathway inhibition

Considerable efforts have been invested in identifying resistance mechanisms of BRAF-mutant melanomas to BRAF inhibition (Hartsough et al., 2014; Lito et al., 2013; Spagnolo et al., 2015). Interestingly, the literature also provides some evidence that a given resistance mechanism can occur in the same or in different tumor types treated with different inhibitors of the same pathway (Corcoran et al., 2010; Duncan et al., 2012; Emery et al., 2009; Johannessen et al., 2010; Johannessen et al., 2013; Little et al., 2011; Nazarian et al., 2010; Shi et al., 2012a). Therefore, I hypothesized that some mechanisms of resistance can be shared by different drugs that target the same pathway, or even by different diseases that show hyperactivity of the same pathway.

Previously, we identified CK2α as a synthetic lethal partner of ERK inhibition in pancreatic ductal adenocarcinoma cells (Hayes et al., 2016). Given that the RAS-RAF-MEK-ERK pathway is strongly activated in both pancreatic cancer and melanoma, we sought to determine whether CK2α also plays a role in resistance to inhibition of this pathway in melanoma. To this end, I defined the GI_{50} values of approved single agent therapies (vemurafenib, dabrafenib, and trametinib) in $BRAF^{V600E}$ melanoma cells ectopically expressing CK2α, or depleted of endogenous CK2α. I also monitored reactivation of ERK phosphorylation as a readout for emergence of resistance (Solit et al., 2006). Work from this study sheds light on the role of a novel candidate of resistance, CK2α, in modulating RAF-MEK-ERK signaling.

Define the roles of wild-type RAS isoforms in the presence of oncogenic NRAS

There is mounting evidence to suggest an important and sophisticated role of wild-type RAS isoforms in oncogenic RAS-driven tumor development. However, studies come to different conclusions as to whether they play a tumor-suppressive or tumor-supporting role, suggesting context dependency (tumor type, stage of tumor development, specific oncogenic RAS isoform) (Jeng et al., 2012; Lim et al., 2008; To et al., 2013; Young et al., 2013). In addition, reported mechanisms of interplay between wild-type and oncogenic RAS are diverse, suggesting not only context dependency, but also complexity of their interaction, which is likely an integration of multiple mechanisms. However, little is known about whether NRAS-mutant cancers require $KRAS^{WT}$ or $HRAS^{WT}$ for tumor initiation and/or maintenance. NRAS mutations preferentially occur in cutaneous melanomas, an aggressive and common skin cancer, whose incidence is still on the rise. NRAS-mutant melanoma patients, who constitute approximately 28-30% of the melanoma population (Cancer Genome Atlas, 2015), not only have worse prognosis than other genetic subtypes, but also lack accessibility to targeted therapeutics. My goal was to determine whether NRAS-mutant melanomas need wild-type KRAS and/or HRAS for cancer cell survival, proliferation or transformed growth. In addition, I aimed to define the mechanisms by which wild-type RAS proteins sustain such phenotypes. I further used an unbiased reverse phase protein array (RPPA) approach to gain molecular insights into isoform-specific regulation of protein activation states. Findings from this study inform us about isoform-selective signaling and functions of RAS proteins in an NRAS-mutant background.

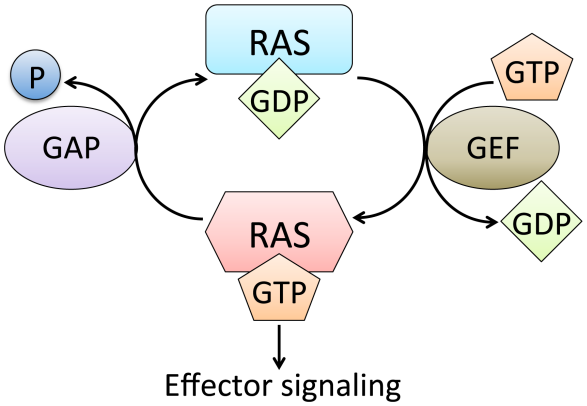

Figure 1.1. The RAS GTPase cycle. RAS cycles between a GDP-bound, inactive state, and a GTP-bound, active state, in which it can signal to its downstream effectors. GDP release is accelerated by guanine nucleotide exchange factors (GEFs), whereas GTP hydrolysis is facilitated by GTPase accelerating proteins (GAPs).

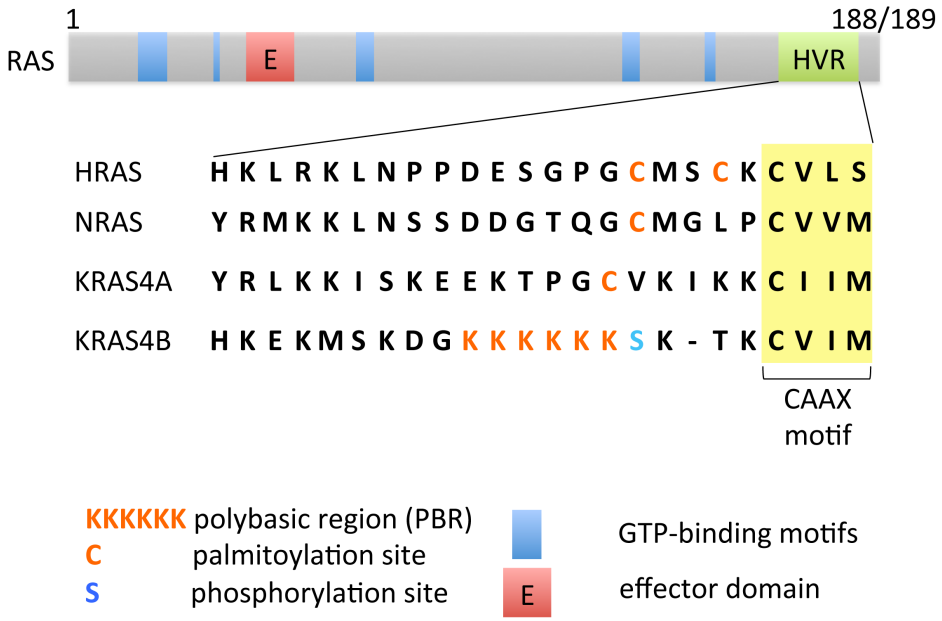

Figure 1.2. Overall domain structure of RAS isoforms. The three RAS genes (HRAS, NRAS, KRAS) encode four 188-189 amino acid proteins (HRAS, NRAS, and splice variants KRAS4A and KRAS4B) that share 82-90% sequence identity. RAS proteins consist of a structured globular G domain, a conserved effector domain (E), and an unstructured hypervariable region (HVR) that serves as the major membrane targeting region. The sequences in the C-terminal CAAX motif dictate prenylation and post-prenyl processing, whereas the elements upstream of the CAAX motif include "second signals" such as a polybasic region (PBR), cysteine acceptor sites for palmitoylation and serine acceptor sites for phosphorylation. (Adapted from Zhou et al. Posttranslational modifications of small G proteins. Springer-Verlag Wien. 2014:99-131)

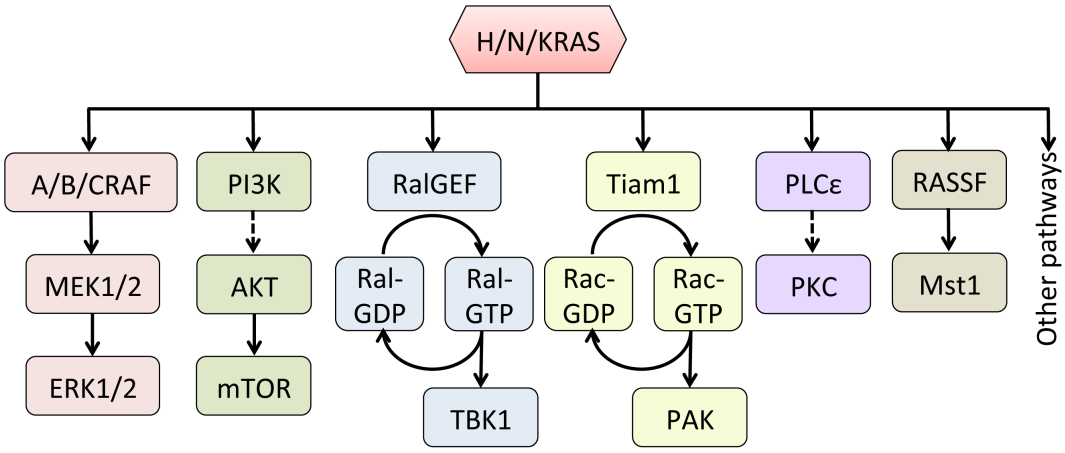

Figure 1.3. RAS effector signaling pathways. The RAF-MEK-ERK, PI3K-AKT-mTOR, RalGEF-Ral-TBK1, Tiam1-Rac-PAK, PLCε-PKC, RASSF-Mst1 pathways are 6 RAS effector pathways are implicated in RAS-driven oncogenesis.

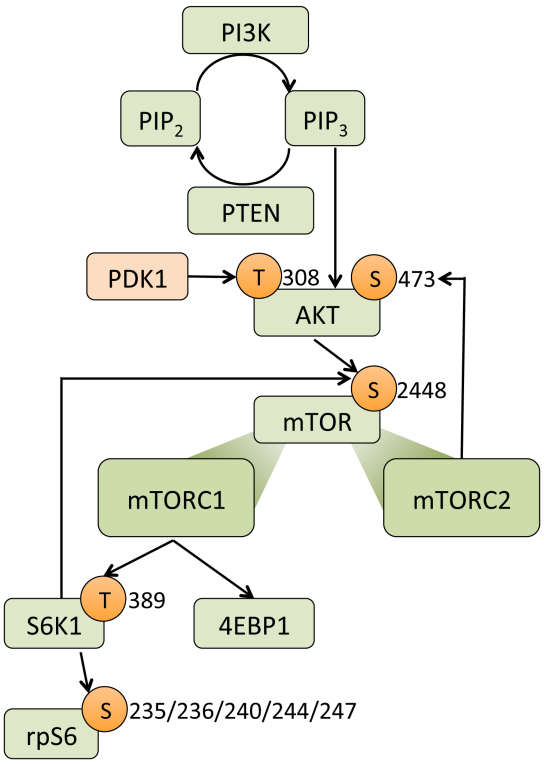

Figure 1.4. The PI3K-AKT-mTOR-S6K pathway. The direct RAS effector phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) converts PIP_2 into PIP_3, the latter recruiting AKT to the plasma membrane. PIP_3 can be dephosphorylated to PIP_2 by phosphatase and tensin homolog (PTEN). Upon translocation to the plasma membrane, AKT can be phosphorylated by PDK1 on its activation loop (T308), which is sufficient to activate the mammalian target of rapamycin complex 1 (mTORC1) by direct phosphorylation on Ser2448. Activation of mTORC1 results in increased protein synthesis and cell survival due to phosphorylation of its effectors, such as 4E-BPs (eIF4E-binding proteins) and p70 ribosomal S6 kinases (S6K1 and S6K2), and consequently of their major targets including several residues of ribosomal protein S6 (rpS6).

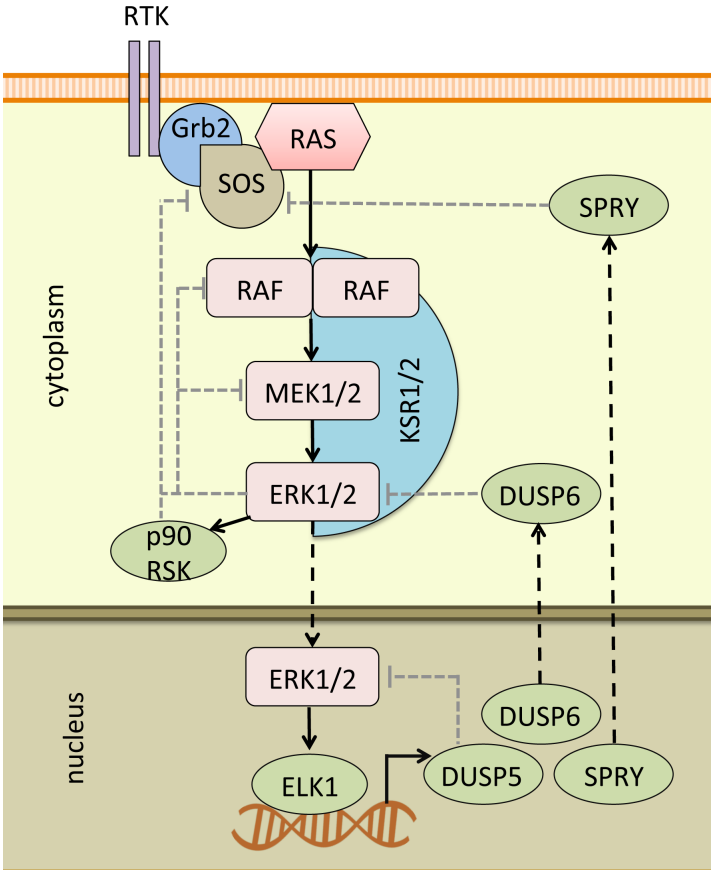

Figure 1.5. Scaffolds and negative feedback signaling fine-tune RAS-RAF-MEK-ERK signaling outcome. The kinase suppressor of RAS 1 (KSR1) scaffold protein facilitates the assembly of RAF, MEK and ERK to enhance signaling efficiency. Rapid and direct feedback by inhibitory phosphorylation on SOS, BRAF, CRAF and MEK1 by ERK1/2, and in some cases, the ERK1/2 effector p90 RSK, limits signal transduction through the pathway. ERK1/2 activation induces the transcriptional expression of ERK-specific dual-specificity phosphatases (DUSPs) and Sprouty (SPRY) proteins. Nuclear DUSP5 and cytoplasmic DUSP6 directly dephosphorylate and inactivate ERK1/2. SPRY proteins are recruited to the plasma membrane upon growth factor stimulation, and interfere with RTK coupling to RAS.

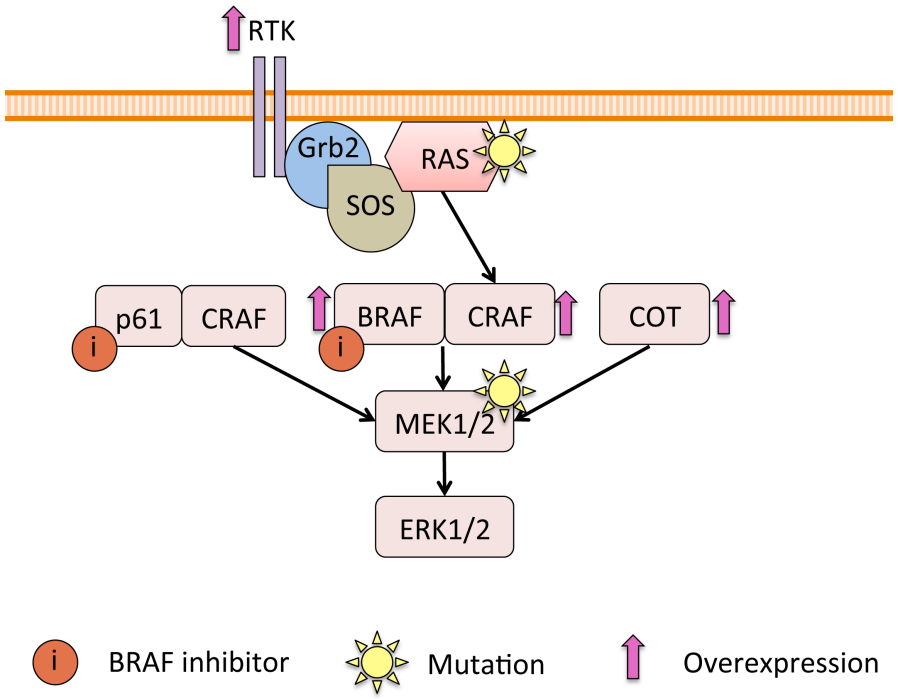

Figure 1.6. Mechanisms of resistance to BRAF inhibition lead to ERK1/2 reactivation. Mutations in NRAS at residue Q61 and in MEK1 at several sites are known to confer resistance to BRAF inhibition. Upregulation of receptor tyrosine kinase signaling, increased expression of CRAF and COT, and amplifications in *BRAF*^{V600E}, also promote BRAF inhibitor resistance. A 61 kDa, alternatively spliced form of BRAF^{V600E} (p61) lacking exons 4-8 (encoding the RAS binding domain of BRAF) dimerizes independently of RAS, thereby facilitating signaling through its wild-type RAF partner.

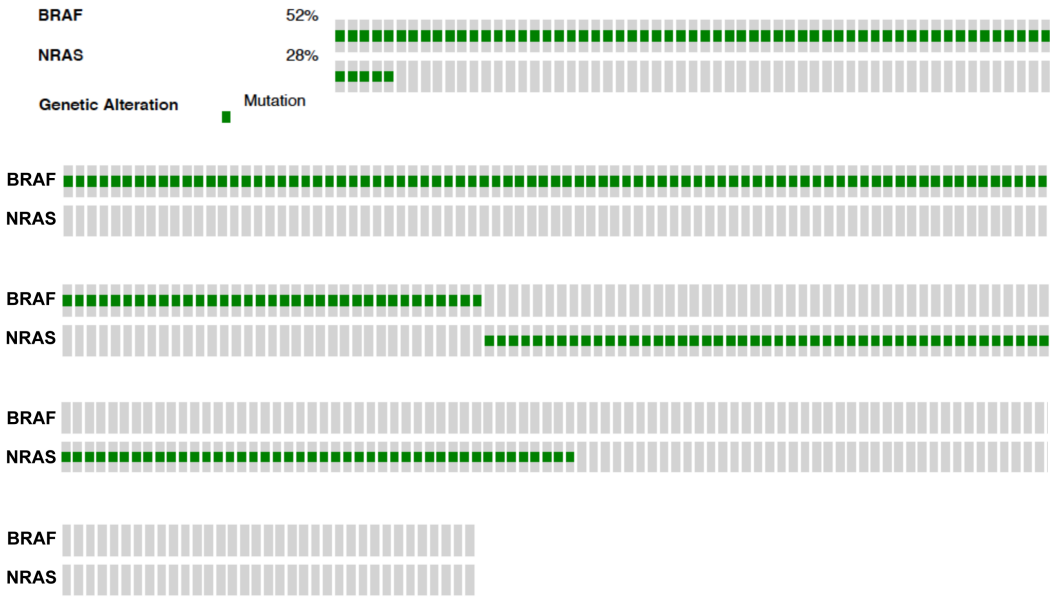

Figure 1.7. BRAF and NRAS mutations in skin cutaneous melanoma. BRAF and NRAS mutations account for 52% and 28% of melanoma cases, respectively. BRAF and NRAS mutations are largely mutually exclusive (mutual exclusivity *p*-value < 0.001). Data acquired from www.cBioPortal.org (345 samples).

CHAPTER II: CK2α MAINTAINS ERK ACTIVITY TO PROMOTE RESISTANCE TO INHIBITORS OF THE RAF-MEK-ERK PATHWAY IN BRAF-MUTANT MELANOMA

Overview

The protein kinase CK2 is a pleiotropic and constitutively active kinase that plays crucial roles in cellular proliferation and survival. Overexpression of CK2, particularly the alpha catalytic subunit (CK2α, CSNK2A1), has been implicated in a wide variety of cancers and is associated with poorer survival and with resistance to both conventional and targeted anticancer therapies. Here, we found that CK2α protein is elevated in melanoma cell lines compared to normal human melanocytes. We then tested the involvement of CK2α in drug resistance to FDA-approved single-agent targeted therapies for melanoma. In *BRAF*-mutant melanoma cells, ectopic CK2α decreased sensitivity to vemurafenib (BRAFi), dabrafenib (BRAFi), and trametinib (MEKi), by a mechanism distinct from that of mutant NRAS. Conversely, knockdown of CK2α sensitized cells to inhibitor treatment. CK2α-mediated RAF-MEK kinase inhibitor resistance was tightly linked to its maintenance of ERK phosphorylation. We found that CK2α post-translationally regulates the ERK-specific phosphatase DUSP6 in a kinase dependent-manner, decreasing its abundance. However, we unexpectedly showed, by using a kinase-inactive mutant of CK2α, that RAF-MEK inhibitor resistance did not rely on CK2α kinase catalytic function, and both wild-type and kinase-inactive CK2α maintained ERK phosphorylation upon inhibition of BRAF or MEK. That both wild-type and kinase-inactive CK2α bound equally well to

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the RAF-MEK-ERK scaffold KSR1 suggested that CK2α increases KSR facilitation of ERK phosphorylation. Accordingly, CK2α did not cause resistance to direct inhibition of ERK by the ERK1/2-selective inhibitor SCH772984 (Morris et al., 2013). Our findings support a kinase-independent scaffolding function of CK2α that promotes resistance to RAF- and MEK-targeted therapies.

Introduction

The protein kinase CK2 (casein kinase 2 or II), is a highly conserved, ubiquitously expressed, pleiotropic, and constitutively active serine/threonine kinase that has crucial roles in cell survival, proliferation and differentiation (Pinna, 2002; Pinna and Allende, 2009; Trembley et al., 2009). The CK2 holoenzyme consists of two regulatory (beta) and two catalytic (alpha or alpha') subunits, the latter of which can also function independently of the tetramer (Hanif et al., 2010). Although CK2 itself does not appear to be a proto-oncogene, its upregulation has been shown to promote growth and prevent apoptosis, both of which promote cancer (Trembley et al., 2009). Indeed, overexpression of CK2 at the transcript and/or protein level has been observed in many cancers (Ortega et al., 2014), including multiple myeloma (Piazza et al., 2006), chronic lymphocytic leukemia (Martins et al., 2010), breast (Giusiano et al., 2011), colorectal (Lin et al., 2011) and liver cancers (Zhang et al., 2015), *etc*., and is correlated with poorer patient survival (Bae et al., 2015; Lin et al., 2011; Ortega et al., 2014). Similarly, CK2 exhibited 2.5-fold higher catalytic activity in metastatic melanoma than in dermal nevi (Mitev et al., 1994).

In addition to its roles in tumor growth and progression, CK2 also promotes drug resistance to both conventional and targeted therapeutics. For example, pharmacological inhibition of CK2 kinase activity reverted multidrug resistance of a human T lymphoblastoid cell line (Di Maira et al., 2007), at least in part by down-regulating P-glycoprotein activity. In addition, siRNA-mediated knockdown of CK2 catalytic subunits enhanced chemosensitivity to gemcitabine in human pancreatic cancer cells (Kreutzer et al., 2010). In cells depleted of CK2α,

gemcitabine induced MKK4/JNK signaling, resulting in cell death (Kreutzer et al., 2010). Further, CK2 displayed elevated protein expression and activity in chronic myeloid leukemia cells that are resistant to the small molecule kinase inhibitor imatinib (Borgo et al., 2013). Either reduction of CK2α expression or pharmacological inhibition of CK2α kinase activity restored imatinib sensitivity, possibly through suppressing Akt activity (Borgo et al., 2013). However, the role of CK2α in drug resistance, particularly to targeted therapeutics, has remained underexplored.

Inhibitors of BRAF and MEK, members of the RAF-MEK-ERK kinase cascade, have achieved remarkable overall response rates in advanced melanomas harboring BRAF V600 mutations, yet a significant proportion of patients is intrinsically resistant to such therapies, and those who respond almost inevitably develop resistance over a matter of months (Menzies and Long, 2014). Considerable efforts have been invested in identifying resistance mechanisms of *BRAF*-mutant melanomas to BRAF inhibition, and some have demonstrated clinical relevance (Lito et al., 2013; Spagnolo et al., 2014; Sullivan and Flaherty, 2013; Wang et al., 2015). In a recent whole-kinome siRNA screen for kinases that could induce resistance to ERK kinase inhibitors in pancreatic ductal adenocarcinoma cells, we identified CK2α as a synthetic lethal partner of ERK inhibition (Hayes et al., 2016). We postulated that kinase inhibitor resistance mechanisms can be shared by diseases that show hyperactivity of the same pathway. Given that the RAF-MEK-ERK pathway is strongly activated in both pancreatic cancer and melanoma, we sought to determine whether CK2 also plays a role in resistance to inhibition of this pathway in melanoma.

In the present study, we found that CK2α overexpression was sufficient to drive resistance to both BRAF and MEK inhibitors in BRAF-mutant melanoma cells. Conversely, depletion of CK2α increased sensitivity to the BRAF inhibitor vemurafenib. Consistent with these results, CK2α sustained ERK phosphorylation under conditions of pathway inhibition. Although we found that CK2 negatively regulated expression of the ERK-specific phosphatase DUSP6 in a kinase-dependent manner, yet the maintenance of ERK phosphorylation was not

due to these decreased levels of DUSP6. Instead, we found that CK2α-mediated maintenance of ERK phosphorylation and drug resistance were kinase-independent. The ability of both wild-type and kinase-inactive CK2α to bind to the key RAF-MEK-ERK pathway scaffold protein KSR1, that is required for optimal ERK phosphorylation and activation, supports a kinase-independent scaffolding role for CK2α in facilitating optimal ERK signaling under conditions of pathway inhibition. That CK2α overexpression did not cause resistance to a direct ERK inhibitor is further evidence that ERK inhibition may overcome resistance mechanisms that shorten the effectiveness of blocking upstream kinases in the RAF-MEK-ERK pathway.

Experimental procedures

Cell culture and reagents

A375, A2058, Sbc12A, Malme-3 and 293T cell lines were grown in DMEM-H supplemented with 10% FBS (HyClone™, Thermo Scientific) and 1% gentamycin/kanamycin (Tissue Culture Facility (TCF), Lineberger Comprehensive Cancer Center (LCCC), University of North Carolina at Chapel Hill (UNC-CH)). SK-MEL-28 and RPMI-7951 were grown in MEM-Alpha (Gibco) supplemented with 10% FBS and 1% gentamycin/kanamycin. Normal human melanocyte pellets were kindly provided by Dr. William Kaufmann, UNC-CH. The BRAFi vemurafenib (PLX4032) was a generous gift from Gideon Bollag (Plexxikon). The BRAFi dabrafenib (GSK2118436) MEKi trametinib (GSK1120212) were purchased from Selleckchem. MG132 was purchased from Calbiochem (#474790).

Plasmid constructs and gateway cloning

The CK2α expression construct, pDONR223-CSNK2A1 (Human ORFeome v5.1), was purchased from UNC's TCF. pHAGE-FLAG (N-terminal tag) empty vector was a generous gift from Ben Major, UNC-CH. Both CK2α and NRAS(Q61K) were cloned into the pHAGE-FLAG vector by Gateway cloning using LR Enzyme Clonase Mix (Invitrogen), according to the

manufacturer's protocol. A set of five shRNAs targeting CK2α (*CSNK2A1*) in pLKO.1 vector was purchased from the Lenti-shRNA Core Facility at UNC-CH. Target sequences are indicated below.

shRNA	TRC Clone ID	Target sequence
#1	TRCN0000000606	5'-GCTGCATTTAGGTGGAGACTT-3'
#2	TRCN0000000607	5'-CGTAAACAACACAGACTTCAA-3'
#3	TRCN0000000608	5'-CAAGAATATAATGTCCGAGTT-3'
#4	TRCN0000000609	5'-AGAATTTGAGAGGAGGTCCCA-3'
#5	TRCN0000000610	5'-CCAAGAATATAATGTCCGAGT-3'

Lentivirus production and infection

To produce lentivirus, 293T cells were transfected with pHAGE vector-based GFP, CK2α or NRAS(Q61K), in combination with psPAX2 and pMD2.G, at a ratio of 4:3:1. After overnight transfection, the culture medium was changed to DMEM-H supplemented with 20% FBS. Thirty-six hours later, viral supernatants were harvested and filtered through a sterile 0.45 μm filter to remove cell debris. Cleared supernatants were aliquotted and frozen at -80°C until use. Cells were infected with 500 μl virus in 5 μg/ml polybrene (Millipore) overnight. Selection of transduced cells in puromycin (1 μg/ml or 2 μg/ml for A375 or SK-MEL-28 cells, respectively) was complete at 48 h after infection.

Western blotting

Cells were washed twice with ice-cold PBS and lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0) containing 1X protease inhibitors (BaculoGold™ Protease Inhibitor Cocktail, BD Biosciences, #51-21426Z) and 1× phosphatase inhibitors (Halt™ Phosphatase Inhibitor Cocktail, Thermo Scientific, #78420).

Lysates were depleted of cell debris by centrifugation at maximum speed (4°C, 10 min), then proteins were quantified by Bradford assay (*DC*™ Protein Assay, Bio-Rad), normalized, reduced, denatured at 95 °C for 5 min, and resolved by SDS gel electrophoresis. Proteins were transferred to PVDF membranes (Millipore, #IPFL00010) and probed with primary antibodies recognizing pERK (Cell Signaling, #4370), ERK (Cell Signaling, #9102), pMEK (Cell Signaling, #9154), pRSK (Cell Signaling, #9344), DUSP6 (Abcam, ab54940), beta-actin (Sigma, A5316), CK2α (Santa Cruz, sc-373894), FLAG-tag (Sigma, F3165; Novus, NBP1-06712SS), or GFP (Roche, 11814460001). A rabbit antibody to the phosphorylated CK2α substrate EEF1D was a generous gift from David W. Litchfield (Western University). A rabbit-anti KSR1 antibody (Morrison Lab) was used to detect KSR1 following immunoprecipitation. After incubation with the appropriate secondary anti-mouse (GE Healthcare, NA931V) or anti-rabbit (GE Healthcare, NA934V) antibody, proteins were detected by chemiluminescence (Thermo Scientific, #34075). Blots were developed by exposure to X-ray film, or by the ChemiDoc™ MP Imaging System (Bio-Rad) for quantification.

Pharmacologic growth inhibition (GI_{50}) assay

Growth inhibition assays were performed as described previously (Johannessen et al., 2013; Nazarian et al., 2010) with minor modifications. Cultured cells were seeded into 96-well plates (2,000 cells per well). Sixteen hours after seeding (baseline), serial dilutions of inhibitors were prepared in DMSO and added to cells, yielding final drug concentrations ranging from 1 nM to 10 μM for vemurafenib, dabrafenib and SCH772984, 0.1 nM to 1 μM for trametinib, with the final volume of DMSO not exceeding 1%. Cells were incubated for 72 h following addition of drug. To measure cell proliferation, 5 mg/ml MTT (Sigma-Aldrich, #M5655) was added 1:10 into wells, and incubated at 37°C for 4 h. Formazan products were solubilized using acidified isopropanol (0.04N HCl in isopropanol), and absorbance was measured at 562 nm, with a

background subtraction at 650 nm. Percent cell growth under each condition was calculated as follows: Cell growth (%)=100×(T-T_0)/(C-T_0), where T_0 is absorbance at baseline, T is absorbance of drug-treated wells at 72 h, C is absorbance of DMSO-treated wells at 72 h. A minimum of three replicates was performed for each cell line and drug combination. Data from growth inhibition assays were modeled using a non-linear regression curve fit with a sigmoidal dose-response (GraphPad Prism, v5.0c). The resulting curves were displayed, and GI_{50} values were also generated, using GraphPad Prism.

Clonogenic assay

Clonogenic assays were performed as described previously (Franken et al., 2006) with slight modifications. Briefly, cells were plated in duplicate wells at 100 cells/well in 6-well plates, and allowed to adhere for 3 h at 37°C, after which culture medium was carefully removed and replaced with medium containing either DMSO vehicle control or inhibitor. Two weeks following drug treatment, cells were washed once with PBS, then fixed and stained with crystal violet/paraformaldehyde for 30 min at room temperature. Stain was decanted, and plates were carefully rinsed with distilled water, until background staining of the wells was minimized. Finally, plates were air-dried, and colonies were counted manually using a cell counter.

Site-directed Mutagenesis

Site-directed mutagenesis of CK2α was performed using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent). A forward primer 5'-GCCATCAACATCACAAATAATGAAAAAGTTGTTGTTA**TG**ATTCTCAAGCCAG -3' and reverse primer 5'- CTGGCTTGAGAAT**CA**TAACAACAACTTTTTCATTATTTGTGATGTTGATGGC -3' were used to introduce a catalytic site mutation (K68M) into pDONR223-CSNK2A1, with bolded

nucleotides showing the site of mutagenesis. Reaction conditions strictly followed the manufacturer's protocol.

RNA isolation, reverse transcription and real-time PCR

Total RNA was isolated from cells using Thermo RNA kit (Thermo Scientific), and then 0.5 μg total RNA was reverse transcribed to generate cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. Real-time PCR was performed using the SsoFast™ EvaGreen® Supermix (Bio-Rad) on the Bio-Rad CFX-96 Real-Time PCR System. Beta-actin was used for normalization. DUSP6 was amplified using forward primer 5'-CGACTGGAACGAGAATACGG-3' and reverse primer 5'-TTGGAACTTACTGAAGCCACCT-3'.

Co-immunoprecipitation

Co-immunoprecipitation assays were performed as described previously (Ritt et al., 2007). In brief, two 10 cm dishes of A375 cells were washed twice with cold PBS and lysed in 1% NP-40 buffer (20 mM Tris [pH 8.0], 137 mM NaCl, 10% glycerol, 1% NP-40 alternative, 0.15 U/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM sodium vanadate, 20 μM leupeptin) using 600 μl lysis buffer/10 cm dish. The lysates were clarified by centrifugation and equivalent amounts of protein lysate were incubated with a mouse-anti-human KSR1 antibody (Sigma-Aldrich, WH0008844M1) and protein G sepharose beads for 3 h at 4 °C. Immunoprecipitated complexes were collected by centrifugation, washed extensively with 1% NP-40 buffer and then examined by immunoblot analysis.

Results

CK2α expression is upregulated in a subset of melanomas

To examine the expression of CK2α in melanoma, we first surveyed the Cancer Genome Atlas (TCGA) skin cutaneous melanoma dataset for CK2α mRNA expression through cBioPortal (http://www.cbioportal.org)**.** We found that the CK2α transcript is upregulated in a subset of those tumors (15% of 278 samples), and that 90% of that subset also harbor mutations in BRAF, NRAS and/or NF1 that lead to hyperactivation of ERK. Next, we measured CK2α protein expression in a panel of normal human melanocytes (NHMs) and melanoma cell lines (5 BRAF-mutant: A375, SK-MEL-28, A2058, RPMI-7951, Malme-3; and 1 NRAS-mutant: Sbc12A). Compared to NHM, all of the melanoma cell lines had higher levels of CK2α protein expression (Figure 2.1). Like the melanoma patient samples evaluated by TCGA, BRAF- and NRAS-mutant melanoma cell lines also exhibit ERK hyperactivation (Shields et al., 2007; Solit et al., 2006).

CK2α promotes resistance to inhibitors of BRAF and MEK in BRAF-mutant melanoma cells

We recently used a whole-kinome siRNA screen to search for mechanisms of resistance to ERK inhibition in pancreatic ductal adenocarcinoma cells, and found that CK2α was one of the hits identified (Hayes et al., 2016). To test whether CK2α promotes resistance to conventional RAF-MEK-ERK pathway inhibition in melanoma cells with hyperactivation of this pathway, we first stably expressed FLAG-tagged wild-type CK2α in A375 melanoma cells (Figure 2.2A,B). These cells possess a homozygous BRAF(V600E) mutation and are sensitive to both BRAFi and MEKi. We then assessed sensitivity to growth inhibition by multiple inhibitors of the pathway, including mutant BRAF-selective inhibitors vemurafenib and dabrafenib, and MEK1/2-selective inhibitor trametinib. Oncogenically activated (mutant) NRAS has been identified in patients with BRAF-mutant melanomas as one mechanism of resistance to BRAF but not MEK inhibition (Nazarian et al., 2010). Therefore, we stably expressed FLAG-tagged

mutant NRAS(Q61K) (Figure 2.2A) as a positive control. Neither CK2α nor mutant NRAS increased the already high basal level of ERK phosphorylation (Figure 2.2C). But, as expected, mutant NRAS promoted resistance to both BRAF inhibitors vemurafenib and dabrafenib, as evidenced by increased values for 50% growth inhibition (GI_{50}) of 5.5-fold and 4.4-fold, respectively, compared to the GFP negative control (Figure 2.2D). In contrast, mutant NRAS only mildly increased the GI_{50} (1.7-fold) for the MEKi trametinib, consistent with findings that BRAF-mutant melanomas with secondary NRAS mutations still remain sensitive to MEK inhibition (Nazarian et al., 2010). Notably, expression of CK2α increased the GI_{50} for vemurafenib, dabrafenib, and trametinib by 4.7-fold, 3.8-fold and 7.4-fold, respectively, indicating reduced sensitivity to all three inhibitors (Figure 2.2D). Expression of CK2α also produced resistance to vemurafenib in another BRAF-mutant melanoma cell line, SK-MEL-28 (data not shown). To further evaluate the effect of CK2α expression on responses to the abovementioned inhibitors, we performed clonogenic cell survival assays. Consistent with the increased GI_{50} values, CK2α enhanced the clonogenic survival of A375 cells in the presence of each inhibitor (Figure 2.2E). In contrast to CK2α, NRAS(Q61K) significantly enhanced clonogenic survival only in response to vemurafenib. The modest enhancement in clonogenic survival in response to dabrafenib did not reach statistical significance. Together, these results indicate that CK2α overexpression, but not NRAS mutation, is sufficient to induce resistance to both BRAF and MEK inhibition, which implies a CK2α-mediated resistance mechanism distinct from that mediated by mutant NRAS.

CK2α depletion sensitizes melanoma cells to BRAF inhibition

Given that CK2α overexpression was sufficient to drive resistance, we asked whether, conversely, depletion of CK2α would enhance sensitivity to pathway inhibition. We employed a set of five shRNAs (#1-#5) to knock down CK2α in A375 cells. Complete knockdown of CK2α was incompatible with cell survival (data not shown). Therefore, to obtain cells for subsequent

experimentation, we utilized shRNAs #4 and #5, which yielded ~60% knockdown and sufficient viability (Figure 2.3A). Even this incomplete depletion of CK2α expression resulted in decreased GI_{50} for vemurafenib (55% and 65% decrease for shRNAs #4 and #5, respectively; Figure 2.3B). This result indicates that CK2α overexpression is necessary for resistance to BRAF inhibition.

CK2α sustains ERK phosphorylation under conditions of RAF-MEK-ERK pathway inhibition

Previous studies have identified multiple mechanisms of resistance to BRAF inhibition, the majority of which are characterized by ERK reactivation (Solit and Rosen, 2014). Given that CK2α and mutant NRAS both promote BRAFi resistance, we hypothesized that they are both capable of facilitating ERK reactivation following inhibition of BRAF and/or MEK. As anticipated, mutant NRAS induced strong ERK reactivation upon inhibition of BRAF with either vemurafenib or dabrafenib (Figure 2.4A,B). CK2α also facilitated ERK rebound, though not as strongly as NRAS(Q61K). Consistent with their trametinib resistance profiles, NRAS(Q61K) failed to reactivate ERK in the presence of trametinib, while CK2α did sustain ERK phosphorylation (Figure 2.4C). The maintenance of ERK phosphorylation by CK2α suggested either maintained upstream activation, or suppressed deactivation mechanisms. MEK is the only known direct activator of ERK (Crews et al., 1992), and phosphorylation of MEK at Ser217 and Ser221 is indicative of MEK activation. DUSP6/MKP3 (dual specificity phosphatase 6/MAP kinase phosphatase 3) is a key ERK-specific phosphatase that reverses MEK phosphorylation at the TEY motif of ERK (Muda et al., 1996). Therefore, we assessed the status of both MEK activation and DUSP6 expression by western blotting. We found that CK2α accelerated the rebound of MEK activation upon MEK inhibition (Figure 2.4C). Intriguingly, DUSP6 expression was also strongly reduced in CK2α-overexpressing cells even without inhibitor treatment (Figure 2.4C). Based on this finding, we initially hypothesized that downregulation of DUSP6 contributed

to the sustained ERK phosphorylation. However, we first needed to confirm whether CK2α truly regulates DUSP6 expression.

CK2α regulates DUSP6 protein levels in a kinase-dependent manner

CK2α has been reported to directly phosphorylate DUSP6 at multiple sites in vitro, the consequences of which are largely unknown (Castelli et al., 2004). To control for CK2α kinase activity, we generated a kinase-inactive mutant of CK2α (Ji et al., 2009), and measured DUSP6 protein levels upon ectopic expression of either kinase-inactive (K68M) or wild-type (WT) CK2α. As expected, CK2α(WT) was constitutively active, and cells overexpressing this form of CK2α exhibited elevated basal phosphorylation of EEF1D (Figure 2.5A), a validated marker of CK2 activity (Gyenis et al., 2011). In contrast, cells expressing kinase-inactive CK2α(K68M) exhibited mildly reduced levels of EEF1D phosphorylation (Figure 2.5A), suggesting a weak dominant-negative effect. Interestingly, CK2α(WT) drastically reduced DUSP6 abundance whereas K68M did not have an effect (Figure 2.5B), indicating that the decrease in DUSP6 protein is likely due to CK2α-mediated phosphorylation. Conversely, shRNA-mediated knockdown of endogenous CK2α enhanced DUSP6 protein levels (Figure 2.5C). To determine whether the reduction in DUSP6 was the result of accelerated degradation or suppressed transcription, we first used MG132 to block proteasome-mediated protein degradation. Six hours after MG132 treatment, DUSP6 abundance was fully rescued (Figure 2.5D). We also examined DUSP6 mRNA levels by qPCR, and found that they did not change upon CK2α expression (Figure 2.5E). These results indicate that CK2α kinase activity regulates DUSP6 abundance by facilitating its proteasomal degradation.

CK2α-mediated maintenance of ERK phosphorylation and pathway inhibitor resistance does not require its kinase function

Given that CK2α kinase activity was essential to the decrease in DUSP6 expression (Figure 2.5B), we hypothesized that kinase-inactive CK2α would not be able to maintain ERK phosphorylation when the pathway was inhibited. Unexpectedly, the kinase-inactive mutant also sustained ERK phosphorylation, comparable to that of the wild-type kinase (Figure 2.6A). We therefore anticipated that kinase-inactive CK2α would also promote resistance in a similar fashion. Consistent with this, we found that CK2α(K68M) promoted resistance to vemurafenib, dabrafenib and trametinib to the same extent as CK2α wild-type (Figure 2.6B), indicating that CK2α-mediated BRAFi and MEKi resistance does not depend on its catalytic kinase activity. Instead, our findings indicate that the ability of CK2α to maintain ERK phosphorylation when the pathway is inhibited and to promote resistance to BRAF and MEK inhibitors is more likely due to a protein-binding or scaffolding function of CK2α.

CK2α(WT) and CK2α(K68M) bind equally well to the RAF-MEK-ERK scaffold protein KSR1

Our previous work (Ritt et al., 2007) uncovered an essential role of CK2α in maximally facilitating RAF-MEK-ERK pathway activation through its direct binding to KSR1 (Kinase Suppressor of Ras 1), within the KSR1 scaffolding complex that also includes RAF, MEK and ERK. CK2α association with KSR enhances RAF phosphorylation of MEK. Given our finding here that kinase-inactive CK2α maintains ERK phosphorylation and resistance to pathway inhibitors to the same extent as its wild-type counterpart, we speculated that it too retains binding to KSR1. Accordingly, when we immunoprecipitated endogenous KSR1 from A375 cells, we detected considerable levels of ectopically expressed CK2α(WT) and CK2α(K68M), but not the GFP control (Figure 2.7). This result supports our hypothesis that CK2α binding to KSR1 is kinase-independent, offering a potential mechanism by which both CK2α(WT) and CK2α(K68M) maintain ERK phosphorylation and resistance to BRAF and MEK inhibitors.

Specifically, our findings are consistent with a model whereby CK2α binding enhances the efficiency of KSR1 scaffolding to facilitate ERK activation.

ERK inhibition avoids CK2α-mediated resistance to RAF-MEK-ERK pathway blockade

Our model predicts that CK2 α should not be able to cause resistance in melanoma cells to an inhibitor downstream of RAF and MEK that acts directly at the level of ERK. To test this model, we evaluated whether CK2α could cause resistance to the ERK inhibitor SCH772984 (Morris et al., 2013). Consistent with our model, neither CK2α nor NRAS(Q61K) caused resistance to the ERKi, as evidenced by the absence of either increased GI_{50} (Figure 2.8A) or enhanced clonogenic survival (Figure 2.8B). For evidence that the inhibitor correctly hit its ERK target, we examined the phosphorylation status of the ERK substrate p90 RSK (Figure 2.8C). We (Hayes et al., 2016) and others (Morris et al., 2013) have shown recently that decreased phospho-RSK (pRSK) is a more reliable marker of decreased flux through ERK than is ERK phosphorylation itself, not least because ERK phosphorylation rebounds quickly whereas pRSK does not. Examination of pRSK in ERKi-treated cells at the same time point (72 h) as the GI_{50} analysis revealed that neither overexpressed CK2α nor mutant NRAS was able to restore ERK pathway activation in the presence of ERKi. Thus, although CK2α induces resistance to BRAFi and MEKi in a kinase-independent manner, even kinase-intact CK2α does not induce resistance to ERKi, which is an effective means of impairing the RAF-MEK-ERK pathway in these BRAF-mutant melanoma cells. This finding is similar to other mechanisms that cause resistance to RAFi or MEKi in BRAF-mutant melanomas, where ERKi sensitivity is retained (Morris et al., 2013).

Discussion

Although targeted therapies in melanoma have substantially improved patient outcomes immediately following treatment in a subset of patients, even responsive patients are confronted

with the inevitable development of resistance months later (Lito et al., 2013; Spagnolo et al., 2014; Sullivan and Flaherty, 2013; Zhang, 2015). Understanding the underlying mechanisms of innate or acquired resistance is key to developing new combination therapies to overcome tumor unresponsiveness or recurrence. In the present study, we demonstrate that abnormally elevated expression of the alpha catalytic subunit of protein kinase CK2 (CK2α, CSNK2A1) is sufficient to cause resistance to each of three small molecule kinase inhibitors of the RAF-MEK-ERK pathway approved for treatment of melanoma: vemurafenib, dabrafenib and trametinib. Further, we show that this resistance correlates with the rebound/maintenance of ERK activity following pathway inhibition.

The distinct resistance profiles of CK2α and NRAS(Q61K) imply different mechanisms of promoting resistance. Specifically, it is known that secondary NRAS mutations that increase flux through the RAS-MEK-ERK pathway via CRAF activation can overcome inhibitor potency (Nazarian et al., 2010). Such a route of reactivation could easily be blocked by MEK inhibition. Consistent with this idea, NRAS(Q61K) did not confer resistance to MEK inhibitor trametinib. However, the fact that CK2α-mediated resistance is MEK inhibitor-inert suggests two possible mechanisms. The first entails some unknown bypass that leads to sustained ERK phosphorylation. This is somewhat unlikely because MEK is still the only known direct activator of ERK. The second involves steric hindrance provided by CK2α that prevents a MEK inhibitor from binding to its target effectively. Such a mechanism can be provided by a scaffolding function of CK2α, as discussed below.

Intriguingly, we found that wild-type CK2α drastically reduced expression of the dual specificity phosphatase 6 (DUSP6), whereas CK2α silencing elevated endogenous DUSP6 protein levels. DUSP6 is a key ERK-specific phosphatase that negatively regulates the RAF-MEK-ERK pathway (Muda et al., 1996). Indeed, DUSP6 has been previously reported to interact with and to be phosphorylated by CK2α (Castelli et al., 2004). We further show here that CK2α-facilitated proteasomal degradation accounts for the decreased abundance of

DUSP6 protein. In light of our results, it would be interesting to know whether DUSP6 expression could serve as a biomarker of CK2 inhibition.

Much to our initial surprise, we determined, using a kinase-inactive mutant of CK2α, that CK2α kinase activity is not required for either CK2α-mediated inhibitor resistance or sustained ERK phosphorylation in the context of these BRAF-mutant melanoma cells. This was unexpected because CK2 is well-known as a constitutively active kinase that has hundreds of endogenous substrates, and its kinase activity has largely been assumed to be responsible for its pleiotropic effects (Pinna, 2002; Pinna and Allende, 2009; Trembley et al., 2009). However, the whole-kinome screen by which we identified CK2α as a potential resistance mechanism capable of inducing at least a 5-fold increase in resistance to ERK inhibitor was not performed by inhibition of the catalytic activity of the CK2α kinase, but rather by siRNA-mediated depletion of expression of the entire protein (Hayes et al., 2016). Therefore this screen would capture effects induced by loss of protein-binding or scaffolding functions as well as by loss of catalytic activities of the depleted kinases.

Our data suggest that the above resistance phenotypes are the result of CK2α-mediated protein-protein interactions rather than CK2α kinase activity. Consistent with this notion, we reported previously that all subunits of CK2 bind to the RAF-MEK-ERK pathway scaffolding protein KSR1 (Ritt et al., 2007), and that binding of the CK2α subunit in particular to KSR1 is critical for maximal activation of the pathway (Ritt et al., 2007). However, we had not tested whether the kinase activity of CK2α was required. Since we have now found that kinase deficiency does not impair CK2α binding to KSR1, we speculate that the catalytic activity-independent binding of CK2α to KSR1 helps to maintain the integrity and function of the RAF-MEK-ERK pathway, enabling the sustained ERK phosphorylation observed in the presence of overexpressed CK2α even when the pathway is inhibited at the level of RAF or MEK. This hypothesis could be further tested in the future once the region of CK2α that mediates KSR1 binding has been identified, as that would allow interrogation of KSR1-binding-deficient mutants

of CK2α, It would also be of great interest to determine whether the onset of BRAFi/MEKi resistance in melanoma patients is associated with increased levels of CK2α.

The importance of CK2α protein-protein interactions versus catalytic kinase activity may differ greatly depending on context. A recent study examined the effects of a CK2α-selective kinase inhibitor, CX-4945, on the viability of BRAF-mutant thyroid cancer cell lines and found synergism of CX-4945 with both the BRAFi vemurafenib and the MEKi selumetinib (Parker et al., 2014), suggesting that the kinase activity of CK2α was important for the response to BRAFi/MEKi in this tumor type. Surprisingly, when they compared the combination of vemurafenib with CX-4945 or with siRNA directed against CK2α in a patient-derived BRAF-mutant melanoma cell line, they found an additive effect of each on cell death (Parker et al., 2014). The equivalent effects on vemurafenib responses of kinase-intact and kinase-inactive CK2α that we observed argues that the kinase activity is not important in the vemurafenib response of BRAF-mutant melanoma, but it is certainly possible that other genetic differences may also affect the relative roles of catalytic activity versus protein-protein interactions. It is also interesting that, while our original siRNA screen identified CK2α as a mediator of resistance to ERKi in KRAS-mutant pancreatic ductal adenocarcinoma cells, CK2α did not mediate resistance to ERKi in BRAF-mutant melanoma cells. Clearly much remains to be elucidated about the role of CK2α in responses to inhibitors of the RAF-MEK-ERK pathway, a role that is likely to be as complex as its hundreds of substrates and numerous biological activities portend.

In summary, our results identify a role for CK2α in promoting resistance to BRAF and MEK but not ERK inhibitors in BRAF-mutant melanoma. We also demonstrate, for the first time to our knowledge, a kinase-independent function of CK2α in modulating cellular signaling. These findings represent a novel mode of innate resistance to RAF-MEK targeted therapy in BRAF-mutant melanoma, which may not be easily addressed by inhibition of the dysregulated CK2α kinase.

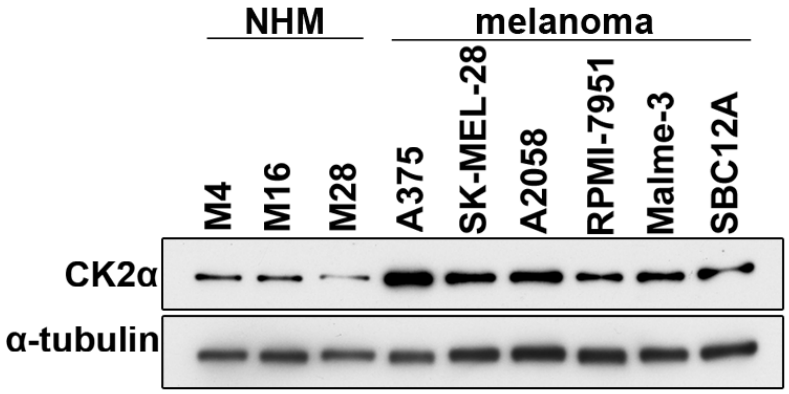

Figure 2.1. CK2α protein expression is elevated in melanoma cell lines compared to normal human melanocytes (NHM). Cell lysates from a panel consisting of 3 NHM, 5 BRAF- and 1 NRAS-mutant melanoma cell lines were probed for CK2α protein by western blotting with anti-CK2α antibody. Alpha-tubulin was used as a loading control.

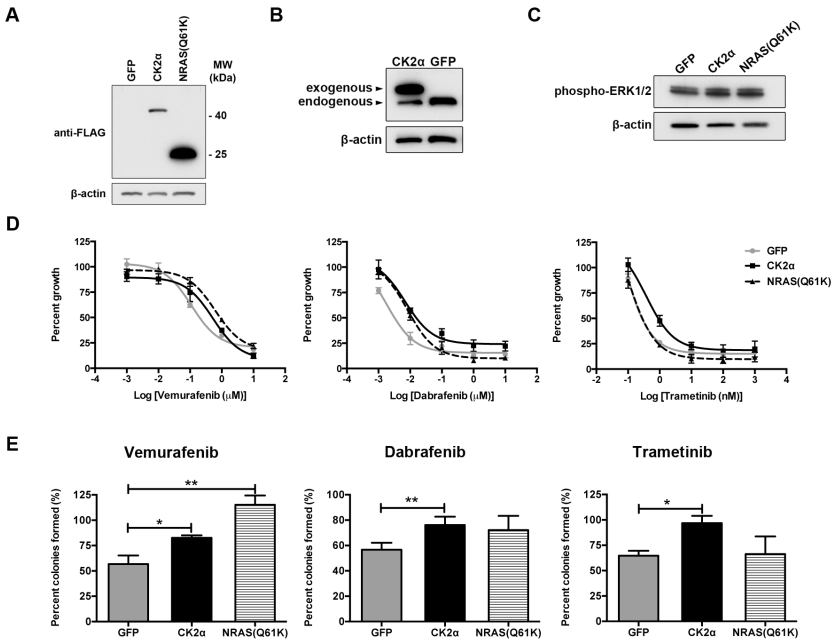

Figure 2.2. Ectopic CK2α promotes resistance to inhibitors of BRAF and MEK. *A*, A375 cells were stably infected with lentiviral vectors to ectopically express GFP negative control, or FLAG-tagged CK2α or NRAS(Q61K), and cell lysates were subjected to western blot using anti-FLAG antibody. β-actin served as a loading control. *B*, To determine the relative expression of exogenous versus endogenous CK2α, the A375 cells expressing either FLAG-tagged CK2α or GFP were immunoblotted with anti-CK2α antibody. *C*, Neither CK2α nor NRAS(Q61K) increases the already elevated basal level of ERK phosphorylation in A375 cells, as determined by western blot using anti-phospho-ERK1/2(T202/Y204) antibody. *D*, CK2α increases GI_{50} for BRAFi vemurafenib, BRAFi dabrafenib and MEKi trametinib. MTT assays were performed after 72 h of treatment with 5 different doses of inhibitors, and GI_{50} curves were generated. Results are presented as means ± S.E.M. (n = 6) *E*, CK2α enhances clonogenic survival of inhibitor-treated A375 cells. Cells as in the previous panels were grown for two weeks on plastic as single colonies in the presence of vemurafenib (1 μM), dabrafenib (100 nM), trametinib (1 nM), or DMSO vehicle control. Shown are the percent of colonies formed in the presence of each inhibitor relative to the vehicle control. Results are presented as means ± S.E.M. **, $p<0.01$; *, $p<0.05$. (n = 3)

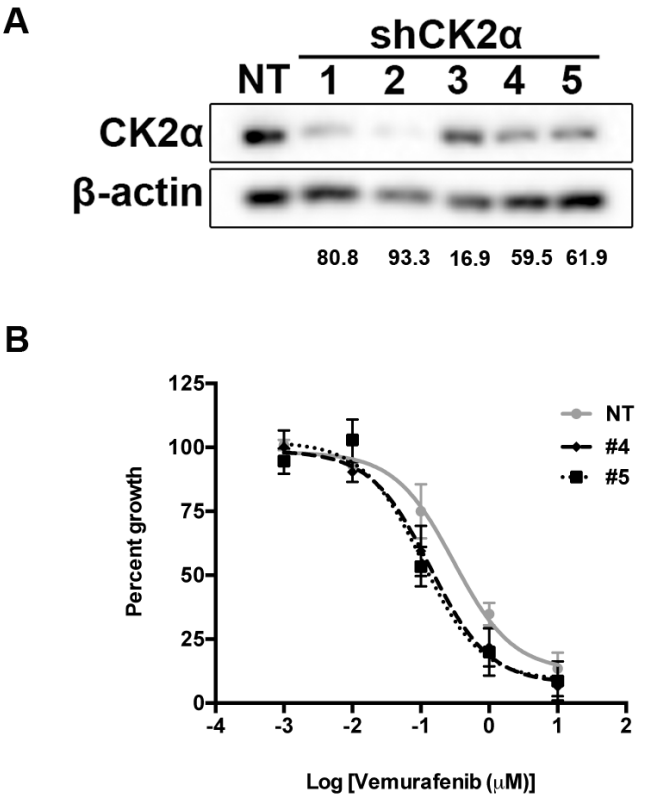

Figure 2.3. Suppression of endogenous CK2α increases sensitivity to the BRAF inhibitor vemurafenib. *A*, Endogenous CK2α was suppressed in A375 cells by using 5 different shRNA sequences, and the degree of knockdown was assessed by western blot using anti-CK2 antibody. β-actin served as a loading control. The percent of knockdown achieved by each shRNA directed against CK2α, normalized to the non-targeting (NT) shRNA, is indicated below each lane. *B*, Knockdown of ~60% of endogenous CK2α (panel A) is sufficient to decrease the GI_{50} for vemurafenib. GI_{50} curves for A375 cells infected with either NT or shRNA#4 (black dashed line) or shRNA#5 (black dotted line) are shown. Results are presented as means ± S.E.M. (n = 3)

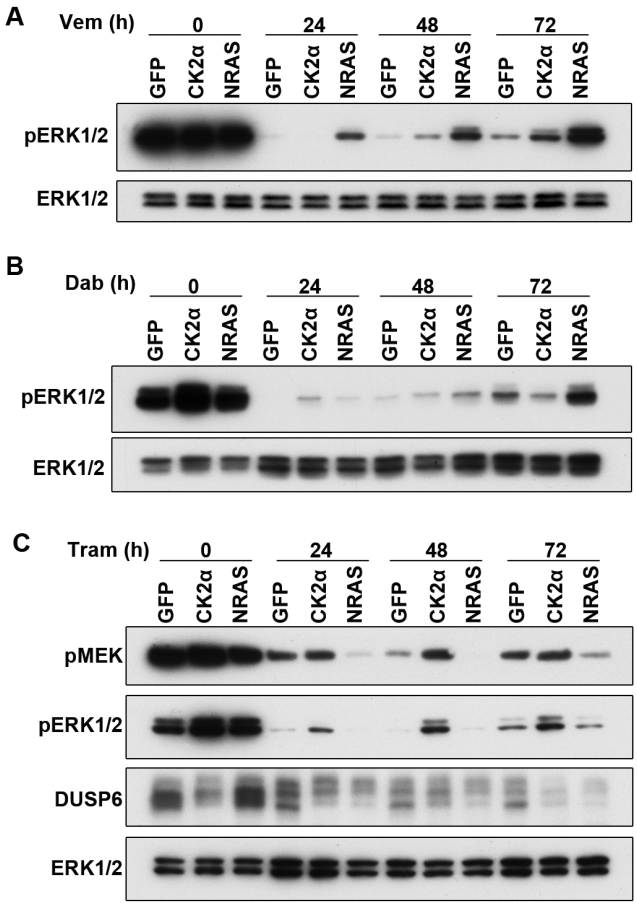

Figure 2.4. Overexpressed CK2α accelerates ERK rebound or sustains ERK phosphorylation in response to RAF-MEK-ERK pathway inhibition. Phosphorylated ERK (pERK1/2) was evaluated by western blot analysis of lysates from A375 cells ectopically expressing GFP, CK2α or NRAS(Q61K), treated for 24, 48 or 72h with *A*, vemurafenib (BRAFi, 1 μM); *B*, dabrafenib (BRAFi, 100 nM), or *C*, trametinib (MEKi, 1 nM). Total ERK1/2 served as a loading control. MEKi (trametinib)-treated cell lysates were additionally immunoblotted for phospho-MEK1/2 (pMEK) and for the ERK1/2-specific phosphatase DUSP6.

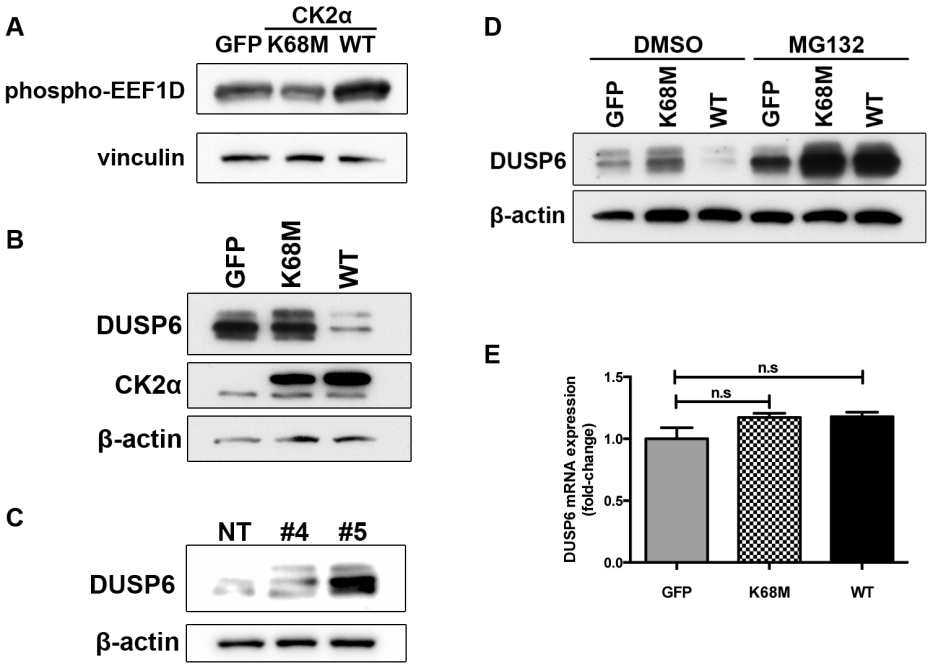

Figure 2.5. CK2α decreases protein stability of the ERK phosphatase DUSP6 in a kinase-dependent manner. *A*, Phosphorylation of the CK2α substrate EEF1D upon expression of wild-type (WT) or kinase-inactive (K68M) CK2α was detected by western blotting with a phospho-EEF1D antibody. *B*, Levels of endogenous DUSP6 protein were determined by western blot of lysates from A375 cells ectopically expressing CK2α(WT) or CK2α(K68M), or *C*, from A375 cells depleted of endogenous CK2α by two different shRNAs (same lysates as shown in Fig. 3A). *D*, To determine whether CK2α regulates DUSP6 protein stability, the same cells as panel *B* were immunoblotted for DUSP6 protein after treatment for 6 h with either the proteasome inhibitor MG132 (10 μM) or DMSO vehicle control. *E*, To determine whether CK2α also regulates DUSP6 at the transcriptional level, qRT-PCR analysis of DUSP6 mRNA levels was done on cells expressing CK2α(WT) or kinase-inactive CK2α(K68M). Results are presented as means ± S.E.M. (n = 3)

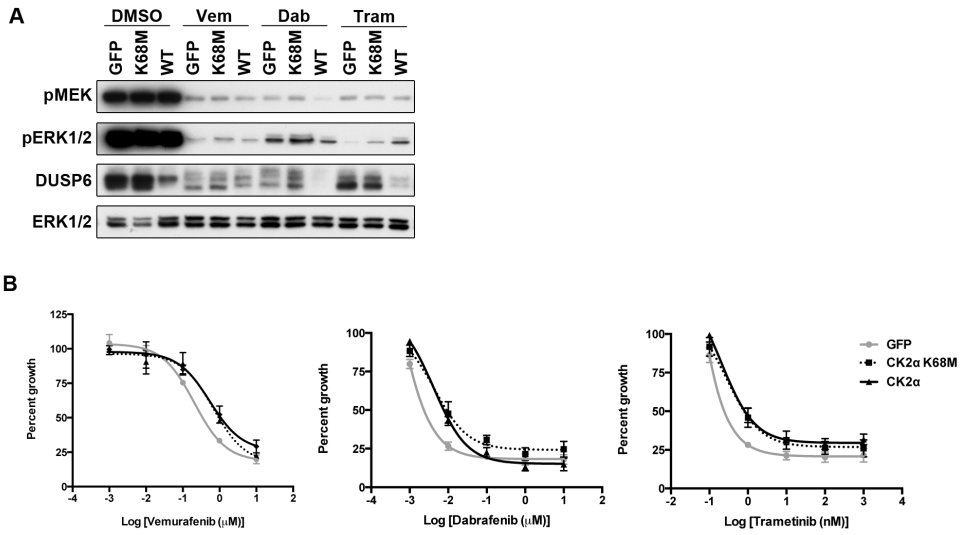

Figure 2.6. CK2α-mediated maintenance of ERK phosphorylation upon pathway inhibition and resistance to BRAFi/MEKi are both kinase-independent. *A*, A375 cells ectopically expressing GFP, CK2α(K68M) or CK2α(WT) were treated with BRAFi and MEKi as in Fig. 4, then lysed and immunoblotted for phosphorylated MEK1/2 (pMEK), phosphorylated ERK1/2 (pERK1/2), and total DUSP6. Total ERK1/2 served as loading control. *B*, Cells were treated as in panel A and GI_{50} curves were generated after 72 h. Results are presented as means ± S.E.M. (n = 4)

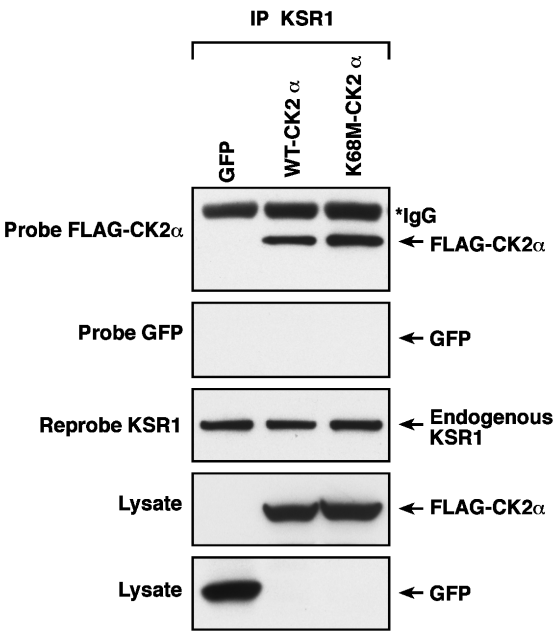

Figure 2.7. Both wild-type and kinase-inactive CK2α interact with the RAF-MEK-ERK scaffold protein KSR1. Endogenous KSR1 was immunoprecipitated from A375 cells expressing the GFP control, or FLAG-tagged CK2α(WT) or kinase-inactive CK2α(K68M). Whole cell lysates and immunoprecipitates were then blotted for the presence of FLAG-CK2α, and reprobed for KSR1 to ensure that equal amounts of KSR1 were immunoprecipitated. GFP served as a negative control to rule out nonspecific co-immunoprecipitation of the ectopic CK2α proteins. (n=2)

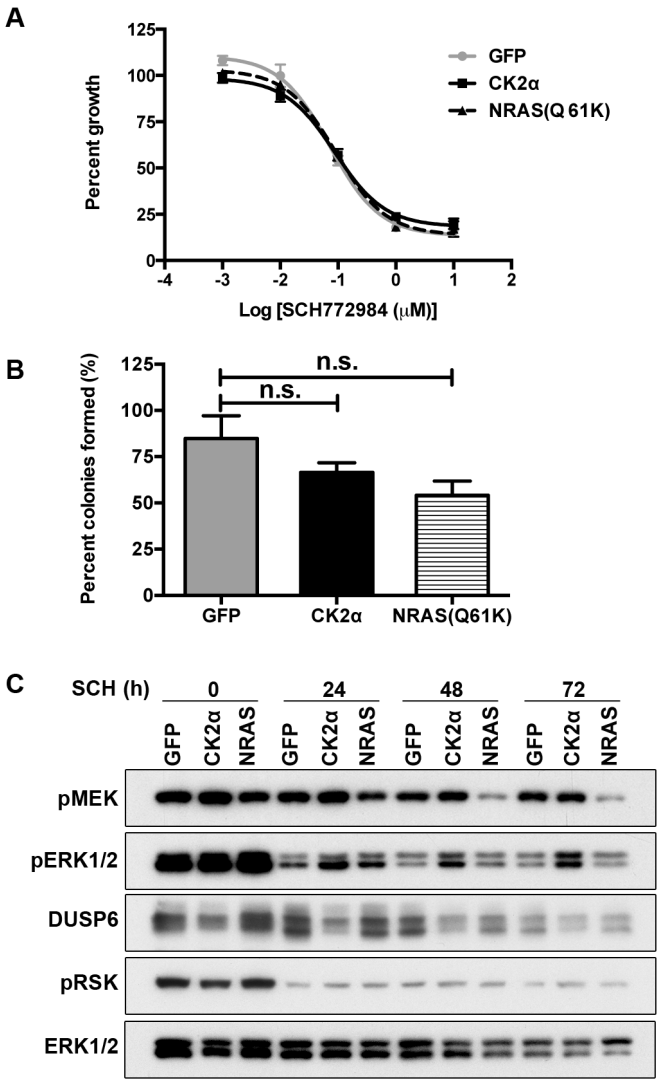

Figure 2.8. ERK inhibitor SCH772984 is insensitive to overexpression of CK2α. *A*, The GI_{50} for ERKi SCH772984 is unchanged by overexpression of CK2α or by mutant NRAS. GI_{50} curves were generated after 72 h of ERKi treatment. Results are presented as means ± S.E.M. (n = 3) *B*, Clonogenic survival in the presence of ERKi is not enhanced by overexpressed CK2α or mutant NRAS. Shown are the percent of colonies formed by A375 cells expressing GFP, CK2α or NRAS(Q61K) and treated with ERKi (100 nM) normalized to DMSO vehicle control. Results are presented as means ± S.E.M. (n = 3). *C*, ERKi treatment shuts down ERK pathway signaling, as indicated by loss of phosphorylated ERK substrate p90RSK (pRSK). A375 cells as in panels A and B were treated for 24, 48 or 72 h with 100 nM SCH772984.

CHAPTER III: AN ESSENTIAL ROLE FOR WILD-TYPE KRAS IN NRAS-MUTANT MELANOMA

Overview

It has now become increasingly clear that wild-type RAS isoforms may also play important roles in the development and maintenance of mutant RAS-driven cancers, although whether these are tumor-promoting or tumor-suppressive roles appears to be context-dependent. This question has largely been studied in KRAS-mutant cancers, and little is known about the roles of wild-type RAS isoforms in NRAS-mutant cancers. Here, I show that, in NRAS-mutant melanoma, which represents 28-30% of all melanoma cases, both wild-type KRAS and HRAS isoforms are needed for cell proliferation and survival, and for transformed growth. Specifically, I demonstrate that depletion of $KRAS^{WT}$ results in G1 cell cycle arrest and induction of apoptosis. In line with this phenotype, I find increases in p53 protein levels and its downstream targets including p21 and PUMA, which are known mediators of p53-dependent cell cycle arrest and apoptosis, respectively. Further, using reverse-phase protein array analysis, I uncover an unanticipated role of $KRAS^{WT}$ in maintaining rpS6 phosphorylation that is likely the result of AKT- and mTOR-independent regulation of the p70 S6 kinase (S6K1). Together, my data demonstrate the importance of wild-type RAS isoforms in NRAS-mutant melanoma, and provide insights into potential mechanistic explanations for this dependency.

This chapter is currently in preparation for submission for publication. All experiments were performed by myself with the exception of Figure 3.4A, which was performed by Mariaelena Pierobon in the laboratory of Dr. Emanuel F. Petricoin III.

Introduction

RAS proteins are the founding members of the RAS superfamily of small GTPases, and are located at the center of a highly-complicated signaling network that controls many aspects of fundamental cellular processes, including cell differentiation, survival and proliferation (Cox and Der, 2010). Alongside their critical importance in normal cell physiology, RAS proteins are also heavily involved in human disease. In particular, point mutations that result in constitutive activation of RAS are found in ~30% of all human cancers (Cox et al., 2014). Since its discovery over three decades ago, RAS has never left the center of the stage of cancer research and drug discovery.

The human genome is home to three RAS genes (HRAS, NRAS, and KRAS), which together encode four RAS proteins (HRAS, NRAS, splice variants KRAS4A and KRAS4B). These proteins share an overall 82-90% amino acid sequence identity: all of the regions required for nucleotide binding and effector interactions are essentially identical. However, they differ significantly in a region termed the “hypervariable region” at their C-termini, which is critical for their respective lipid modification, and thus determines their distinct membrane binding and trafficking kinetics (Cox et al., 2015). As a result, each RAS isoform shares overlapping but distinct localizations at the plasma membrane and on endomembranes (Prior and Hancock, 2001). This allows RAS engagement of different pools and concentrations of activators and effectors, and therefore has been proposed to contribute to isoform-specific signaling properties (Omerovic and Prior, 2009; Plowman and Hancock, 2005). Indeed, studies have repeatedly demonstrated that RAS proteins are not created equal (Fotiadou et al., 2007; Haigis et al., 2008; Parikh et al., 2007; Prior et al., 2012; Whitwam et al., 2007), thus adding another layer of complexity and challenge in addition to the already daunting task to inhibit RAS activity in cancer.

The current focus of RAS research has extended beyond oncogenic RAS isoforms to their wild-type counterparts. Accumulating evidence suggests a significant but complex role of

wild-type RAS isoforms in oncogenic RAS-driven tumorigenesis, an interaction network that appears to be highly context-dependent. Such studies largely fall into two broad categories: 1) studies of the wild-type counterpart of the oncogenic RAS isoforms, and 2) studies of the remaining two wild-type RAS isoforms in the presence of the oncogenic isoform.

Bergo and colleagues used the *Kras2*^{LSL}Mx1-Cre (KM) mouse model to study *Kras*^{G12D}-induced leukemia, and found that all T-cell acute lymphoblastic leukemia (T-ALL) tumors in bone marrow-transplanted mice demonstrated loss of the wild-type *Kras2* allele, suggesting a tumor suppressive role of wild-type KRAS in this model (Staffas et al., 2015). Similarly, Balmain and colleagues also reported tumor suppressive functions of HRAS^{WT} and KRAS^{WT} in experimental models of HRAS-driven non-melanoma skin cancer and KRAS-driven lung cancer, respectively (To et al., 2013). In delineating the effects of *Kras* copy number changes on tumor formation, a tumor suppressor effect of wild-type *Kras* was proposed for a carcinogen-induced mouse model of colorectal neoplasms, based on the observation that compared to wild-type (Kras+/+) mice, hemizygous Kras (Kras+/-) mice developed more and bigger colorectal tumors (Luo et al., 2014). Of note, this study was not investigating wild-type KRAS function in the presence of oncogenic KRAS. To determine the role of KRAS in regulating the tumor suppressor and RAS effector RASSF1A, Barbacid, Kolch and co-workers compared signaling of an isogenic cell line pair HCT-116 (KRAS$^{G13D/WT}$) and Hke3 (KRAS$^{-/WT}$), and found that *KRAS*^{G13D} activates the proapoptotic MST2 pathway. By using siRNAs selectively targeting *KRAS*^{WT}, the authors determined that it antagonizes the activation of the MST2 pathway by mutant KRAS, and concluded that in colorectal cancer, wild-type KRAS supports mutant KRAS transformation (Matallanas et al., 2011). Therefore, the role of wild-type RAS in oncogenesis differs depending on the model system.

Studies that primarily focused on studying wild-type RAS isoforms other than the wild-type counterpart of oncogenic RAS began with a landmark study by the Counter laboratory, where they revealed a RAS^{mut}-PI3K-Akt-eNOS-RAS^{WT} activation mechanism in an HRAS^{mut}-

expressing human kidney cell-derived cell line and in a panel of $KRAS^{mut}$ human pancreatic ductal adenocarcinoma cell lines (Lim et al., 2008). In a similar vein, Jeng *et al*. proposed that allosteric stimulation of SOS by $KRAS^{mut}$ activates RAS^{WT} (Jeng et al., 2012). $KRAS^{mut}$ has been shown to depend on RAS^{WT} for activation of the G2 DNA damage checkpoint and timely progression through mitosis (Grabocka et al., 2014). Work by McCormick et al. also suggested the need for the other two RAS^{WT} isoforms for proliferation in a third isoform-mutated cancer cell line. In this study, oncogenic and wild-type RAS isoforms have reported to be responsible for regulating different aspects of signal transduction, with oncogenic RAS modulating basal mitogen-activated protein kinase (MAPK) pathway signaling, and WT isoforms controlling response to growth factor signaling (Young et al., 2013). However, *in vivo* studies by To *et al*. illustrated a more complex picture of wild-type RAS biology. While $HRAS^{WT}$ suppressed $KRAS^{mut}$-driven lung carcinogenesis, $KRAS^{WT}$ cooperated with $HRAS^{mut}$ to drive the formation of skin papillomas (To et al., 2013). Intriguingly, $NRAS^{WT}$ had opposing effects in different tumor types: it acted as a tumor suppressor in $KRAS^{mut}$ lung tumors, but supported $HRAS^{mut}$-induced skin carcinogenesis (To et al., 2013). Hence, in either type of wild-type RAS study, the function of wild-type RAS in RAS-mutant cancers is not uniform, suggesting strong context dependency.

Surprisingly little is known about whether NRAS-mutant cancers require $KRAS^{WT}$ or $HRAS^{WT}$ for tumor initiation and/or maintenance. Given the fact that RAS isoforms themselves are distinct in their functions (Castellano and Santos, 2011; Newlaczyl et al., 2014), NRAS-mutant diseases may have different functional requirements for the other two wild-type isoforms, and thus require independent investigation.

Although the majority of RAS-mutant cancers harbor KRAS mutations, NRAS mutations are highly enriched in certain types of cancers, such as melanoma, thyroid cancer, and acute myeloid leukemia (AML) (Cox et al., 2014). Melanoma is the deadliest form of skin cancer, and the sixth most common cancer in the United States. Mutations in NRAS arise in 28-30% of all melanoma patients, rendering them the second largest genetic subpopulation (Cancer Genome

Atlas, 2015). In addition, compared to patients with *BRAF*-mutant melanoma or without mutations in *NRAS* or *BRAF*, patients with NRAS-mutated melanoma have a worse prognosis (Jakob et al., 2012), and there are no FDA-approved targeted therapies for this disease, indicating a strong incentive for an in-depth molecular characterization of this subclass. Therefore, *NRAS*-mutant melanoma not only represents an excellent disease model to study the interplay of WT and oncogenic RAS, but also is in dire need of a deeper understanding of its biology to lay the foundation for identifying druggable targets.

In the present study, I sought to determine the roles of wild-type (WT) RAS isoforms in NRAS-mutant melanoma. Cellular assays examining cell proliferation and clonogenic survival in both 2D and 3D culture revealed a need for both wild-type RAS isoforms ($KRAS^{WT}$ and $HRAS^{WT}$) in all NRAS-mutant melanoma cells. To further understand the causes of cell death, I examined cell cycle distribution and Annexin V staining intensities of cells following $KRAS^{WT}$ depletion, and showed that loss of $KRAS^{WT}$ led to both G1 cell cycle arrest and apoptosis. To delineate the molecular underpinnings of these effects, I first employed a candidate approach, and examined one of the best-known regulators of cell cycle arrest and apoptosis, p53. I found that p53 protein stability and hence protein abundance was increased upon loss of KRAS, leading to an upregulation of many of its target genes. Contemporaneously, my collaborators also performed an unbiased reverse-phase protein array (RPPA) analysis to identify proteins that are selectively regulated in an isoform-specific manner. One such protein was ribosomal protein S6 (rpS6), a component of the 40S ribosomal subunit. Specifically, KRAS knockdown strongly suppressed phosphorylation of rpS6 at Ser235 and Ser236, which are substrates of S6K1. Dynamic dissection of rpS6 phosphorylation demonstrated the involvement of S6K1 but not ERK in phosphorylating rpS6 with or without depletion of $KRAS^{WT}$. Together, these findings unveil the dependence of NRAS-mutant melanoma on RAS^{WT}, and provide insight into the critical role of $KRAS^{WT}$ in this disease.

Methods and Materials

Cell culture and reagents

SK-MEL-147, SK-MEL-173, SK-MEL-119, SK-MEL-103, Mel224, Sbc12, and 293T cell lines were grown in DMEM-H (Gibco) supplemented with 10% FBS (HyClone^{TM}, Thermo Scientific) and 1% gentamycin/kanamycin (Tissue Culture Facility (TCF), Lineberger Comprehensive Cancer Center (LCCC), University of North Carolina at Chapel Hill (UNC-CH)).

shRNA constructs

Two hairpins sequence targeting (shNRAS-3 and shHRAS-3) were synthesized by Integrated DNA Technologies, and were cloned into a pLKO.1 puro vector (Addgene, #8453). The shRNA plasmids targeting p53 and the shNT plasmid were kind gifts from Dr. Yanping Zhang and Dr. Channing Der, respectively (UNC-Chapel Hill). All other shRNA constructs were purchased from the Lenti-shRNA Core Facility at UNC-Chapel Hill. Target sequences for all hairpins used in this study are summarized below.

shRNA	TRC Clone ID	Target Sequence
shNT	N/A	CAACAAGATGAAGAGCACCAA
shNRAS-1	TRCN0000033254	CGCACTGACAATCCAGCTAAT
shNRAS-2	TRCN0000033258	CAAGAGTTACGGGATTCCATT
shNRAS-3	N/A	GTGCCATGAGAGACCAATACA
shKRAS-1	TRCN0000010369	CAGTTGAGACCTTCTAATTGG
shKRAS-2	TRCN0000033260	GAGGGCTTTCTTTGTGTATTT
shHRAS-1	TRCN0000040089	AAGAGTGCGCTGACCATCCAC
shHRAS-2	TRCN0000040091	GACGTGCCTGTTGGACATCCT
shHRAS-3	N/A	GAGTGGAGGATGCCTTCTACA
shp53-1	TRCN0000003753	CGGCGCACAGAGGAAGAGAAT

shp53-2	TRCN0000003755	GTCCAGATGAAGCTCCCAGAA
shp53-3	TRCN0000003756	CACCATCCACTACAACTACAT
shp53-4	TRCN0000003757	CTGTAGCCCATGTTGTAGCAA

Lentivirus production and infection

To produce lentivirus, 293T cells were transfected with pLKO.1 vector-based non-targeting shRNA (shNT) or shRNA against a specific gene, in combination with psPAX2 and pMD2.G (packaging vectors), at a ratio of 4:3:1. After overnight transfection, the culture medium was changed to DMEM-H supplemented with 20% FBS. Thirty-six hours later, viral supernatants were harvested and filtered through a sterile 0.45 μm filter to remove cell debris. Cleared supernatants were aliquotted and frozen at -80°C until use. Cells were infected with an appropriate amount of virus in 5 μg/ml polybrene (Millipore) overnight. Selection of transduced cells in puromycin (1 μg/ml) was complete at 48 h after infection.

Western blotting

Cells were washed twice with ice-cold PBS and lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0) containing 1X protease inhibitors (BaculoGold™ Protease Inhibitor Cocktail, BD Biosciences, #51-21426Z) and 1× phosphatase inhibitors (Halt™ Phosphatase Inhibitor Cocktail, Thermo Scientific, #78420). Lysates were depleted of cell debris by centrifugation at maximum speed (4°C, 10 min), then proteins were quantified by Bradford assay (*DC*™ Protein Assay, Bio-Rad), normalized, reduced, denatured at 95°C for 5 min, and resolved by SDS gel electrophoresis. Proteins were transferred to PDVF membranes (Millipore, #IPFL00010) and probed with primary antibodies at 4°C overnight. After incubation with the appropriate secondary anti-mouse (GE Healthcare, NA931V) or anti-rabbit (GE Healthcare, NA934V) antibody, proteins were detected by

chemiluminescence (Thermo Scientific, #34075). Below is a list of primary antibodies used in this study.

Protein	Manufacturer	Catalog #
ERK	Cell Signaling Technology	9102
p-ERK (T202/Y204)	Cell Signaling Technology	4370
MEK	Cell Signaling Technology	4694
p-MEK (Ser217/221)	Cell Signaling Technology	9154
RSK	Cell Signaling Technology	9355
p-RSK (T259/S263)	Cell Signaling Technology	9344
Akt	Cell Signaling Technology	9272
p-Akt (Ser473)	Cell Signaling Technology	9271
p-Akt (Thr308)	Cell Signaling Technology	9275
mTOR	Cell Signaling Technology	2972
p-mTOR (S2448)	Cell Signaling Technology	5536
S6K1/p70 S6K	Cell Signaling Technology	9202
p-S6K1 (T389)	Cell Signaling Technology	9205
ribosomal protein S6 (rpS6)	Cell Signaling Technology	2217
p-rpS6 (S235/236)	Cell Signaling Technology	2211
p-rpS6 (S240/244)	Cell Signaling Technology	2215
β-actin	Sigma-Aldrich	A5316
NRAS (F155)	Santa Cruz Biotechnology	sc-31
KRAS	Calbiochem	OP-24
HRAS (C-20)	Santa Cruz Biotechnology	sc-520
PUMA (H-136)	Santa Cruz Biotechnology	sc-28226
p21	Santa Cruz Biotechnology	sc-397

p53 (DO-1)	Santa Cruz Biotechnology	sc-126
cyclinD1	Cell Signaling Technology	2922
Bax	BD Biosciences	554104
p73	Santa Cruz Biotechnology	sc-7957
FoxO3a	Bethyl Laboratories	A300-453A-T

RNA isolation, reverse transcription and real-time qPCR

Total RNA was isolated using a QIAshredder™ homogenizer (Qiagen, #79654) and an RNeasy® Mini Kit (Qiagen, #74104). Reverse transcription was performed using the High Capacity RNA-to-cDNA kit (Thermo Fisher Scientific, #4387406). Samples were prepared using the TaqMan Gene Expression Master Mix (Thermo Fisher Scientific, #4369016) and real-time quantitative Taqman PCR was performed on the QuantStudio 6 Flex (Thermo Fisher Scientific) with FAM/MGB labeled probes against NRAS (Hs00180035_m1), KRAS (Hs00364284_g1), HRAS (Hs00978050_g1), or PUMA/BBC3 (Hs00248075_m1), normalized to a human ACTB (β-actin) endogenous control (Thermo Fisher Scientific, #4310881E).

MTT proliferation assay

Cells were plated at 1-2 $X10^3$ cells per well in 96-well plates, and stained with 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich M5655) the next day (day 1), and then either on days 3, 5, and 7 or on days 4, 7, and 10, depending on the proliferation rate of each cell line. Formazan products were solubilized using acidified isopropanol (0.04N HCl in isopropanol), and absorbance was measured at 570 nm, with background subtraction at 650 nm. Absorbance was normalized to the average value for day 1 and growth curves were generated from the average of all experiments.

2D clonogenic assay

Clonogenic assays were performed as described previously (Franken et al., 2006) with minor modifications. Briefly, cells were plated in triplicate wells at 500 - 1000 cells/well in 6-well plates, and incubated at 37°C for 2 weeks. After removal of culture medium, cells were fixed and stained with a crystal violet staining solution (0.5% crystal violet, 50% methanol, 10% acetic acid) at room temperature for 30 min. The stain was decanted, and plates were carefully rinsed with distilled water until background staining of the wells was minimized. Colonies were imaged, and then quantified with ImageJ 1.45s.

Soft agar assay

Anchorage-independent growth was measured as described previously (Alan et al., 2010). Single-cell suspensions of cells (1 X 10^4 cells per well in 6-well plates) were suspended in 0.4% agar (BD Biosciences) in complete medium and layered on top of 0.6% agar. After 2-4 weeks, colonies were stained with 2 mg/ml MTT, and colony numbers were quantified with ImageJ 1.45s.

Cell cycle analysis

Samples were prepared according to the manufacturer's protocol (Roche, #11348639001) with slight modifications. Briefly, ~1 X 10^6 cells were dissociated with TrypLE™ Express (ThermoFisher Scientific), washed twice with cold phosphate-buffered saline (PBS), fixed with 70% ice-cold ethanol, and stored at -20°C until use. Fixed cells were washed again with cold PBS, stained with 1 ml of propidium iodide staining solution (0.02 mg/ml propidium iodide, 0.2 mg/ml RNase A) per condition, and incubated at 4°C overnight. Cells were filtered through 30 μm CellTrics® filters prior to analysis. Measurement of cell cycle distribution was

performed on a Beckman Coulter CyAn ADP flow cytometer (Flow Cytometry Core Facility, UNC-Chapel Hill), and analyzed using ModFit Software (Verity Software House).

Annexin-V/PI staining

Cells were collected and stained using the Annexin-V-FLUOS Staining Kit (Roche, #11858777001). In brief, ~1 X 10^6 cells were washed with PBS, spun down at 200 X *g* for 5 min, resuspended in 100 μl of Annexin-V-FLUOS labeling solution, and incubated at room temperature for 15 min. Fluorescence was then analyzed by a Beckman Coulter CyAn ADP flow cytometer (Flow Cytometry Core Facility, UNC-Chapel Hill).

Reverse-phase protein array analysis

Reverse-phase protein array (RPPA) analysis was performed as described previously (Federici et al., 2013). Briefly, samples from the triplicate sets of five melanoma cell lines (SK-MEL-147, SK-MEL-173, SK-MEL-119, Mel224, and SK-MEL-103) were printed in triplicate spots on nitrocellulose-coated glass slides (GRACE Bio-Labs) using an Aushon 2470 arrayer equipped with 185 μm pins (Aushon Biosystems), according to the manufacturer's instructions. Reference standard lysates, composed of HeLa + pervanadate (BD), Jurkat + etoposide (Cell Signaling), and Jurkat + calyculin A (Cell Signaling) cell lysates, were printed in 10-point dilution curves as procedural controls and as positive controls for antibody staining. Each reference standard curve was printed in triplicate at concentrations of 0.5 μg/μl and 0.125 μg/μl. A selected subset of the printed array slides was stained with SYPRO Ruby Protein Blot Stain (Invitrogen) to estimate sample total protein concentration, and the remaining slides were stored desiccated at −20°C. Just before antibody staining, printed slides were treated with 1× ReBlot Mild Solution (Chemicon) for 15 minutes, washed 2 times for 5 minutes with PBS (Invitrogen), and incubated for 1 hour in blocking solution (2% I-Block (Applied Biosystems), 0.1% Tween-20 in PBS). Immunostaining was completed on an automated slide stainer using a catalyzed signal

amplification kit (DAKO). The arrays were probed with a library of almost 200 antibodies against total, cleaved, and phosphoprotein endpoints. Primary antibody binding was detected using a biotinylated goat anti-rabbit immunoglobulin G (IgG) H+L (1:7500; Vector Laboratories) or rabbit anti-mouse IgG (1:10; DAKO) followed by streptavidin-conjugated IRDye680 fluorophore (LI-COR Biosciences). Before use, primary antibodies were extensively validated for single-band specificity by Western immunoblotting with complex cellular lysates. Negative control slides were incubated with secondary antibody only. All SYPRO- and immuno-stained slides were scanned using a Revolution 4550 scanner (Vidar Corp.), and acquired images were analyzed with MicroVigene v4.0.0.0 (VigeneTech), which conducted spot detection, local background subtraction, negative control subtraction, replicate averaging, and total protein normalization, producing a single value for each sample. Unsupervised hierarchical clustering was conducted with JMP v5.1 (SAS Institute). Endpoint relative intensity correlation plots were conducted with GraphPad Prism v5 (GraphPad Software Inc.).

Results

$KRAS^{WT}$ is essential for cell survival and proliferation in NRAS-mutant melanoma cells

Previous studies in KRAS-mutant cancers have identified both tumor-promoting and tumor-suppressing roles of wild-RAS isoforms (Grabocka et al., 2014; Jeng et al., 2012; Lim et al., 2008; To et al., 2013; Weyandt et al., 2015; Young et al., 2013); NRAS-mutant cancers have not been evaluated. Therefore, I first tested whether wild-type KRAS ($KRAS^{WT}$) was necessary for cell proliferation in a panel of NRAS-mutant melanoma cells. I used a non-targeting (NT) shRNA and two independent short hairpins targeting NRAS, KRAS, or HRAS to selectively knock down each isoform. Knockdown efficiency was confirmed at both the mRNA level and protein level (Figure 3.1A,B). In all cell lines tested, KRAS depletion significantly impeded cell proliferation (Figure 3.1C). I also observed similar effects on cell proliferation upon HRAS depletion (data not shown). Additionally, KRAS knockdown strongly inhibited 2D clonogenic

growth (Figure 3.1D), as well as 3D anchorage-independent growth of NRAS-mutant cells (Figure 3.1E), indicating a loss of the transformed phenotype. Similar phenotypes were observed with HRAS depletion (Figure 3.5). Next, I determined whether cell cycle arrest and/or apoptosis could explain the reduced cell survival and proliferation. Propidium iodide (PI) staining revealed a marked increase in the G1 phase of the cell cycle (Figure 3.1F) upon KRAS knockdown, suggesting a G1 cell cycle arrest. In addition, KRAS silencing greatly increased the proportion of cells that were Annexin V(+)/PI(-), indicating induction of apoptosis. Together, these results show that $KRAS^{WT}$ is essential for cell proliferation, survival and the transformed phenotype of NRAS-mutant melanoma cells, and that its loss results in cell cycle arrest and apoptosis.

RAS isoforms play divergent roles in NRAS-mutant melanoma

Earlier reports had not explicitly distinguished between the WT RAS isoforms (i.e., $NRAS^{WT}$ and $HRAS^{WT}$) in KRAS-mutant cells. However, given that RAS isoforms exhibit different biological activities in various contexts (Fotiadou et al., 2007; Haigis et al., 2008; Parikh et al., 2007; Whitwam et al., 2007), by means of distinct effector utilization, I postulated that they perform different roles in NRAS-mutant cancer. In the present study, I asked whether the wild-type isoforms (i.e., $KRAS^{WT}$ and $HRAS^{WT}$) have different biological functions in NRAS-mutant melanoma cells. First, I observed changes in cellular morphology, and surprisingly, found distinct morphologies upon knockdown of each RAS isoform: cells with KRAS knockdown appeared highly rounded and refractile, consistent with the early stages of apoptotic death. In contrast, NRAS depletion gave rise to a flattened morphology, whereas HRAS knockdown led to an elongated, fibroblastic appearance (Figure 3.2A). These distinct morphological consequences imply distinct consequences of the knockdown of each isoform on effector utilization, resulting in different signaling outcomes. I therefore examined activation of the RAF-MEK-ERK and AKT-mTOR-S6 pathways, two of the best-studied canonical effector pathways of

RAS proteins. As predicted by the different morphological consequences, loss of the three RAS isoforms resulted in different consequences to these effector pathways (Figure 3.2B). In SK-MEL-147 cells, NRAS knockdown resulted in mild loss of both total and phosphorylated MEK, ERK, and RSK, whereas KRAS depletion elevated phosphorylation of those proteins without affecting their total protein levels. HRAS silencing had no effect on MEK or ERK signaling, but suppressed RSK phosphorylation. In SK-MEL-147 (Figure 3.2B) and three other NRAS-mutant cell lines (SK-MEL-119, SK-MEL-173 and Sbc12; Figure 3.6B), knockdown of NRAS consistently reduced phosphorylation of ERK, whereas knockdown of KRAS increased it, and knockdown of HRAS had little effect. In contrast, although phosphorylation of AKT at S473 was diminished in SK-MEL-147 cells (Figure 3.2B), AKT phosphorylation did not exhibit a consistent alteration across the cell lines (Figure 3.6B). Overall, these observations indicate that RAS isoforms are not redundant, and have at least some functionally distinct roles, despite their high degree of sequence identity and a shared signaling network. Additionally, the finding that alterations in phosphorylation of ERK but not AKT were consistent across the cell lines upon knockdown of each RAS isoform implies that RAS isoforms may control the RAF-MEK-ERK pathway more tightly than the PI3K-AKT-mTOR-S6 pathway in NRAS-mutant melanoma. Interestingly, although phosphorylation of both AKT and rpS6 was reduced in SK-MEL-147 cells, phosphorylation of mTOR was if anything elevated (Figure 3.2B). This suggests that regulation of rpS6 may be more complex than currently described.

KRAS depletion increases p53 stability

The observation that $KRAS^{WT}$ depletion resulted in both G1 cell cycle arrest and apoptotic cell death implied possible impact on a master regulator of cell survival. To test this hypothesis, I examined the expression of p53, the guardian of the genome (Lane, 1992), and a known regulator of both cell cycle progression and apoptosis (Chen, 2016), in cells with KRAS knockdown. As anticipated, KRAS silencing with shKRAS-1 and shKRAS-2 increased levels of

p53 protein by 4.5- and 7.4-fold, respectively (Figure 3.3A). Accordingly, well-known p53 target genes, such as Mdm2, p21 and PUMA were also elevated at the protein level (Figure 3.3B). PUMA, a potent killer (Yu and Zhang, 2003, 2008), was also strongly upregulated at the mRNA level in both SK-MEL-147 (Figure 3.7) and other NRAS-mutant melanoma cell lines (data not shown). Since p53 is not usually regulated at the transcriptional level (Meek, 2015), I tested whether increased protein stability was the cause of p53 upregulation. Indeed, by using cycloheximide to inhibit de novo protein synthesis, I observed that the half-life of p53 protein increased 2.3-fold upon KRAS knockdown (Figure 3.3C). These data demonstrate that NRAS-mutant melanoma cells respond to $KRAS^{WT}$ loss by upregulating p53, indicating a stress response. Whether the observed increases in cell cycle arrest and/or apoptosis are dependent on this upregulation of p53 remain to be determined.

KRAS depletion impairs phosphorylation of ribosomal protein S6

To obtain a more global view of the signaling outcomes upon knockdown of each RAS isoform, my collaborators Emanuel F. Petricoin III and Mariaelena Pierobon performed reverse phase protein array (RPPA) analysis (Federici et al., 2013) on my cell lysates to detect the activation state of nearly 200 proteins in a high-throughput manner. Using RPPA, we observed an unexpectedly strong correlation between knockdown of KRAS and low-level phosphorylation of the ribosomal protein rpS6 at residues S235/236 (Figure 3.4A). I then validated this decreased phosphorylation by western blotting of additional cell lysates (Figure 3.4B).

Phosphorylation of rpS6 downstream of RAS is typically thought to result from activity of the AKT-mTOR-S6K1-S6 signaling pathway. Yet in these NRAS-mutant melanoma cell lines, although loss of KRAS was strongly correlated with reduced phosphorylation of rpS6 (Figure 3.4A,B), it was not strongly correlated with reduced phosphorylation of AKT (Figure 3.6B). Although rpS6 can also be activated downstream of RAF-MEK-ERK-RSK, in these cells, loss of KRAS elevated phospho-ERK and phospho-RSK (Figures 3.2, 3.6B) rather than reduced them.

One possible explanation for these results could be an indirect effect on other kinases that can phosphorylate rpS6 at S235/236. Serine residues 235 and 236 are two of five serines at the C-terminus of rpS6 that become phosphorylated in response to multiple physiological, pathological and pharmacological stimuli (Meyuhas, 2008). All five of these serines (S235, S236, S240, S244, S247) are substrates for S6K1 (Ballou et al., 1991; Chung et al., 1992), whereas both p90RSK (Pende et al., 2004; Roux et al., 2007) and PKA (Moore et al., 2009) can also phosphorylate S235 and S236. In contrast, CK1 exhibits specificity towards S247 (Hutchinson et al., 2011). To determine the major kinase responsible for phosphorylating S235/236 in NRAS-mutant melanoma cells, I examined the kinetics of rpS6 phosphorylation at both S235/236 and S240/244 in the presence or absence of $KRAS^{WT}$ depletion. In cells treated with non-targeting shRNA, rpS6 phosphorylation at both S235/236 and S240/244 increased gradually over 60 min (Figure 3.4C). As expected, phosphorylation of rpS6 at the S6K1-only sites of S240/244 tracked closely with S6K1 activation, as measured by S6K1 phosphorylation at T389. Importantly, rpS6 phosphorylation at S235/236 also followed the same trend. In contrast, the signal amplitudes of ERK phosphorylation in KRAS-depleted cells were higher at all time points, and followed different kinetics. These observations indicate that rpS6 phosphorylation in this system is largely regulated by S6K1, and not the ERK pathway. Notably, phosphorylation of both S6K1 and rpS6 was much less robust and peaked earlier (at 30 min) in cells treated with shRNA directed against KRAS (Figure 3.4E), indicating reduced activation and enhanced deactivation of this pathway upon depletion of $KRAS^{WT}$. These findings, together with the observations of a variable AKT response (Figure 3.6B) suggest that, in NRAS-mutant melanoma, KRAS may bypass AKT-mTOR to more directly control S6K1 and thus rpS6 activity.

I next asked whether this phenotype is unique to NRAS-mutant melanoma. To this end, I measured rpS6 phosphorylation upon RAS isoform knockdown in a panel of five non-small cell lung cancer cell lines (H2228, H358, H441, H23, H1299), and found that all five showed a striking loss of phosphorylation at S235 and S236 upon depletion of KRAS, but not NRAS or

HRAS (Figure 3.8A). Of note, H358, H441 and H23 all harbor a $KRAS^{G12}$ mutation, H1299 harbors an $NRAS^{Q61K}$ mutation, and H2228 does not have a RAS mutation. These results demonstrate that the loss of rpS6 phosphorylation at S235/236 is independent of both tissue of origin and RAS mutation status.

Several studies have shown that rpS6 gene dosage is closely linked to p53 activation (Fumagalli et al., 2009; McGowan et al., 2011; Panic et al., 2007; Panic et al., 2006; Sulic et al., 2005). Further, a pancreatic cancer study used $rpS6^{P-/-}$ mice (lacking all five phosphorylatable sites in rpS6) to demonstrate the effect of rpS6 phosphorylation on tumor development, and found that those mice had increased p53 tissue staining (Khalaileh et al., 2013). Therefore, to understand whether the increased levels of p53 that I observed upon depletion of $KRAS^{WT}$ (Figure 3.3) were due to impaired rpS6 phosphorylation, I treated cells with rapamycin, and found that, while rpS6 phosphorylation quickly declined to an undetectable level, p53 remained steady until 24 h after treatment (Figure 3.8B), indicating that the increase in p53 and suppression of rpS6 phosphorylation lie in two independent pathways. Overall, my findings uncover a critical role of $KRAS^{WT}$ in NRAS-mutant melanoma, loss of which leads to upregulation of p53 and its target genes, and, in a parallel fashion, to severe impairment of rpS6 phosphorylation that is S6K1-dependent but AKT-mTOR-independent.

Discussion

The roles of wild-type RAS isoforms in *RAS*-mutant cancers re-entered the spotlight when several groups demonstrated that they play important roles in *RAS*-mutant cancers (Grabocka et al., 2014; Jeng et al., 2012; Lim et al., 2008; To et al., 2013; Weyandt et al., 2015; Young et al., 2013). Although the majority of studies in KRAS-mutant cancers described a need for wild-type HRAS and/or NRAS in tumor initiation and/or progression (Grabocka et al., 2014; Lim et al., 2008; Young et al., 2013), those observations cannot simply be generalized to other cancer types and different oncogenic RAS isoforms, given the highly complex and context-

dependent nature of interactions between wild-type and oncogenic forms of different RAS family members. In this study, I investigated the roles of wild-type RAS in *NRAS*-mutant melanoma, with a special focus on $KRAS^{WT}$, and show here that it is critical not only in maintaining the transformed phenotype, but also in fundamental cellular processes such as proliferation and survival. Furthermore, I provide evidence that the biological functions of the wild-type RAS isoforms are not identical, different from findings that suggested that the wild-type RAS proteins are functionally redundant (Grabocka et al., 2014; Jeng et al., 2012; Young et al., 2013). Specifically, these studies showed that the two wild-type RAS isoforms responded the same way both phenotypically and mechanistically. Discrepancies among studies are very likely due to the specific disease model, and the specific RAS isoform that drives the disease. In fact, I noticed that knockdown of NRAS and HRAS behave more similarly to one another than knockdown of KRAS. For example, KRAS depletion resulted in cell death more rapidly than NRAS or HRAS depletion, based on cellular morphology. The highly rounded, refractile phenotype shown in Figure 3.2A may reflect the initiation of cellular death. Furthermore, NRAS and HRAS also more mirror each other in terms of modulation of downstream signaling (Figure 3.2B, Figure 3.7 and Figure 3.4A-C). It is therefore not entirely surprising that studies in KRAS-mutant cancers all show similar effects of knocking down either NRAS or HRAS. In addition, such differences in conclusions also heavily rely on the exact definition of "functional redundancy". It is known that there is functional overlap (Johnson et al., 1997) and difference (Fotiadou et al., 2007) among the RAS isoforms. Thus, depending on the specific biological endpoint examined, there may or may not be a distinction between the two wild-type RAS proteins.

Our findings are also in line with previous observations that demonstrated a potential need for wild-type RAS in NRAS-mutant cells. Cell proliferation defect of the RD rhabdomyosarcoma cell line ($NRAS^{Q61H}$) was observed upon knockdown of $HRAS^{WT}$ and/or $KRAS^{WT}$(Young et al., 2013). In the SK-MEL-103 ($NRAS^{Q61L}$) melanoma cell line, knockdown of

HRAS^{WT} was shown to enhance γH2AX levels, indicating DNA damage regulation by HRAS^{WT} (Grabocka et al., 2014).

Another interesting observation is the upregulation of p53 protein expression upon KRAS knockdown in some *NRAS*-mutant cell lines. Using cycloheximide to block *de novo* protein synthesis, I showed that p53 protein was stabilized, and its half-life increased more than 2-fold. Melanoma is different from many other cancers in that it has a relatively low frequency of TP53 mutation (19%) (Petitjean et al., 2007; Vultur and Herlyn, 2013). Indeed, all cell lines used in my study are *TP53*^{WT}. The increase in p53 protein may not be a direct consequence of KRAS knockdown, and may instead indicate a more general stress response of the cells when KRAS is depleted. A wide range of cellular stresses induce p53, including DNA damage, oncogene activation, ribosomal stress, etc. (Colman et al., 2000; Haupt, 2004; Meek, 2015; Xu, 2003). In my system, I did not observe significant DNA damage as measured by γH2AX staining, nor did I observe induction of Ser15 phosphorylation upon treatment with shKRAS-1, which is indicative of DNA-damage (Shieh et al., 1997) (data not shown). No changes in ribosomal proteins L5, L11, or L23 were detected (data not shown), indicating a lack of ribosomal stress (Deisenroth and Zhang, 2010). Therefore, it remains unclear how KRAS depletion leads to accumulation. Noteworthy to mention, although I observed increased stabilization of p53 protein, I counterintuitively found enhanced Mdm2 interaction with p53 and increased ubiquitination of p53 upon treatment with shKRAS-2 (data not shown). These findings suggest an unconventional means of p53 protein induction. Future studies are necessary to elucidate the precise mechanism of p53 stabilization upon KRAS depletion.

Using an unbiased approach, we identified a novel link between KRAS and ribosomal protein S6 (rpS6) phosphorylation state. In all cell lines tested for validation, knockdown of KRAS resulted in the strongest suppression of rpS6 phosphorylation at Ser235 and Ser236. rpS6 is one of the 33 proteins that constitute the 40S small subunit of eukaryotic ribosomes. It has been more thoroughly studied than many other ribosomal proteins because it was the first

one shown to undergo inducible phosphorylation (Meyuhas, 2015). As a ribosomal protein, phosphorylated rpS6 has been assumed to exert significant impact on global protein synthesis. However, contradictory to this postulation, several studies demonstrated that rpS6 phosphorylation apparently lacks an important role in global protein synthesis (Garelick et al., 2013; Johnson and Warner, 1987; Ruvinsky et al., 2005). Similarly, genetic studies deleting its upstream kinases, S6K1 and S6K2 (Chauvin et al., 2014; Mieulet et al., 2007), also reported no effect on global protein synthesis. Other physiological roles of rpS6 phosphorylation include regulation of cell size, normal muscle function, hypertrophic responses, cell proliferation, clearance of apoptotic cells, and tumorigenicity (Meyuhas, 2015). Thus, the functional outcome of KRAS-knockdown-induced rpS6 hypophosphorylation warrants further detailed analysis. Overall, this isoform-selective control of downstream signaling events may offer mechanistic insights into isoform-related phenotypes.

However, contrary to the common perception that rpS6 phosphorylation serves a reliable marker of mTOR pathway activity (Andreoli et al., 2015; Iwenofu et al., 2008), I show that, in the context of KRAS depletion, rpS6 phosphorylation is suppressed regardless of AKT or mTOR activity, but its phosphorylation kinetics still followed that of S6K1. One possible explanation is that KRAS utilizes a bypass route to directly modulate S6K1 activity. Aside from phosphorylation by mTORC1 at Thr389 (Jacinto et al., 2004; Kim et al., 2003), S6K is also directly phosphorylated by PDK1 at Thr229 (Alessi et al., 1998; Pullen et al., 1998). It is therefore possible that KRAS regulates the ability of PDK1 to phosphorylate S6K1. KRAS-specific regulation of PP1, the only known phosphatase responsible for dephosphorylating rpS6, may also play a role in dictating its final phosphorylation state.

In summary, I show dependence of *NRAS*-mutant melanoma cells on wild-type KRAS for survival, proliferation and a transformed phenotype. While both wild-type HRAS and wild-type KRAS are needed for these cellular processes, they exhibit distinct impacts on cellular morphology and canonical effector pathway signaling. Importantly, I uncover a novel link

between KRAS and rpS6 that neither NRAS nor HRAS shares. Future work investigating both regulation and functional consequences of impaired rpS6 phosphorylation may shed light on the unique function of $KRAS^{WT}$ in *NRAS*-mutant melanoma.

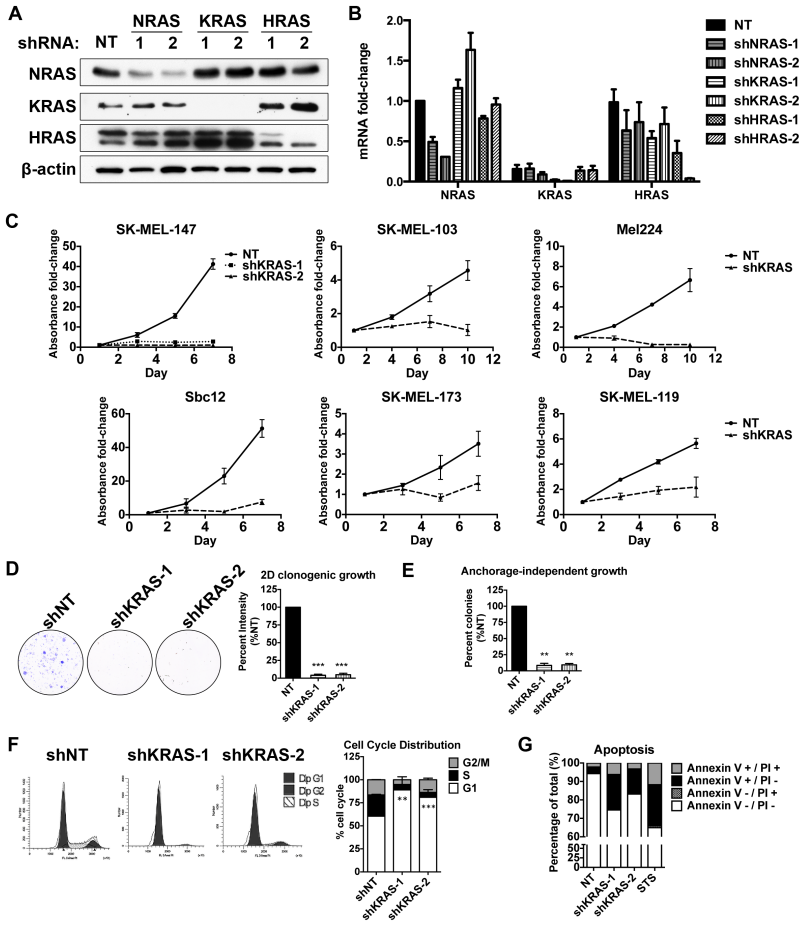

Figure 3.1. KRAS^{WT} is essential for cell proliferation and survival in NRAS-mutant melanoma cells.

A. Western blots of NRAS, KRAS, and HRAS protein expression upon shRNA knockdown. SK-MEL-147 cells were infected with lentivirus expressing short-hairpins against each of the RAS isoforms. Protein was harvested 48 h post puromycin selection. Beta-actin was used as a loading control.

B. Real-time qPCR of NRAS, KRAS, and HRAS mRNA expression upon shRNA knockdown. SK-MEL-147 cells were infected with lentivirus expressing short-hairpins against each of the RAS isoforms. Total RNA was harvested 48 h post puromycin selection. Beta-actin was used as an internal control.

C. MTT proliferation curves of a panel of NRAS-mutant melanoma cells (SK-MEL-147, SK-MEL-103, Mel224, SK-MEL-173, SK-MEL-119, and Sbc12) with (shNT) or without KRAS^{WT} (shKRAS).

D. 2D clonogenic growth of SK-MEL-147 with (shNT) or without KRAS^{WT} (shKRAS). Colonies were stained with a 0.5% crystal violet stain, and quantified by ImageJ. *** $p < 0.0005$.

E. Anchorage-independent growth of of SK-MEL-147 with (shNT) or without KRAS^{WT} (shKRAS). Colonies were stained with 2mg/ml MTT, and quantified by ImageJ. ** $p < 0.005$.

F. Propidium iodide staining of fixed SK-MEL-147 cells to determine cell cycle distribution following KRAS depletion. ** $p < 0.001$; *** $p < 0.0001$.

G. Annexin V/propidium iodide staining of SK-MEL-147 cells following KRAS depletion.

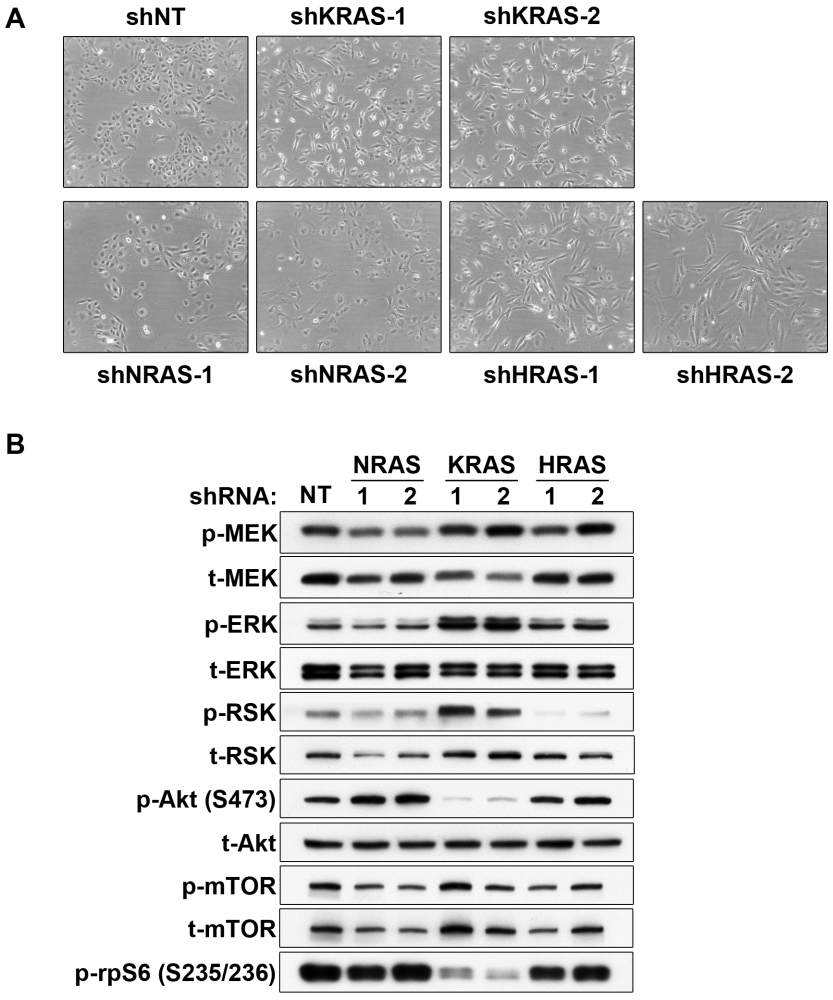

Figure 3.2. RAS isoforms perform divergent functions in NRAS-mutant melanoma cells.
A. Microscopic images (4X magnification) of SK-MEL-147 cells treated with either a non-targeting (shNT) shRNA or shRNA targeting each of the RAS isoforms.
B. Western blot analysis of canonical downstream signaling pathway activities of RAS (RAF-MEK-ERK-RSK and PI3K-AKT-mTOR-S6).

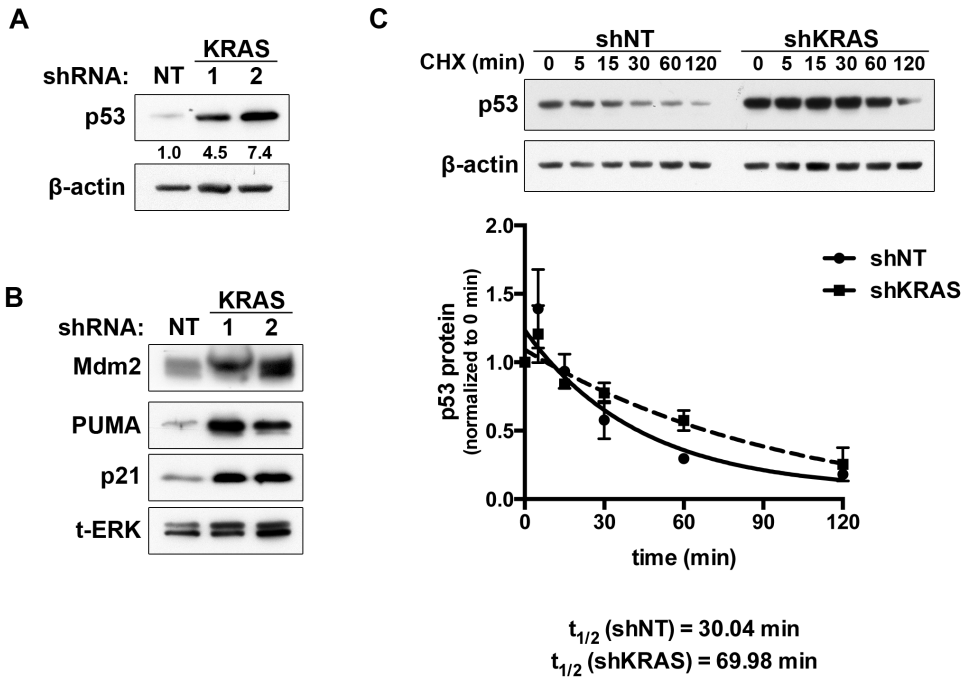

Figure 3.3. KRAS depletion increases p53 protein stability and activation of its target genes.

Western blot of p53 (A) and its target genes Mdm2, PUMA, and p21 (B) following KRAS depletion by shRNA in SK-MEL-147.

C. To determine the half-life ($t_{1/2}$) of p53 protein in SK-MEL-147 cells, 50 μg/ml cycloheximide (CHX) was added to cells, and protein was harvested at the indicated times. $T_{1/2}$ was calculated using one-phase decay analysis in Prism 6.

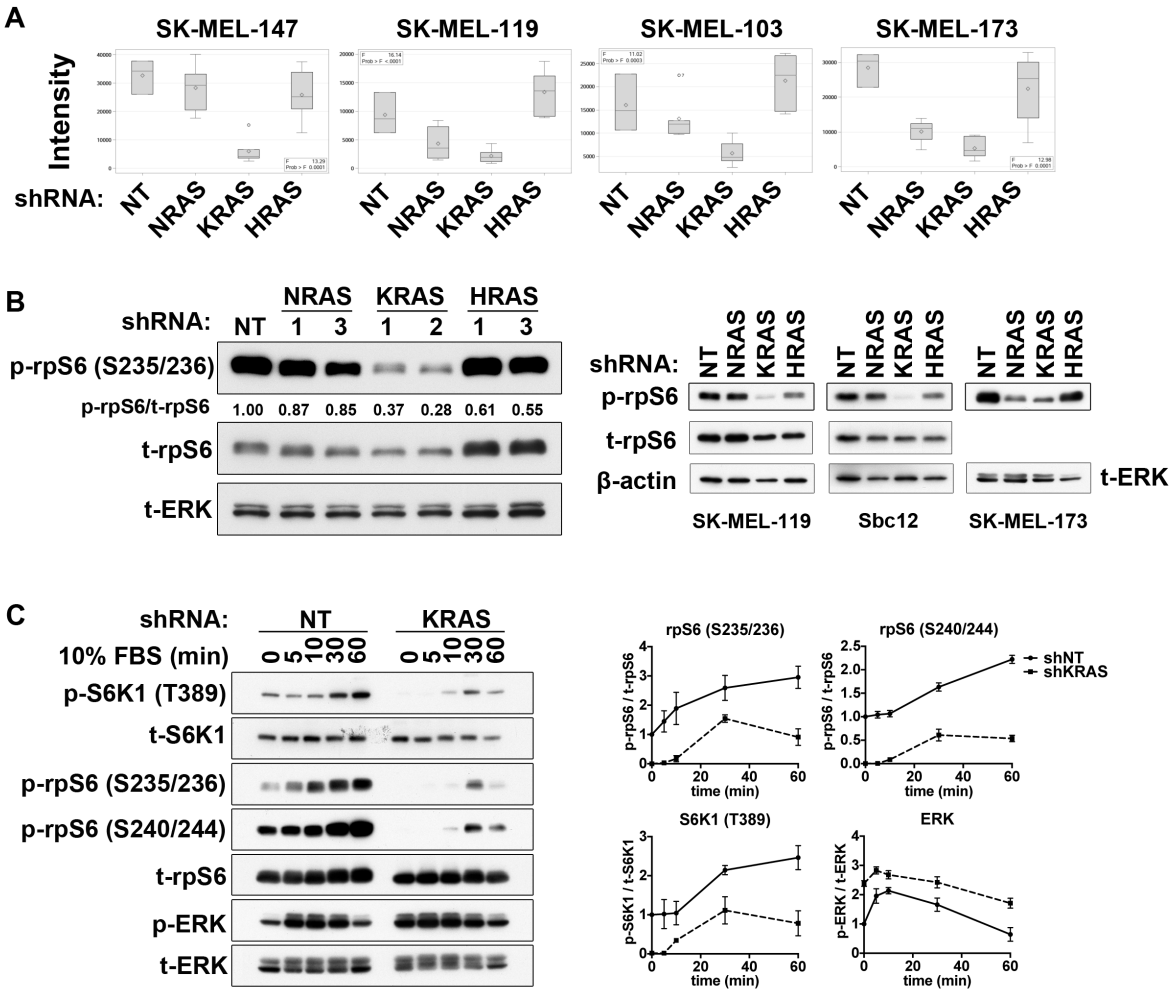

Figure 3.4. KRAS, but not NRAS or HRAS depletion leads to severe impairment of rpS6 phosphorylation at Ser235/236.

A. Endpoint relative intensity correlation plots of phosphorylation of Ser235/236 of ribosomal protein S6 based on RPPA analysis. Beta-actin was used as a loading control.

B. Western blots of phosphorylated rpS6 (S235/236) in multiple NRAS-mutant cell lines. Total ERK or beta-actin was used as a loading control.

C. SK-MEL-147 cells with or without KRAS knockdown were serum-starved for 18 h and stimulated with 10% FBS for the indicated times. Phosphorylation of rpS6 (S235/236 and S240/244), S6K1 (T389) and ERK (T202/Y204) was monitored over time. Graphs represent signal intensities of phosphorylated proteins normalized to their total counterpart from three independent experiments.

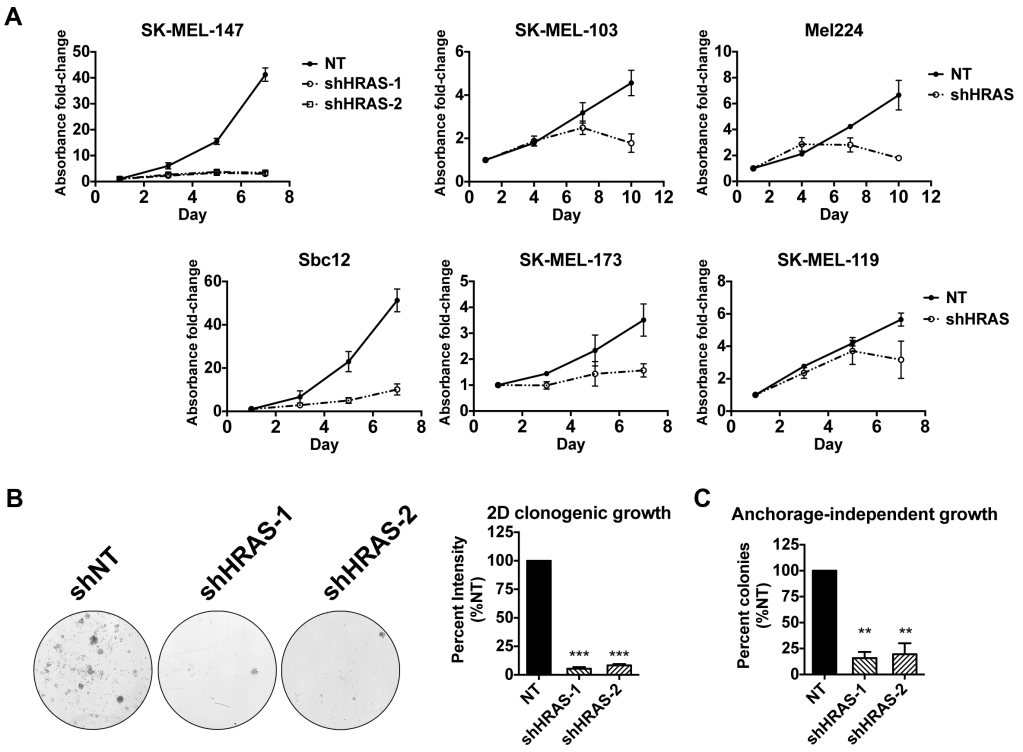

Figure 3.5. HRAS^{WT} is essential for cell proliferation, survival and transformed growth in NRAS-mutant melanoma cell lines.

A. MTT proliferation curves of a panel of NRAS-mutant melanoma cells (SK-MEL-147, SK-MEL-103, Mel224, SK-MEL-173, SK-MEL-119, and Sbc12) with (shNT) or without HRAS^{WT} (shHRAS). Growth curves for NT cells are identical to those shown in Fig. 1B, because all knockdowns were evaluated in the same set of experiments.

B. 2D clonogenic growth of SK-MEL-147 with (shNT) or without HRAS^{WT} (shHRAS). Colonies were stained with a 0.5% crystal violet stain, and quantified by ImageJ. *** $p < 0.0005$.

C. Anchorage-independent growth of of SK-MEL-147 with (shNT) or without HRAS^{WT} (shHRAS). Colonies were stained with 2mg/ml MTT, and quantified by ImageJ. ** $p < 0.005$.

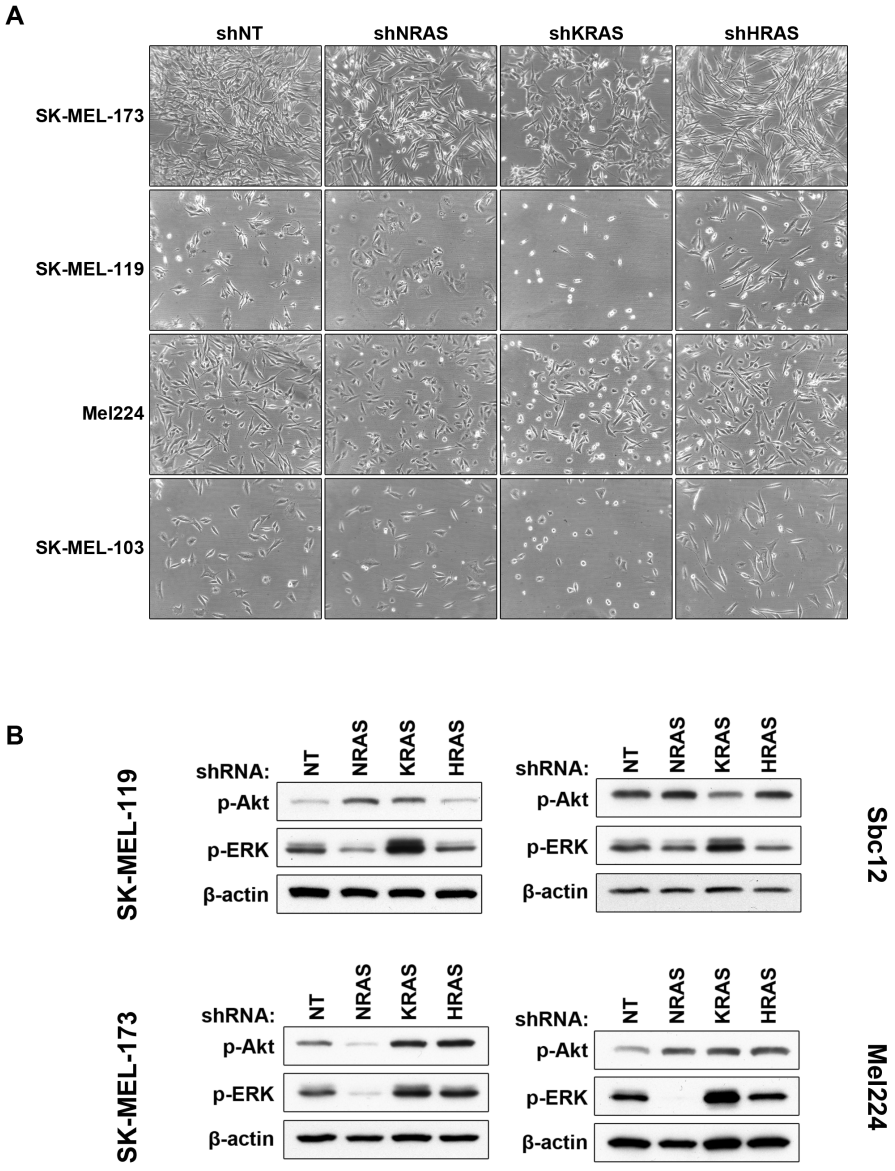

Figure 3.6. RAS isoforms differentially regulate cell morphology and downstream signaling.

A. Microscopic images (4X magnification) of NRAS-mutant melanoma cells treated with either a non-targeting (shNT) shRNA or shRNA targeting each of the RAS isoforms.

B. Western blot analysis of AKT and ERK phosphorylation upon RAS isoform knockdown in NRAS-mutant melanoma cells.

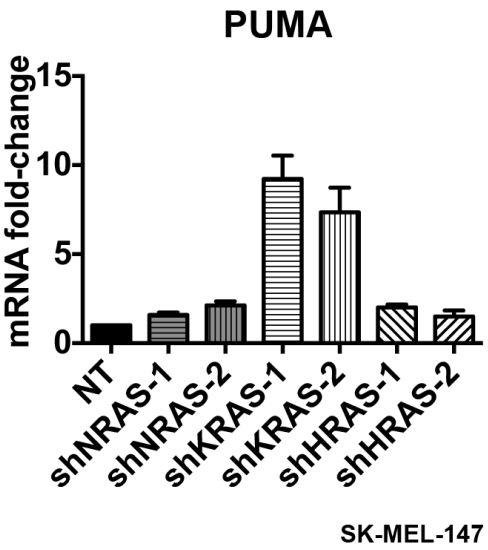

Figure 3.7. KRAS^{WT} depletion specifically upregulates PUMA mRNA.
Real-time qPCR of PUMA mRNA expression upon shRNA knockdown of each RAS isoform. SK-MEL-147 cells were infected with lentivirus expressing short-hairpins against each of the RAS isoforms. Total RNA was harvested 48 h post puromycin selection. Beta-actin was used as an internal control.

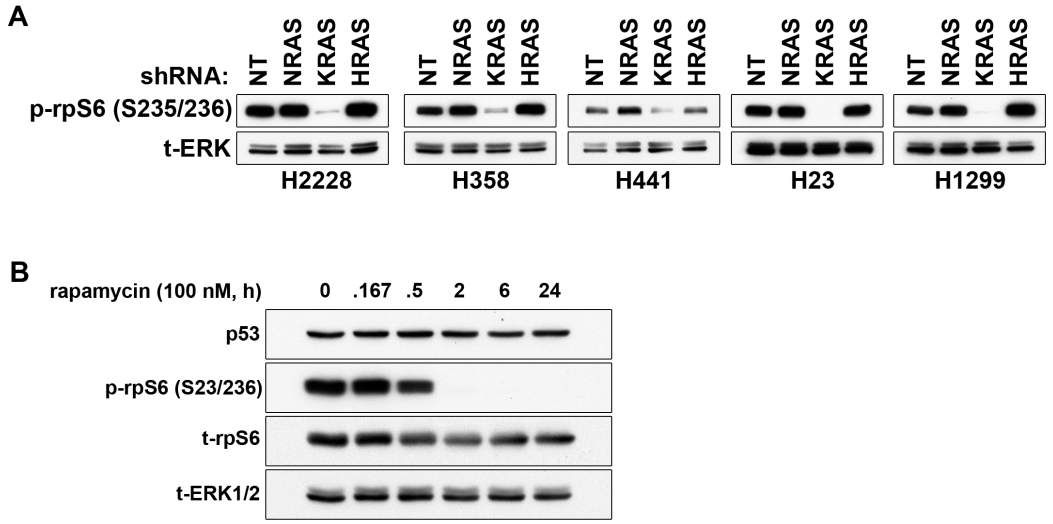

Figure 3.8. rpS6 phosphorylation is decreased upon KRAS depletion in NSCLC cells and does not cause p53 protein upregulation.
A. Western blot analysis of rpS6 phosphorylation at S235/236 in non-small cell lung cancer cell lines.
B. To determine whether p53 increases as a result of suppressed rpS6 phosphorylation at S235/236, 100 nM rapamycin was added to SK-MEL-147 culture, and cell lysates were harvested at indicated times for western blot analysis.

CHAPTER IV: CONCLUSIONS AND FUTURE DIRECTIONS

Summary and Conclusions

Situated at the center of a vast signaling network, RAS, an oncogene found in almost a third of human cancers, poses several major challenges to researchers investigating both RAS biology and RAS therapeutic targeting: exponentially increasing complexities due to the existence of multiple RAS isoforms and post-translational modifications; incomplete understanding of the interactions and relative significance of its many downstream effector pathways; the difficulty of targeting RAS directly; and clinical resistance to existing and upcoming targeted therapies directed against RAS effector pathways. My studies described herein addresses two aspects of these challenges. The first study uncovers a novel mechanism of resistance to inhibitors of the RAF-MEK-ERK kinase cascade in BRAF-mutant melanoma. The second study demonstrates the importance of WT RAS isoforms, especially $KRAS^{WT}$, in NRAS-mutant melanoma.

Melanoma presents an excellent disease model for the study of RAS-directed drug resistance, not least in part because four out of seven approved RAS effector pathway inhibitors are approved for melanoma patients, and therefore the vast majority of clinical inhibitor resistance data have been obtained from treated melanomas. Identified mechanisms of resistance largely fall into two categories (Lito et al., 2013): activation of RAS-RAF-MEK-ERK pathway components that result in ERK reactivation (e.g., *RAS* or *MEK* mutations, *BRAF* alternative splicing, *BRAF* amplification, increased expression of CRAF or COT, etc.) or diminution of cell dependence on ERK signaling (e.g., *PTEN* and *RB1* loss, activation of RTKs, *etc*.). In my study, I identified an atypical mechanism that does not directly belong to either of

these two classes. My findings show that overexpression of either the wild-type or a kinase-inactive mutant form of the catalytic subunit of protein kinase CK2 (CK2α) promotes resistance to BRAF and MEK inhibitors, but can be reverted by an ATP-competitive ERK inhibitor, SCH772984. My observations were surprising in two ways. First, although CK2α promotes ERK reactivation, it is not an integral component of the RAS-RAF-MEK-ERK pathway. Hence, the exact mechanism of this enhanced ERK activity needs to be defined, but can be promiscuous owing to the huge repertoire of CK2α substrates. Secondly, all reported biological activities of CK2α to date are attributed to its kinase activity. However, my results indicate that the kinase function of CK2α is not required for promoting resistance. The only study, to my knowledge, that has linked CKα to scaffolding also suggested a contribution of its kinase function (Ritt et al., 2007). Collectively, my findings provide insight into possibly neglected roles of CK2 in modulating the RAS-RAF-MEK-ERK pathway.

Another area that has attracted much attention in the past several years is dissection of the roles of wild-type RAS isoforms in the presence of oncogenic RAS (Grabocka et al., 2014; Jeng et al., 2012; Lim et al., 2008; To et al., 2013; Weyandt et al., 2015; Young et al., 2013). As part of my doctoral work, I interrogated the roles of wild-type RAS, especially wild-type KRAS, in NRAS-mutant melanoma. Based on my data, I conclude that $KRAS^{WT}$ plays a significant role in the maintenance of the transforming capabilities, as well as in cell proliferation and survival, of NRAS-mutant melanoma. I also demonstrate divergent contributions of RAS isoforms to cellular morphology and to canonical pathway signaling. Furthermore, KRAS depletion induces p53 stabilization and concomitant increases in p21and PUMA, which are known to be key mediators of p53-induced G1 cell cycle arrest (Waldman et al., 1995) and apoptosis (Yu and Zhang, 2003), respectively. Unbiased analysis of protein activation states following RAS isoform knockdown by reverse phase protein array (RPPA) unexpectedly revealed that rpS6 phosphorylation is selectively impaired in KRAS-depleted cells. Intriguingly, rpS6

phosphorylation at S235/236 in KRAS-depleted cells does not correlate with AKT-mTOR activity or ERK-RSK signaling, implying a more direct means of controlling S6K1 signaling. These data provide evidence for a previously unappreciated role of RAS isoform-specific regulation of rpS6 phosphorylation in NRAS-mutant cancer. Taken together, my work, for the first time to my knowledge, illustrates the dependence of an NRAS-mutant cancer on wild-type RAS isoforms, and particularly on wild-type KRAS.

Future Directions

Is CK2α-mediated resistance to RAF/MEK inhibition clinically relevant?

My work shows the sufficiency of CK2α to promote resistance to BRAF and MEK inhibitors in melanoma cells through regulating the RAS-RAF-MEK-ERK pathway. To ascertain the degree to which this finding exhibits clinical relevance, it would be interesting to learn whether CK2α is also required for resistance in a subset of treatment-resistant BRAF-mutant melanoma. If so, the use of inhibitors to disrupt CK2α functions could be of clinical value. Although my data sheds some light on the necessity of CK2α in intrinsic resistance in cell culture, the effects were relatively modest, likely due to the already very high sensitivity of the A375 melanoma cell line to treatment with BRAF/MEK inhibitors. It is almost impossible to find a cell line that is both an appropriate candidate for treatment with BRAF inhibitors and also intrinsically resistant to BRAF inhibition. This is owing to the points that only BRAF-mutant cells are sensitive to BRAF inhibitors, and that non-BRAF-mutant cells are not candidates for BRAF inhibition (Holderfield et al., 2014b). However, it is possible to test the involvement of CK2α in acquired resistance. To do this, I could generate BRAF inhibitor-resistant cell lines by exposing sensitive cells to increasing concentrations of inhibitor for a long period of time, and then determine whether sensitivity is restored upon depletion of CK2α protein expression. I could also determine whether CK2α protein levels are increased in resistant cells, and thereby serve as a biomarker for resistance.

Although CK2 has been reported to promote drug resistance to both conventional and targeted therapeutics, all the reported studies have been performed in cell lines or in animal models (Borgo et al., 2013; Di Maira et al., 2007; Kreutzer et al., 2010; Salizzato et al., 2016; Stolarczyk et al., 2012; Wang et al., 2015). My TCGA-based data analysis revealed a significant increase in CSNK2A1 mRNA expression when comparing metastatic to primary melanoma (Figure 4.1). However, although this increase suggests that CK2α may be involved in disease progression in melanoma patients, these data are not linked to patient response to treatment with RAF or MEK inhibitors. Unfortunately, patient inhibitor treatment data are lacking in TCGA samples. Therefore, no direct conclusions can be drawn on the role of CK2α in resistance to RAF or MEK inhibitors from these data. One approach to testing the clinical relevance of CK2α in resistance to RAF or MEK inhibitors at a smaller scale would be to perform immunohistochemical staining for CK2α on tumor tissue samples from patients not responding to initial therapy (intrinsic resistance), and from patients who have progressed after treatment (acquired resistance), and comparing them both to treatment-naïve sensitive tumor samples. Additionally, shRNA delivery of CK2α knockdown constructs into tumors of patient-derived xenografts would be useful in studying whether CK2α is a driver of resistance or simply a passenger.

In addition to resistance to RAF/MEK inhibitors, CK2 may also play a role in immune modulation. Melanoma is an immunogenic tumor that overcomes the control of the immune system through impairment in dendritic cell maturation, loss of dendritic cells (Failli et al., 2013), and excess production of tolerogenic cytokines and growth factors in the microenvironment (Umansky and Sevko, 2012), which is known as tumor-induced immunosuppression. This led to decades of research into the development of antibodies that elicit effective anti-tumor responses through sustained activation of the immune system. Accordingly, immune checkpoint inhibitors (ipilimumab, pembrolizumab, and nivolumab) have been approved by the FDA for the treatment of advanced melanoma. However, CD4+ regulatory T cells (T_{reg} cells), which are essential for

peripheral tolerance, have been shown to possess high CK2 activity (Ulges et al., 2015). The authors found that T_{reg} cell–specific deficiency in CK2β resulted in the spontaneous development of excessive T helper type 2 (T_H2) responses in the lungs (Ulges et al., 2015). Therefore, I posit that CK2 hyperactivity leads to increased immune suppression. If so, CK2 overexpression/hyperactivity may reduce the effectiveness of immunotherapies in the clinic. I postulate that CK2 levels may also modulate the new treatment regimens combining RAF-MEK inhibitors with checkpoint modulators that are now being tested in melanoma patients (www.clinicaltrials.gov).

How does kinase-inactive CK2α promote resistance to inhibitors of RAF or MEK?

My study unexpectedly showed that a kinase-inactive mutant of CK2α (K68M) promotes resistance to BRAF or MEK inhibition, similar to wild-type CK2α. In Chapter 3, I provided a possible explanation: that wild-type and kinase-inactive CK2α bind equally well to the RAF-MEK-ERK pathway scaffold Kinase Suppressor of Ras 1 (KSR1). I hypothesized that the kinase function of CK2α is not critical to the scaffolding efficiency of KSR, and that kinase-deficient CK2α binds to KSR1 and promotes scaffolding as efficiently as wild-type CK2α, therefore ensuring maximal signal transduction efficiency. To test this hypothesis, I would first need to identify the region of CK2α that binds to KSR1, then mutate it to generate a KSR binding-deficient CK2α. Using this construct, I could then ask whether the resistance profiles associated with wild-type and/or kinase-inactive CK2α are dependent on their binding to KSR1.

An alternative possibility is grounded in the intricate interaction between CK2 subunits. As mentioned earlier, protein kinase CK2 is a heterotetrameric enzyme that is comprised of two regulatory subunits (beta), and two catalytic subunits (alpha or alpha'). Mounting evidence suggests CK2α can exist free of CK2β, and vice versa, raising the possibility that the regulation and function of monomeric CK2 subunits may be distinct from those of the tetramer (Guerra et al., 1999; Heller-Harrison and Czech, 1991; Lebrin et al., 1999; Martel et al., 2001; Theis-Febvre

et al., 2005). In most cases, the catalytic activity of the holoenzyme is somewhat higher than the isolated subunits, but exceptions exist. For example, calmodulin is only phosphorylated by the catalytic subunits but not the holoenzyme (Pinna, 2002). It is interesting to speculate that resistance caused by CK2α overexpression is dependent only on "free" CK2α and not on CK2α in the form of the holoenzyme. In this hypothetical model, kinase-inactive CK2α(K68M) competes with CK2α(WT) for CK2β binding, thereby releasing CK2α(WT) monomers. In this scenario, with the kinase function important in regulating resistance, one could enhance sensitivity by using a CK2α inhibitor, such as CX-4945 (Siddiqui-Jain et al., 2010). Indeed, I have preliminary data suggesting that this could be true (Figure 4.2). In my preliminary experiment, a fixed concentration (15 μM) of CX-4945 enhanced the sensitivity of CK2α-expressing A375 cells to vemurafenib, reducing the GI_{50} from 0.79 μM to 0.12 μM.

However, two premises must be fulfilled to support this bold speculation. First, given the expression levels of ectopic CK2α(K68M) versus endogenous CK2α(WT) (~10-fold), the endogenous molarity of tetrameric CK2 needs to reach at least approximately 10-times the molarity of monomeric CK2α. There is scarce evidence in the current literature on the relative abundance of CK2 subunits in cells or on the equilibrium between tetrameric and monomeric CK2. Even if available, this ratio can be highly context-specific, changing with tissue, disease state, etc. (Ferrer-Font et al., 2015; Stalter et al., 1994). Therefore, at present it is difficult to extrapolate any published values to those in my cell lines. Second, the dissociation constant (K_d) of CK2α(K68M) binding to CK2β needs to be smaller than that of CK2α(WT). Goldberg and colleagues were able to demonstrate with CK2α(K68A) that this kinase-inactive mutant has the capacity to integrate the endogenous CK2 subunit pool both as an isolated kinase-inactive alpha subunit and when associated with the beta subunit in a kinase-inactive tetramer, implying that the K_ds of wild-type and K68-mutated CK2α may be comparable (Lebrin et al., 2001). Another kinase-inactive variant of CK2α, i.e., CK2α A156, is known to bind tightly to CK2β,

efficiently compete with wild-type CK2α, and act as a dominant-negative (Cosmelli et al., 1997; Korn et al., 2001). One way to test my model would be to titrate in increasing amounts of the CK2β regulatory subunit, while overexpressing a fixed amount of CK2α. If sensitivity of cells to RAF/MEK inhibition gradually increases in parallel with CK2β expression, then that would support my speculation that it is the "free" pool of CK2α that is primarily responsible for resistance.

How does CK2α regulate DUSP6 protein levels?

An unexpected and interesting preliminary finding in the first part of my doctoral work is the regulation of DUSP6, an ERK-specific phosphatase, by CK2α. Specifically, overexpression of wild-type but not kinase-inactive CK2α dramatically decreased DUSP6 protein, which could be rescued by the addition of MG132, and did not correlate with DUSP6 mRNA (Figure 3.5). These data suggest that the kinase function of CK2α is required for regulation of DUSP6 at the post-translational level, possibly through phosphorylating and thus targeting DUSP6 for proteasomal degradation. In one study, the authors transfected equal amounts of MKP3 (DUSP6) and increasing amounts of CK2α into COS-7 cells, and observed a slight increase in ERK phosphorylation (Castelli et al., 2004). However, they failed to note the reduction in MKP3/DUSP6 protein, but instead concluded that the increase in ERK phosphorylation was due to reduced MKP3/DUSP6 phosphatase activity. They did show that CK2α phosphorylates MKP3/DUSP6 *in vitro*, but did not test whether this phosphorylation occurs in cells or affects MKP3/DUSP6 phosphatase activity on ERK. Further experiments will be needed to identify the specific phosphorylation sites and the effect(s) of phosphorylation on DUSP6.

It would also be worthwhile to investigate the contribution of CK2β to DUSP6 regulation, since, as mentioned above, the CK2 holoenzyme and the catalytic subunits can exert different effects on substrates (Pinna, 2002). My preliminary data show that, while CK2α expression diminished DUSP6 protein, CK2β dramatically increased it. Concurrent expression of both alpha

and beta subunits resulted in a compromised expression of DUSP6 (Figure 4.3). These results indicate that CK2α and CK2β or the holoenzyme play opposing roles in DUSP6 regulation. Although CK2β is regarded only as a regulatory subunit, it is endowed with independent physiological and pathophysiological roles (Filhol et al., 2015; Huillard et al., 2010; Lee et al., 2009; Lin et al., 2010; Pallares et al., 2009; Yde et al., 2008). Curiously, direct sequence analysis of CK2β did not show any significant similarity of this protein to other proteins in the databank with the exception of the stellate protein from Drosophila (Bozzetti et al., 1995). However, other methods of analysis may be more appropriate. For example, PropSearch is a program that is not based on direct sequence comparison but rather on the aggregate analysis of many properties and characteristics of proteins such as size, amino acid composition, isoelectric points and frequency of amino acid pairs (Hobohm and Sander, 1995). PropSearch revealed a high level of similarity between the CK2β subunits and some protein phosphatases, including protein phosphatase 2A, and ion transport ATPases (Korn et al., 1999). Future experiments using more physiologically relevant systems that enable fine-tuning of subunit expression to dissect the functions of CK2α, CK2α', CK2β, and the CK2 holoenzyme will provide further insight into the regulation of the kinase activity of CK2 and the sophisticated interplay among its subunits.

Why is $KRAS^{WT}$ more critical to cell survival than oncogenic NRAS in NRAS-mutant melanoma?

In the second part of my thesis work, I revealed that NRAS-mutant melanoma cells are dependent on $KRAS^{WT}$ for cell survival, proliferation, and various aspects of the transformed phenotype. Since NRAS is the oncogenic RAS isoform in this tumor type, one might assume that depleting cells of NRAS would lead to a stronger effect in all phenotypes, as was reported for an NRAS-mutant rhabdomyosarcoma cell line (Young et al., 2013). However, my data suggest otherwise. As shown in Figure 4.4, the more rapidly proliferating (high fold-changes in

absorbance over time) NRAS-mutant cells SK-MEL-147 and Sbc12 did not appear to distinguish among the RAS isoforms with respect to their requirements for proliferation. In contrast, the more slowly proliferating lines SK-MEL-119 and Mel224 (low fold-changes in absorbance over time) tended to respond more strongly to $KRAS^{WT}$ knockdown than to $NRAS^{mut}$ knockdown during the first 5-6 days, the time frame used by other studies (Grabocka et al., 2014; Young et al., 2013). This observation implies that cells may tolerate more cell divisions upon NRAS depletion than upon KRAS depletion. In addition, when monitoring cells in culture upon RAS isoform knockdown, I also observed that KRAS-depleted cells undergo apoptotic cell death faster than others (data not shown). Accordingly, 2 days post viral transduction, *PUMA* was selectively induced at the mRNA level in KRAS-depleted cells (Figure 3.8). Of note, although both NRAS and HRAS were compensating for the loss of KRAS by upregulating their own mRNA and/or protein expression (Figure 3.1), they were incapable of attenuating cell death. These findings all point to a critical role of KRAS in maintaining fundamental cellular functions such as survival.

Nevertheless, technical issues may confound these observations. It is possible that KRAS knockdown is more efficient than NRAS knockdown in these cells. However, such a conclusion cannot be drawn based solely on western blots or qPCR, due to differences in antibody and primer efficiencies. To better address this question, I could use CRISPR-Cas9-mediated knockout to eliminate the desired RAS isoform. This method, however, is further complicated by the necessity of selecting clones in which the gene of interest has been successfully knocked out. This not only gives rise to cells that have fully reprogrammed to accommodate loss of a given RAS isoform, but also creates an additional variable, that of clonal variation.

In addition, genetic depletion of oncogenic NRAS may also target any wild-type NRAS copies in the cells, which could affect the consequences of depletion of mutant RAS either negatively or positively. For example, $HRAS^{WT}$ and $KRAS^{WT}$ act as tumor suppressors in

experimental models of HRAS-driven non-melanoma skin cancer and KRAS-driven lung cancer, respectively (To et al., 2013). If $NRAS^{WT}$ also acts as a tumor suppressor in the context of NRAS-mutant melanoma, then depleting it may partially rescue loss of oncogenic NRAS. This could help to explain why knockdown of NRAS in these NRAS-mutant melanoma lines did not impair their growth and survival to the same extent as knockdown of $KRAS^{WT}$.

What are the functional consequences of the suppression of rpS6 phosphorylation that is induced upon KRAS silencing?

Using an unbiased reverse phase protein array (RPPA) approach, we identified an unexpected link between KRAS and the phosphorylation state of ribosomal protein S6 (rpS6). Compared to silencing of either NRAS or HRAS, depletion of KRAS resulted in the strongest suppression of rpS6 phosphorylation at Ser235 and Ser236 (Figure 3.4A,B). rpS6 is one of the 33 proteins that constitute the 40S small subunit of eukaryotic ribosomes. It was the first, and for many years the only subunit that has been shown to undergo inducible phosphorylation (Meyuhas, 2015). As part of the translation machinery, rpS6 and its phosphorylation have been thought to be primarily involved in protein synthesis. However, several studies demonstrated an apparent lack of a role for rpS6 phosphorylation in protein synthesis (Garelick et al., 2013; Johnson and Warner, 1987; Ruvinsky et al., 2005). Similarly, genetic studies deleting its upstream kinases, S6K1 and S6K2 (Chauvin et al., 2014; Mieulet et al., 2007), also reported no effect on global protein synthesis. Although not significantly involved in global protein synthesis, rpS6 phosphorylation has been reported to promote cap-dependent translation, polysome assembly (Roux et al., 2007), and ribosomal genesis (Chauvin et al., 2014). Other physiological roles of rpS6 phosphorylation include cell size regulation, normal muscle function, hypertrophic responses, cell proliferation, clearance of apoptotic cells, tumorigenicity (Meyuhas, 2015), and metabolic signaling in CD8 T cells (Salmond et al., 2009). To examine whether impaired rpS6 phosphorylation contributes to the defects that I observed in cell proliferation and cell cycle

progression of NRAS-mutant melanomas in which KRAS had been knocked down (Volarevic et al., 2000), I could generate a series of phosphorylation-mimetic or -deficient mutants of rpS6, and compare their abilities to rescue cell proliferation and/or cell cycle arrest to those of wild-type rpS6. There is, however, a caveat to this method: ectopic expression of those constructs may disrupt the endogenous stoichiometry, and lead to unwanted effects that may complicate interpretation of data. Therefore, it will be critical to express these constructs at levels similar to the endogenous proteins. Additional experiments along the same lines but elucidating other functional outcomes of rpS6 hypophosphorylation induced by knockdown of KRAS will advance our understanding of the currently elusive roles of such modifications.

How do RAS isoforms differentially engage effector signaling?

My results suggest that RAS isoforms differ in their ability to engage downstream effector signaling (Figure 3.2). Their differential regulation of AKT and ERK phosphorylation was even more prominent at later time points (Figure 4.5). In fact, differences among RAS isoforms in signaling to the canonical effector pathways RAF-MEK-ERK and PI3K-AKT-mTOR have been reported (Newlaczyl et al., 2014; Omerovic et al., 2007; Voice et al., 1999; Yan et al., 1998), but these results are contradicted by other studies that did not show specific coupling to these major pathways (Omerovic et al., 2008). In COS cells, compared to $HRAS^{G12V}$, $KRAS^{G12V}$ more efficiently recruited RAF-1 to the plasma membrane, and was also a more potent activator of membrane-recruited RAF-1. In contrast, $HRAS^{G12V}$ was more potent in activating PI3K than $KRAS^{G12V}$ (Yan et al., 1998). A more thorough study compared the relative ability of oncogenic forms (G12V) of all four RAS homologs (HRAS, NRAS, KRAS4A, and KRAS4B), to activate Raf-1, induce foci formation, enable anchorage-independent growth, and stimulate cell migration in COS-1 cells, NIH 3T3 mouse fibroblasts, Rat-1 fibroblasts, and RIE-1 rat intestinal epithelial cells (Voice et al., 1999). The RAS isoforms displayed very different potentials to promote each phenotype. For Raf-1 activation, KRAS4B > KRAS4A >>> NRAS > HRAS; for

focus formation, HRAS ≥ KRAS4A >>> NRAS = KRAS4B; for anchorage-independent growth, KRAS4A ≥ NRAS >>> KRAS4B = HRAS = no growth; and for cell migration, KRAS4B >>> HRAS > NRAS = KRAS4A = no migration. Walsh and Bar-Sagi compared the abilities of *HRAS*^{G12V} and *KRAS4B*^{G12V} to activate the Tiam1-Rac-PAK pathway in REF-52 rat fibroblasts, using three Rac-dependent readouts: induction of membrane ruffling and pinocytosis, stimulation of cell motility, and PAK binding. In all assays, *KRAS4B*^{G12V} displayed superior ability to stimulate the pathway than did *HRAS*^{G12V} (Walsh and Bar-Sagi, 2001). A comprehensive analysis of mutant RAS isoforms *HRAS*^{G12V}, *NRAS*^{G12D}, and *KRAS*^{G12V} to regulate gene expression in preneoplastic rat 208F cells revealed 26 genes whose expression was regulated in a RAS isoform-specific manner (Zuber et al., 2000). Therefore, in my system, future experiments will need to address the following questions: Do RAS isoforms differentially impact Tiam1-Rac-PAK signaling, as I might expect, given that the morphological features that I observed (Figure 3.2) were unique to knockdown of each isoform? How effective are RAS isoforms in transmitting signals through RAF-MEK-ERK or PI3K-AKT-mTOR pathways? Why do mutant NRAS and wild-type KRAS regulate ERK signaling in opposite directions? *etc*.

Another issue worth highlighting is that "KRAS" is a simplified representation of two very distinct isoforms, KRAS4A and KRAS4B. As shown in Figure 1.2, KRAS4A possesses a very different hypervariable region than does KRAS4B. In addition to the CAAX motif for farnesylation, KRAS4B contains a stretch of polybasic residues that promotes electrostatic interactions with the negatively charged membrane phospholipids, whereas KRAS4A has both a bipartite polybasic region as well as a palmitoylatable cysteine. KRAS4A was, and sometimes still is, the ignored KRAS isoform, for various reasons, mainly because it was shown to be much less important than KRAS4B in during mouse embryonic development (Plowman et al., 2003), and also due to the proposedly lower expression of this variant (Capon et al., 1983; Pells et al., 1997; Plowman et al., 2006). However, a recent study in human colorectal cells revealed that both mRNA and protein levels of KRAS4A and KRAS4B are relatively similar (Tsai et al., 2015),

calling for a new view of RAS isoforms (Nussinov et al., 2016). Remarkably, KRAS4A was found to be active at the plasma membrane even in the absence of the palmitoyl lipid (Tsai et al., 2015), suggesting that KRAS4A may exist in two distinct states. In state1, KRAS4A is only farnesylated, resembling KRAS4B; in state 2, KRAS4A is both farnesylated and palmitoylated, like NRAS. My knockdowns of KRAS in NRAS-mutant melanoma cells depleted both splice variants. Future studies could determine whether loss of either KRAS splice variant alone would have similar consequences as loss of both.

How does KRAS control rpS6 phosphorylation?

I observed that, whereas neither the MEK inhibitor trametinib nor the ERK inhibitor SCH7782984 significantly impacted rpS6 phosphorylation in cells with a non-targeting control knockdown construct, either inhibitor abolished the residual phosphorylation of rpS6 remaining after KRAS knockdown (Figure 4.5). This result suggests that the MEK-ERK pathway is not primarily responsible for S235/S236 phosphorylation in this system, and that KRAS mainly utilizes another kinase, notably S6K1 (Figure 3.4C), to control rpS6 activity.

Contrary to the widely-held perception that rpS6 phosphorylation serves as a reliable readout of mTOR pathway activity (Andreoli et al., 2015; Iwenofu et al., 2008), I showed that rpS6 phosphorylation is suppressed upon KRAS depletion in NRAS-mutant melanoma cells regardless of AKT or mTOR activity (Figure 3.2B). One obvious explanation is that KRAS signals to other intermediates to regulate S6K1 activity. Aside from phosphorylation by mTORC1 at Thr389 (Jacinto et al., 2004; Kim et al., 2003), S6K1 is also directly phosphorylated by PDK1 at Thr229 (Alessi et al., 1998; Pullen et al., 1998). While Thr389 phosphorylation is required for phosphorylation at Thr229, the ability of the latter to induce S6K activation is in turn controlled by phosphorylation of Thr389 (Alessi et al., 1998). Therefore, it is possible that KRAS reglates the ability of PDK1 to phosphorylate S6K1, which can be partially tested by examining the level of PDK1 phosphorylation on Ser241 upon KRAS depletion, whose autophosphorylation

is necessary for PDK1 activity (Casamayor et al., 1999). Further, I can measure the effects of mTORC1 or PDK1 inhibition on rpS6 phosphorylation at S235/236 by using rapamycin and a PDK1 inhibitor (e.g., GSK2334470 (Najafov et al., 2011)), respectively, and determine their relative contributions. An alternative hypothesis is that KRAS depletion induces PP1, the only known phosphatase of rpS6 (Belandia et al., 1994; Hutchinson et al., 2011), enhancing dephosphorylation of rpS6. To this end, I could assess whether Calyculin A, a potent inhibitor of PP1, rescues the rpS6 hypophosphorylation that is a result of KRAS knockdown. Other regulators of rpS6 phosphorylation at S235/236 include protein kinase A (PKA) (Biever et al., 2015; Chowdhury and Kohler, 2015; Moore et al., 2009), and less commonly, protein kinase C (PKC) (House et al., 1987) and Death-Associated Protein Kinase (DAPK) (Schumacher et al., 2006). The involvement of PKA can be tested with forskolin, a stimulator of cyclic AMP (cAMP) and hence PKA.

In summary, the specific pathway or pathways by which KRAS selectively controls rpS6 phosphorylation is currently unknown. Future studies unraveling the detailed mechanisms will be an important step towards delineating a RAS isoform-specific engagement of downstream signaling.

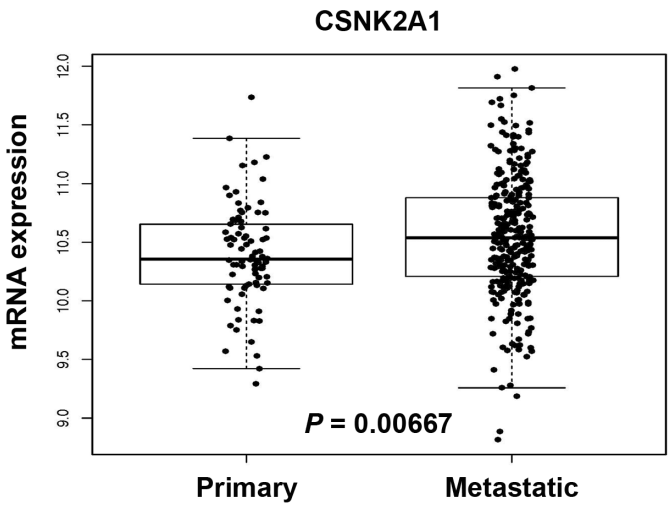

Figure 4.1. CK2α (CSNK2A1) mRNA expression is associated with melanoma progression.
CK2α (CSNK2A1) mRNA expression in primary and metastatic melanomas was analyzed based on data from The Cancer Genome Atlas (Cancer Genome Atlas, 2015). *p*-values were calculated by Student's *t*-test.

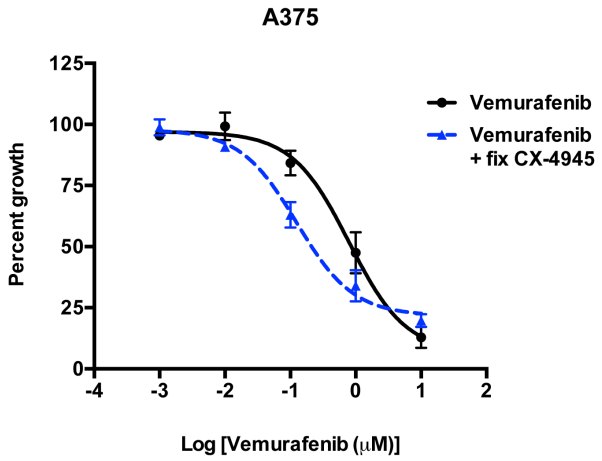

Figure 4.2 CK2α inhibition sensitizes CK2α-overexpressing A375 cells to BRAF inhibition by vemurafenib.
A375 melanoma cells stably overexpressing CK2α (A375-CK2α) were treated with a fixed-dose of CX-4945 (15 μM) plus varying concentrations of vemurafenib. A dose-response curve was generated by MTT staining on day 3, and GI_{50} values were calculated with Prism 6 (GraphPad). GI_{50}(vemurafenib) = 0.7902 μM; GI_{50}(vemurafenib + CX-4945) = 0.1251 μM.

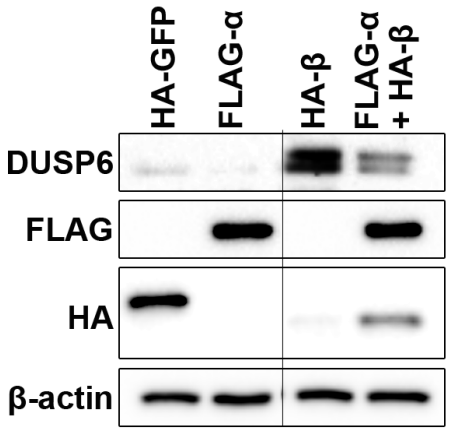

Figure 4.3. Opposing effects of CK2α and CK2β on DUSP6 protein expression.
To determine the effects of CK2 subunits on DUSP6, A375 cells were virally transduced with HA-GFP (control), FLAG-CK2α, HA-CK2β, or FLAG-CK2α + HA-CK2β. Two days post infection, cells were harvested, and blotted for DUSP6 expression.

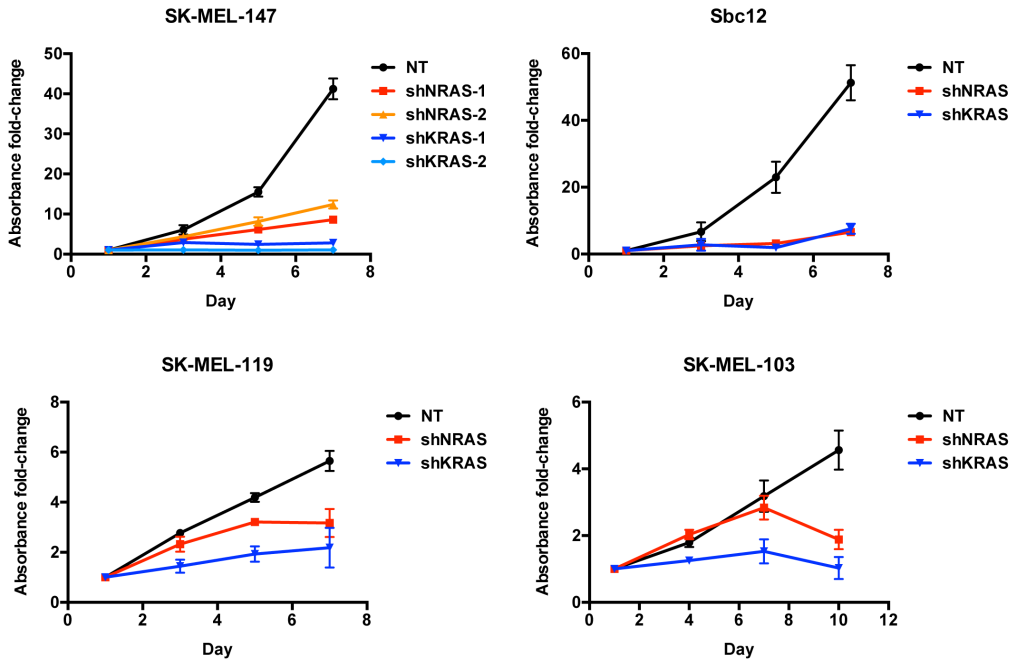

Figure 4.4. Proliferation of rapidly dividing and slowly dividing NRAS-mutant melanoma cells depends differentially on NRAS and KRAS.
MTT proliferation curves for a panel of NRAS-mutant melanoma cells (SK-MEL-147, Sbc12, SK-MEL-119, and SK-MEL-103) upon NRAS or KRAS depletion.

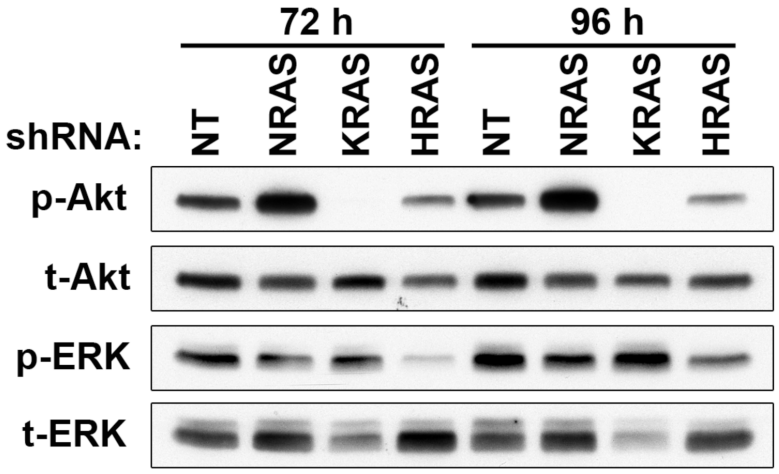

Figure 4.5. AKT and ERK phosphorylation is differentially regulated by RAS isoforms. shRNA depletion of NRAS, KRAS or HRAS was performed in SK-MEL-147 cells, and proteins were harvested on day 3 and day 4 post infection. Expression of both total and phosphorylated forms of AKT and ERK was examined by western blotting.

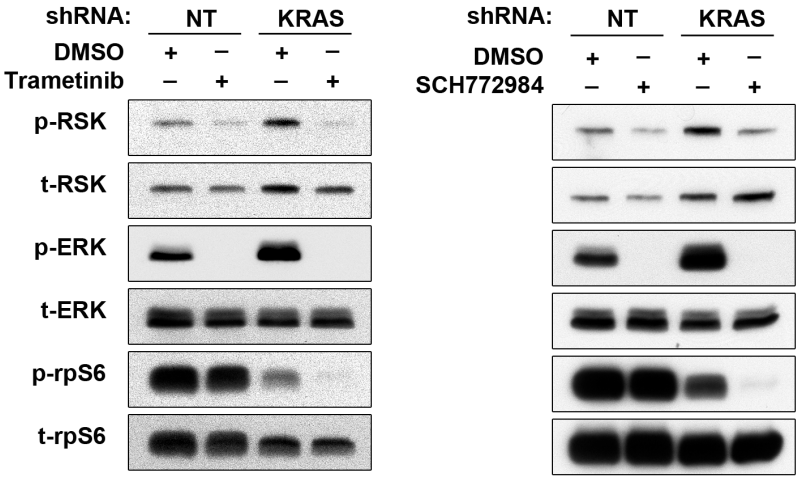

Figure 4.6. ERK pathway signaling controls residual rpS6 phosphorylation after KRAS depletion. SK-MEL-147 cells were treated with MEK inhibitor trametinib (2 nM, left) or ERK inhibitor SCH772984 (1 μM, right) for 1 hour in the presence or absence of KRAS, and lysates were probed for rpS6 phosphorylation at Ser235/236.

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