TARGETING THE ERK MAPK PATHWAY IN RAS-DRIVEN CANCERS

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ABSTRACT

Meagan Bridget Ryan: Targeting the ERK MAPK cascade in RAS-driven cancers
(Under the direction of Adrienne D. Cox and Channing J. Der)

RAS mutations are frequently found in the deadliest cancers in the United States, and there is a renewed interest in identifying therapeutic strategies to target RAS-driven cancers. While recent strategies to directly target mutant RAS have identified provocative small molecules, whether these can be developed into clinically potent and selective drugs remains to be seen. Arguably, among the most promising directions have been efforts to target protein kinase components of the effector pathways downstream of RAS. One of the best characterized effector pathways is the ERK mitogen-activated protein kinase (MAPK) cascade, a pathway that is critical in both the initiation and maintenance of NRAS- and BRAF-mutant melanoma as well as KRAS-mutant pancreatic ductal adenocarcinoma (PDAC). My research has focused on two aspects of the ERK MAPK cascade in these cancers: ERK regulation of the RAC small GTPase guanine nucleotide exchange factor (RACGEF) PREX1 in melanoma, and synergy between p38 MAPK and ERK inhibitors in PDAC.

My studies in melanoma are focused on the RacGEF PREX1, a protein that has been previously identified as a driver of metastasis in an NRAS-driven genetically engineered mouse model of cancer. PREX1 is an activator of RAC1, also

mutationally activated in melanoma. Previous work from our lab identified PREX1 as one of 82 genes regulated downstream of the ERK MAPK pathway in BRAF-mutant melanomas. Our lab also found that mice deficient in Prex1 were impaired in Nras-driven melanoma metastasis. My work has extended these studies on PREX1 to a broader panel of both NRAS- and BRAF-mutant melanomas. I found that expression of PREX1 protein is elevated in malignant melanomas compared to benign nevi and that high PREX1 protein expression is correlated with high levels of phosphorylated ERK. Loss of PREX1 reduced invasion in a context dependent manner and reduced levels of active RAC1-GTP, but not of the related GTPase CDC42. Also, the expression of PREX1 was regulated by ERK both transcriptionally and post-translationally. I found that the mechanisms of ERK driven overexpression of PREX1 in melanomas differs from those of PREX1 regulation previously identified in prostate cancer and breast cancer. Finally, my studies provide a mechanistic basis for a connection between the ERK MAPK cascade and RAC1, two pathways critical for the maintenance of melanomas. Since ERK MAPK pathway inhibitors are currently the standard of care in BRAF mutant melanomas, this connection warrants further study, especially in the context of therapeutic resistance.

Therapeutic resistance to ERK MAPK cascade inhibition arises not only in BRAF-mutant melanomas, but also in other cancers driven by activation of the ERK MAPK cascade. KRAS-mutant pancreatic cancer is the third deadliest cancer in the United States and is dependent on the ERK MAPK cascade for both tumor

development and maintenance. A recent study from our group found that a subset of KRAS-mutant PDAC cell lines and tumors are sensitive to the ERK inhibitors SCH772984 and BVD-523. I sought to validate a resistance mechanism to ERK inhibition first identified by this study, MAPK14 (p38α). Similar to ERK, p38 is the terminal kinase of a three-tiered MAPK cascade. We employed a novel CRISPR/Cas9 screen to identify mechanisms of resistance to the ERK inhibitor SCH772984 in KRAS-mutant pancreatic, lung, and colorectal cancers. MAPK14 was identified as a sensitizer to ERK inhibition and I validated that pharmacologic inhibition of p38 with the clinical candidate p38α/β inhibitor LY2228820 also sensitized PDAC to ERK inhibition. Concurrent p38 inhibition sensitized PDAC cell lines to the ERK inhibitors SCH772984 and BVD-523 in both anchorage-dependent and anchorage-independent growth. Concurrent p38 and ERK inhibition also led to an increase in G_0/G_1 cell cycle arrest vs ERK inhibitor treatment alone, while no enhancement in apoptosis was seen with dual inhibition vs ERK inhibitor alone. Mechanistically, ERK inhibitor treatment induced activation of the p38 MAPK cascade, including induction of expression of the p38 downstream substrate HSP27. Finally, concurrent p38 and ERK inhibition also enhanced loss of MYC, an oncogene critical for maintaining PDAC growth and previously identified by our group as a marker of sensitivity or resistance to ERK inhibition. My studies provide a mechanistic basis for synergy between p38 and ERK inhibition in PDAC that can be extended to additional KRAS-mutant cancers.

In summary, my studies provide a rationale for the importance of the ERK MAPK cascade in RAS-driven cancers. ERK plays many roles in initiating and

maintaining tumors of diverse genetic backgrounds, encompassing NRAS, KRAS, and BRAF mutations. Finally, my studies reveal the value in direct pharmacologic inhibition of ERK in RAS-driven cancers and in understanding resistance mechanisms to enhance ERK inhibitor therapeutic benefit.

To my father, who fought bravely not once, but twice against cancer.

I will continue your fight.

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LIST OF ABBREVIATIONS AND SYMBOLS

AKT	Protein kinase B (PKB)
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
Cas	CRISPR associated proteins
Cdc42	Cell division cycle 42 small GTPase
cDNA	Complementary deoxyribonucleic acid
C-terminus	Carboxyl-terminus
CRISPR	Clustered regularly interspaced short palindromic repeats
DMEM-H	High glucose Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSRT	Drug sensitivity and resistance testing
EC_{50}	Effective concentration 50, concentration of drug to cause 50% of maximal reduction in proliferation
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FTI	Farnesyltransferase inhibitor
GAP	GTPase-activating protein

GDP	Guanine diphosphate
GEF	Guanine nucleotide exchange factor
GI_{50}	Growth inhibitory 50, concentration of drug to cause 50% reduction in proliferation
GTP	Guanine triphosphate
GTPase	Guanosine triphospatase
h	Hour
HDAC	Histone deacetylase
HSP27	heat shock protein beta-1 (HSPB1)
HRP	Horseradish peroxidase
JNK	c-Jun N-terminal kinase
KRAS	Kirsten rat sarcoma viral oncogene homolog
MAPK	Mitogen-activated protein kinase
MAPK1	ERK2
MAPK3	ERK1
MAPK14	p38α
MEK	Mitogen-activated protein kinase kinase (MKK1/2)
MITF	Microphthalmia-associated transcription factor
MK2	MAPK-activated protein kinase-2
MKK	Mitogen-activated protein kinase kinase
mL	Milliliter
MM	Mis-match siRNA
mM	Millimolar
mRNA	Messenger ribonucleic acid

mTOR	Mammalian target of rapamycin
MTT	3-[4,5-dimthylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
MYC	v-Myc avian myelocytomatosis viral oncogene homolog
NF1	Neurofibromin 1
nM	Nanomolar
NSCLC	Non-small cell lung cancer
NS	Non-specific shRNA
N-terminus	Amino-terminus
PAGE	Polyacrylamide gel electrophoresis
PAK	p21 activated kinase
PARP	Poly (ADP-ribose) polymerase
P90RSK	p90 ribosomal kinase
PDAC	Pancreatic ductal adenocarcinoma
PDGFR	Platelet derived growth factor receptor
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PREX1	Phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1
PREX2	Phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 2
qPCR	Quantitative polymerase chain reaction

RAC	Ras-related C3 botulinum toxin substrate
RacGEF	Rac guanine nucleotide exchange factor
RAF	Rapidly accelerated fibrosarcoma kinase
RAS	Rat sarcoma viral oncogene homolog
RB	Retinoblastoma protein
Rho	Ras homologous protein
RhoGEF	Rho guanine nucleotide exchange factor
RNA	Ribonucleic acid
RPPA	Reverse phase protein array
RTK	Receptor tyrosine kinase
SEM	Standard error of the mean
SDS	Sodium dodecyl sulfate
shRNA	Short hairpin ribonucleic acid
siRNA	Short interfering ribionucleic acid
SOS	Son of sevenless
SP1	Specificity protein 1
Tiam1	T-cell invasion and metastasis gene 1
μm	Micron
μM	Micromolar
μL	Microliter
VEGFR	Vascular endothelial growth factor receptor

Chapter I: INTRODUCTION[1]
ADAPTED FROM: Targeting *RAS*-mutant cancers: Is ERK the key?

Introduction

The three *RAS* genes comprise the most frequently mutated oncogene family in cancer. With significant and compelling evidence that continued function of mutant *RAS* is required for tumor maintenance, it is widely accepted that effective anti-RAS therapy will have a significant impact on cancer growth and patient survival. However, despite more than three decades of intense research and pharmaceutical industry efforts, a clinically effective anti-RAS drug has yet to be developed. With the recent renewed interest in targeting RAS, exciting and promising progress has been made. My dissertation studies focused on inhibiting the RAF-MEK-ERK cascade in *NRAS*- and *BRAF*-mutant melanoma and the role it plays in regulating expression of the RacGEF PREX1, as described in Chapter 2, and on identification of a potential resistance mechanism to direct ERK inhibition in *KRAS*-mutant PDAC, as described in Chapter 3. Below, these studies are placed into the larger context of the prospects and challenges of drugging oncogenic RAS. In particular I focus here on new inhibitors of RAS effector signaling and on the ERK mitogen-activated protein kinase cascade.

[1] This Chapter is adapted from previously published work. Authors are Meagan B. Ryan, Channing J. Der, Andrea Wang-Gillam, and Adrienne D. Cox

RAS: in fashion, again

The discoveries in 1982 that human *RAS* genes are mutationally activated in cancer (Figure 1-1) initiated intensive efforts to identify pharmacological strategies that could disrupt the aberrant function of the corresponding RAS proteins[1]. Two decades later, when it became disappointingly apparent that farnesyltransferase inhibitors (FTIs) were not the answer, enthusiasm diminished dramatically. This failure coincided with the dawn of the current post-genomic era of cancer research, when sequencing of the cancer genome began to reveal the complexities of the genetic basis of cancer[2,3]. What these studies did not yield, however, were attractive new targets for cancer drug discovery. Instead, exome sequencing of colorectal, lung and pancreatic cancers verified that *RAS* mutations are the most prevalent gain-of-function genetic alterations in the cancers that comprise three of the top four causes of cancer deaths in the United States[2-4]. With this reality check, it became apparent that further efforts to seek an effective anti-RAS therapy, long an elusive holy grail of cancer research, must become a renewed priority, however difficult the task[5]. In this review, we provide an overview and perspective on the most promising directions for these efforts. We then focus on the direction where the greatest promise lies in the near future: with inhibitors already under clinical evaluation, there is guarded optimism that blocking RAS effector signaling may produce a clinically effective anti-RAS drug. In particular, we focus on the prospects and challenges faced by inhibitors of what is arguably the most significant signaling network driving cancer growth, the RAF-MEK-ERK protein kinase cascade.

Targeting RAS in cancer

The three *RAS* genes (*HRAS*, *NRAS* and *KRAS*) comprise the most frequently mutated gene family in cancer, with *KRAS* by far the most commonly mutated of these[2,3] (Figure 1-2a). There is substantial experimental evidence in cell culture and mouse model studies that mutant *RAS* is a critical driver of cancer initiation and maintenance. Thus, an effective anti-RAS therapy is expected to significantly impact cancer growth. Oncogenic RAS mutations are typically found in hotspots critical for the GTP/GDP on-off switch (Figure 1-2b), so the mutated RAS proteins escape normal regulation and are constitutively GTP-bound and active (Figure 1-2c). Unlike the successful development of ATP-competitive inhibitors of protein kinases, similar strategies to disrupt persistent GTP binding to mutant RAS have been seen as unsuccessful due to the apparent high picomolar binding affinities of RAS for GTP. Moreover, the smooth topology of RAS proteins originally discouraged efforts to search for small molecules that bound RAS directly, prompting perceptions that RAS is “undruggable”. Yet recent intriguing success in this area includes identification of cell- active small molecules that do bind directly to RAS and disrupt RAS interaction with regulators and/or effectors[6-8]. Particularly significant are the small molecules that target a specific KRAS mutation (G12C)[9,10], although it remains uncertain as to whether these can be advanced to clinically active and selective inhibitors of mutant RAS.

In addition to the challenging attempts to directly inhibit RAS itself, four approaches to inhibit RAS involve indirect targeting of proteins that support mutant RAS function (Figure 1-3). These approaches include: *i) Inhibition of RAS*-

membrane association. RAS proteins undergo posttranslational modification and covalent addition of prenyl and fatty acid lipids that promote association with the plasma membrane[11]. Inhibitors of the enzyme farnesyltransferase (FTIs) effectively disrupt plasma membrane association of HRAS, but not KRAS or NRAS. Therefore, it was not surprising that FTIs were clinically ineffective in pancreatic and colon cancer, where there is nearly exclusive mutation of KRAS. Another recent approach is to inhibit phosphodiesterase delta (PDEδ), a chaperone that is thought to facilitate RAS membrane trafficking[12]. A potential limitation of these approaches is that the proteins targeted also support the functions of numerous other proteins; *ii) Inhibition of synthetic lethality interaction*. Functional genetic screens have identified synthetic lethal interactors of mutant RAS, proteins whose functions are critical only in the context of *RAS*-mutant cancer cells[13]. However, the initial excitement in this area was dampened considerably when follow-up analyses failed to support the strong association of these proteins specifically with mutant RAS. Despite mixed opinions on the ultimate promise of this direction, ongoing studies still seek to improve the methodologies and biological screens in hopes of overcoming earlier limitations; *iii) Inhibition of RAS-regulated metabolic processes.* A recent new direction has been prompted by findings that mutant RAS function deregulates cellular processes (e.g., autophagy, glucose and glutamine metabolism) that support the increased metabolic needs of cancer cells[14]. These efforts are still in their infancy, with attractive targets and selective inhibitors for those targets still to be developed. A key limitation of the latter three approaches is that these proteins do not support RAS function exclusively and hence, their inhibition can have significant non-RAS cellular effects.

Currently, the area with the most advanced activity is the *iv) Inhibition of RAS effector signaling*. Numerous candidate inhibitors are presently under clinical evaluation, including inhibitors of the RAF and PI3K effector pathways[3] (RAF-MEK-ERK inhibitors detailed in Table 1). While conceptually simple, in practice this approach is complicated by the diversity of RAS downstream signaling networks, extensive signaling crosstalk and the highly dynamic nature of these networks. In this review, we ask, "Can inhibitors of the RAF-MEK-ERK mitogen-activated protein kinase (MAPK) cascade fulfill the promise of targeting RAS?".

The RAF-MEK-ERK cascade: sufficient and necessary for mutant RAS-driven tumor development

Active RAS-GTP can bind to and regulate a spectrum of catalytically diverse effectors (Figure 1-2c). Of these, the three-tiered RAF-MEK-ERK protein kinase cascade is the best characterized and validated driver of normal and mutant RAS function (Figure 1-4). The RAF-MEK-ERK cascade is under tight spatio-temporal regulation, dictating both quantitative and qualitative differences in ERK signaling output and biological outcomes. Among the numerous ERK substrates are components that comprise negative feedback mechanisms to attenuate the strength of ERK signaling. While ERK activation generally stimulates growth, excessive ERK activation can instead cause growth arrest[15]. Thus, finely tuned dynamic regulation of signaling flux through this cascade is critical in dictating the cellular consequences of ERK activation. Accordingly, there are diverse mechanisms of ERK feedback inhibition (Figure 1-5). One key mechanism involves ERK phosphorylation of CRAF

and BRAF, thereby decreasing RAF dimerization and association with activated RAS[16].

The importance of the RAF-MEK-ERK cascade as a therapeutic target in cancer is supported by several lines of evidence. *BRAF* is frequently mutationally activated (19%; COSMIC). The non-overlapping occurrence of *RAS* and *BRAF* mutations in cancer types where both are found is consistent with equivalent driver roles for each activated oncogene. Supporting a key driver role of *BRAF* in *KRAS*-driven oncogenesis, mutationally activated *Braf*^{V600E} but not *Pik3ca*^{1047R} was sufficient to phenocopy activated *Kras*^{G12D} in a mouse model of pancreatic cancer, and to induce pancreatic ductal adenocarcinoma together with mutant *Tp53*^{R270H}[17]. Genetic ablation of components of this pathway further supports the therapeutic value of targeting each level of this cascade. For example, in a *Kras*-driven mouse model of lung tumorigenesis, loss of either *Mek1* or *Mek2* increased survival by ~20%, while loss of both genes induced a near 100% increase in survival[18]. Also, the loss of *Erk1* or *Erk2* increased survival by 20% and 16%, respectively, and deficit of both genes increased survival by 40%[18]. More importantly, the few tumors that did arise in the *Erk1* null background were "escapers" that continued to express Erk2[18]. However, the complete genetic ablation of both *Erk1* and *Erk2* was deleterious for normal adult tissue homeostasis[18]. Genetic ablation of *Craf* alone (but not *Braf*) impaired mutant *Kras*-driven lung tumor formation and increased survival[18,19]. However, *Craf* deficiency did not impair mutant *Kras*-driven pancreatic cancer development, indicating that there are cancer-type differences in RAF isoform dependencies[20]. These genetic studies support both the sufficiency and

necessity of the RAF-MEK-ERK cascade in mutant *RAS*-driven tumor initiation and progression. However, since each MAPK component was ablated concurrently with RAS activation, their requirement in tumor maintenance was not addressed. Additionally, genetic loss of an entire protein may not accurately model the consequences of the pharmacologic inhibition of its catalytic kinase domain and activity. How far along is the development of RAF-MEK-ERK inhibitors and how are these drugs performing in the clinic?

RAF Inhibitors

The FDA-approved drug sorafenib was developed originally as an ATP-competitive CRAF inhibitor, but its clinical efficacy is attributed to its unspecific multi-kinase inhibitory activity, particularly the inhibition of receptor tyrosine kinases (RTKs) that drive tumor angiogenesis [21]. While sorafenib can inhibit ERK signaling, the degree of ERK inhibition may not be sufficient for effective suppression of ERK-driven cancer growth[21]. Second generation ATP-competitive BRAF-selective inhibitors, vemurafenib and dabrafenib, have been approved by the FDA for use in *BRAF*-mutant malignant melanoma and lead to clinically significant progression-free and overall survival[22-24]. However, while both cause initial rapid tumor regression in 70 to 80% of *BRAF*-mutant melanoma patients, mechanisms of resistance leading to relapse also occur rapidly in the majority of cases. Additionally, many *BRAF*-mutant colorectal, thyroid, and lung cancers exhibit *de novo* resistance to these BRAF-selective inhibitors[25]. Identifying resistance mechanisms will therefore be critical to more effectively applying these inhibitors in the clinic.

Much of the information regarding mechanisms that drive *de novo* and/or acquired resistance to inhibitors of RAF-MEK-ERK inhibition (Figure 1-6) comes from cell culture experiments in which resistance is induced by long-term treatment with inhibitors. These mechanisms include activation of upstream components (e.g., *NRAS* mutation, *NF1* inactivation, increased RTK expression and/or activation)[26,27] or increased RAF activity (via truncation and increased BRAF dimerization or increased BRAF expression) that lead to ERK reactivation. Since more than 80% suppression of ERK is required for a clinical response[28], increased flux through the cascade and increased ERK activation is sufficient to render cancer cells drug-insensitive. Other resistance mechanisms that reactivate the pathway downstream of the inhibitor blockade include activating mutations in MEK1 and MEK2[29] or amplification of TPL2/COT[30], which phosphorylates and activates MEK1/2. Additional mechanisms that do not restore ERK activation, but that instead decrease dependency on ERK-driven growth, include activation of PI3K-AKT-mTOR signaling and mutational activation of the small GTPase RAC1[31,32]. The clinical significance of some mechanisms remains to be established.

In contrast to their efficacy in *BRAF*-mutant cancers, vemurafenib and dabrafenib not only are ineffective in *RAS*-mutant cancers, but instead stimulate their growth[33-37]. This effect is due to paradoxical activation, rather than inhibition, of ERK. In this setting, drug-inactivated BRAF forms a heterodimer with drug-free CRAF that complexes with mutant RAS, which causes allosteric activation of CRAF by the inactive BRAF dimerization partner, thereby increasing ERK signaling (Figure 1-6).

Third-generation BRAF inhibitors (Table 1) are not limited by this activation and are known as "paradox breakers"., Currently, there is one paradox-breaker inhibitor, PLX8394, in clinical Phase I evaluation (NCT02428712). Compared to vemurafenib, PLX8394 has unique binding sites in the BRAF activation site and is also a superior inhibitor of CRAF[38,39]. PLX8394 can also effectively block ERK activation and the growth of *RAS*-mutant vemurafenib-resistant melanoma cells[39]. Pan-RAF inhibitors -MLN2480, HM95573 and LY3009120- have also entered Phase I trials[40-42]. LY3009120 has shown *in vitro* and *in vivo* efficacy in inhibiting the ERK pathway without eliciting the effect of paradoxical activation [42]. An alternative strategy for effective RAF inhibition in *RAS*-mutant cancers may be the use of small molecule inhibitors of RAF dimerization[43]. While these strategies can overcome upstream signaling resistance mechanisms, they will still be, however, susceptible to downstream mechanisms of resistance (e.g., mutational activation of MEK) or to those that reduce ERK dependency (e.g., increased PI3K-AKT-mTOR activity).

MEK Inhibitors

Currently, there is one FDA-approved MEK1/2 inhibitor for the treatment of *BRAF*-mutant melanoma, trametinib, and at least 11 other agents in clinical trial evaluation (Table 1). Trametinib and the majority of MEK drugs are allosteric non-ATP-competitive inhibitors and, consequently, exhibit greater target selectivity than ATP-competitive protein kinase inhibitors. These drugs work by blocking the ability of activated MEK to phosphorylate and activate ERK. In preclinical studies, MEK inhibitors that were effective in *BRAF*-mutant cancer cell lines were not effective in a

majority of *KRAS*- or *NRAS*-mutant tumor lines[44-46]. Consistent with this, clinical trials showed limited to no response of *RAS*-mutant NSCLC patients to these drugs [47,48]. Phase II trials failed to show an advantage of combining trametinib with gemcitabine in *KRAS*-mutant pancreatic cancer[49]. In contrast, the MEK inhibitor selumetinib plus docetaxel showed increased overall survival (9.4 months) compared with docetaxel alone (5.2 months) in Phase II trials for *KRAS*-mutant lung cancer patients[50,51]. Mutation-selective trends were seen, in that patients with G12V mutation-positive cancers responded better than others[50,51]. Other clinical Phase II studies have shown that trametinib induces similar progression-free survival and response rates as docetaxel in patients with *KRAS*-mutant- positive NSCLC [52]. MEK162 also showed limited activity in *NRAS*-mutant melanomas[53], where a partial response was seen in 20% of *NRAS*-mutant patients, although the response was transient, with rapid onset of resistance. Collectively, the clinical data suggest that combination therapies will likely be warranted.

Like RAF inhibitors, MEK inhibitors are also limited by mechanisms of drug resistance that typically involve the loss of multiple ERK-driven negative feedback loops that normally modulate flux through the cascade (Figure 1-5). Further, while initial treatment with MEK inhibitors effectively blocks ERK activation, kinome reprogramming (sometimes described as the rewiring of kinase signaling networks) drives a rebound in ERK activity within 24 h[54]. Acute inhibition of ERK impairs its ability to regulate stability of the MYC oncoprotein[54,55], resulting in loss of RTK suppression by this nuclear transcription factor. Upregulation of RTK expression and signaling then overcomes MEK inhibitor activity (Figure 1-6).

Two novel MEK inhibitors have distinct mechanisms of action that reduce their vulnerability to the loss of ERK-dependent negative feedback loops, and consequently may be more effective against *RAS*-mutant tumors. The clinical candidate GDC-0623 stabilizes the RAF-MEK complex in cells, preventing the activation of MEK by RAF[44,45]. GDC-0623 showed greater efficacy than conventional MEK inhibitors in *KRAS*-mutant cancer cells. Similarly, the clinical candidate RO5126766 forms a stable RAF-MEK-drug complex in cells, preventing both MEK and ERK phosphorylation[44,56,45,57,58]. However, these inhibitors remain susceptible to resistance mechanisms at the levels of MEK and ERK, as well as non-ERK mechanisms.

ERK Inhibitors

Until recently, it was assumed that RAF and/or MEK inhibitors would be sufficient to inhibit ERK1/2 activity and that there would be no additional benefit of directly blocking ERK. Thus, development of ERK inhibitors lagged behind RAF and MEK drugs. However, because the majority of resistance mechanisms to RAF and MEK drugs results in reactivation of ERK1/2, blocking ERK1/2 directly may overcome the current limitations of RAF or MEK inhibitors. Furthermore, although reactivation of ERK alone can overcome the loss of MEK function, it is likely that no single ERK substrate will be capable of restoring loss of ERK function. Hence, the mechanisms of resistance to ERK inhibitors will likely be both diverse and distinct from those of resistance to MEK inhibitors.

To date, two potent and selective cell-active preclinical ERK inhibitors have been described in the literature: VTX-11e and SCH772984, an analog of the orally available clinical candidate MK-8353/SCH900353 (Table 1)[59,60]. VTX-11e is a type I ATP-competitive inhibitor, whereas SCH772984 has a dual mechanism of action, causing the allosteric inhibition of MEK1/2 binding and ERK phosphorylation and also the ATP-competitive inhibition of ERK phosphorylation of its substrates. SCH772984 binding adjacent to the ATP binding pocket induces formation of a new allosteric pocket that then optimally accommodates the inhibitor[61]. Although VTX-11e and SCH772984 exhibit different interactions with ERK and distinct mechanisms of ERK inhibition[61], both inhibitors exhibit a slow off-rate[61,62], a property that prolongs their cellular inhibitory activities.

In in vitro studies, SCH772984 inhibited cellular proliferation in a subset of 121 *RAS*- (49%) or *BRAF*- (88%) mutant cancer cell lines[60]. Further, the majority (11 of 14) *NRAS*-mutant melanoma cell lines were sensitive to SCH772984 but not to vemurafenib [63]. Four ERK1/2 inhibitors are currently undergoing Phase I or I/II clinical evaluation (Table 1). GDC-0994[64] and BVD-523 (ulixertinib)[65] have shown potency in *RAS*-mutant cancer cells. In a Phase I dose escalation in patients with advanced solid tumors, BVD-523 achieved ERK inhibition and showed manageable tolerability, with adverse events most commonly including diarrhea, nausea, vomiting or constipation[66]. Ongoing trials will demonstrate whether sufficient inhibition can be achieved for therapeutic benefit.

Vertical inhibition of the RAF-MEK-ERK cascade

Current evidence indicates that inhibition of RAF or MEK alone is not sufficient for prolonged arrest of *RAS*-mutant cancers. Furthermore, the emergence of tumor cell resistance and normal tissue toxicity due to blockade of the critical RAF-MEK-ERK cascade are anticipated to pose additional limitations. Instead, combination approaches will be needed to effectively 1) overcome bypass of inhibitor action that drive ERK reactivation, 2) block ERK-independent mechanisms that overcome cancer cell addiction to ERK, and 3) concurrently block other RAS effector pathways important for cancer growth. Which combined therapies may provide the answer?

The restricted number of substrates of RAF and MEK led to the earlier perception that the RAF-MEK-ERK kinase cascade was a simple linear unidirectional pathway. However, there is now greater appreciation that there are multiple input and output signals at different levels and that ERK activation stimulates feedback inhibitory mechanisms to reduce flux through the pathway. Consequently, concurrent inhibition of the pathway at multiple levels may induce a more effective inhibition of ERK, .In fact, the combination of the BRAF inhibitor dabrafenib with the MEK inhibitor trametinib enhanced progression-free survival and reduced toxicity as compared to dabrafenib alone in *BRAF*-mutant melanoma[67-69], leading to FDA approval of this combination for these tumors. Also, in *KRAS*-mutant tumor cells, unbiased shRNA screening showed that genetic ablation of *CRAF* enhanced MEK inhibitor response[44,70,45]. And the combination of a pan-RAF

inhibitor (PRi, Amgen Compd A) with trametinib showed a synergistic effect on the growth inhibition of *NRAS*-mutant melanoma cells[71].

Although combining RAF and MEK inhibitors has shown greater clinical efficacy in *BRAF*-mutant melanoma cancers than either drug alone, reactivation of ERK signaling limits the long-term effectiveness of this combination[72,29]. *BRAF*-mutant melanomas acquired resistance to combined dabrafenib and trametinib treatment by several alterations (*BRAF* amplification and *NRAS* or *MEK1/2* mutational activation) that ultimately led to ERK reactivation. These results prompted studies to evaluate if blockade of ERK can overcome resistance to RAF and/or MEK inhibition. Data from multiple studies in different cancers has shown that this is the case. In fact, resistance of a *BRAF*-mutant melanoma cell line to concurrent vemurafenib and trametinib treatment was overcome by the ERK-selective inhibitor SCH772984[60]. Similarly, a *BRAF*-mutant melanoma cell line resistant to a RAF/MEK inhibitor combination due to MEK2 mutation remained sensitive to the preclinical ERK inhibitor VTX-11e[72,29]. Further, *KRAS*-mutant tumor cell lines resistant to MEK inhibitor (PD0325901) retained sensitivity to VTX-11e[73]. Co-treatment with VTX-11e enhanced the growth inhibitory activity of selumetinib and trametinib by preventing RAF-dependent rebound of flux through the RAF-MEK-ERK cascade, and caused apoptosis in *NRAS*-mutant melanoma cells[74]. Finally, SCH772984 was also effective in both *NRAS*- and *BRAF*-mutant melanoma cell lines, and synergized with vemurafenib in *BRAF*-mutant lines[63]. Thus, ERK inhibition in combination with RAF and/or MEK inhibition may be a superior therapeutic strategy to inhibition of any single step alone.

Despite these promising findings, ERK inhibitors will also likely be limited by both de novo and acquired mechanisms of resistance. A recent study found that experimentally induced mutations in ERK1 and ERK2 conferred resistance to VTX-11e or SCH772984 treatment[15]. However, the fact that these mutations did not confer cross-resistance to RAF or MEK inhibitors supports the value of combining ERK inhibitors with RAF or MEK inhibitors.

ERK substrates

ERK1/2 kinases undergo nuclear/cytoplasmic shuttling and translocate to the nucleus upon phosphorylation. ERK subcellular localization is further regulated by dimerization and by interaction with scaffold proteins (e.g., kinase suppressor of Ras (KSR)), and this localization in turn regulates ERK selectivity towards its substrates [75]. Unlike the restricted substrate profile for RAF and MEK, >200 nuclear and cytoplasmic ERK substrates have been identified[76,77]. The specific ERK substrates that are critical for ERK-dependent cancer growth remain poorly understood, with opposing conclusions reached regarding whether nuclear or cytoplasmic substrates, or both, are critical for cancer progression For example, the multi-functional protein PEA-15 binds and sequesters ERK in the cytoplasm, and genetic ablation of PEA-15 increased ERK nuclear localization and promoted cellular proliferation[78]. In a study where whole-body *Kras*^{G12D} activation was induced, tumorigenesis was driven in a subset of mouse tissues that was associated with nuclear accumulation of activated ERK and activation of nuclear substrates. In contrast, nonresponsive tissue was associated with cytoplasmic ERK[79]. Further, the nuclear import protein importin7

facilitates ERK nuclear translocation by recognition of the phosphorylated nuclear translocation signal (NTS), and an NTS-derived phosphomimetic peptide that blocks nuclear translocation of ERK impairs the growth of *RAS*- or *BRAF*-mutant tumor cell lines[80]. Because many nuclear ERK substrates are associated with cell proliferation whereas ERK negative feedback targets are cytosolic (Figure 1-5), the selective inhibition of phosphorylation of ERK nuclear substrates might favor inhibition of tumor growth. Among the multitude of nuclear transcription factors that are ERK substrates, MYC is likely a critical mediator of ERK effects in *RAS*-mutant cancers. Substantial evidence shows that MYC is essential for *RAS*-driven cancer initiation and growth[81-83]. MYC is a critical driver of *Kras*G12D-dependent up-regulation of genes that support the increased glycolytic and metabolic needs of pancreatic tumors[84], and ERK phosphorylation of MYC prevents MYC protein degradation[85].

In contrast, the therapeutic response to vemurafenib correlated with reduction in cytoplasmic rather than nuclear ERK phosphorylation, arguing for a critical role of cytoplasmic ERK substrates[28]. This finding is consistent with observations that ERK dimerization is essential for the activation of cytoplasmic but not nuclear substrates, and that preventing ERK dimerization impaired the tumorigenic growth of *RAS*-mutant cancer cell lines[86]. Recent efforts have demonstrated that pharmacologically targeting ERK dimerization can lead to a significant reduction in RAS-driven tumor growth by potently inhibiting phosphorylation of ERK cytoplasmic substrates. The ERK dimerization inhibitor DEL-22379 reduced tumor growth in mutant KRAS xenograft models and was able to overcome upstream resistance

mechanisms, including NRAS overexpression and MEK mutation[87]. Among ERK1/2 cytoplasmic substrates that drive tumorigenesis are the RSK serine/threonine kinases. RSKs are major effectors of the ERK1/2 kinases and have been identified as drivers of motility and invasiveness in cancer, as regulators of mTOR in *BRAF*-mutant cancers, and as drivers of chemoresistance[88,89]. Clearly, further work is needed to fully understand the importance of the diverse spectrum of ERK substrates in *RAS*-driven cancers.

Combined inhibition of RAF-MEK-ERK and PI3K-AKT-mTOR signaling

In addition to ERK reactivation downstream of RAF and MEK inhibitors, increased activation of the PI3K-AKT-mTOR pathway has also been observed. This can occur by increased RTK signaling[90] and therefore concurrent treatment with RTK inhibitors may enhance inhibition of RAF-MEK-ERK signaling. Combining inhibitors of the PI3K-AKT-mTOR pathway with MEK inhibitors effectively inhibited *NRAS*-mutant melanoma growth both *in vitro* and *in vivo*[91]. In *KRAS*-mutant pancreatic cancer, the dual PI3K-mTOR inhibitor BEZ235 enhanced MEK/ERK signaling, which could be reversed by the addition of a MEK inhibitor, leading to enhanced growth suppression compared to targeting either pathway alone[92]. Similarly, combination of the PI3K inhibitor GDC-0973 with the MEK inhibitor GDC-0941 was able to confer a greater survival advantage in a *Kras*^{G12D}-driven mouse model of pancreatic cancer than either inhibitor alone[93]. Pre-clinical findings with combined PI3K-AKT-mTOR and RAF-MEK-ERK inhibition have been followed by early clinical trials in a small series of *KRAS*-driven cancers including NSCLC, colorectal, pancreatic and ovarian[94,95]. Occasional partial responses were noted,

particularly in ovarian cancers, although normal tissue toxicity remains a concern[94,95].

The RAC effector pathway

Many additional effector pathways can contribute to the tumorigenic potential of RAS , including the T-cell Tiam1-RAC1 pathway. RAC1 is a GTPase which cycles between and active GTP bound state and an inactive GDP bound state, in a similar fashion to wilde-type RAS. RAC1 can be activated by many upstream guanine nucleotide exchange factors (GEFs), including Tiam1, which is directly activated by RAS[96,97]. In a RAS(V12)-driven model of skin cancer, *Tiam1* deficient mice were found to be resistant to the formation of tumors, emphasizing an essential role for RAC1 in RAS signaling[98]. Additional GEFs can also interact with and activate RAC1 in cancers, including VAVs, ECT2, and PREXs[99]. The RAC pathway, and its downstream substrates, p21 activated kinases (PAKs), also hold promise as an anti-cancer therapeutic strategy in RAS-driven cancers[100].

The RAC1 pathway has been found to play an essential role in melanoma progression downstream of either mutant *NRAS* or *BRAF* as well as representing a potential resistance mechanism to RAF-MEK-ERK cascade inhibition. RAC1 mutation can accelerate melanoma development and a fast cycling mutant of RAC1 (P29S) was found to confer resistance to the RAF-inhibitor vemurafenib[31,101,32]. The RacGEF PREX2 was also found to be frequently mutated in melanoma and altered RAC signaling[102,103]. Work from our lab has shown a role for the highly related protein PREX1 in regulating metastasis in an *Nras*-mutant mouse model and identified PREX1 as a gene potentially regulated downstream of the RAF-MEK-ERK

cascade[104,105]. My studies described in Chapter 2 focused on the role of ERK in regulating PREX1 in both *NRAS*- and *BRAF*-mutant melanomas.

MAPK cascades, more than just RAF-MEK-ERK

While the RAF-MEK-ERK cascade is the best characterized effector downstream of RAS, it is just one of many MAPK cascades in mammalian cells. All MAPK cascades follow a three tiered structure of MAP kinase kinase kinase (MAP3K), MAP kinase kinase (MAP2K) and MAPK and can be grouped into three families, ERK, Jun amino-terminal kinases (JNKs), and p38 kinases[16]. Collectively, the JNK and p38 MAPK cascades are known as the stress activated MAPKs and are activated in response to both intracellular and extracellular stimuli, such as UV radiation, osmotic shock, RTK activation, and response to chemotherapy[106].

My studies described in Chapter 3 focused on the p38 MAPK cascade as a potential mechanism of resistance to ERK inhibition in *KRAS*-mutant PDAC. The ERK and p38 MAPK cascades regulate diverse cellular processes, with p38 and ERK representing the terminal node of their respective MAPK cascades[107,108]. In cancer, p38 can play both a tumor promoting role or a tumor suppressing role, depending on the cellular and tumor type context[109]. The p38 MAPK cascade can also play a role in response to both chemotherapy and targeted therapy in cancer. In *KRAS*-mutant PDAC, the p38 MAPK cascade can paradoxically play both a tumor suppressive role while also acting in a tumor promoting role by contributing to resistance to the nucleoside analog gemcitabine[110,111]. In Chapter 3, I explore the

role of p38 in conferring resistance to ERK inhibition and demonstrate synergy between concurrent p38 and ERK inhibition in PDAC.

Concluding Remarks

While direct inhibitors of RAS remain the ideal strategy for clinically active anti-RAS drug discovery, inhibitors of the RAF-MEK-ERK cascade arguably hold the greatest promise for the immediate future. With earlier perceptions that this protein kinase cascade operated as a simple linear unidirectional pathway, initial efforts centered on MEK inhibitors, and subsequently on RAF inhibitors, to block ERK activation. As the development of RAF and MEK inhibitors progressed, it became painfully apparent that cancer cells can dynamically rewire their signaling networks to restore ERK activity and override the actions of inhibitors that act upstream of ERK. These revelations have led the field to consider ERK itself as perhaps the "best" node for effective disruption of ERK signaling.

As ERK inhibitors transit through clinical evaluation, new issues will likely arise that will challenge the usefulness of ERK inhibitors for cancer treatment. While ERK is clearly a key driver of cancer growth, it is also an essential component in normal cell physiology. Therefore, achieving a therapeutic index and minimizing normal tissue toxicity will be one challenge. Another will be acquired mechanisms of cancer cell resistance to ERK inhibition. However, unlike RAF or MEK, ERK action cannot be attributed to a single substrate. Thus, mechanisms of resistance to ERK inhibitors will likely be distinct from those that overcome the actions of RAF or MEK inhibitors, and likely more complex and varied as well. Defining combination

approaches with ERK inhibitors that might overcome cancer cell resistance and normal cell toxicity will be key challenges for the development of ERK inhibitors. Innovative chemical library or genetic functional screens will provide helpful unbiased functional strategies to address this need[112,113].

Other strategies beyond protein kinase inhibitors to block growth dependent on RAF-MEK-ERK signaling, for example inhibitors of RAF or ERK dimerization, are also being pursued. Defining the key ERK substrates critical for ERK-dependent cancer growth remains to be fully elucidated and may provide additional targets for effective blockade of ERK activation in cancer. Additional pathways, such as RAC1 and the p38 MAPK cascade can also be utilized as therapeutic strategies to target RAS dependency and overcome resistance to RAF-MEK-ERK cascade inhibitors, including ERK inhibitors.

Finally, even if direct inhibitors of RAS can be developed, given experimental evidence that cancers can overcome their addiction to mutant RAS, defining the mechanisms by which they accomplish this will also be important. Nevertheless, despite the considerable uncertainty ahead (see Outstanding Questions), there is renewed albeit cautious optimism that an effective anti-RAS strategy may finally be at hand.

My dissertation studies focused on two aspects of ERK MAPK signaling as a potential anti-RAS strategy: ERK regulation of the RacGEF PREX1 in *NRAS*- and *BRAF*-mutant melanoma, described in Chapter 2, and synergy between p38 and ERK inhibitors in *KRAS*-mutant PDAC, described in Chapter 3.

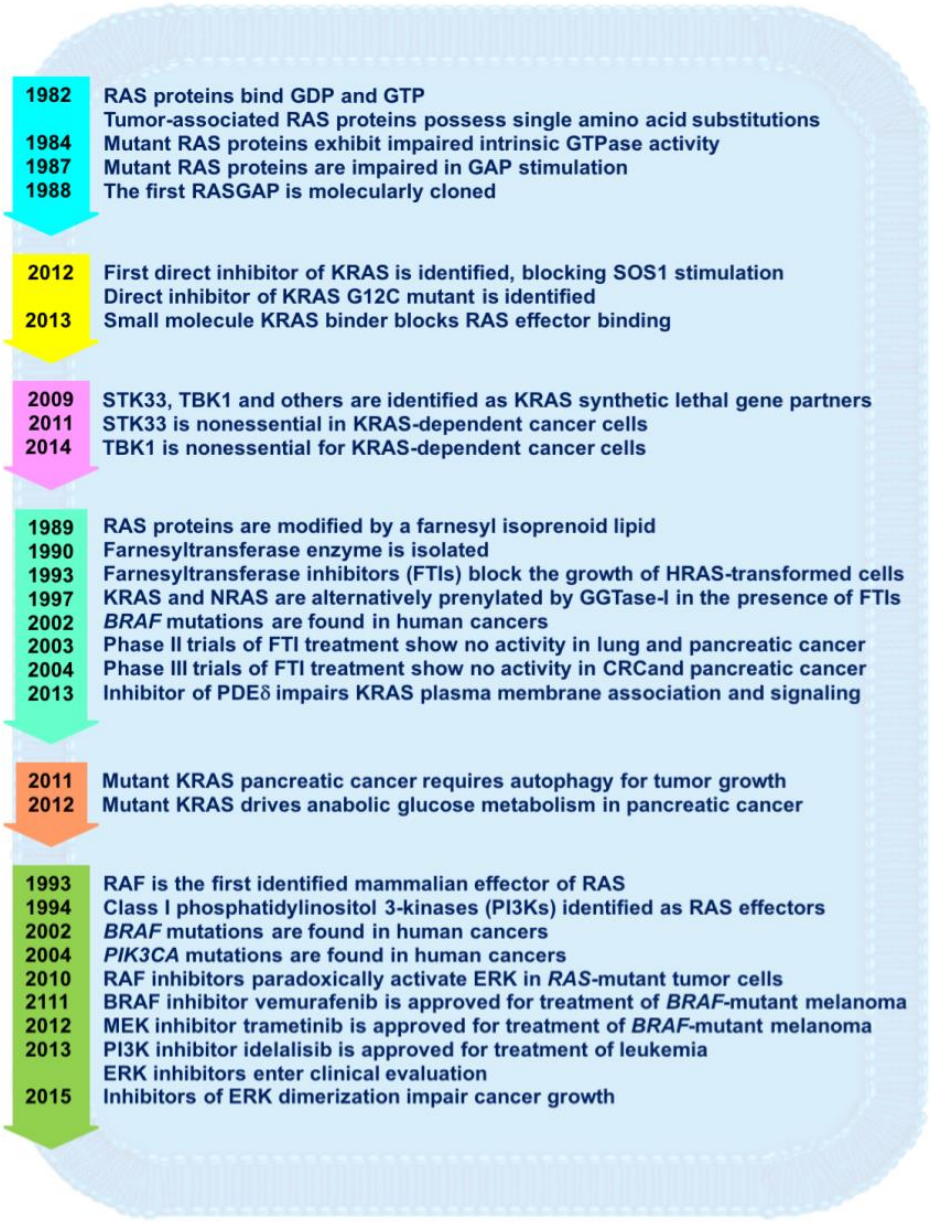


Figure 1-1 History of anti-RAS drug discovery

Summary of key representative events in the search for the still-elusive anti-RAS drugs.

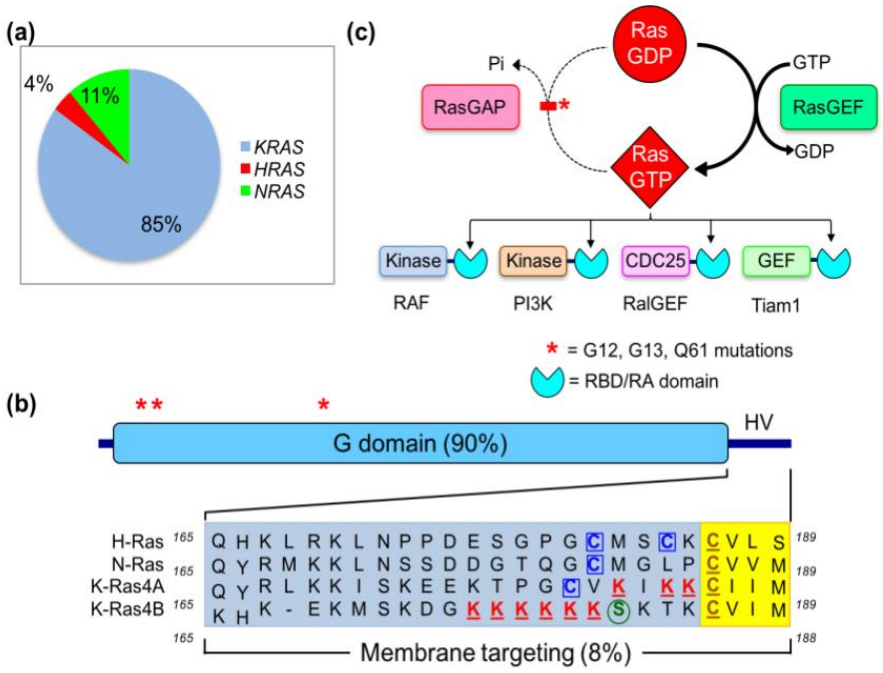


Figure 1-2. The RAS proteins (a) RAS oncogenes (HRAS, NRAS and KRas) comprise the most frequently mutated gene family in cancer[2,3]. Overall, RAS mutations are found in ~25% of human cancers (COSMIC v73). The mutation frequency is not uniform, with frequencies highest in three of the four most deadly cancers in the United States- lung (30%), colorectal (50%) and pancreatic (95%) carcinomas. The frequency of mutation of each RAS isoform is also not uniform, with 85% of all RAS mutations found in KRAS, followed by NRAS (11%), whereas HRAS is infrequently mutated (4%). (b) The three RAS genes encode four highly related proteins of 188-189 amino acids (82-90% sequence identity): HRAS, KRAS4A, KRAS4B and NRAS. RAS proteins are comprised of a highly conserved N-terminal G domain (90% amino acid sequence identity) involved in GTP binding and hydrolysis and a C-terminal membrane-targeting hypervariable (HV) sequence. Underlined C, cysteine of the CAAX motif (highlighted in yellow, the site for farnesylation; see Figure 1). Underlined K, lysine(s) comprising the polybasic domain. Boxed C, site of palmitoylation. Circled S, site of phosphorylation by PKC. (c) RAS proteins function as GDP-GTP regulated binary on-off switches. In normal quiescent cells, RAS is predominantly GDP-bound and inactive. Growth factors activate RAS-selective guanine nucleotide exchange factors (RASGEFs; e.g., SOS1) to promote nucleotide exchange and formation of active RAS-GTP. Once in the active, GTP-bound conformation, RAS can bind to a variety of effector proteins that contain Ras Binding or RAS Association Domains (RBDs/RAs), in order to transmit its downstream signals. RAS-selective GTPase accelerating proteins (RASGAPs; e.g., NF1, neurofibromin) then promote GTP hydrolysis to return RAS to its GDP-bound resting state. Mutated RAS genes in cancer harbor missense mutations primarily at three hotspots (G12, G13 and Q61, marked by asterisks); they encode mutant RAS proteins that are GAP-insensitive and are persistently GTP-bound and active.

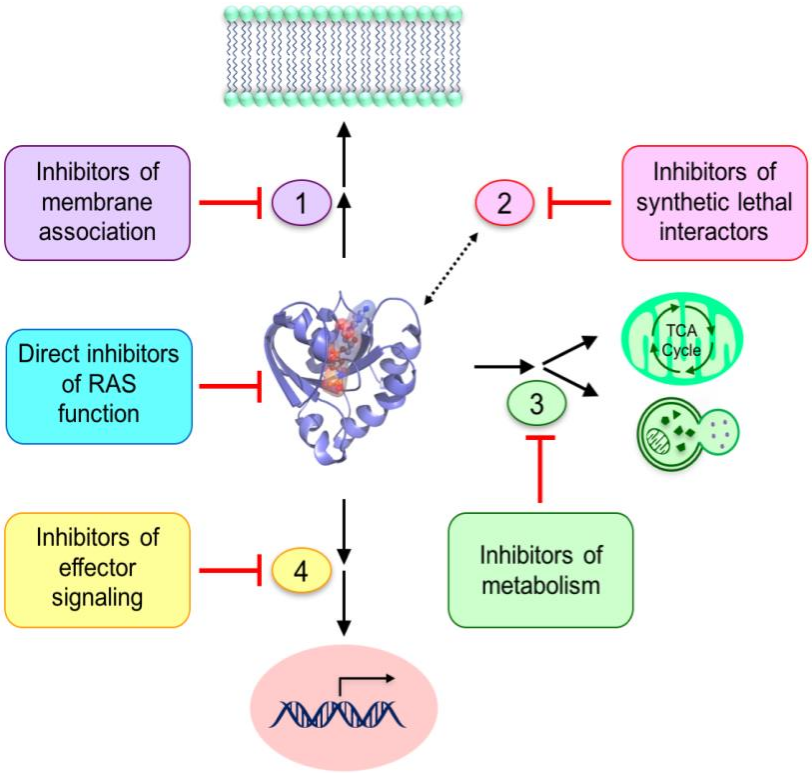


Figure 1-3. Pharmacological strategies to inhibit aberrant RAS function. RAS proteins (center, structure of KRAS4B) must associate with membranes (top) to be biologically active. Once activated, RAS proteins signal to effector cascades that ultimately alter gene transcription (bottom). Shown are one direct and four indirect strategies (1-4) to inhibit the function of RAS in cancer. See text for details.

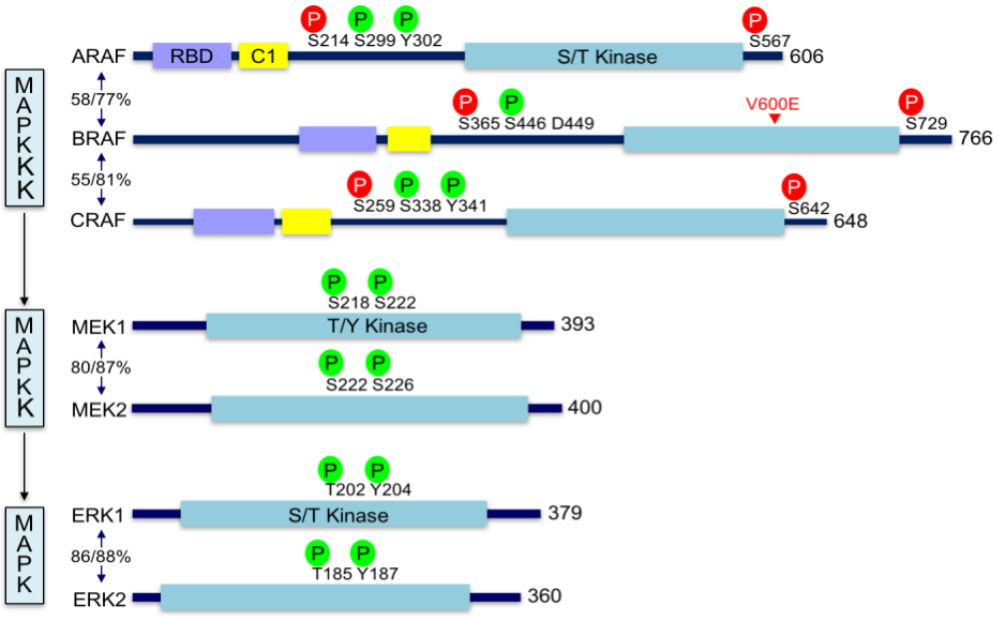


Figure 1-4. Components of the RAF-MEK-ERK MAPK cascade. The RAF-MEK-ERK mitogen-activated protein kinase (MAPK) cascade comprises three sequentially activated protein kinase events: RAF (MAPKKK)→ MEK (MAPKK)→ ERK (MAPK). There are three highly identical human RAF MAPKKK isoforms (ARAF, BRAF, and CRAF), and RAS-mediated homo- or hetero-dimerization of RAF is essential for their full activation[114]. Binding of activated RAS-GTP to the N-terminal RAS-binding domain (RBD) of RAF relieves the N-terminal auto-inhibition of the C-terminal RAF kinase domain and promotes association of the normally cytosolic RAF protein with the plasma membrane, where complex subsequent activation events lead to activation of RAF kinase activity. A still incompletely understood complex set of both negative (red) and positive (green) phosphorylation events regulate RAF catalytic activity[115] (representative sites shown). In the inactive configuration, a 14-3-3 dimer binds to conserved phosphorylation sites in N- and C-terminal residues flanking the kinase domain (ARAF, pS214 and pS576; BRAF, pS365 and p729; CRAF, pS259 and pS621). Protein kinase A and other kinases can phosphorylate these sites. Phosphorylation events that promote kinase activation occur at residues including S338 and Y341 in CRAF (S299 and Y302; ARAF). However, the analogous positions in BRAF are either constitutively phosphorylated (S446) or encode a phosphomimetic residue (D449), explaining why BRAF but not ARAF or RAF can be rendered constitutively activated by a single missense mutation in cancer (V600E). Each activated RAF isoform phosphorylates and activates the highly related MEK1 and MEK2 dual-specificity MAPKKs. Activated MEK1/2 phosphorylate and activate the highly related ERK1 and ERK2 serine/threonine kinases. Total protein and kinase domain sequence identities are indicated (%/%) as determined by CLUSTALW multiple sequence alignment. In stark contrast to the limited substrates of A/B/CRAF and MEK1/2, >200 cytoplasmic and nuclear substrates of ERK1/2 have been described[76].

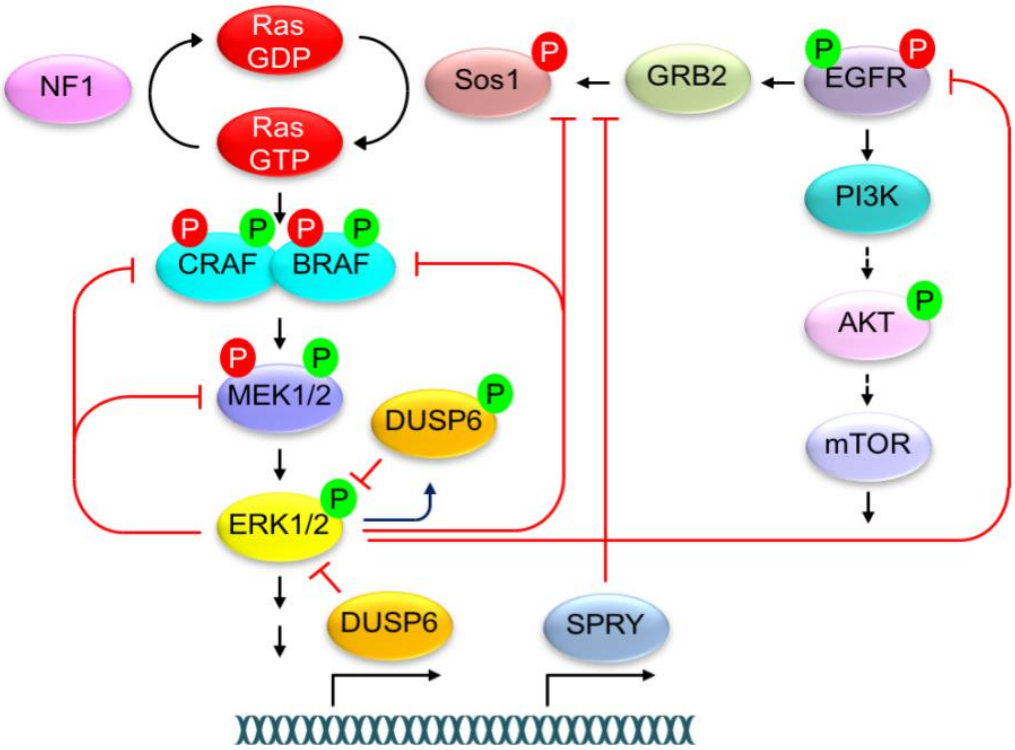


Figure 1-5. Regulatory mechanisms of ERK negative feedback regulation. ERK phosphorylation of CRAF disrupts interaction with RAS. ERK phosphorylation of BRAF disrupts dimerization and interaction with RAS. ERK phosphorylation of MEK1 promotes heterodimerization with MEK2. ERK phosphorylation of SOS1 disrupts interaction with GRB2. ERK phosphorylation of the dual specificity phosphatase DUSP6 regulates its protein stability. ERK-activated transcription factors promote expression of DUSP6[116] and the scaffold protein SPRY, with SPRY disrupting SOS1 interaction with GRB2. ERK phosphorylation of T669 in the EGFR juxtamembrane region is important for EGFR dimerization and activation[117], promoting activation of RAS[118] and PI3K[119].

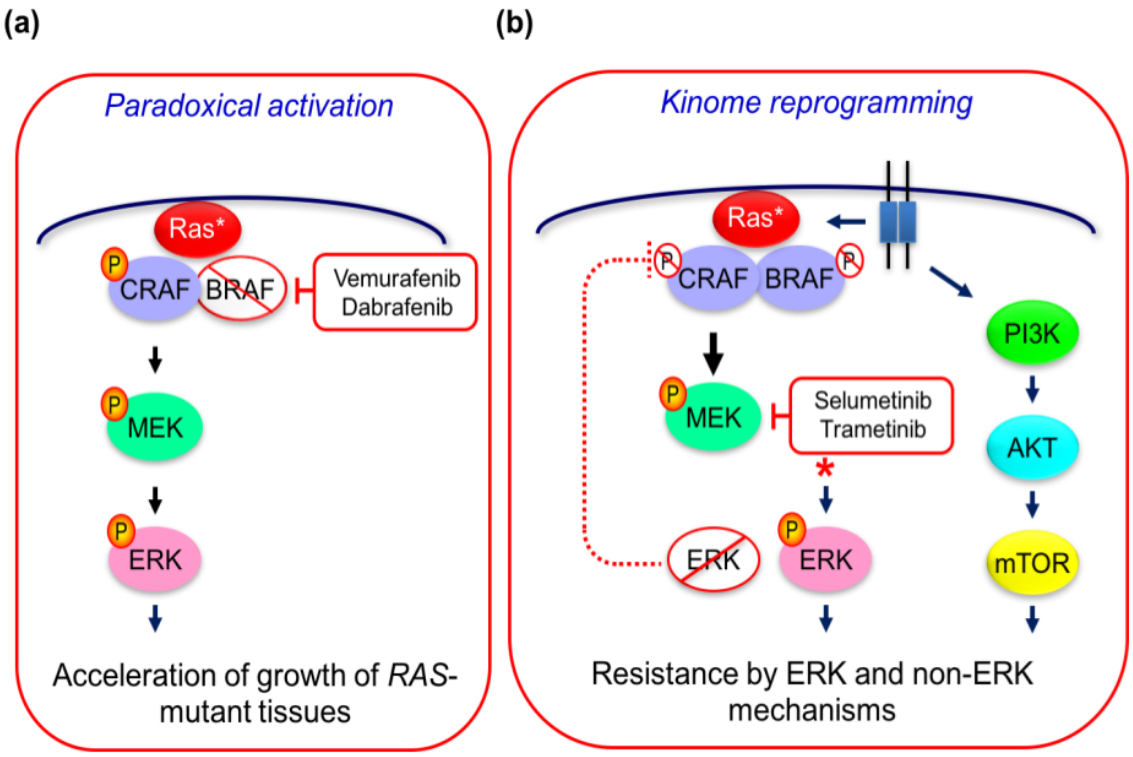


Figure 1-6. Mechanisms of resistance to RAF and MEK inhibitors in RAS-mutant cancers. Second generation RAF inhibitors such as vemurafenib and dabrafenib are BRAF-selective and cause paradoxical activation of ERK (panel a). The inhibitor-blocked BRAF forms a heterodimer with active CRAF, and complexes with activated RAS. MEK inhibitors transiently block ERK activation. Since high ERK activation can be deleterious for cell proliferation, ERK activation stimulates negative feedback mechanisms that dampen upstream signaling through the pathway (panel b). Kinome reprogramming results in rewiring of the signaling networks to increase flux through non-RAF-MEK-ERK pathways such as PI3K-AKT-mTOR.

Table 1. RAF-MEK-ERK inhibitors under clinical evaluation

Agent	Other Names	Phase[a]	Targets	Mechanism and properties[e]
RAF				
BGB-283		Phase I[b]	RAF, EGFR	Dual RAF dimer and EGFR inhibitor[b]
BMS-908662	XL281	Phase I/ II[c]	RAF	ATP-competitive, pan-RAF
Dabrafenib	GSK2118436	Approved for BRAF V600E melanoma	RAF	Type I ATP-competitive, BRAF-selective
Encorafenib	LGX818	Phase II	RAF	ATP-competitive, BRAF-selective
HM95573		Phase I	RAF	Pan-RAF
LY3009120		Phase I	RAF	ATP-competitive, "paradox breaker"
MLN2480	BIIB-024	Phase I	RAF	Pan-RAF
RAF265	CHIR-265	Phase I/ II[c]	RAF, VEGFR	ATP-competitive, multi-kinase
Regorafenib	BAY 73-4506	Approved for metastatic colorectal cancer and advanced gastrointestinal stroma tumors	RAF, KIT, VEGFR	Type II ATP-competitive, multi-kinase
Sorafenib	BAY 43-9006	Approved for unresectable hepatocellular carcinoma, advanced renal cell carcinoma and thyroid cancer	VEGFR2, PDGFRβ, KIT, FLT3, CRAF	Type II ATP-competitive, multi-kinase
Vemurafenib	PLX4032, RG7204, RO5185426	Approved for BRAF V600E melanoma		Type I ATP-competitive, BRAF-selective
MEK				
ARRY-300		Phase I[c]	MEK1/2	Type III allosteric, non-ATP-competitive; analog of MEK162
AS703988	MSC2015103B	Phase I[c]	MEK1/2	Type III allosteric, non-ATP-competitive
AZD8330	ARRY-424704, ARRY-704	Phase I[c]	MEK1/2	Type III allosteric, non-ATP-competitive
Binimetinib	ARRY-438162, ARRY-162, MEK162	Phase II	MEK1/2	Type III allosteric, non-ATP-competitive
Cobimetinib	XL-518, GDC-0973, RG7421	Phase III	MEK1	Non-ATP competitive, 100-fold selectively for MEK1 over MEK2
E6201	ER 806201	Phase I/II	MEK1, MEKK1 FLT3	Synthetic, fungal metabolite analogue
GDC-0623	RG7420, G-868	Phase I[c]	MEK1/2	Type III allosteric, non-ATP-competitive; analog of CI-1040; stabilizes a RAF-MEK complex
PD-0325901		Phase II	MEK1/2	Type III allosteric, non-ATP-competitive
Pimasertib	AS703026, SAR245509, EMD 1036239, MSC1936369B	Phase II	MEK1/2	Type III allosteric, non-ATP-competitive
Refametinib	RDEA119, BAY86-9766	Phase II	MEK1/2	Allosteric, non-ATP-competitive
RO4987655	CH4987655, RG7167	Phase I[c]	MEK1/2	Allosteric, non-ATP-competitive
RO5126766	CH5126766, RG7304	Phase I[c]	Raf, MEK1/2	Type III allosteric, non-ATP-competitive; binds to MEK1/2, forms a stable Raf-MEK-RO5126766 complex, preventing both MEK and ERK phosphorylation
Selumetinib	AZD6244, ARRY-142886	Phase III	MEK1/2	Type III allosteric, non-ATP-competitive
TAK733		Phase I[c]	MEK1/2	Type III allosteric, non-ATP-competitive
Trametinib	GSK1120212, JTP-74057	Approved for BRAF V600E melanoma	MEK1/2	Type III allosteric, non-ATP-competitive
WX-554		Phase I/II[d]		
ERK				
CC-90003		Phase I	ERK1/2	
GDC-0994	RG7842	Phase I	ERK1/2	ATP-competitive
MK-8353	SCH900353	Phase I[d]	ERK1/2	Allosteric and ATP-competitive
Ulixertinib	BVD-523	Phase I/II	ERK1/2	ATP-competitive

[a]Compiled from ClinicalTrials.gov
[b]http://www.beigene.com/
[c]Completed
[d]Terminated

[e]ATP-competitive inhibitors are broadly classified as type I or II, that target the active “in” or inactive “out” conformation of the ATP/Mg^{2+}-coordinating three amino acid DFG motif, highly conserved among most protein kinases and located N-terminal to the activation loop. Type III inhibitors bind to a hydrophobic pocket directly adjacent to the ATP-binding site.

Chapter II: ERK/MAPK SIGNALING DRIVES OVEREXPRESSION OF THE RAC-GEF, PREX1, IN BRAF- AND NRAS-MUTANT MELANOMA[2]

OVERVIEW

Recently we identified that PREX1 overexpression is critical for metastatic but not tumorigenic growth in a mouse model of NRAS-driven melanoma. In addition, a PREX1 gene signature correlated with and was dependent on ERK mitogen-activated protein kinase (MAPK) activation in human melanoma cell lines. In the current study, the underlying mechanism of PREX1 overexpression in human melanoma was assessed. PREX1 protein levels were increased in melanoma tumor tissues and cell lines compared with benign nevi and normal melanocytes, respectively. Suppression of PREX1 by siRNA impaired invasion but not proliferation *in vitro*. PREX1-dependent invasion was attributable to PREX1-mediated activation of the small GTPase RAC1 but not the related small GTPase CDC42. Pharmacologic inhibition of ERK signaling reduced PREX1 gene transcription and additionally regulated PREX1 protein stability. This ERK-dependent upregulation of PREX1 in melanoma, due to both increased gene transcription and protein stability, contrasts with the mechanisms identified in breast

[2] Adapted from previously published work. Authors are Meagan B. Ryan, Katherine H. Pedone, Alexander J. Finn, Nancy E. Thomas, Channing J. Der, and Adrienne D. Cox. All Figures except Figures 2-1, 2-7 and 2-8 represent the work of Meagan B. Ryan.

and prostate cancers, where PREX1 overexpression was driven by gene amplification and HDAC-mediated gene transcription, respectively. Thus, although PREX1 expression is aberrantly upregulated and regulates RAC1 activity and invasion in these three different tumor types, the mechanisms of its upregulation are distinct and context-dependent.

INTRODUCTION

Driver roles in cancer have been identified for several members of the Dbl family of Rho guanine nucleotide exchange factors (RhoGEFs), most prominently ECT2, TIAM1, VAV1/2/3 and PREX1/2 [120,99]. Increased expression and activation of these RhoGEFs result in enhanced activity of their Rho family small GTPase substrates in a context-dependent manner. For example, we recently identified overexpression of ECT2 protein in ovarian cancer, mediated by gene amplification, that resulted in activation of RHOA in the cytosol and RAC1 in the nucleus[121]. The critical importance of RAC1 in cancer cell migration and invasion[122] has further focused attention on the mechanisms regulating activators of RAC1, such as the Dbl family of RhoGEFs.

The highly related Dbl RhoGEFs PREX1 and PREX2 (56% overall amino acid identity), which are GEFs for RAC1 and other Rho family small GTPases such as CDC42[123], have been implicated as cancer drivers in several tumor types. The first cancer-driving role for PREX1 was described in prostate cancer[124], where limited analyses of tumor tissue revealed elevated levels of PREX1 protein. PREX1 was also elevated in metastatic but not primary prostate tumor cell lines. Suppression of *PREX1* by RNA interference in PC-3 human prostate cancer cells decreased the

levels of activated RAC and impaired tumor cell migration and invasion *in vitro*. Conversely, ectopic expression of PREX1 stimulated RAC activation, and promoted metastatic but not primary tumor growth of CWR22Rv1 prostate tumor cells. A follow-up study identified a histone deacetylase (HDAC)-mediated increase in *PREX1* gene transcription as a basis for the increased levels of PREX1 protein in prostate cancer[125].

PREX1 overexpression was also identified in estrogen receptor-positive luminal and HER2-positive breast cancers[126-128]. PREX1 protein was detected in ~60% of breast tumors but not in normal breast tissue. *PREX1* is located in a chromosomal region frequently amplified in breast cancers and *PREX1* gene amplification was detected in breast cancer cell lines, supporting gene amplification as a mechanism for PREX1 protein overexpression in these tumor types. Silencing of *PREX1* expression by RNA interference reduced HER2-stimulated activation of RAC1, and impaired tumor cell motility and invasion *in vitro* and tumorigenic growth in vivo[126-128].

A role in cancer for the related RhoGEF PREX2 has also been identified, but not by overexpression. Instead, missense mutations in PREX2 have been identified in 25% of malignant melanomas[102]. Although no clear mutational hotspots have been seen in melanomas, experimental studies support a gain-of-function consequence of these mutations[102,103]. *PREX2* missense mutations have also been found in 38% of cutaneous squamous cell carcinomas[129] and 17% of stomach adenocarcinomas[130]. To date, a similar level of activating missense mutations in *PREX1* in cancer has not been reported. However, the occurrence of activating

missense mutations (e.g., P29S) in the PREX1 substrate, RAC1, in ~11% of melanomas[31,131] is also consistent with a driver role for overexpressed PREX1 in this disease.

Our previous studies revealed overexpression of PREX1 protein in melanoma cell lines and tumor tissue[105]. Further, in a mouse model of melanoma, we determined that *Prex*1-deficient mice were impaired in forming tumor metastases but not primary tumors. Here, extending our mouse model studies, we demonstrate that PREX1 protein is increased in human melanoma tumor tissue and that PREX1 is required for human melanoma cell invasion but not proliferation.

In a separate earlier study, we also identified *PREX1* as a gene upregulated by the ERK mitogen-activated protein kinase (MAPK) in melanoma[104]. The RAF-MEK-ERK protein kinase cascade is aberrantly activated in up to 80% of melanomas through *BRAF* or *NRAS* mutation, and serves as a critical therapeutic target in this disease[15,132-134]. These observations supported the possibility that PREX1 protein overexpression in melanoma is driven by ERK activation. Additionally, since PREX1 has a demonstrated driver role in other cancers, PREX1 overexpression may be a key driver of ERK-dependent melanoma growth. In the present study, we determined that PREX1 protein overexpression is blocked by pharmacologic inhibitors of RAF-MEK-ERK signaling, and that ERK regulates not only *PREX1* gene transcription but also PREX1 protein stability. Thus, there are significant cancer type-distinct mechanisms that drive PREX1 overexpression in cancer.

MATERIALS AND METHODS

Human melanoma tissue and immunohistochemistry (IHC)

Following institutional review board approval, primary and metastatic melanomas were retrieved from a series of patients treated at UNC Healthcare. Immunohistochemical staining was performed in the UNC Department of Dermatology Dermatopathology Laboratory as we have recently described[135]. Briefly, freshly cut 4-µm thick sections of formalin-fixed and paraffin-embedded melanoma tissue blocks were stained using the fully automated Leica Bond III system. Sections were pretreated using an onboard heat-induced epitope retrieval in EDTA buffer. Following incubation with PREX1 antibody (6F12; provided by Marcus Thelen, IRB, Switzerland), chromogenic detection was performed using the Leica Refined Red polymer detection system (Leica Microsystems). Some sections were also counterstained with hematoxylin and eosin (H&E). PREX1 antibody staining intensity was scored in a blinded manner by a pathologist (AJ Finn) as high, medium, low or none.

Tissue culture

Cutaneous melanoma, breast cancer and prostate cancer cell lines were obtained from ATCC. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and were not cultured for longer than 6 months after receipt from cell banks.

siRNA transfection, proliferation, and invasion assays

For siRNA knockdown, A375, WM2664, SK-MEL-119 and Mel224 cells were plated in 6-well plates. Cells were transfected with 10 nM siRNA against *PREX1* (Thermo Fisher s33364, s33365, s33366; PREX1 #1, #2, #3, respectively), *RAC1* (Thermo Fisher s11711, s11712, s11713; RAC1 #1, #2, #3, respectively) or mismatch control (Dharmacon #D-001210-05), using Lipofectamine RNAimax (Life Technologies). Cells were serum-starved overnight for 18 h and then seeded for invasion assays after 48 h of siRNA knockdown. For the proliferation assay, cells were seeded at 2-3 x 10^3 cells/well in 96-well plates and allowed to grow for 72 h before incubation for 3 h in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). MTT was solubilized in DMSO and the absorbance was read at A570. For the Boyden chamber invasion assay, 1-3 x 10^4 cells were seeded into the upper chamber of Matrigel-coated invasion chambers in duplicate (Corning BioCoat) and allowed to invade towards 20% FBS in DMEM for 24 h. Invasion chambers were fixed and stained using a Diff-Quik staining kit (GE). Invasion chambers were imaged using a 10x objective lens on a Nikon TS100 microscope at 5 fields per insert, and images were analyzed to calculate invaded cells per field using ImageJ software. For the collagen spheroid invasion assay, we slightly modified a published protocol[136,137]. Briefly, 5-10 x 10^3 cells were seeded in ultra-low attachment round-bottomed 96-well plates (Corning) for 96 h, a sufficient time for the cells to organize into spheroids. Spheroids were then transferred to 48-well plates and embedded in collagen (1 mg/ml rat-tail collagen, BD). Spheroids were imaged at 0 h and after 72 h of invasion using a 5x objective on a Nikon TS100 microscope. Total spheroid

area was calculated as fold-change in area of 72 h outgrowth versus 0 h spheroid area, using ImageJ.

Pulldown assay to detect GTPase activity

Levels of active, GTP-bound RAC1 and CDC42 were assessed by an affinity pulldown assay as we described previously[138]. Briefly, after 48 h of siRNA-mediated PREX1 knockdown, whole cell lysates were exposed to GST-PAK-PBD, which contains the binding domain of the shared RAC1/CDC42 effector PAK1. After resolving pulldown samples on 15% SDS-PAGE gels and western blotting for RAC1 (clone 23A9, BD) and CDC42 (BD), levels of each GTP-bound GTPase were normalized to both total protein and the vinculin loading control (Sigma) by densitometry analysis performed in ImageJ.

Drug treatment and western blotting

BRAF-mutant A375 and WM2664 cells were treated with BRAF inhibitor vemurafenib (Selleckchem) or ERK inhibitor SCH772984 (Merck, kindly provided by Ahmed Samatar), and *NRAS*-mutant SK-MEL-119 and Mel224 cells were treated with MEK inhibitor trametinib (Selleckchem) or SCH772984 for 24 and 48 h before samples were collected in RIPA lysis buffer. For PREX1 protein stability experiments, cells were co-treated with cycloheximide (50 µg/ml) and SCH772984 for a 24 h timecourse before samples were collected in RIPA. Whole cell lysates were resolved on 10% SDS-PAGE gels and western blotting was performed using antibodies to phospho-ERK1/2 (Thr202/Tyr204), total ERK1/2, phospho-RSK (Ser308), phospho-RSK (Thr359/Ser363), total RSK1/2/3, and c-myc (MYC) (Cell Signaling); β-actin and vinculin (Sigma), and PREX1 (6F12)[139]. IRDye800-

conjugated anti-mouse and anti-rabbit secondary antibodies were from Rockland Immunochemicals.

Quantitative PCR

Total RNA was isolated using an RNeasy kit (Qiagen) and reverse transcription was performed using the High Capacity RNA-to-cDNA kit (Thermo Fisher). Real time quantitative Taqman PCR was performed on the QuantStudio 6 Flex (Thermo Fisher) with FAM/MGB labeled probes against PREX1 (Hs00368207_m1, Hs_001031512, Thermo Fisher) and endogenous control VIC/TAMRA labeled β-actin (Thermo Fisher).

Statistical analysis

Data were analyzed using GraphPad Prism 6 software and statistical analyses were performed as indicated in the Figure Legends.

RESULTS

PREX1 overexpression is correlated with elevated ERK activation

We recently identified overexpression of PREX1 protein in melanomas, determined that *Prex1* deficiency impaired mouse melanoblast migration in vivo, and demonstrated that *Prex1* expression is required for metastasis in an *Nras*-mutant genetically engineered mouse model of cutaneous melanoma[105]. Since our previous gene array analyses identified *PREX1* as an ERK activation-dependent gene[104], here we assessed a relationship among *BRAF* and *NRAS* mutation status, ERK activation and PREX1 protein overexpression in human melanoma. We first

investigated the expression of PREX1 in a panel of human melanoma cell lines that did or did not harbor *BRAF* or *NRAS* mutations. The majority of *BRAF*- (3 of 4) or *NRAS*- (3 of 4) mutant cell lines exhibited substantially higher PREX1 protein expression when compared with normal melanocytes or with *BRAF/NRAS* wild type cell lines (Figure 2-1A). Generally, the level of activated, phosphorylated ERK (pERK) correlated with the level of PREX1. In our analyses, normal melanocytes may exhibit low pERK levels[104], or they may also display low PREX1 protein levels even in the presence of high pERK levels, as shown here.

We next utilized immunohistochemistry (IHC) to evaluate PREX1 protein expression and pERK levels in melanoma patient tissues. We first compared PREX1 expression in benign melanocytic nevi (n=35) and human melanoma tumors (n=33) (Figure 2-1B). A range of expression was seen in nevi, with ~75% expressing low-to-medium levels of PREX1. Since *BRAF* and *NRAS* mutations are found in a high percentage of nevi[140,141], it is not surprising to find PREX1 in nevi as well as in melanoma tissue. However, high level PREX1 expression was detected only in melanomas (~10%, Figure 2-1B).

ERK can phosphorylate numerous substrates present in both the nucleus and the cytoplasm[76,142], few of which have been firmly linked to specific outcomes of ERK-mediated signaling. Melanoma responses to pharmacological inhibitors of the RAF-MEK-ERK pathway (e.g., clinically, to BRAF inhibition[28] and preclinically, to inhibitors of ERK dimerization[86,87]) correlated with suppression of cytoplasmic

pERK. We therefore evaluated the distribution of pERK in our human melanoma tissues, and found that levels of pERK were correlated with those of

PREX1 both in the nucleus (Figure 2-1C) and in the cytoplasm (Figure 2-1D). Both nuclear and cytoplasmic ERK activity may contribute to increased expression of PREX1.

PREX1 regulates invasion in a complex manner in both *BRAF*- and *NRAS*-mutant melanoma cell lines

An unexpected observation in our studies of PREX1 function in a mouse model of melanoma was that *Prex1* deficiency greatly impaired metastatic but not tumorigenic growth. This result contrasts with studies evaluating the role of PREX1 overexpression in human breast cancer cells, where stable shRNA-mediated suppression of *PREX1* reduced their tumorigenic growth[126-128]. We therefore compared the effect of PREX1 suppression in human melanoma cell lines on both proliferation and invasion *in vitro*.

To evaluate the role of PREX1 overexpression in cell growth, we first used three independent siRNAs to knock down *PREX1* in two *BRAF*-mutant (A375 and WM2664) and two *NRAS*-mutant cell lines (SK-MEL-119 and Mel224) (Figure 2-2A, upper panels). We found that transient (72 h) suppression of *PREX1* did not significantly reduce their proliferation *in vitro* (Figure 2-2A, lower panels), consistent with the lack of effect of *Prex1* deficiency on the growth of primary melanomas in mice. Next, we evaluated the role of PREX1 in invasion by analysis of invasion through Matrigel towards serum as a chemoattractant. We observed a surprisingly heterogeneous response to PREX1 knockdown that was independent of *BRAF* or

NRAS mutational status. For example, the *NRAS*-mutant line SK-MEL-119 exhibited a 60-70% decrease in invasion upon knockdown of PREX1 ($p<0.0001$, Figure 2-2B) whereas the *BRAF*-mutant cell line A375 conversely exhibited a ~2-fold increase ($p<0.001$). In contrast, the already very low degree of directed invasion of the BRAF-mutant line WM2664 was unaffected by PREX1 knockdown. Similarly, the invasive NRAS-mutant cell line Mel224 was largely unaffected, despite efficient knockdown of PREX1. These data demonstrate that PREX1 plays a complex and variable role in directed invasion towards an attractant.

Next, we investigated the role of PREX1 in a three-dimensional spheroid formation and collagen invasion assay, which mimics the *in vivo* tumor environment of human skin[143]. Figure 2-2C illustrates both spheroid formation and the subsequent invasion of cells from the spheroid into the surrounding collagen matrix. In three of the four cell lines, knockdown of PREX1 impaired spheroid invasion into collagen, either trending (SK-MEL-119) or significantly so (Mel224, WM2664). In contrast, A375 spheroids were defective in formation and did not invade the surrounding collagen matrix. The nearly doubled total spheroid area of PREX1-knockdown A375 cells compared to mismatch control cells observed after 4 days in culture was caused by a flattening of the three-dimensional spheroid structure and not by increased invasion or by increased proliferation; no change in proliferation occurred upon loss of PREX1 (Fig. 2-2A).

Collectively, our results suggest a complex and context-dependent role for PREX1 in driving both directed invasion and three-dimensional spheroid collagen

outgrowth of human melanomas, and one that is not dependent on *BRAF* or *NRAS* mutational status.

PREX1 regulates active, GTP-bound RAC1 but not CDC42 in melanoma cells

Although PREX1 is considered a RAC-selective GEF[144], PREX1 is also active on CDC42[139]. We therefore investigated which Rho family small GTPases are activated downstream of PREX1 in human melanoma cells. We found that knockdown of PREX1 decreased the levels of activated RAC1, as measured by RAC1-GTP pulldown, in both *BRAF*-mutant A375 and *NRAS*-mutant SK-MEL-119 cells (Figure 2-3). Despite the continued presence of other RacGEFs capable of inducing nucleotide exchange on RAC1, even incomplete loss of PREX1 was sufficient to cause a substantial decrease in RAC1-GTP (Figure 2-3). This effect was selective for RAC1, as the levels of activated CDC42 did not decrease (Figure 2-3). These results support a role for PREX1 in regulating RAC1 activity and subsequent RAC1-driven invasive behavior of melanomas.

We next asked if the loss of RAC1 was sufficient to phenocopy the impairment of invasion that we observed upon loss of PREX1. We found that knockdown of either PREX1 or RAC1 with three independent siRNAs for each (Figure 2-4A) was sufficient to substantially impair spheroid formation of A375 cells, as demonstrated by an increase in the flattened spheroid area (Figure 2-4B,C). The degree of impairment upon knockdown of RAC1 was highly significant ($p<0.0001$, Figure 4C) and comparable to the degree of impairment observed upon knockdown of PREX1 ($p<0.0001$, Figure 2-4C). Next, we observed that loss of RAC1 (Figure 2-4D) was sufficient to prevent the vast majority of SK-MEL-119 cell invasion in the

Boyden chamber assay (Figure 2-4E). This decrease in invasion was similar to the decrease seen upon PREX1 knockdown ($p<0.0001$, Figure 2-4F). The ability of RAC1 to phenocopy PREX1 in impairing both spheroid formation and directed invasion supports the idea that RAC1 is the most critical Rho family small GTPase downstream of PREX1 in regulating invasive melanoma behavior. Of note, other Rho family small GTPases such as RND3 have also been shown to be regulated by the RAF-MEK-ERK pathway and to contribute to melanoma invasion and spheroid outgrowth[145,146]. However, as a Rho-like rather than a Rac-like GTPase, RND3 is unlikely to be a target of PREX1 in this context[147].

PREX1 protein levels are positively regulated by ERK activity in melanoma

Our evaluation of human melanoma cell lines and tumor tissue found a correlation between phosphorylated ERK and PREX1 protein overexpression. To directly address whether ERK activation is required for PREX1 overexpression, we evaluated whether pharmacologic inhibition of RAF-MEK-ERK signaling would reduce PREX1 protein levels in *BRAF*- and *NRAS*-mutant melanoma. We first treated *NRAS*-mutant SK-MEL-119 cells with increasing concentrations of the MEK inhibitor trametinib. The more effective the inhibition of MEK, as measured by decreasing levels of phosphorylated and activated ERK (pERK) and total MYC (an ERK substrate; ERK phosphorylation blocks degradation), the greater the decrease in PREX1 protein (Figures 2-5A,B), suggesting a direct correlation between ERK activity and PREX1 protein levels. We next wanted to determine whether this dose-dependent decrease in PREX1 protein would also occur when the ERK MAPK cascade was inhibited at different nodes, and whether such an effect is time-

dependent. We therefore treated SK-MEL-119 cells with two different concentrations (1 x EC_{50} and 5 x EC_{50} for growth) of either trametinib or the ERK inhibitor SCH772984, for either 24 or 48 h (Figures 2-5C,D). PREX1 protein levels tracked closely with the level of pERK at each concentration of inhibitor, and this was sustained for 48 h. Next, we investigated whether PREX1 protein was similarly regulated downstream of the ERK-MAPK cascade in *BRAF*-mutant melanoma cells. We treated A375 cells similarly but with the BRAF inhibitor vemurafenib or SCH772984 for 24 or 48 h (Figures 2-5E,F). Similarly to SK-MEL-119 cells, levels of PREX1 in A375 cells tracked closely with the levels of pERK and demonstrated a time-dependent effect. In both SK-MEL-119 and A375 cells, phosphorylation of the ERK substrate RSK (pRSK) and total MYC served as effective markers to demonstrate inhibition of ERK, as we have observed in other settings[148]. These results indicate that ERK MAPK activity is an important contributor to the total amount of PREX1 protein in melanoma cells.

PREX1 levels are regulated by ERK both transcriptionally and post-transcriptionally in melanoma

To determine if the loss of PREX1 protein upon blockade of the ERK MAPK cascade was due to loss of PREX1 mRNA, we treated two *BRAF*-mutant and two *NRAS*-mutant melanoma cell lines with ERK MAPK cascade inhibitors. *BRAF*-mutant A375 and WM2664 cells were treated for 24 h with vemurafenib or SCH772984 as above. Taqman quantitative PCR analysis revealed that *PREX1* mRNA, measured by two independent probes, did not change upon inhibition of

BRAF or ERK in A375 cells (Figure 2-6A) but decreased dose-dependently in WM2664 cells upon inhibition of either BRAF or ERK (Figure 2-6B). *NRAS*-mutant cells were treated with trametinib or SCH772984 as above. We observed that PREX1 mRNA also decreased upon ERK inhibition in both SK-MEL-119 (Figure 2-6C) and Mel224 cells (Figure 2-6D). Additional melanoma lines also exhibited reduced *PREX1* mRNA levels when treated with inhibitors of the ERK MAPK cascade, including *BRAF*-mutant SK-MEL-28 and *NRAS*-mutant SK-MEL-147 cells (Figures 2-9A,B). Thus, in the majority of melanoma cell lines, ERK MAPK regulates PREX1 protein levels both transcriptionally and post-transcriptionally.

That ERK MAPK activity altered PREX1 protein levels in A375 melanoma cells without changes at the transcriptional level suggested a post-transcriptional mechanism for ERK MAPK-mediated regulation of PREX1 protein in these cells. To investigate this possibility, we treated A375 cells with vehicle or SCH772984 in the presence of the protein synthesis inhibitor cycloheximide at various time points over 24 h (Figures 2-6E,F). Inhibition of ERK led to greater loss of PREX1 protein in the presence of cycloheximide compared to vehicle-treated cells. Similar results were obtained upon treatment of SK-MEL-119 cells with trametinib in the presence of cycloheximide (Figures 2-9C,D). These results indicate that ERK can regulate protein stability as well as transcription of PREX1 in melanoma cell lines.

Finally, we tested the possibility that PREX1 is not only an ERK target but also an ERK activator. Since it has been demonstrated that PREX1 can regulate MEK-ERK signaling through RAC1 in breast cancer[149,128], we also examined whether PREX1 can regulate ERK1/2 phosphorylation in our melanoma lines. We

found that knockdown of PREX1 did not alter ERK1/2 phosphorylation in the *BRAF*-mutant cell lines, A375 and WM2664, or the *NRAS*-mutant cell lines, SK-MEL-119 and Mel224 (Figures 2-10A,B and S4C,D, respectively).

To determine the generality of ERK regulation of PREX1 expression in non-melanoma tumor types, we also tested whether they held true in breast and prostate cancer cell lines. PREX1 has been shown to be overexpressed in these tumor types, but the role of ERK in its expression has not been explored. Unlike our observations in melanoma cells, we observed that inhibition of the ERK MAPK cascade in T47D and MCF7 breast cancer cells did not reduce PREX1 protein (Figures 2-11A,C) or mRNA (Figures 2-11B,D). Conversely, in PC-3 prostate cancer cells, inhibition of the ERK MAPK cascade reduced PREX1 mRNA levels (Figure S5F) but had minimal effects on PREX1 protein (Figure 2-11E). These results support distinct mechanisms of regulating PREX1 expression in melanoma through ERK1/2 that do not apply in breast or prostate cancer, cancers in which BRAF and RAS mutation frequencies are low. Overall, our results support that both ERK regulation of PREX1 abundance and PREX1 regulation of ERK phosphorylation are context-dependent, and may differ between breast and prostate cancers and cutaneous melanoma.

DISCUSSION

We determined that the ERK MAPK cascade plays an important role in driving PREX1 protein overexpression in both *BRAF*- and *NRAS*-mutant melanomas. In contrast, ERK is not the key driver for PREX1 overexpression in

prostate[124,125] or in breast carcinomas[126,127,150,128], where its abundance is associated with HDAC-dependent [125] *PREX1* gene transcription and with HDAC- and methylation-dependent *PREX1* gene transcription[150] and gene amplification[126,150,128], respectively. Thus, there are striking cancer-type differences in mechanisms driving PREX1 overexpression. In support of this idea, PREX1 and PREX2 display distinct expression and mutation patterns in breast cancer, prostate cancer, and melanoma (Figure 2-7), and PREX1 in particular is differentially amplified in breast cancer, prostate cancer and cutaneous melanoma (Figure 2-8). Our findings also suggest that loss of PREX1-RAC1 signaling may contribute to the clinical response of patients with *BRAF*-mutant melanomas to BRAF and MEK inhibitors.

The effectiveness of these inhibitors provides compelling evidence that aberrant ERK signaling is a major driver of melanoma growth. Despite this clear driver role, the ERK targets important for melanoma growth remain poorly characterized. The ERK1/2 kinases can phosphorylate more than 200 known substrates[76,142] and serve as master regulators of numerous transcription factors[16], both directly and indirectly, through both transcriptional and post-translational mechanisms[151,152,16,148] We have determined that ERK regulates PREX1 expression levels in part via protein stability, a mechanism also not observed in other cancers. ERK regulation of PREX1 protein stability presents a previously unknown mechanism of maintaining PREX1 protein expression and may explain the basis for the relatively high PREX1 expression in malignant melanomas where the ERK MAPK cascade is upregulated.

Finally, the PREX1-related RhoGEF PREX2 is activated by missense mutations in 25% of metastatic melanomas, especially by truncating mutations mutations[102,103], and mutational activation of the PREX1/2 target RAC1 has been observed in ~11% of melanomas[31,131]. The rarity of PREX1 truncating mutations and lack of apparent hotspots among the few missense mutations argue that this is not a significant mechanism of PREX1 activation in melanoma. Instead, our determination that PREX1 is overexpressed and regulated at multiple levels in response to the ERK MAPK cascade characterizes a third mechanism for driving aberrant RAC1 signaling in melanoma. Our findings that loss of RAC1 phenocopies loss of PREX1 with respect to invasive behavior regardless of BRAF or NRAS mutation status supports the importance of the PREX1-RAC1 relationship as a promoter of melanoma cell invasion. Interestingly, downregulation of the RacGEF TIAM1 by mutant BRAF was shown to enhance invasion of human melanoma cells[153]. Although PREX1 was not examined in that particular study, PREX1 has consistently demonstrated a positive role in invasion[124,105,154], whereas TIAM1 can be either a positive or a negative regulator of this process[155,156,153,99,157]. Thus, the relative input from different upstream activators of RAC1 can have a profound influence on melanoma invasion.

In summary, we have demonstrated that the ERK MAPK cascade mediates overexpression of PREX1 in melanoma at multiple levels, and by mechanisms that are distinct from those identified previously in other cancer types. Our results contribute to a better understanding of how RacGEFs are modulated in distinct cancer contexts.

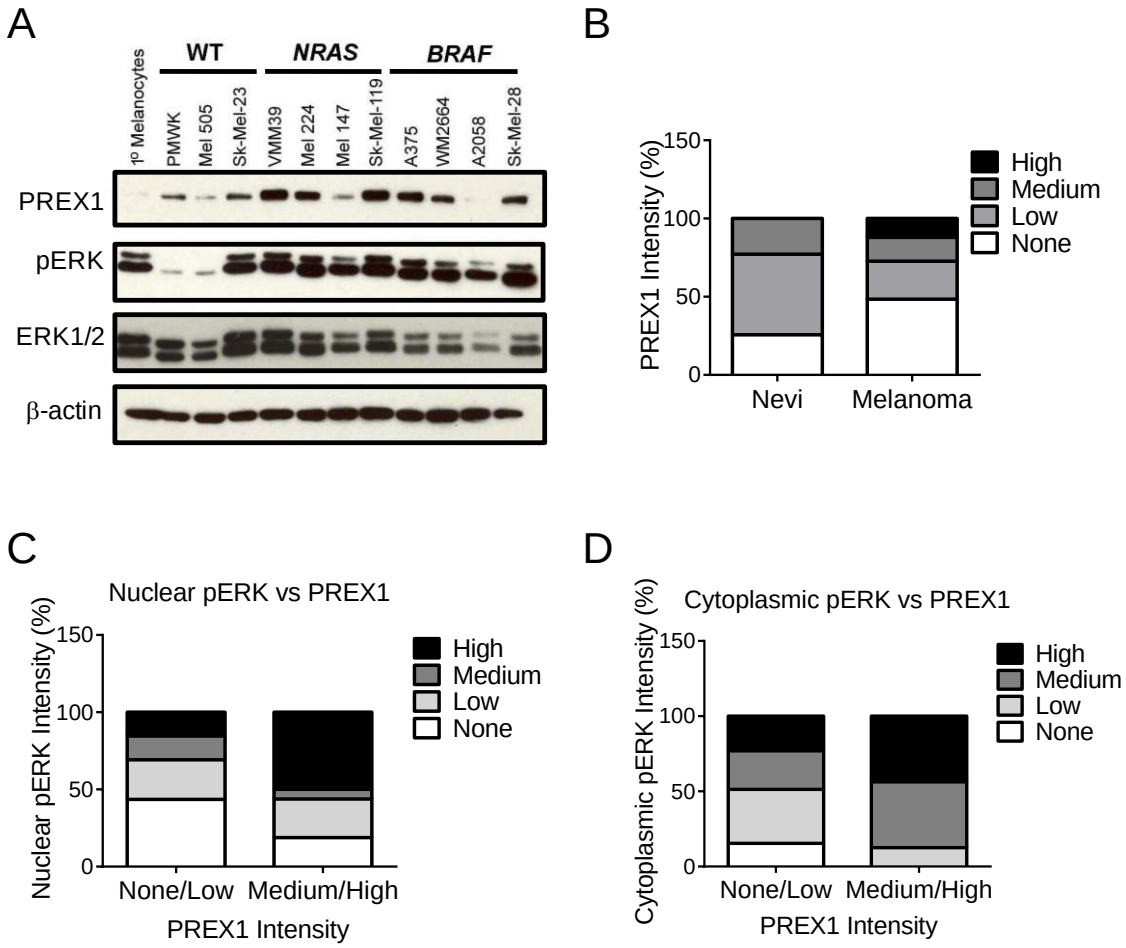
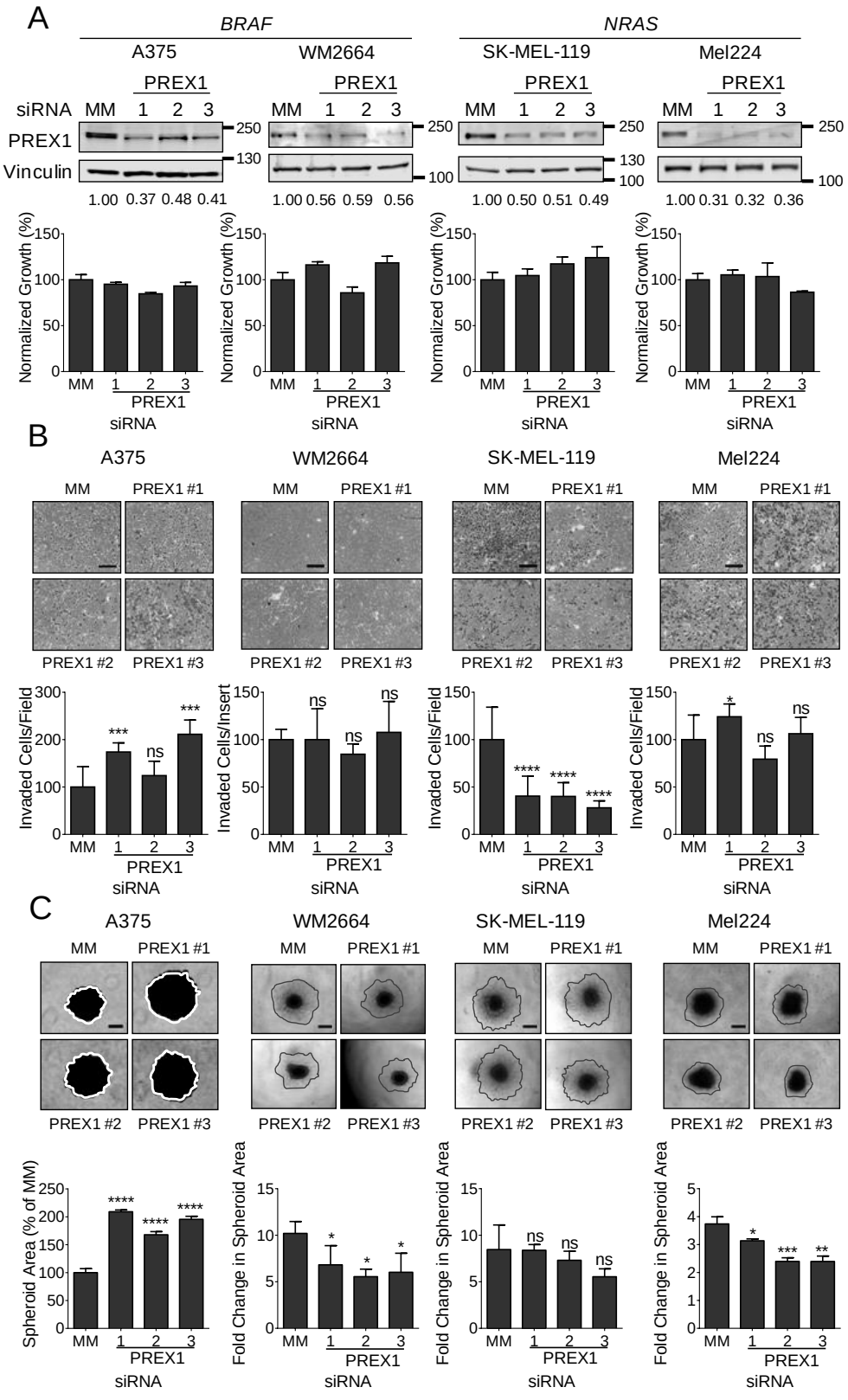


Figure 2-1. PREX1 protein levels are elevated in melanoma patient tumor tissues and cell lines, along with phospho-ERK. (A) Western blot analysis of PREX1 protein, phospho-ERK (pERK) and total ERK1/2 in a panel of WT, BRAF- or NRAS-mutant human melanoma tumor cell lines. (B-D) Human tissue samples of benign melanocytic nevi and malignant skin cutaneous melanoma were subjected to IHC for PREX1 and pERK. Shown are (B) the distribution of PREX1 expression in nevi versus melanoma samples as measured by IHC; n=35 and 33, respectively. Samples were first binned according to no, low, medium or high staining intensity for each protein, and then the distribution was graphed to show the relationship between PREX1 and the percent of samples that stained positive for (C) nuclear pERK or (D) cytoplasmic pERK.


A
BRAF
NRAS
A375
WM2664
SK-MEL-119
Mel224
PREX1
siRNA MM 1 2 3
PREX1
Vinculin
250
130
100
1.00 0.37 0.48 0.41
1.00 0.56 0.59 0.56
1.00 0.50 0.51 0.49
1.00 0.31 0.32 0.36
Normalized Growth (%)
150
100
50
0
MM 1 2 3
PREX1
siRNA
B
A375
WM2664
SK-MEL-119
Mel224
MM
PREX1 #1
PREX1 #2
PREX1 #3
Invaded Cells/Field
Invaded Cells/Insert
300
200
100
0
***
ns
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*
MM 1 2 3
PREX1
siRNA
C
A375
WM2664
SK-MEL-119
Mel224
MM
PREX1 #1
PREX1 #2
PREX1 #3
Spheroid Area (% of MM)
250
200
150
100
50
0
****
Fold Change in Spheroid Area
15
10
5
0
*
ns
MM 1 2 3
PREX1
siRNA

Figure 2-2. PREX1 regulates spheroid formation and invasion, but not proliferation, of *BRAF*- and *NRAS*-mutant melanoma cells in a context-dependent manner. (A) BRAF-mutant A375 and WM2664 and NRAS-mutant SK-MEL-119 and Mel224 cells were transfected with siRNA against PREX1 or a mismatch control (MM) for 48 h, and knockdown was confirmed by western blot (upper panels). Apparent molecular weights are indicated to the right of each panel; vinculin served as a loading control. Fold changes in protein expression compared to MM control are shown in numbers below each blot. Effects of PREX1 knockdown on growth in monolayer culture were determined by MTT assay at 72 hr (lower panels). (B) To determine the effects of PREX1 knockdown on invasion, cells were seeded in the upper chamber of a Matrigel-coated Boyden chamber, and allowed to invade towards serum for 24 h, then stained and imaged. ImageJ was used to quantitate invaded cells per field for 5 fields per insert in duplicate inserts (A375, SK-MEL-119, Mel224) or invaded cells over both inserts (WM2664). (C) For spheroid collagen invasion assays, spheroids were allowed to form for 4 days. Total spheroid area was normalized to that of mismatch control-treated cells; impaired spheroid formation is indicated by increased area of the flattened spheroid. WM2664, SK-MEL-119, and Mel224 spheroids were embedded in a collagen matrix and imaged (day 0) and the extent of cell outgrowth/invasion was imaged 3 days later (thick gray lines). Fold change in area from day 3 to day 0 was calculated in ImageJ. Data are represented as mean ± SD and statistical significance was evaluated by Student's t-test, where *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$. Scale bar represents 250 µm for invasion assays and 500 µm for spheroids. Experiments shown are representative of two (WM2664, SK-MEL-119) or three (A375, SK-MEL-119) independent experiments.

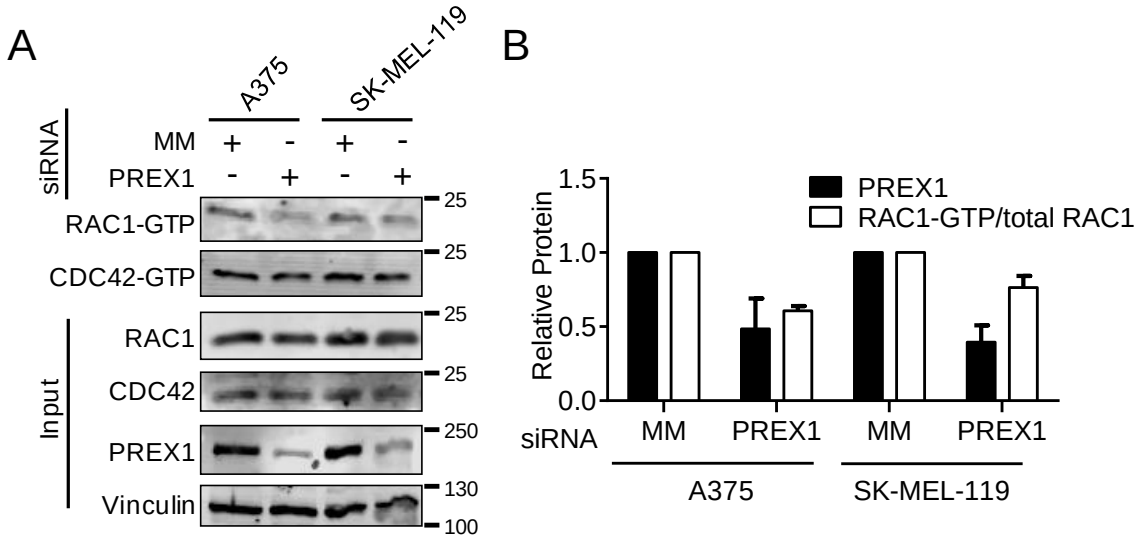


Figure 2-3. PREX1 regulates active RAC1-GTP, but not active CDC42-GTP, in melanoma cells. A375 and SK-MEL-119 cells were transfected with pooled siRNAs #1-3 against PREX1 or MM control for 48 h, then starved overnight (18 h). RAC1-GTP and CDC42-GTP were measured by GST-PAK-PBD pulldown (A). Apparent molecular weights are indicated to the right of each panel; vinculin served as a loading control. Quantification (mean ± SD) using ImageJ (B) is representative of two independent experiments.

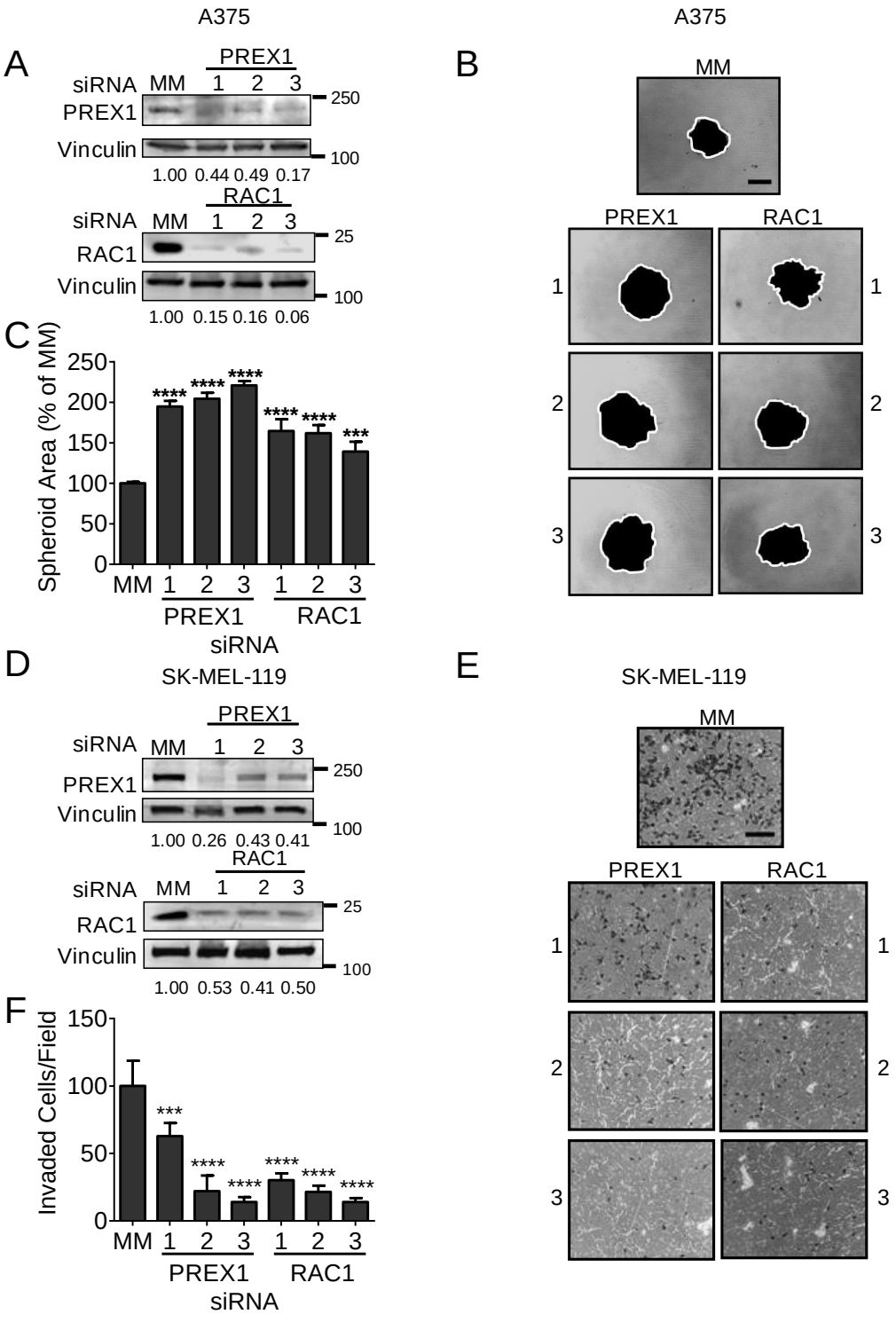


Figure 2-4. RAC1 phenocopies PREX1 in regulating spheroid formation in A375 and invasion in SK-MEL-119. A375 and SK-MEL-119 cells were transfected with siRNA against PREX1, RAC1, or MM control for 48 h before seeding into invasion chambers. Knockdown was confirmed by western blot in A375 (panel A) and SK-MEL-119 (panel D).

Apparent molecular weights are on the right of each panel; vinculin was a loading control. Total spheroid area of A375 cells was quantified after 4 days using ImageJ (white line); increased flattened spheroid area indicates impaired spheroid formation (panels B,C). SK-MEL-119 cells were starved overnight and seeded in a Boyden chamber assay with 20% serum as a chemoattractant and allowed to invade for 24h (10x magnification) (E). Stained inserts were quantified for invaded cells/field, 10 fields per condition, using ImageJ (F). Data are represented as mean ± SD. Student's t-test, where *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$). Scale bar represents 250 µm for invasion assays and 500 µm for spheroids.

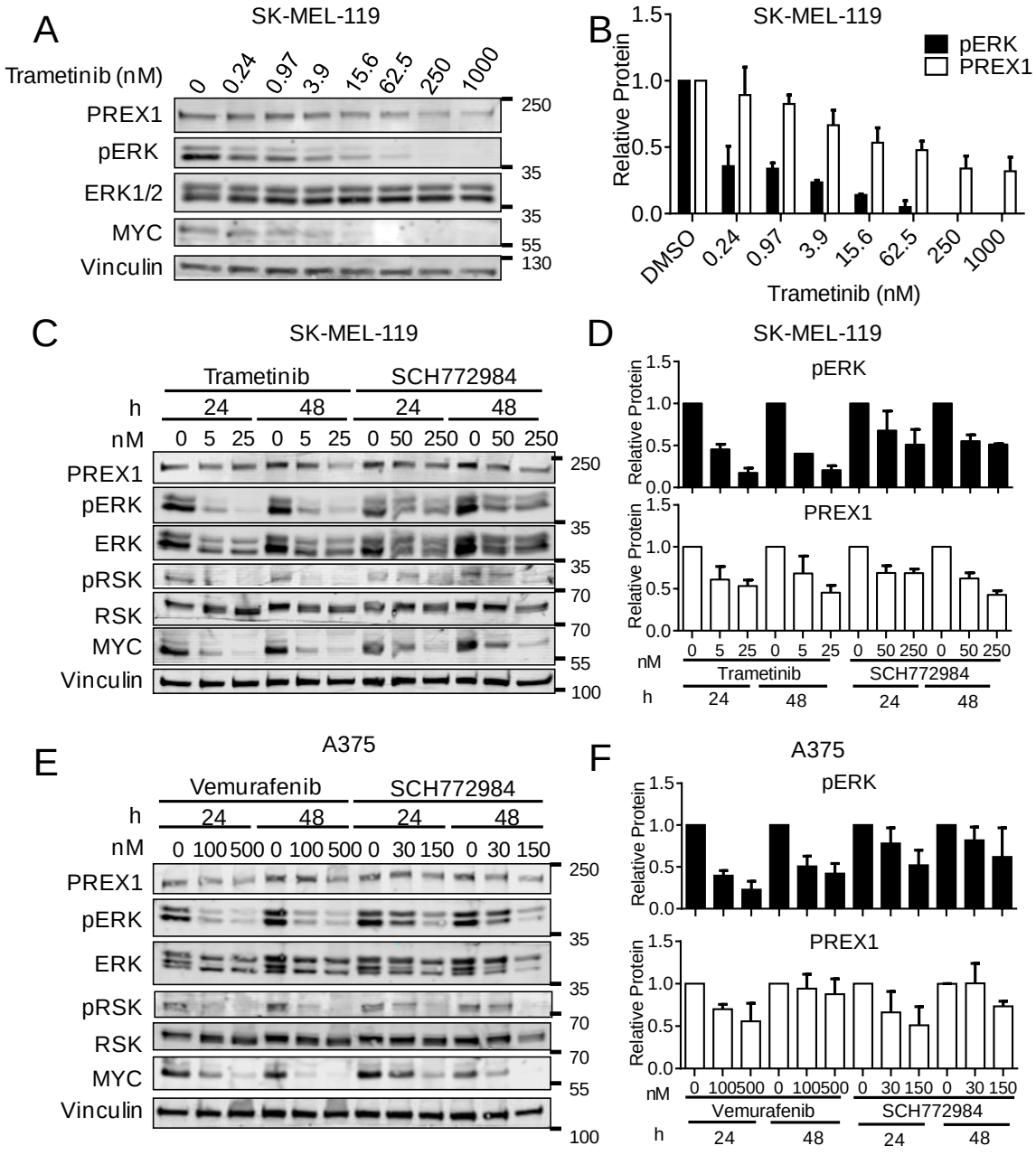


Figure 2-5. PREX1 protein levels are regulated by the ERK kinase cascade. *NRAS*-mutant SK-MEL-119 cells were first treated with the indicated concentrations of MEKi trametinib for 48 h, and lysates immunoblotted for PREX1, pERK and MYC (A; quantified in B). SK-MEL-119 cells were next treated with trametinib or the ERK inhibitor SCH772984 for 24 or 48 h, and lysates probed for pERK and PREX1 (C; quantified in D), and for pRSK and total MYC to monitor ERK pathway inhibition (C). Similarly, *BRAF*-mutant A375 cells were treated with the BRAF inhibitor vemurafenib or with SCH772984 for 24 or 48 h and lysates probed as above (E; quantified in F). Quantification is of n=3 experiments for SK-MEL-119 and n=4 for A375. Data are represented as mean ± SD.

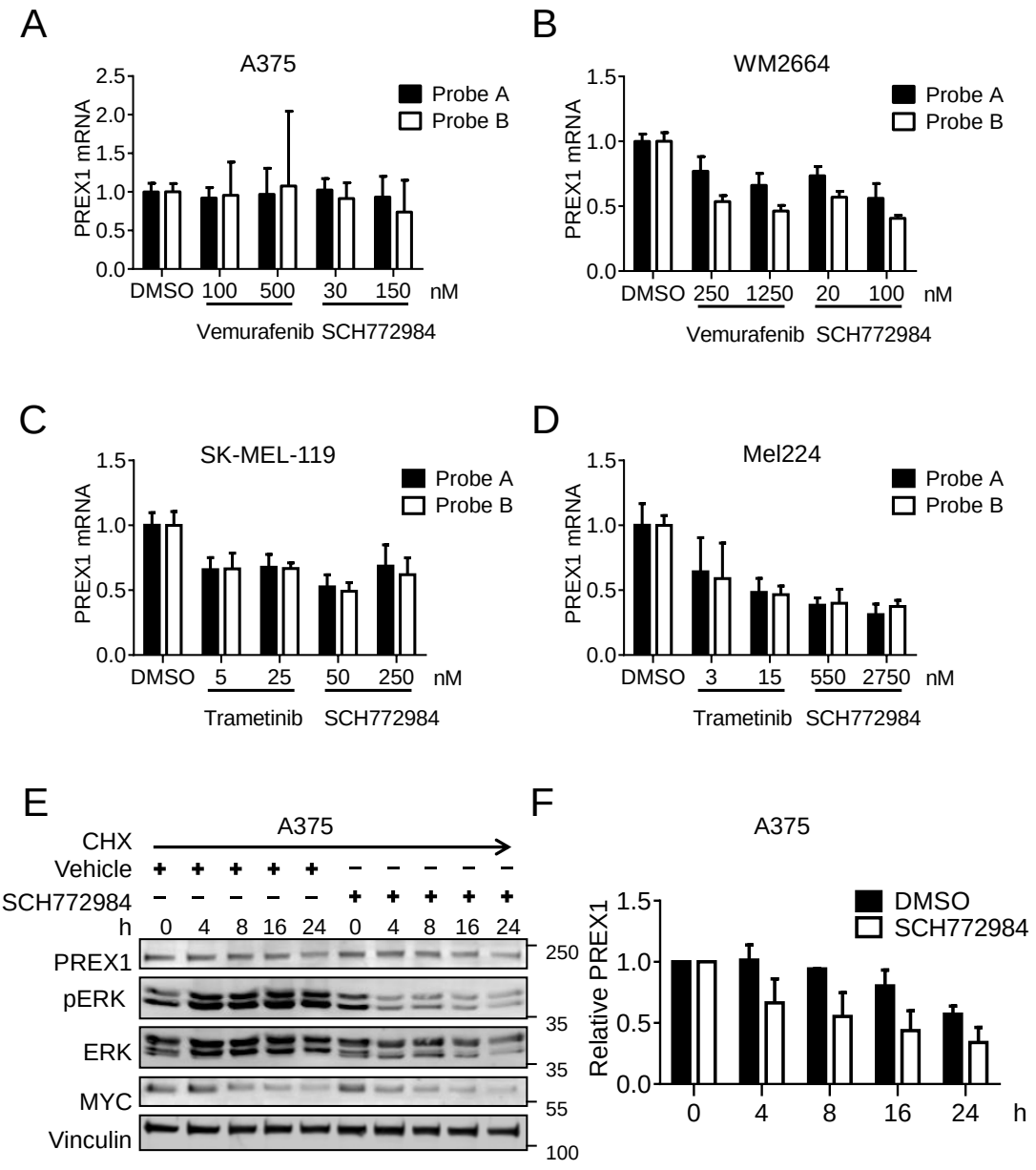


Figure 2-6. PREX1 levels are regulated by ERK both transcriptionally and post-transcriptionally. *BRAF*-mutant A375 and WM2664 cells were treated with vemurafenib or SCH772984 for 24 h and PREX1 mRNA levels were measured by Taqman qPCR using two independent probes (A,B). *NRAS*-mutant SK-MEL-119 and Mel224 cells were treated with trametinib or SCH772984 for 24 h, and PREX1 mRNA measured as above (C,D). Taqman analyses indicate compiled results of n=2 experiments for WM2664 and Mel224, and n=3 experiments for A375 and SK-MEL-119. To test posttranscriptional regulation, A375 cells were treated with vehicle or SCH772984 in the presence of 50 µg/ml cycloheximide, and lysates were probed by western blot for PREX1, pERK and MYC (E). Quantification of PREX1 levels using ImageJ (F) is representative of n=2 independent experiments. Data are represented as mean ± SD.

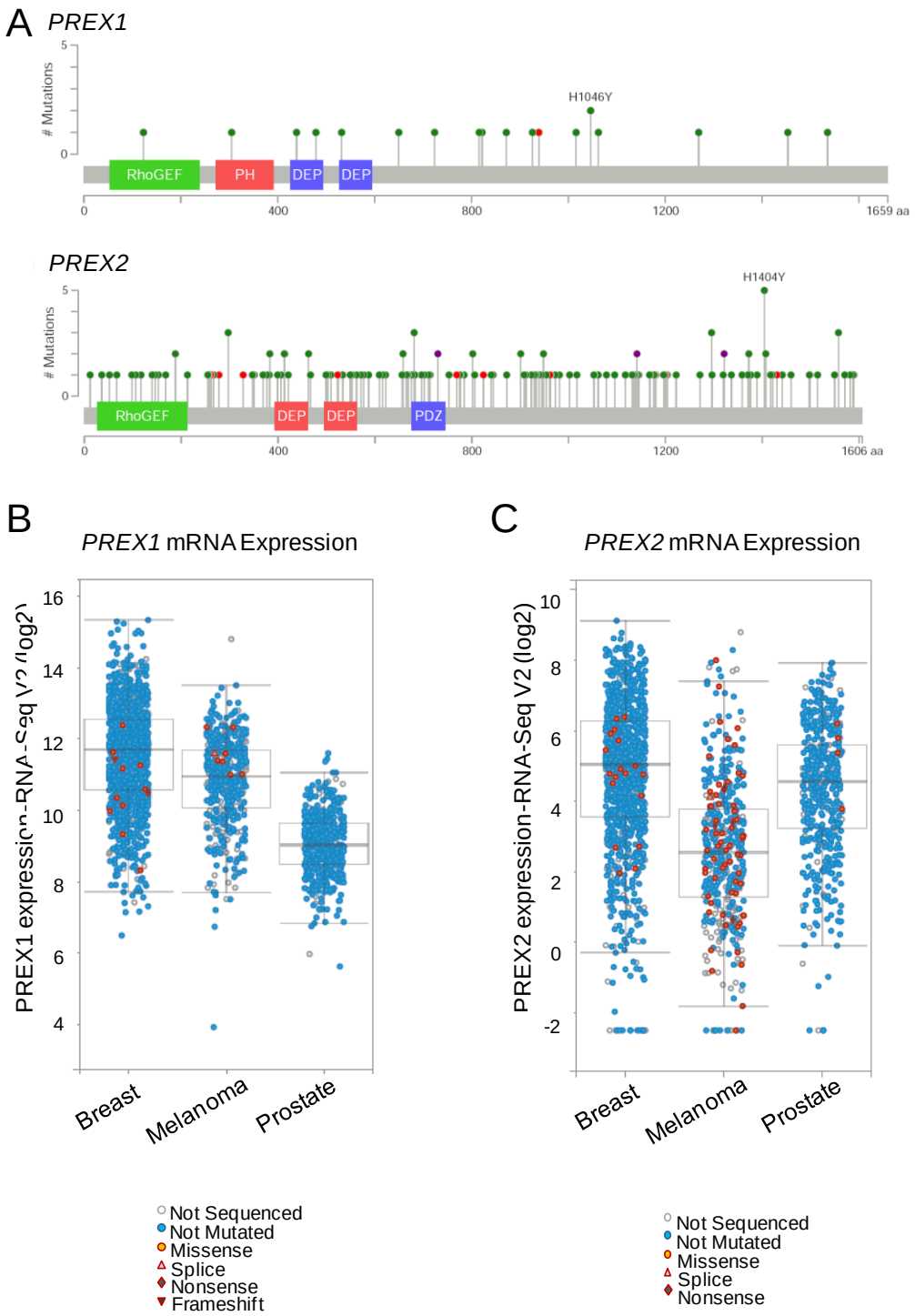
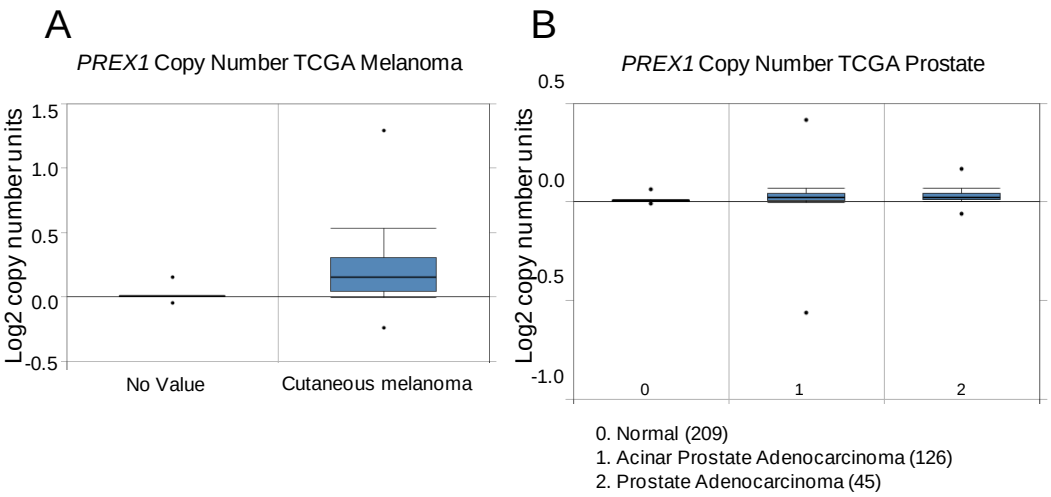
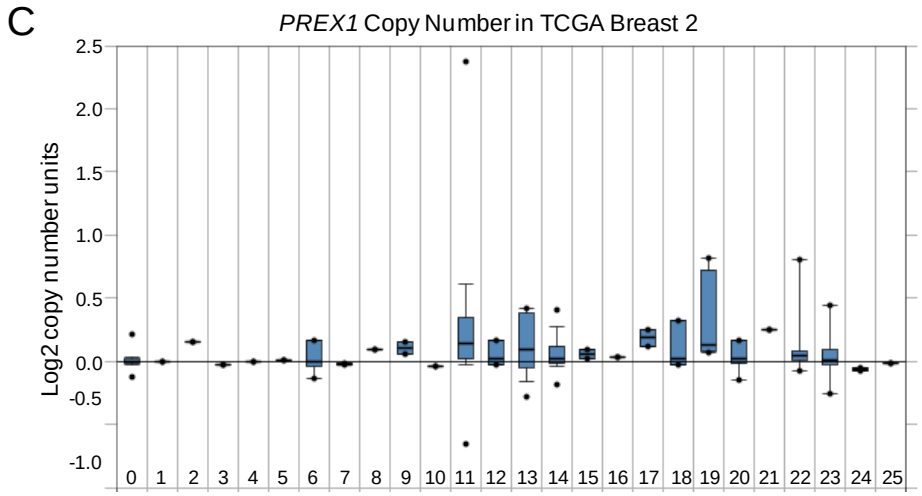


Figure 2-7. PREX1 and PREX2 display distinct expression and mutation patterns in breast cancer, prostate cancer, and melanoma. cBioPortal was used to generate: (A) Alignment of PREX1 and PREX2 proteins with their mutation profiles in cutaneous melanoma. Distribution of PREX1 (B) and PREX2 (C) mRNA expression and mutation status in breast cancer, prostate cancer, and cutaneous melanoma TCGA samples.



0. Normal (209)
1. Acinar Prostate Adenocarcinoma (126)
2. Prostate Adenocarcinoma (45)



0. Normal (813)
1. Adenoid Cystic Breast Carcinoma (1)
2. Apocrine Breast Carcinoma (1)
3. Breast Adenocarcinoma with Squamous Metaplasia (1)
4. Breast Large Cell Neuroendocrine Carcinoma (1)
5. Breast Neuroendocrine Carcinoma (1)
6. Ductal Breast Carcinoma (5)
7. Intraductal Cribriform Breast Adenocarcinoma (2)
8. Intraductal Micropapillary Breast Carcinoma (1)
9. Invasive Breast Carcinoma (2)
10. Invasive Cribriform Breast Carcinoma (1)
11. Invasive Ductal Breast Carcinoma (642)
12. Invasive Ductal and Invasive Lobular Breast Carcinoma (3)
13. Invasive Ductal and Lobular Carcinoma (14)
14. Invasive Lobular Breast Carcinoma (71)
15. Invasive Lobular Breast Carcinoma, Pleomorphic Variant (2)
16. Invasive Lobular Breast Carcinoma, Tubulolobular Variant (1)
17. Invasive Micropapillary Breast Carcinoma (2)
18. Invasive Papillary Breast Carcinoma (3)
19. Male Breast Carcinoma (9)
20. Medullary Breast Carcinoma (4)
21. Metaplastic Breast Carcinoma (1)
22. Mixed Lobular and Ductal Breast Carcinoma (9)
23. Mucinous Breast Carcinoma (9)
24. Papillary Breast Carcinoma (2)
25. Secretory Breast Carcinoma (1)

Figure 2-8. PREX1 is differentially amplified in breast cancer, prostate cancer and cutaneous melanoma. PREX1 copy number analysis for TCGA melanoma (A), prostate (B), and breast cancer subtypes (C), generated from Oncomine.

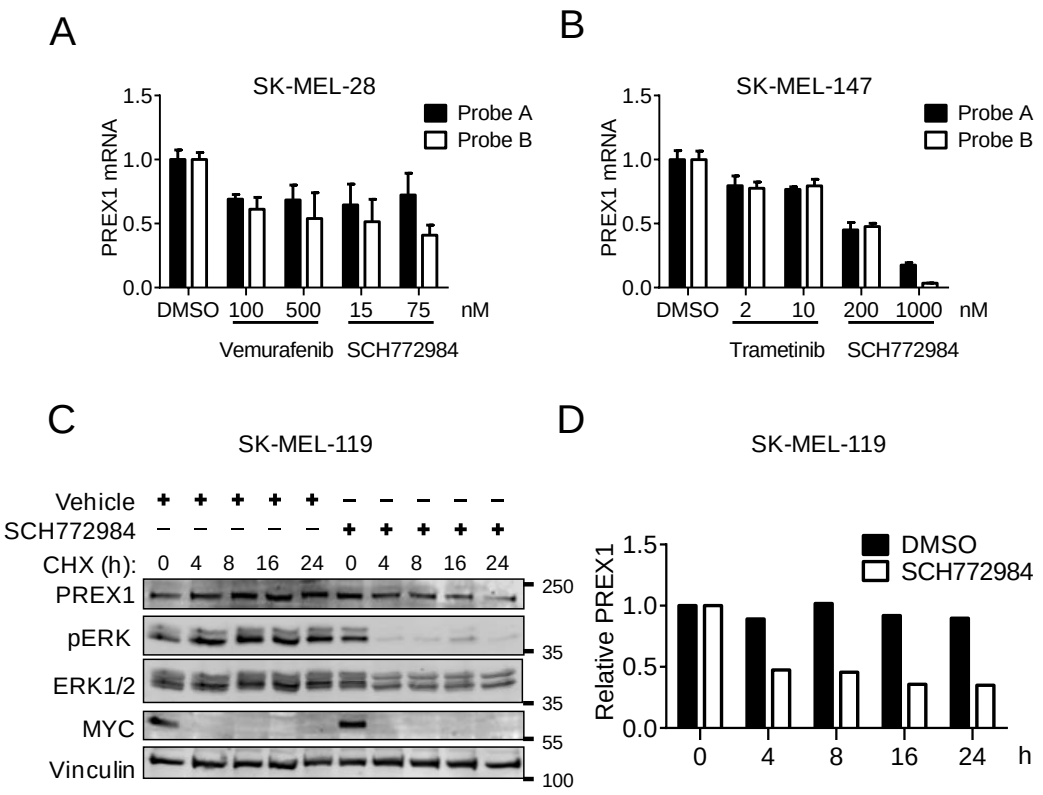


Figure 2-9. PREX1 levels are regulated by ERK both transcriptionally and post-transcriptionally. (A) *BRAF*-mutant SK-MEL-28 cells were treated with or without vemurafenib or SCH772984 for 24 h and PREX1 mRNA levels were measured by Taqman qPCR using two independent probes. (B) *NRAS*-mutant SK-MEL-147 cells were treated with trametinib or SCH772984 for 24 h, and PREX1 mRNA levels were measured as above. (C) *NRAS*-mutant SK-MEL-119 cells were treated with vehicle or trametinib in the presence of 50 µg/ml cycloheximide for the indicated time points, and lysates were probed by western blot for PREX1, pERK/total ERK and MYC. Results were quantified using ImageJ (D). Data are represented as mean ± SD.

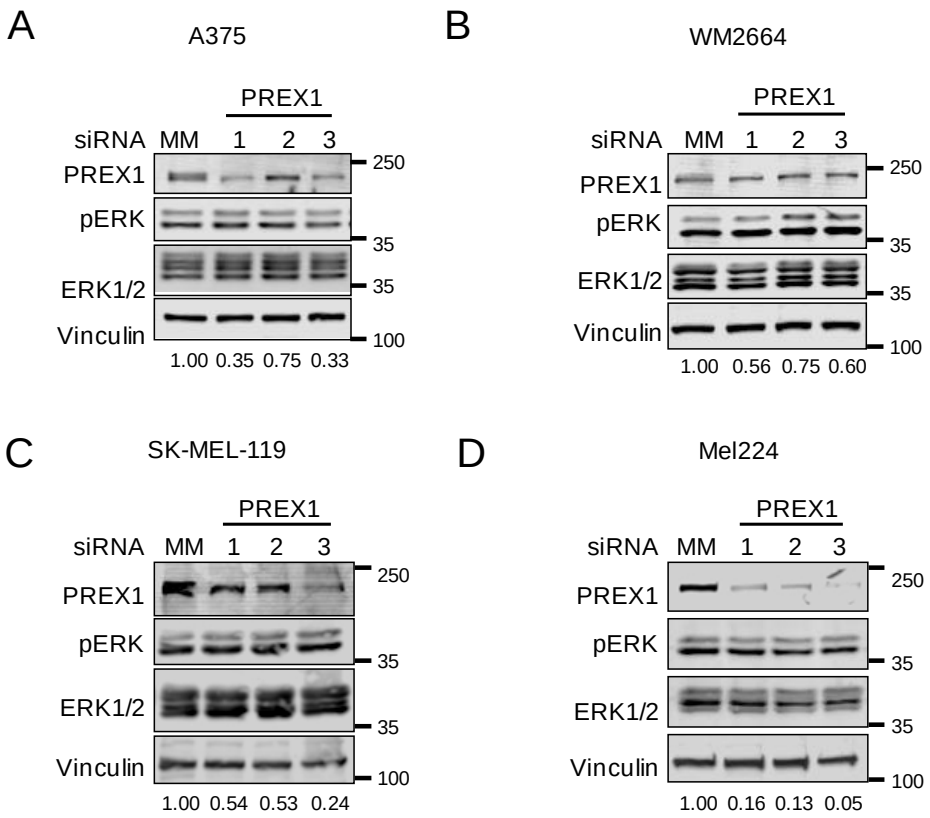


Figure 2-10. Loss of PREX1 does not alter ERK phosphorylation. Cells were first treated with siRNA against PREX1 or a mismatch control (MM) for 48 h. Lysates of *BRAF*-mutant cell lines A375 (panel A) and WM2664 (panel B), and *NRAS*-mutant cell lines SK-MEL-119 (panel C) and Mel224 (panel D), were analyzed by western blot for PREX1 and pERK/total ERK. Apparent molecular weights are indicated to the right of each panel; vinculin served as a loading control.

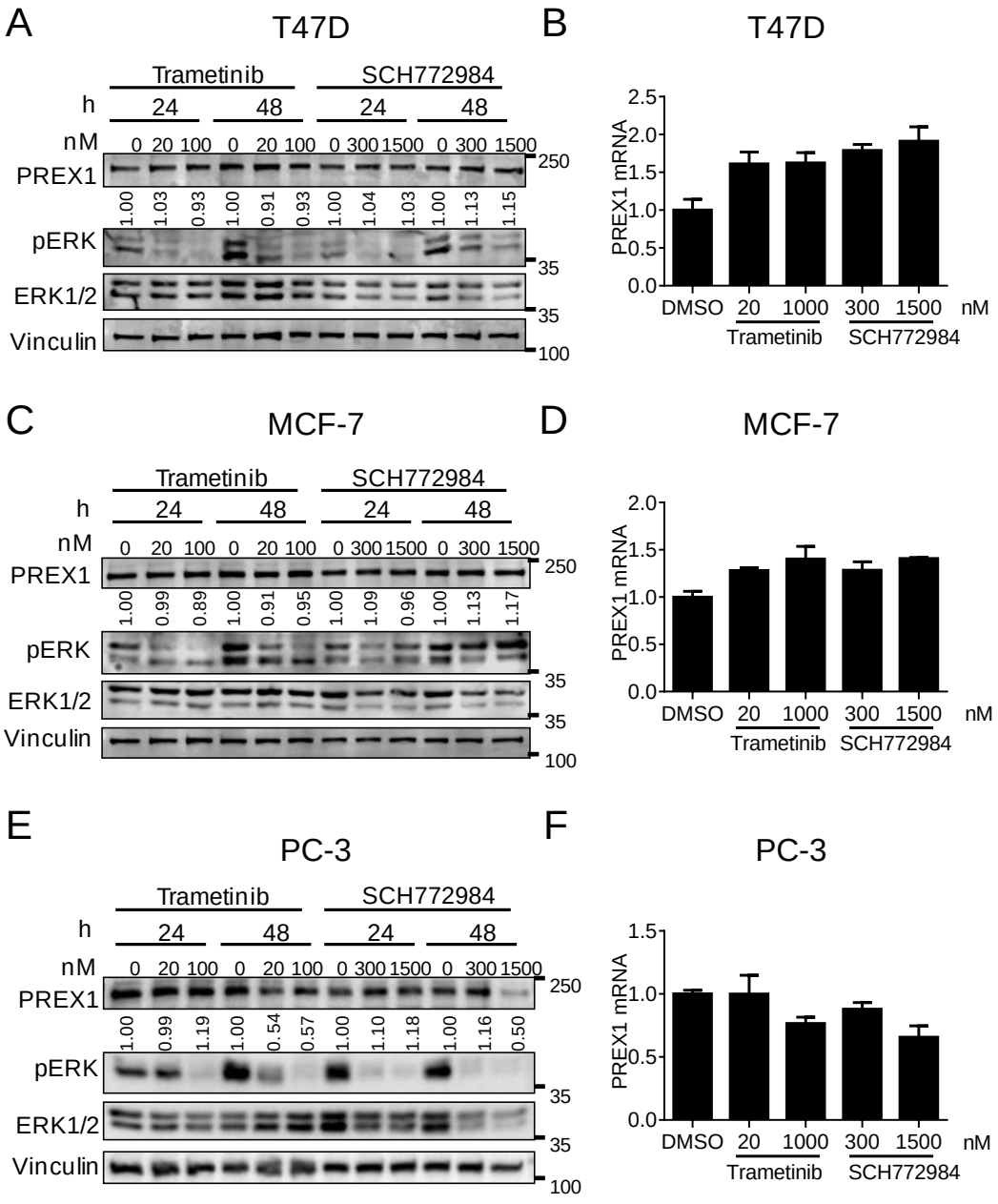


Figure 2-11. ERK differentially regulates PREX1 in breast and prostate cancer cells. T47D (A,B) and MCF7 (C,D) breast cancer cells and PC-3 (E,F) prostate cancer cells were treated with or without trametinib or SCH772984 for 24 or 48 h. Lysates were probed by western blot for PREX1, pERK/total ERK and MYC (A,C,E). Apparent molecular weights are indicated to the right of each panel; vinculin served as a loading control. Fold-change in PREX1 protein levels are indicated by the numbers under the PREX1 panels. PREX1 mRNA levels were measured by Taqman qPCR after 24 h of drug treatment (B,D,F). Data are represented as mean ± SD.

CHAPTER III: CONCURRENT P38 MAPK INHIBITION ENHANCES ERK INHIBITOR ANTI-TUMOR ACTIVITY IN KRAS-MUTANT CANCERS[3]

OVERVIEW

We recently demonstrated that pharmacologic inhibition of the ERK mitogen-activated protein kinases (MAPKs) may be an effective therapeutic approach for the treatment of *KRAS*-mutant pancreatic ductal adenocarcinoma (PDAC). Since we anticipate that treatment-induced resistance will likely limit the success of ERK inhibitor therapy, we applied a CRISPR/Cas9-based genetic loss-of-function screen to identify genetic drivers of resistance to ERK inhibition. We identified loss of MAPK14, encoding the p38α MAPK, as causing increased sensitivity to the ERK1/2-selective inhibitor SCH772984 (ERKi) in *KRAS*-mutant PDAC, lung and colorectal carcinoma cell lines. We then focused on *KRAS*-mutant PDAC to assess p38 signaling as a modulator of ERK inhibitor (ERKi) sensitivity. Conversely, ectopic overexpression of p38α reduced ERKi sensitivity. Cotreatment with a clinical candidate pharmacologic inhibitor of p38α, LY2228820 (ralimetinib, p38i), synergistically enhanced ERKi activity in both anchorage-dependent and anchorage-independent growth and led to increased cell cycle arrest but not apoptosis. Finally, we assessed a mechanistic basis for p38 modulation of ERKi sensitivity. We found

[3] This chapter is currently under revision for publication. The other authors are Peter S. Winter, Andrew M. Waters, Kris C. Wood, Adrienne D. Cox, and Channing J. Der. All figures represent the work of Meagan B. Ryan, with the exception of Figures 3-1 and 3-4.

that ERKi alone enhanced p38 signaling and that concurrent p38i accelerated ERKi-mediated loss of MYC protein. We conclude that concurrent p38i treatment may be an effective combination therapy to enhance ERKi anti-tumor activity in PDAC and other *KRAS*-mutant cancers.

INTRODUCTION

Pancreatic cancer is currently the third leading cause of cancer deaths in the United States with treatments limited to surgical resection or chemotherapy with gemcitabine, FOLFIRINOX (fluorouracil, folinic acid [leucovorin], irinotecan, and oxaliplatin) or gemcitabine plus nanoparticle albumin-bound paclitaxel (nab-paclitaxel)[158,159]. Pancreatic ductal adenocarcinomas (PDAC) are driven by mutant *KRAS* in >95% of cases[14]. Currently, although no treatments have successfully targeted mutant KRAS in the clinic, there has been a renewed interest in developing direct KRAS inhibitors to block mutant-RAS function[3]. To date, the most effective and promising efforts targeting KRAS-dependence in PDAC and other RAS-mutant cancers involve blocking KRAS effector signaling, in particular blocking the RAF-MEK-ERK mitogen activated protein kinase (MAPK) signaling cascade[133]. Activation of the RAF-MEK-ERK cascade is sufficient and necessary for both the formation and maintenance of PDAC[160,17].

Pharmacologic inhibition of the RAF-MEK-ERK cascade presents a promising therapeutic option in *KRAS*-mutant cancers, including PDAC, based on genetic studies showing dependency on the RAF, MEK or ERK nodes of the cascade[161,19,20]. There are currently over 30 inhibitors of the RAF-MEK-ERK cascade under clinical evaluation in cancer[3,133]. RAF phosphorylates and activates

MEK, which in turn phosphorylates and activates ERK, which can phosphorylate >200 cytoplasmic and nuclear substrates[76,142]. RAF and MEK inhibitors have had limited success in the clinic due to paradoxical activation of the RAF-MEK-ERK cascade upon treatment with BRAF-selective inhibitors in RAS-mutant cancers as well as due to kinome reprogramming after treatment with RAF or MEK inhibitors, all which lead to the re-activation of ERK[162,33,54,37]. Thus, direct pharmacologic inhibition of ERK may be the answer to successfully inhibiting the RAF-MEK-ERK cascade in *KRAS*-mutant pancreatic cancer. The allosteric and ATP-competitive ERK1/2 inhibitor SCH772984 effectively reduces the growth of cancers harboring NRAS, BRAF, or KRAS mutations, including melanomas resistant to the BRAF inhibitor vemurafenib[60]. Recent work from our group has shown that SCH772984 is effective in reducing the growth of a subset of PDAC cell lines, through a MYC dependent mechanism[148]. PDAC cell lines that were insensitive to MEK inhibition were sensitive to SCH772984 and the ERK inhibitor BVD-523, and both ERK inhibitors synergized with the AKT inhibitor AZD8186. Additional potential mechanisms of resistance to SCH772984, including PI3K, Notch, and the stress activated MAPK p38α (MAPK14), were also identified through drug sensitivity and resistance testing (DSRT) and cancer toolkit screening (CTK)[112,113,148].

MAPK14 (p38α), along with MAPK11 (p38β), are MAP kinases strongly activated by cellular stress, cytokines, and other exogenous stimuli. In cancer, p38α has been found to play both a tumor promoting and a tumor suppressive role in regulating the balance between cell survival and cell death, in a cancer type dependent manner [109,163]. In PDAC, p38 can paradoxically play a growth

suppressive role in untreated tumors while also conferring resistance to gemcitabine through p38 activation in both the tumor cells and cancer associated fibroblasts [110,111,164]. Evidence in PDAC and other tumor types suggests a broader role for p38 in conferring therapeutic resistance to radiotherapy, chemotherapy, and targeted therapies, and consequently p38 yields a potential target for combination therapies [165,27,166,167]. A number of inhibitors of p38 are under clinical evaluation in inflammatory diseases and cancer, including BIRB-796 (doramapimod), GW-856553 (losmapimod), and LY2228820 (ralimetinib)[168,169]. In this study, I validated MAPK14 (p38α) as a potential mechanism of resistance to ERK inhibition and evaluated the clinical candidate p38 inhibitor LY2228820 as a combination strategy with ERK inhibitors in *KRAS*-mutant PDAC.

MATERIALS AND METHODS

Cell lines and inhibitors

PDAC cell lines were obtained from ATCC and maintained in either DMEM or RPMI-1640 supplemented with 10% fetal calf serum, and were not cultured longer than 6 months from receipt from cell banks. LY2228820, SCH772984, and BVD-523 were purchased from Selleckchem.

CRISPR/Cas9

A barcoded lentiviral library consisting of sgRNA against 2390 genes, 5 constructs per gene, was packaged, pooled, and infected at a multiplicity of infection (MOI) of 0.3 in PDAC, colorectal, or lung cell lines and cells were selected with

puromycin (2 µg/ml) for 48 h. Stably selected sgRNA infected cells were divided into vehicle and SCH772984 treatment groups and treated at GI_{50} of proliferation (SCH) or equivalent concentration of DMSO. Drug was refreshed every 3 days and cell populations were expanded for 4 weeks. Genomic DNA was isolated using the Qiagen DNeasy Blood and Tissue Kit. Genomic DNA samples were prepared for Illumina Sequencing by PCR amplification of individual construct barcodes, using a common P5 Illumina adapter primer (PGK-Illumina-F) and a unique P7 Illumina barcoded adapter primer. Illumina sequencing and analysis of vehicle treated samples vs. drug treatment samples was performed as outlined previously[113].

Inhibitor treatment assays

Sensitivity of PDAC cells to LY2228820, alone or in combination with SCH772984, was determined by MTT assay. Briefly, LY2228820 was serially diluted from 10 µM to 0.002 µM in a 96 well plate. For anchorage-dependent inhibitor studies, SCH772984 was serially diluted from 30 µM to 0.007 µM in the presence of 0, 0.4, 2, or 10 µM LY2228820. Cells were seeded at a density of 2-3 x 10^3 cells per well and allowed to proliferate at 37°C and 5% CO2 for 72 h. before incubation for 3 h in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). MTT was solubilized in DMSO and the absorbance was read at A570. For anchorage-independent inhibitor studies, cells were treated with inhibitor as in anchorage-dependent assays. Cells were seeded at a density of 5-10 x 10^3 cells per well in 1% SeaPrep Agarose (Lonza) in plates coated with 0.6% Bacto Agar and allowed to proliferate at 37°C and 5% CO2 for 72 h before incubation with AlamarBlue for 2-3 h.

Drug treatment and western blotting

PDAC cells were treated with LY2228820, SCH772984, or a combination for 2, 6, 24, or 72 h before samples were collected in RIPA lysis buffer. Whole cell lysates were resolved on 10-12% SDS-PAGE gels and western blotting was performed using antibodies to phospho-MKK3 (Ser189)/-MKK6 (Ser207), phospho-p38 (Thr180/Tyr182), total p38, phospho-MK2 (Thr334), total MK2, phospho-HSP27 (Ser82), total HSP27, phospho-ERK1/2 (Thr202/Tyr204), total ERK1/2, phospho-RSK (Thr359/Ser363), total RSK1/2/3, c-Myc (MYC), phospho-Rb (Ser807/811), cyclin B1, cyclin D1, and PARP (Cell Signaling); vinculin (Sigma); and p16 (Abcam). HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were from Thermo Fisher.

Apoptosis and cell cycle analysis

PDAC cell lines were treated with LY2228820 (2 μM), SCH7772984 (625 nM) or combination for 72 h before apoptosis and cell cycle analysis by flow cytometry. Apoptosis analyses were performed with the TACS® Annexin V-FITC Kit (Trevigen) following the manufacturer's protocol. Briefly, spent culture medium containing detached cells was collected and mixed with trypsinized cells and centrifuged at 300 x g for 5 min. Cells were washed once in ice-cold 1X PBS and incubated in Annexin V Incubation Reagent (1% Annexin V-FITC, 1X Propidium Iodide Solution, in 1X calcium-containing binding buffer) at room temperature for 15 min in the dark. Cells were analyzed on a BD LSRFortessa flow cytometer. For cell cycle analysis, cells were harvested, washed once in 1X PBS, and resuspended in fresh PBS. Ten volumes of ice cold 70% ethanol were added to each tube dropwise while vortexing gently. Cells were incubated overnight at 4°C. The fixed cells were then washed

once in 1X PBS, resuspended in 1X PBS containing 40 µg/ml propidium iodide and 100 µg/ml RNase A (both from Life Technologies) and incubated at 37°C overnight. Cells were analyzed on a BD LSRFortessa flow cytometer.

Quantitative PCR

Total RNA was isolated using an RNeasy kit (Qiagen) and reverse transcription was performed using the High Capacity RNA-to-cDNA kit (Thermo Fisher). Real time quantitative Taqman PCR was performed on the QuantStudio 6 Flex (Thermo Fisher) with FAM/MGB labeled probes (Thermo Fisher) against HSPB1 (Hs00356629_g1), MYC (Hs00153408_m1) and endogenous control VIC/TAMRA labeled β-actin.

Graphical analysis

Data were analyzed using GraphPad Prism 6 software and curve fit and GI50 values were generated as indicated in the Figure Legends.

RESULTS

CRISPR/Cas9 screening identifies MAPK14 (p38α) as a sensitizer to the ERK inhibitor SCH772984

We recently demonstrated that direct inhibition of ERK with the inhibitor SCH772984 is effective in reducing proliferation of a subset of *KRAS*-mutant pancreatic cancer cell lines. However, mechanisms of resistance to this new class of inhibitors targeting ERK have not been fully characterized[148]. Our previous

efforts have identified the PI3K-AKT-mTOR, Notch, and p38 pathways as a potential mechanism of resistance to ERK inhibitors in PDAC. We next sought both to validate previously identified hits and also to identify additional pathways that can confer resistance to the ERK inhibitor SCH772984 in KRAS-mutant lung, colorectal, and pancreatic cancers. In an unbiased genetic loss-of-function screen, we found that CRISPR/Cas9 deletion of MAPK14, encoding p38α, increased the sensitivity of KRAS-mutant lung, colorectal and pancreatic cell lines to the growth inhibitory activity of the ERK inhibitor SCH772984.

p38 inhibition does not impair the proliferation of PDAC cell lines

To determine basal levels of p38 MAPK and ERK MAPK pathway activity in PDAC, I performed Western blot analyses on a panel of 7 established and 7 patient-derived xenograft (PDX) cell lines. Despite similar levels of total p38 protein across all cell lines, levels of phosphorylated p38 (pp38) varied from low (AsPC-1, CFPAC-1, HPAC, HPAF-II, and Pa18c) to high (MIA PaCa-2, Panc-1, Panc10.05) (Figure 3-2A). Phosphorylated MKK3 and MKK6, the upstream activator of p38 also varied highly, as did both phosphorylated and total MAPKAPK-2 (MK2) and HSP27 downstream of p38. Likewise, expression and phosphorylation of ERK MAPK signaling components also varied widely among PDAC cell lines (Figure 3-2A). High levels of phosphorylated ERK (Pa16c) did not correlate with high levels of phosphorylated RSK, a well characterized substrate of ERK[170]. Levels of MYC, both a substrate of ERK and a protein transcriptionally regulated by ERK, also varied widely among the panel of cell lines (Figure 3-2A).

To determine if pharmacologic inhibition of p38 is sufficient to inhibit PDAC growth, I used the clinical candidate p38α/β inhibitor LY2228820 to treat cells in 2D growth assays. LY2228820 (ralimetinib) is a potent and selective ATP-competitive inhibitor of p38α/β and has a 15-50 fold selectivity over JNK1/2/3, while not inhbiting p38δ/γ, ERK1/2, or 176 other kinases[168]. In a panel of 5 established (AsPC-1, HPAC, HPAF-II, MIA PaCa-2, and Panc-1) and 3 PDX (Pa02c, Pa03c,and Pa16c) PDAC cell lines, LY2228820 did not appreciably reduce anchorage-dependent growth, with no GI_{50} calculated after 72 h of up to 10 μM inhibitor treatment (Figure 3-2B). However, LY2228820 potently inhibited the p38 MAPK signaling cascade at both 6 and 24 h in the established cell lines AsPC-1, HPAC, and MIA PaCa-2 (Figure 3-2C). Phosphorylation of HSP27, a well characterized protein regulated by p38, was reduced in a dose dependent fashion in all three cell lines, with maximum reduction in signal at 24 h of LY2228820 treatment. Treatment with LY2228820 also induced rapid phosphorylation of the upstream activator of p38, MKK3/6, which in turn led to an increase in phosphorylation of p38; however, inhibition of the downstream target HSP27 was maintained, suggesting effective inhibition of p38. These results collectively suggest that p38α/β activity is not necessary for maintaining PDAC growth and that it may act in a compensatory pathway when ERK is inhibited.

Concurrent inhibition of p38 enhances ERK inhibitor impairment of PDAC growth

Although the p38 inhibitor LY2228820 did not impair PDAC cell line growth as a single agent, it potently inhibited the pathway at nanomolar concentrations and

thus presents a novel potential combination treatment with ERK inhibitors. PDAC cell lines exhibited varying sensitivity to the ERK inhibitor SCH772984 when measured by maximal inhibition of growth and GI_{50} concentration: the AsPC-1 cell line exhibited the least sensitivity to SCH772984, while the HPAC and MIA PaCa-2 cell lines were highly sensitive to the inhibitor (Figure 3-3A,B). To determine whether LY2228820 sensitizes PDAC cell lines to SCH77284, AsPC-1, HPAC, and MIA PaCa-2 cells were treated with a constant dose of LY2228820 in the presence of a dose titration of SCH772984. After 72 h of growth on plastic, LY2228820 sensitized cells to SCH772984 in a dose dependent fashion as measured by GI_{50} shift (Figure 3-3A). I also determined that LY2228820 sensitized AsPC-1, HPAC, and MIA PaCa-2 cell lines to SCH772984 in a 3D soft agar assay. After 7 days of growth in soft agar, LY2228820 sensitized cells to SCH772984 in a dose dependent fashion as measured by GI_{50} shift (Figure 3-3B). The fold shift in sensitivity to SCH772984 was greater in soft agar compared to growth on plastic for all cell lines.

I also expanded the study to an additional ERK inhibitor BVD-523, a clinical candidate inhibitor currently under investigation in combination with nab-paclitaxel and gemcitabine in pancreatic cancer (NCT02608229). Previous work from our group has demonstrated that BVD-523, an ATP-competitive ERK inhibitor, has a similar efficacy in inhibiting growth in a subset of PDAC cell lines, which generally exhibit sensitivity or resistance to both inhibitors[148]. To determine whether LY2228820 sensitizes PDAC cell lines to BVD-523, AsPC-1, HPAC, and MIA PaCa-2 cells were treated with a constant dose of LY2228820 in the presence of a dose titration of SCH772984. After 72 h of growth on plastic, LY2228820 sensitized cells

to BVD-523 in a dose dependent manner as measured by GI_{50} shift (Figure 3-7A, B). These results demonstrate that concurrent inhibition with p38 sensitizes PDAC cell lines to structurally and mechanistically distinct ERK inhibitors and that ERK inhibitors may share p38 as a common mechanism of therapeutic resistance.

Concurrent inhibition of both p38 and ERK causes G0/G1 cell cycle arrest

PDAC cell lines exhibit varying sensitivity to ERK inhibitors such as SCH772984. Work from our group has shown that treatment with SCH772984 causes apoptosis after short term treatment whereas it induces a senescence like phenotype after longer term treatment[148]. We first asked if the p38 inhibitor LY2228820 enhances the early induction of apoptosis by the ERK inhibitor SCH772984 in the same three PDAC cell lines as above. After 72 h of treatment with SCH772984, both AsPC-1 and HPAC showed a modest increase in apoptotic cells, as measured by cells staining positive for Annexin V (early apoptosis), or for both Annexin V and propidium iodide (late apoptosis) (Figure 3-4A-B). MIA PaCa-2 cells displayed a much stronger induction of apoptosis, with only 30% of cells remaining healthy (PI-/Annexin V-) after treatment with SCH772984. In all three cell lines, concurrent LY2228820 and SCH772984 treatment did not enhance the level of apoptosis induced by SCH772984 alone. These findings were supported by the presence of cleaved PARP in only the MIA PaCa-2 cells treated with SCH772984 alone or with concurrent SCH772984 and LY2228820 (Figure 3-4C). AsPC-1 and HPAC cells did not exhibit cleaved PARP under any inhibitor treatment condition.

Since enhanced induction of apoptosis was not observed with concurrent p38 and ERK inhibition in PDAC cell lines, we next determined if LY2228820 could perturb their cell cycle progression in the presence of SCH772984. Treatment with SCH772984 for 72 h reduced markers of cell cycle progression as measured by phospho-Rb, cyclin D1 (G_1 progression), cyclin B1 (progression through M) in all three cell lines (Figure 3-4C). Loss of these cell cycle progression markers was enhanced by LY2228820 in both AsPC-1 and MIA PaCa-2 cells. However, the negative cell cycle regulator p16 was elevated in the HPAC cell line only upon SCH772984 treatment. Using flow cytometry to measure total DNA content, we observed that treatment with ERK inhibitor alone was sufficient to induce an almost complete G_0/G_1 cell cycle arrest in HPAC cells, and a modest increase in G_0/G_1 in MIA PaCa-2 cells, whereas the percentage of AsPC-1 cells arrested in G_0/G_1 did not increase upon treatment with SCH772984 alone (Figure 3-4D). Adding concurrent inhibition of p38 increased the percentage of MIA PaCa-2 and AsPC-1 cells arrested in G_0/G_1. Collectively, our results show that concurrent inhibition of the ERK and p38 MAPK cascades converges on cell cycle regulation in PDAC cell lines.

SCH772984 inhibition of ERK causes increased p38 signaling

Since CRISPR/Cas9 genetic manipulation and pharmacologic inhibition of p38 signaling both modulated sensitivity to SCH772984 and enhanced G_0/G_1 cell cycle arrest, we speculated that ERK inhibition may cause alterations in p38 signaling. The p38 MAPK cascade is one of the stress activated MAPK cascades and can play dual roles in cancer by mediating both cell survival and cell death, depending on tumor type and mutational background[109]. The p38 MAPK cascade

can also contribute to therapeutic response to both targeted therapies and chemotherapeutics. In hepatocellular carcinoma, MAPK14 (p38α) was found to confer resistance to the RAF and VEGFR inhibitor sorafenib, with activation of the p38 signaling cascade occurring after long term treatment[166]. In $BRAF^{V600E}$-mutant melanomas, MAPK hyperactivation, including both p38 and JNK pathways, contributes to therapeutic resistance to the BRAF-selective inhibitor vemurafenib[27]. MEK inhibitors have also been shown to induce dynamic kinome reprogramming in triple negative breast cancers, leading to the activation of multiple kinases including p38α[54]. Since treatment with both RAF and MEK inhibitors can lead to activation of p38 MAPK in multiple tumor types, we wanted to know if treatment of PDAC cell lines with the ERK inhibitor could also activate the p38 MAPK cascade.

Three PDAC cell lines, AsPC-1, HPAC, and MIA PaCa-2, were treated with the ERK inhibitor SCH772984 for 24 or 72 h. In all three cell lines, ERK was inhibited in a dose-dependent fashion as seen in the reduction of phospho-RSK and total levels of MYC. Concurrently, levels of phospho-p38 and phospho-HSP27 increased in both a time- and dose-dependent fashion, with maximal pathway induction occurring at 72 h (Figure 3-5A). Except in the MIA PaCa-2 cell line, phosphorylation of MKK3/6, the upstream activator of p38α/β, was also increased, as was total HSP27 protein. Similar results were seen after treatment with the ERK inhibitor BVD-523, where inhibition of ERK signaling was associated with increased phospho-p38, phospho-HSP27 and increased total HSP27 protein (Figure 3-7B).

HSP27 expression can be regulated by the ERK pathway through the transcription factor HSF1 and HSP27 is itself a potential mechanism of resistance in

pancreatic cancer[110]. The increased protein levels of HSP27 following SCH772984 treatment are due to an increase in HSP27 (HSP27) mRNA in AsPC-1, HPAC, and MIA PaCa-2 cells, where increasing expression of HSP27 mRNA correlated with an increasing dose of ERK inhibitor (Figure 3-5B). Collectively, our results suggest that pharmacologic inhibition of ERK signaling led to increased p38 MAPK cascade activity, both upstream and downstream of p38.

Concurrent inhibition of p38 can enhance ERK inhibitor treatment-induced loss of MYC protein expression

Both ERK and p38 are the terminal nodes of their respective MAPK signaling cascades. They can phosphorylate many overlapping downstream substrates and subsequently regulate diverse cellular processes, including cell survival and proliferation and response to cellular stress[108]. Previous work from our lab has identified MYC as a critical substrate downstream of ERK and a marker of sensitivity or resistance of PDAC to SCH772984[148]. Therefore, we evaluated if MYC protein loss was a possible mechanism for synergy of concurrent ERK and p38 inhibition in PDAC. The AsPC-1 cell line exhibited enhanced downregulation of total MYC protein in the presence of constant SCH772984 and increasing concentrations of LY2228820 starting at 2 h of concurrent inhibitor treatment (Figure 3-6A). The loss of MYC protein was enhanced over time, with maximal loss of MYC occurring after 72 h of treatment. Both the ERK and p38 signaling pathways remained fully inhibited, as indicated by phospho-RSK and phospho-HSP27, respectively. However, the same synergistic loss of MYC protein was not seen in two other PDAC

cell lines, HPAC and MIA PaCa-2, indicating that the basis for synergy between ERK and p38 inhibition is not always dependent on MYC (Figure 3-8).

Although MYC presents a potential mechanism for synergy of concurrent ERK and p38 inhibition in PDAC, it was not known whether LY2228820 alone could regulate MYC protein levels, or whether the loss of MYC protein seen upon combination treatment was due to transcriptional, post-transcriptional or post-translational mechanisms. To address these questions, we treated AsPC-1 cells for 24 h with increasing doses of LY2228820 in the presence or absence of SCH772984 and then assessed the levels of MYC protein and mRNA. LY2228820 treatment alone was not sufficient to regulate MYC protein; MYC protein levels were reduced only in the presence of SCH772984 (Figure 3-6B). Similarly, increased loss of MYC transcript was seen only in the presence of both LY2228820 and SCH772984 after 6 and 24 h of inhibitor treatment (Figure 3-6C). Loss of MYC in the presence of SCH772984, LY222820, or a combination of the two inhibitors was not due to post-translational regulation, as shown by similarly decreased protein levels over time in the presence of the translational inhibitor cycloheximide (Figure 3-9A,B). Further, although the p38 pathway has been found to regulate mRNA stability under conditions of cell stress[171], the loss of MYC transcript seen upon concurrent treatment with LY2228820 and SCH772984 was not due to destabilization of MYC mRNA, as shown by similarly decreased message levels over time in the presence of the transcriptional inhibitor, actinomycin D (Figure 3-9C). These short term results differ from a previous finding by our group showing that long term treatment with SCH772984 resulted in post-translational loss of MYC protein in ERK inhibitor

sensitive PDAC cell lines. Collectively, my findings reveal an additional novel regulatory mechanism for maintenance of MYC protein levels downstream of the ERK MAPK and p38 MAPK signaling cascades.

DISCUSSION

We have shown that p38 inhibition can synergize with ERK inhibition in *KRAS*-mutant PDAC and that MAPK14 (p38α) presents a novel potential mechanism of resistance to ERK inhibitors. Targeting the RAF-MEK-ERK cascade presents a promising approach to treating *KRAS*-mutant PDAC, as well as other RAS-driven cancers, emphasizing the need to characterize potential resistance mechanisms, such as p38, in order for this treatment approach to become successful. Re-activation of ERK by various means are key mechanisms of resistance to upstream pathway inhibitors, such as EGFR, RAF and MEK inhibitors[172,173,27,72,174,175], and thus, direct ERK inhibition can overcome these resistance mechanisms[73,60]. Resistance to ERK inhibitors can also arise, through mutation in ERK itself and through activation of a parallel pathway such as the PI3K-AKT-mTOR pathway[15,148]. Our study demonstrates that compensatory activation of the parallel p38 MAPK pathway occurs in response to the ERK inhibitors SCH772984 and BVD-523 and that inhibition of p38 can sensitize PDAC cell lines to ERK inhibition.

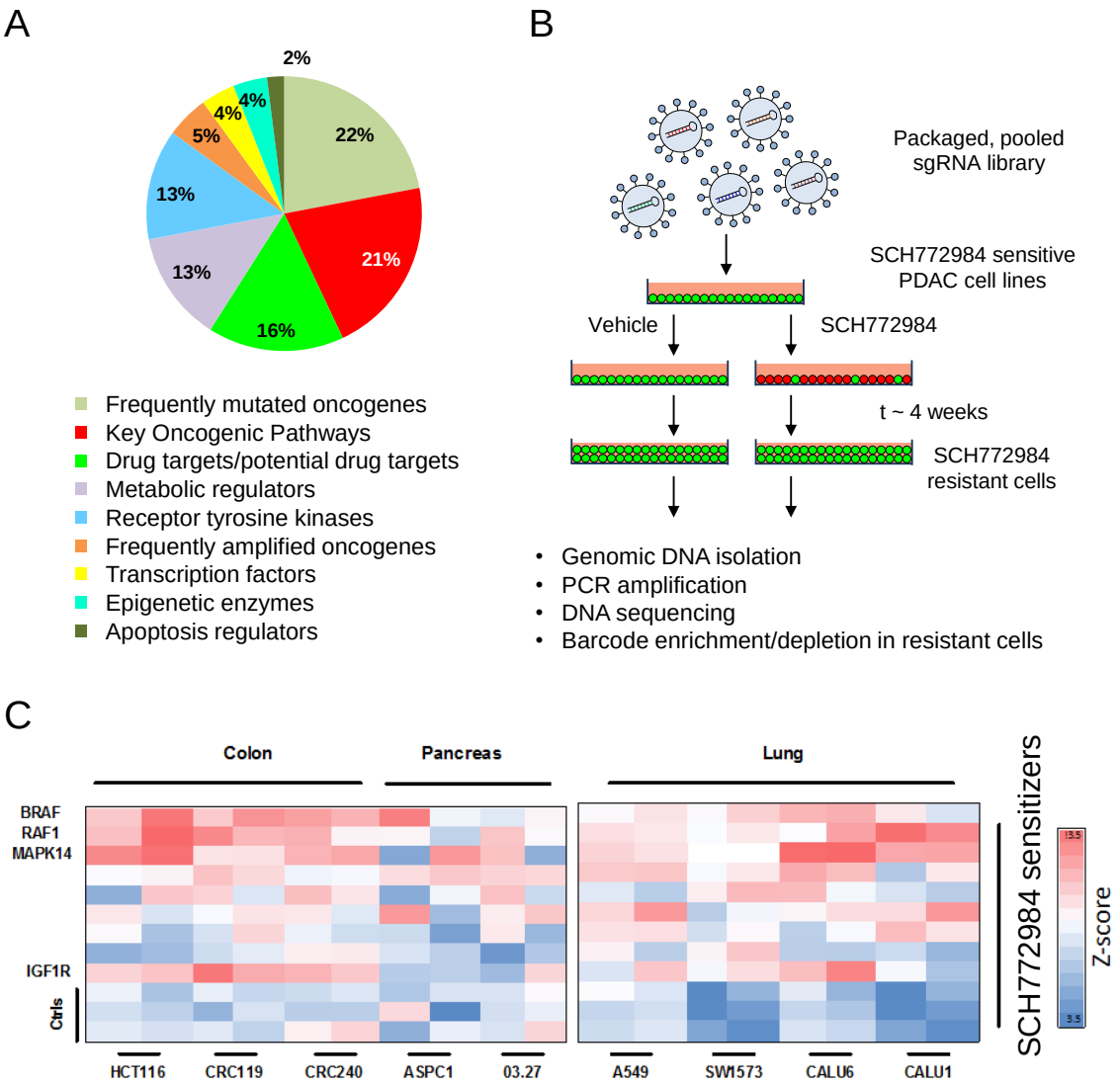
The p38 MAPK pathway and its downstream components have been identified previously as a potential resistance mechanism for both cytotoxic chemotherapeutics and targeted inhibitors. Inhibition of p38 or its substrate MK2

synergized with SMAC mimetics in leukemias and MK2 inhibition synergized with Chk1 inhibition in *KRAS*-mutant cancers[176,177], and MK2 was found to confer resistance to cisplatin in non-small cell lung cancer[165]. Phosphorylated and total protein levels of the heat shock protein HSP27, a substrate of MK2, were elevated in our PDAC cell lines in response to SCH772984 or BVD-523 treatment. Heat shock proteins, including HSP27, are generally elevated in cancer and increased basal levels, or increase in expression levels in response to cancer therapies, can lead to therapeutic resistance[178]. Further, HSP27 can contribute to gemcitabine resistance in pancreatic cancer, which can be overcome by inhibition of HSP27 with OGX-427[179,110]. Our study suggests that HSP27 may play a key role in p38 mediated resistance to ERK inhibitors in PDAC.

Our findings also support a role for cell cycle arrest but not apoptosis in reducing growth upon concurrent ERK and p38 inhibition. Although blocking p38 in some cellular contexts can induce apoptosis, including when combined with MEK inhibitors[180], we did not see levels of apoptosis induced by SCH772984 increase upon the addition of LY2228820. However, we did see enhanced percentages of cells in G0/G1 upon concurrent p38 and ERK inhibition. The cell cycle arrest seen upon concurrent p38 and ERK inhibition may be due in part to enhanced loss of MYC protein, although this was not seen in all cell lines. An enhanced downregulation of phosphorylation of the cell cycle marker Rb was seen with both inhibitors, suggesting a convergence point of both pathways. ERK has been found to regulate the cell cycle, and inhibition of ERK downregulates phophorlyation of Rb and cyclin D1 expression in PDAC[181,148]. Phosphorylation of Rb by p38 has been

found to also regulate its activity independent of the cyclin-dependent kinases[182-184]. These results both support our previous findings that ERK inhibitors regulate the cell cycle and senescence and demonstrate that the addition of a p38 inhibitor can enhance these effects and lead to a marked reduction in PDAC cell growth.

p38 inhibitors have seen limited success as single agent therapies in cancer yet have shown promise as combination strategies both with cytotoxic chemotherapies and with targeted therapies against oncogenic pathways. We have demonstrated that the p38 inhibitor LY2228820 can overcome ERK inhibitor resistance and enhance the efficacy of SCH772984 in PDAC, providing a rationale for combined treatment with these agents that can potentially be applied to other KRAS-mutant cancers.



Intensity represents Z-Score of the depletion metric, that is sequencing reads in drug/ reads in vehicle

Figure 3-1. Loss-of-function genetic screen identifies MAPK14 (p38α) as a regulator of ERK inhibitor sensitivity in *KRAS*-mutant cancer cell lines. (A) Gene targets of our CRISPR/Cas9 sgRNA library. The library was comprised of lentivirus expression vectors encoding five single-guide RNAs (sgRNAs) targeting distinct sequences in 2,390 genes. Shown are the protein classes encoded by these genes. (B) SCH772984 sensitive cell lines were infected with the lentivirus sgRNA library, followed by selection in growth medium supplemented with SCH772984 at the GI_{50} for each cell line, for approximately 4 weeks. Genomic DNA was then isolated, followed by DNA sequencing to identify DNA barcode enrichment/depletion. (C) Heatmap indicating enrichment (blue) or depletion (red) of barcode sequences in SCH772984 treated versus control cell populations.

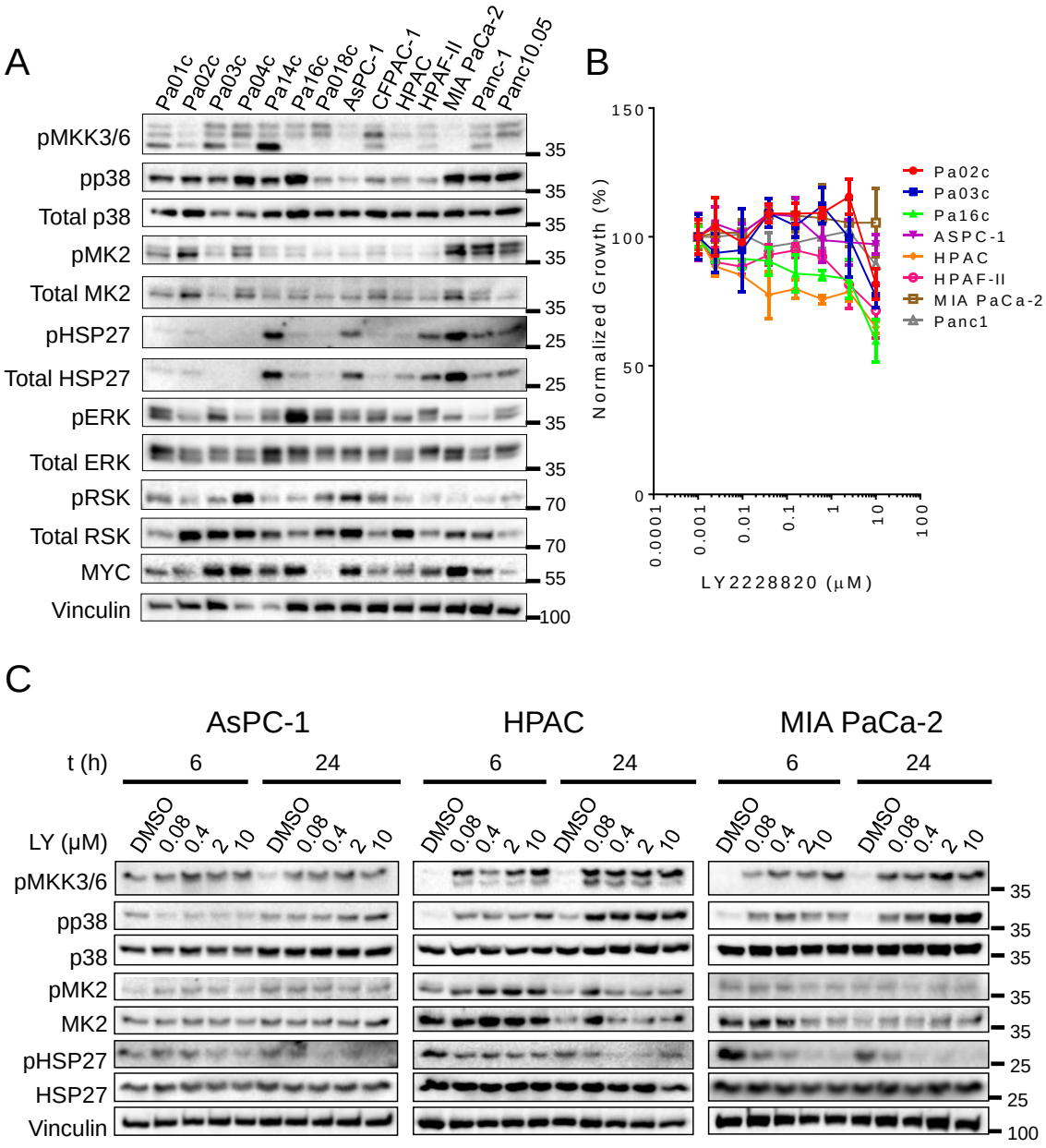


Figure 3-2. Inhibition of p38 does not impair the growth of PDAC cell lines. (A) PDAC cell lines exhibit variable levels of basal p38 signaling. Western blot analyses were performed for phosphorylated and total p38 pathway proteins, including MKK3/6, p38, MK2, HSP27, and for ERK pathway proteins, including phosphorylated and total ERK, RSK, and total MYC. Vinculin served as a loading control. (B) PDAC cell lines were treated for 72 h with a range of LY2228820 concentrations and anchorage-dependent growth was monitored by the MTT viability assay. (C) PDAC cell lines were treated with the indicated concentrations of LY2228820 for 6 or 24 h and western blot analysis was performed for p38 pathway proteins as in panel A.

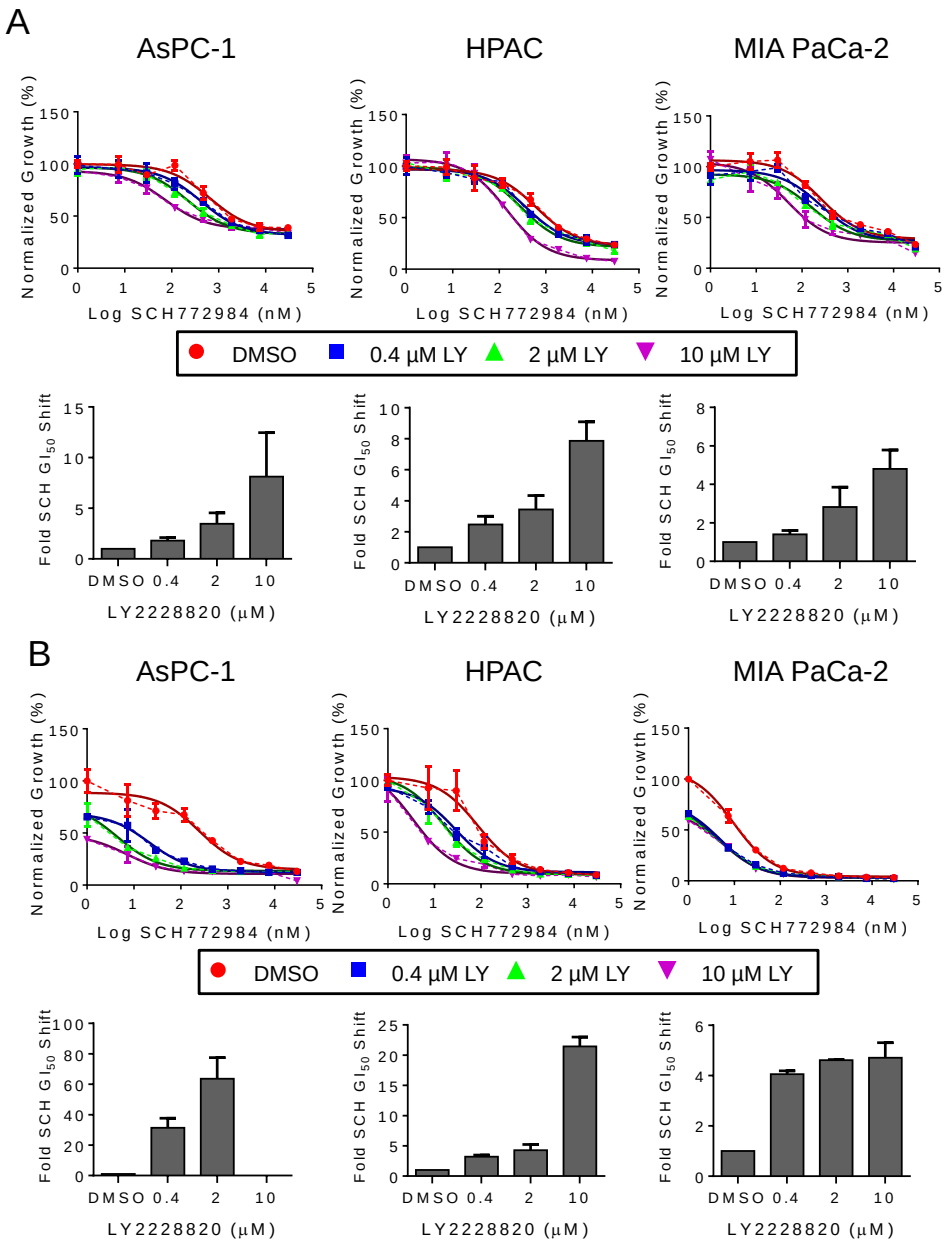


Figure 3-3. Concurrent inhibition of p38 enhances ERK inhibitor-mediated reduction of anchorage-dependent and -independent growth in PDAC cell lines. (A) 72 h MTT assay of AsPC-1, HPAC, and MIA PaCa-2 cell lines treated with indicated concentrations of the p38 inhibitor LY2228820 and a dose titration of the ERK inhibitor SCH772984. Each growth assay is representative of n=3 experiments. Lower panels, compiled results of MTT assays shown in each upper panel, presented as fold GI_{50} shift, n=3 assays. (B) AlamarBlue readout of a 7 day soft agar assay in AsPC-1, HPAC, and MIA PaCa-2 cell lines treated with the indicated constant concentration of the p38 inhibitor LY2228820 and a dose titration of the ERK inhibitor SCH772984. Each growth assay is representative of n=3 experiments. Lower panels, quantification of AlamarBlue growth curves shown in each upper panel B, presented as fold GI_{50} shift, n=3 assays. GI_{50} values are presented as mean ± SEM.

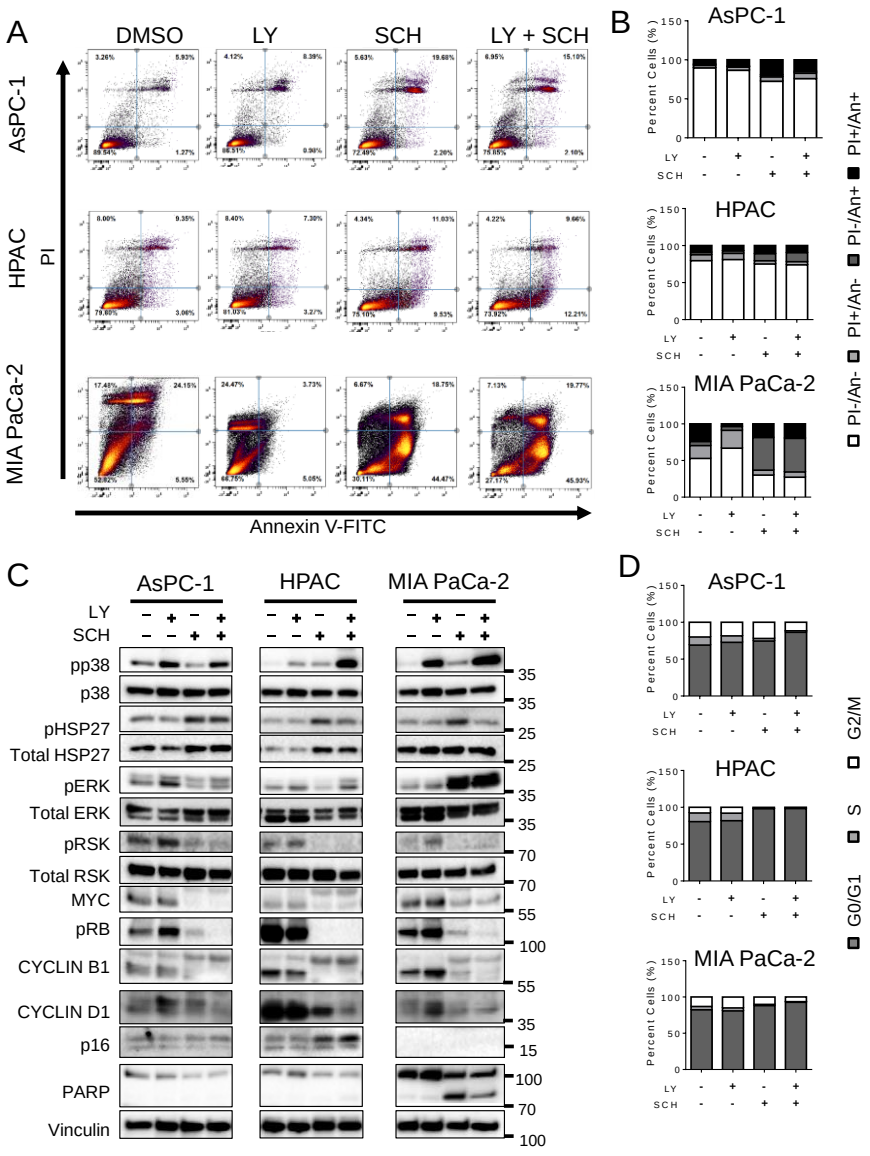


Figure 3-4. Concurrent inhibition of p38 and ERK induces G0/G1 cell cycle arrest and downregulation of markers of cell cycle progression. (A) AsPC-1, HPAC, and MIA PaCa-2 cells were treated with LY2228820 (2 μM), SCH772984 (625 nM), or combination for 72 h before staining with propidium iodide (PI) and Annexin V-FITC and analysis by flow cytometry. (B) Quantification of cells from (A) represented as the percentage of cells in each quadrant: PI-,Annexin V- (healthy); PI+,Annexin V- (necrosis); PI-, Annexin V+ (early apoptosis); PI+, Annexin V+ (late apoptosis). (C) AsPC-1, HPAC, and MIA PaCa-2 cells treated as in (A). Western blot analyses were performed for phosphorylated and total ERK, RSK, p38 and HSP27 as well as phosphorylated Rb, total MYC, Cyclin B1, Cyclin D1, p16, and PARP. Vinculin levels were determined to verify equivalent loading of total cellular protein. (D) AsPC-1, HPAC, and MIA PaCa-2 cells treated as in (A) and stained with propidium iodide for total DNA content. Graphs represent the percentage of cells in G0/G1, S, and G2/M of the cell cycle.

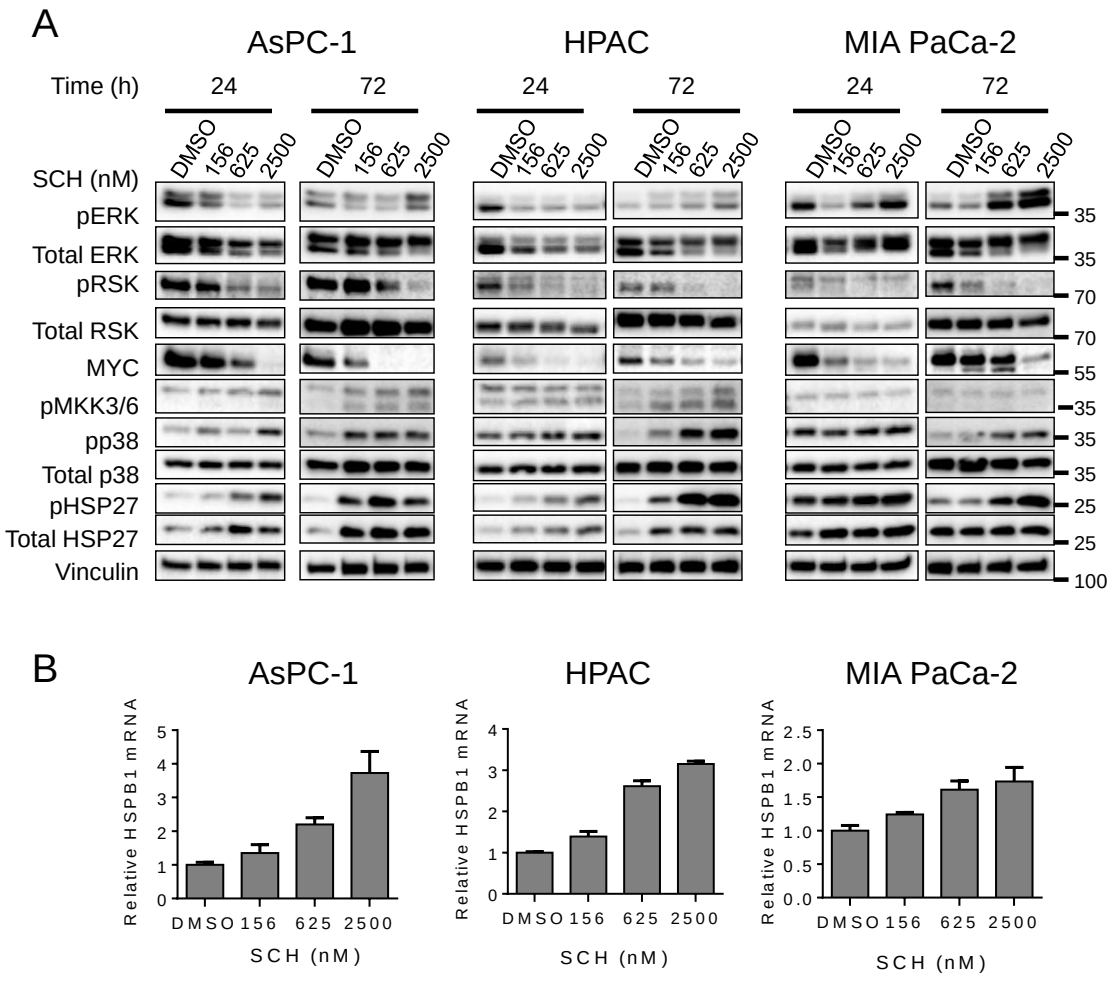


Figure 3-5. Inhibition of ERK causes increased signaling through the p38 pathway. (A) AsPC-1, HPAC, and MIA PaCa-2 cell lines were treated with the indicated concentrations of SCH772984 for 24 or 72 h. Western blot analyses were performed for phosphorylated and total ERK, RSK, MKK3/6, p38 and HSP27 as well as total MYC. Vinculin levels were determined to verify equivalent loading of total cellular protein. (B) AsPC-1, HPAC, and MIA PaCa-2 cell lines were treated with the indicated concentrations of SCH772984 for 24 h. Taqman quantitative PCR was performed to monitor changes in HSPB1 (HSP27) gene transcription and β-actin was used as an endogenous control.

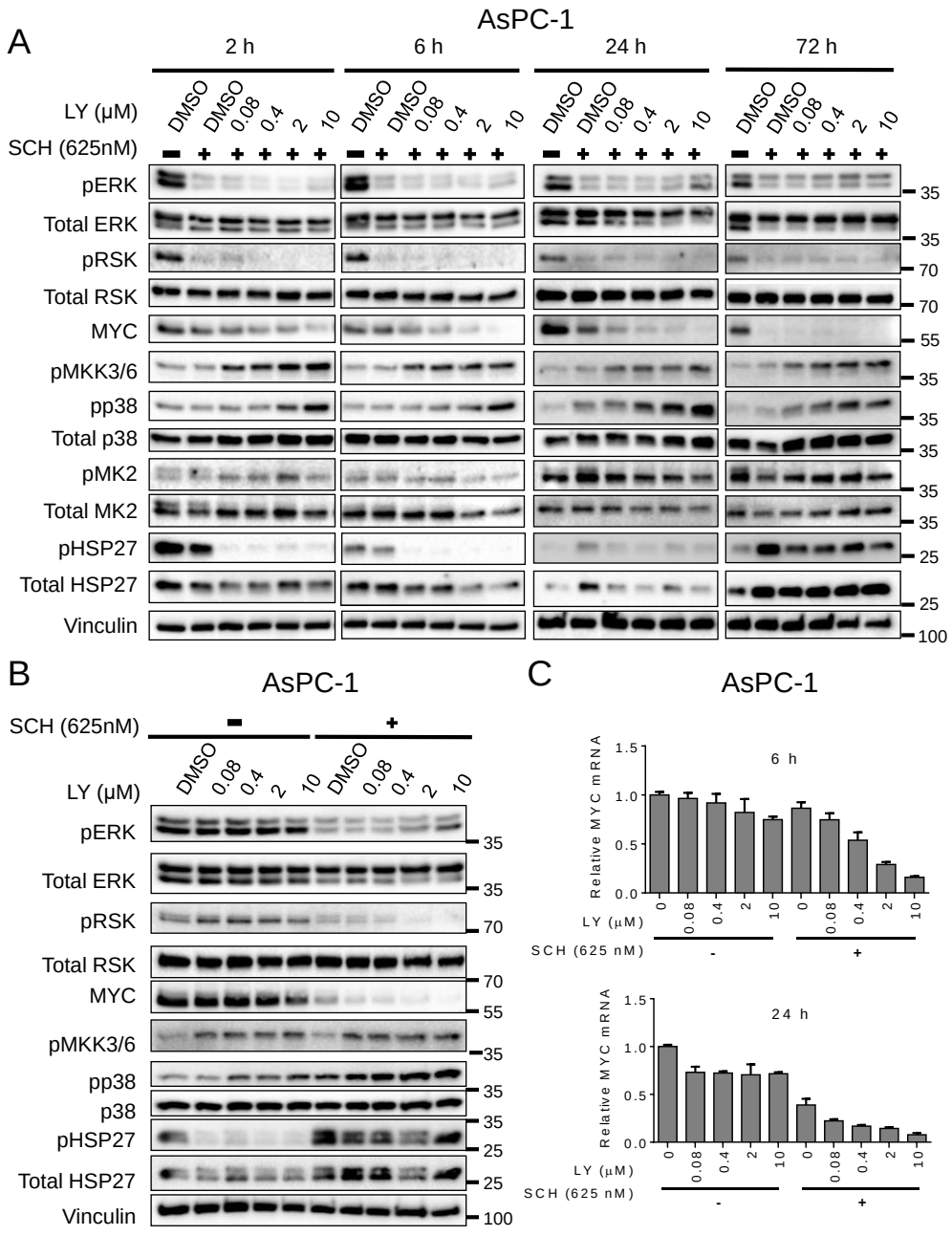


Figure 3-6. Concurrent inhibition of p38 enhances the loss of MYC protein induced by ERK inhibitor SCH772984. (A) AsPC-1 cells were co-treated with LY2228820 and SCH772984 at the indicated concentrations and times. Western blot analyses were done for phosphorylated and total ERK, RSK, MKK3/6, p38, MK2 and HSP27, and total MYC and vinculin. (B) AsPC-1 cells were co-treated with LY2228820 and SCH772984 at the indicated concentrations for 24 h. Western blot analyses were done for phosphorylated and total ERK, RSK, MKK3/6, p38, MK2 and HSP27, and total MYC and vinculin. (C) AsPC-1 cells were co-treated with LY2228820 and SCH772984 at the indicated concentrations for 6 or 24 h. Taqman quantitative PCR was performed to monitor changes in MYC gene transcription and β-actin was used as an endogenous control.

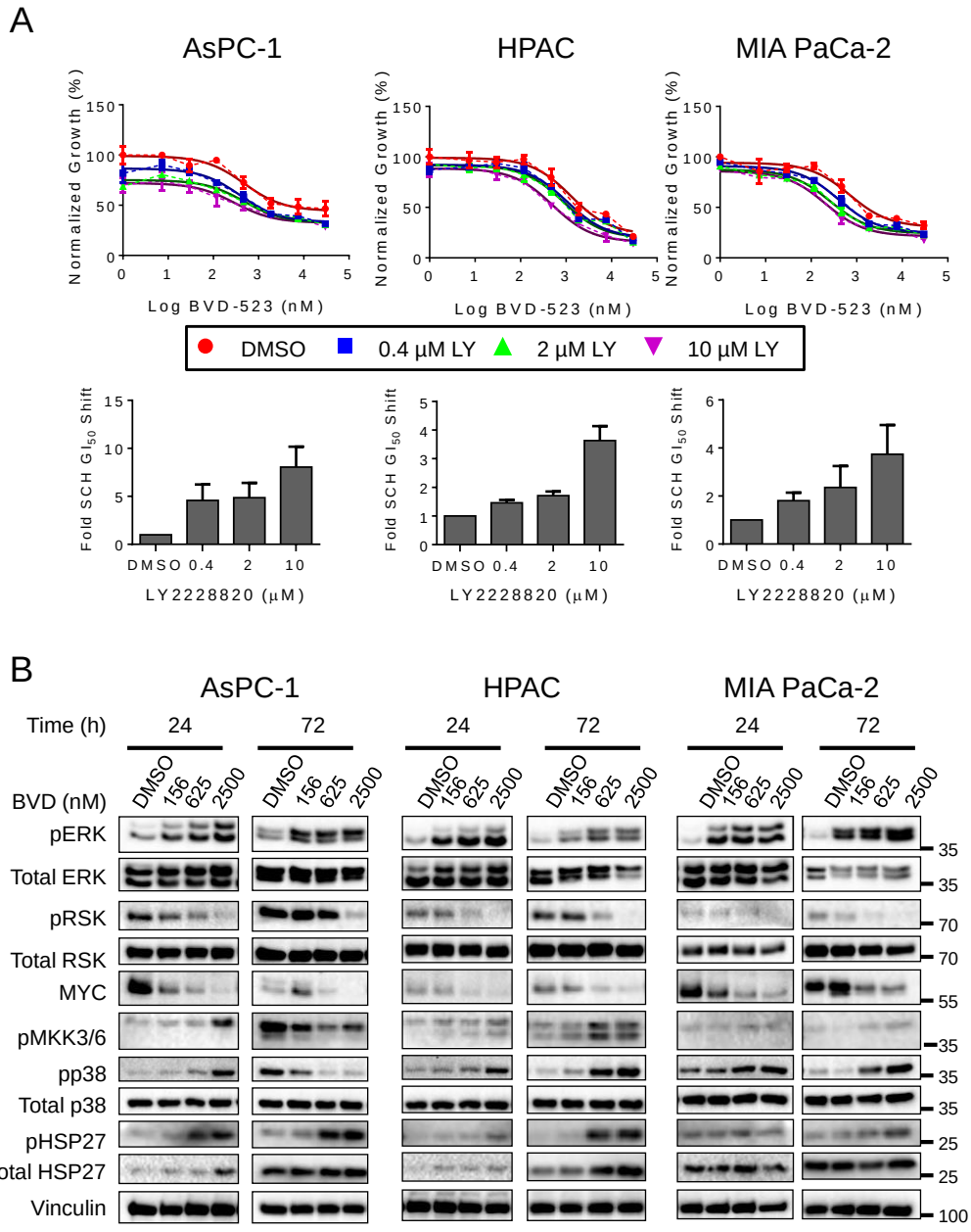


Figure 3-7. Concurrent inhibition of p38 also enhances the reduction in anchorage-dependent and -independent growth induced by ERK inhibitor BVD-523. (A) 72 h MTT assay of AsPC-1, HPAC, and MIA PaCa-2 cell lines treated with indicated concentrations of the p38 inhibitor LY2228820 and dose titration of the ERK inhibitor BVD-523. Each growth assay is representative of n=3 experiments. Lower panels show compiled MTT assay results, represented as fold GI_{50} shift, n=3 assays. (B) AsPC-1, HPAC, and MIA PaCa-2 cell lines were treated with the indicated concentrations of BVD-523 for 24 or 72 h. Western blot analyses were performed for phosphorylated and total ERK, RSK, MKK3/6, p38 and HSP27 as well as total MYC. Vinculin levels were determined to verify equivalent loading of total cellular protein.

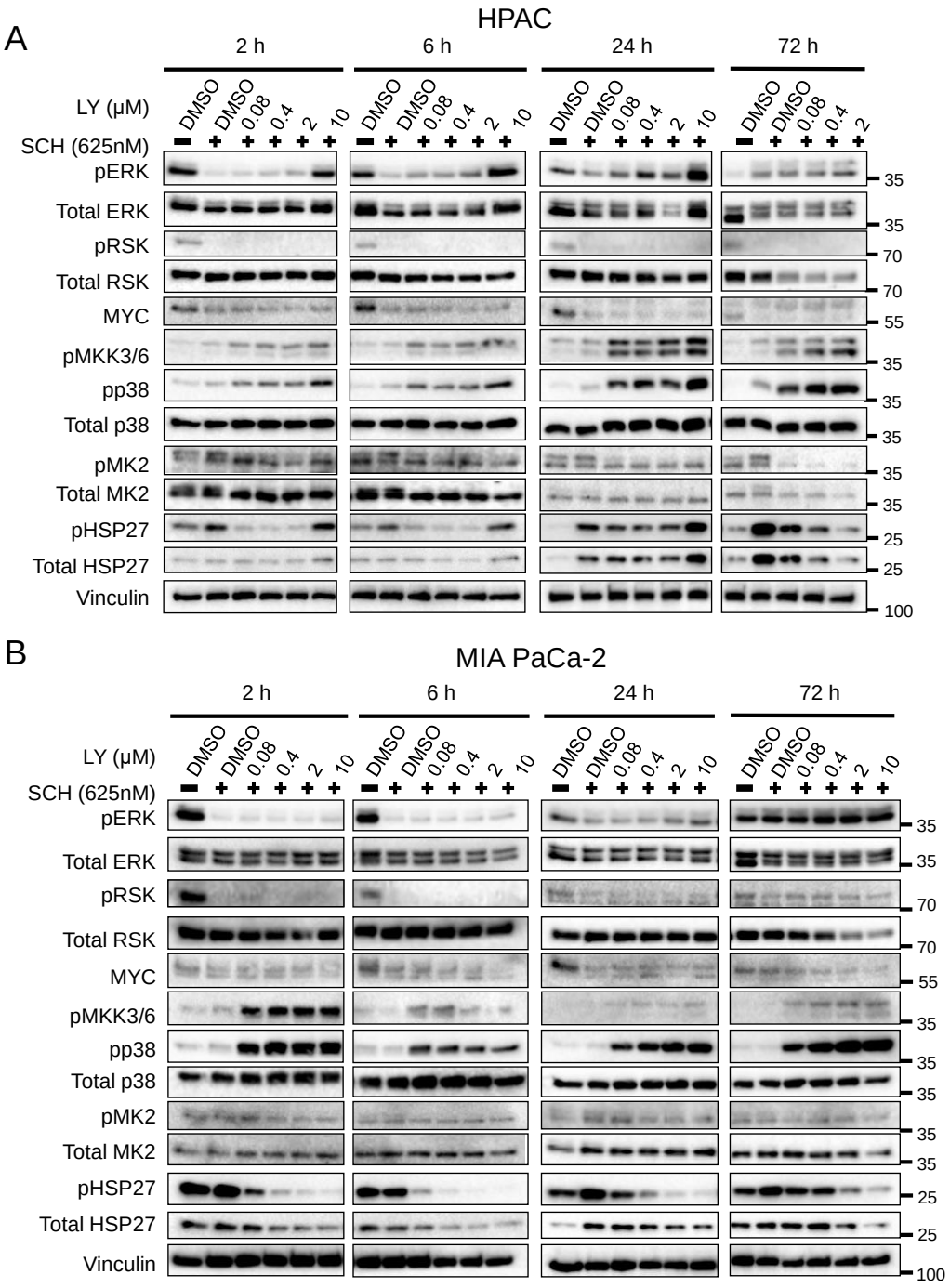


Figure 3-8. Concurrent inhibition of p38 can enhance ERK inhibitor treatment-induced loss of MYC protein. HPAC cells (A) and MIA PaCa-2 cells (B) were co-treated with LY2228820 and SCH772984 at the indicated concentrations and for the indicated times. Western blot analyses were done for phosphorylated and total ERK, RSK, MKK3/6, p38, MK2 and HSP27, and total MYC and vinculin.

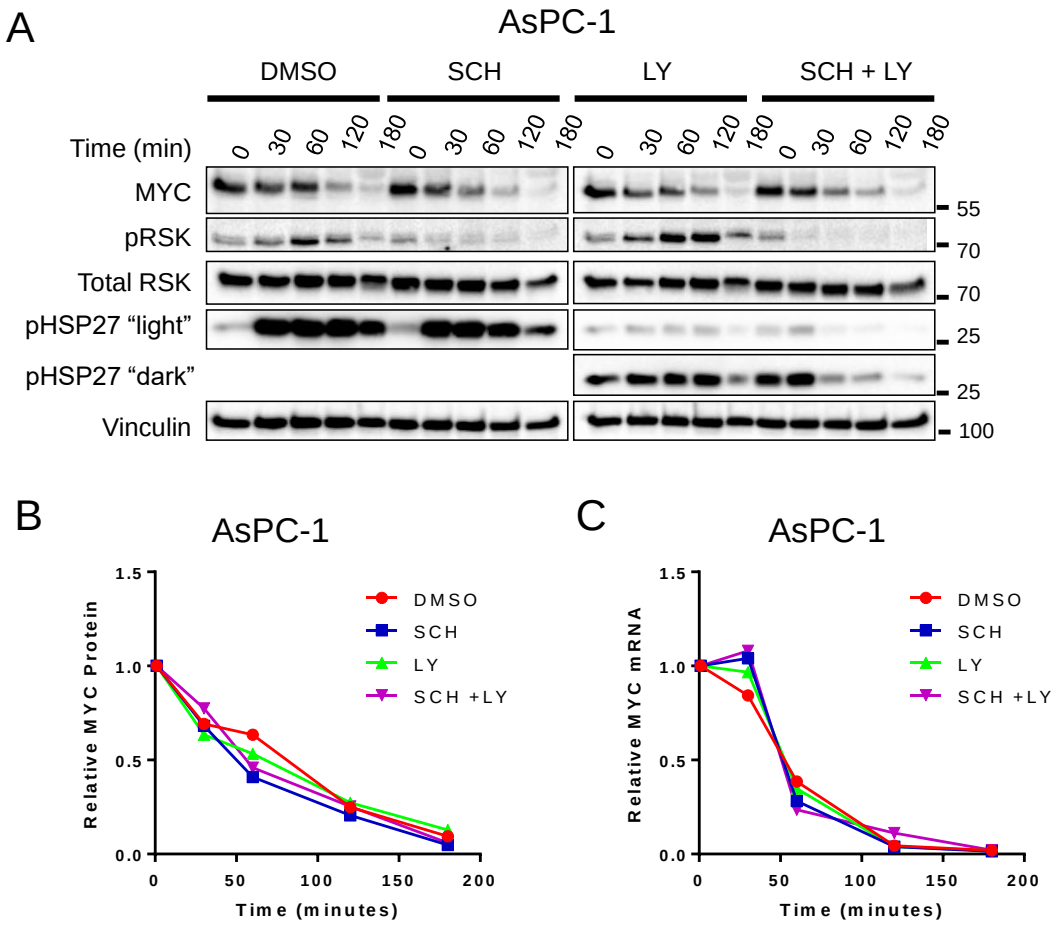


Figure 3-9. Concurrent p38 and ERK inhibition does not regulate MYC protein post-translationally or regulate MYC transcript stability. (A) AsPC-1 cells were treated with LY2228820 (2 µM), SCH772984 (625 nM), or combination in the presence of cycloheximide (50 µg/ml). Lysates were probed for MYC, pRSK and total RSK, and pHSP27. Vinculin served as a loading control. (B) Densitometric calculation of MYC protein levels from blot in (A). (C) AsPC-1 cells were treated with LY2228820 (2 µM), SCH772984 (625 nM), or combination in the presence of Actinomycin D, and quantitative PCR analysis of MYC mRNA was performed.

Chapter IV: CONCLUSIONS AND FUTURE DIRECTIONS

SUMMARY AND CONCLUSIONS

My work has addressed the role of ERK in both melanoma and pancreatic cancers harboring either *RAS* or *RAF* mutations. The RAF-MEK-ERK protein kinase cascade is one of the best characterized effector pathways downstream of RAS and has been the focus of pharmaceutical development at each node of the pathway, with inhibitors approved or in clinical trials for inhibition of RAF, MEK, or ERK. First, I used pharmacologic inhibitors of RAF, MEK or ERK to interrogate the role of ERK and showed that ERK drives the overexpression of PREX1 in both *NRAS*- and *BRAF*-mutant melanoma. Second, I extended my studies of pharmacologic inhibition of ERK to *KRAS*-mutant pancreatic cancer, as our lab has shown ERK inhibition to be an effective treatment for a subset of PDAC, and I identified MAPK14 (p38α) as a novel sensitizer to ERK inhibition. Pharmacologic inhibition of p38α/β with the clinical candidate inhibitor LY2228820 sensitizes PDAC cell lines to the ERK inhibitor SCH772984, and treatment with SCH772984 leads to the activation of the p38 MAPK cascade. Although my work has shown the importance of ERK in melanoma and PDAC, there are many unanswered questions about the role of ERK in each cancer type.

FUTURE DIRECTIONS

What transcription factor regulates PREX1 expression in *NRAS*- and *BRAF*-mutant melanomas?

My work demonstrates that ERK regulates the expression of PREX1 at both a transcriptional and post-transcriptional level in *NRAS*- and *BRAF*-mutant melanomas. However, the transcription factor that regulates PREX1 downstream of ERK in melanoma has not been identified. ERK1/2 can phosphorylate greater than 200 known substrates, including transcription factors such as MYC and ELK-1[142]. Previous work identified the transcription factor specificity factor 1 (SP1) as the regulator of PREX1 expression in prostate cancer and subsequent work found that SP1 also regulates PREX1 in breast cancer[125,150]. ERK directly phosphorylates SP1 and regulates its binding to DNA and transcriptional activity[185]. However, in both *NRAS*- (SK-MEL-119, Mel224) and *BRAF*-mutant (A375, WM2664) melanoma cell lines, knockdown of SP1 did not regulate the expression of PREX1 transcript (Figure 4-1A). This supports my previous findings that PREX1 overexpression in melanoma is regulated by a different mechanism than in either breast cancer or prostate cancer.

To further interrogate the question of how ERK regulates PREX1 transcriptionally, I also investigated additional transcription factor candidates. Microphthalmia-associated transcription factor (MITF) and MYC are two transcription factors which have been found to be regulated by ERK phosphorylation, with MITF

in particular playing a role in melanoma development[151,186,187]. I knocked down both MITF and MYC individually with shRNA in a panel of *NRAS*- and *BRAF*-mutant melanoma cell lines. As was seen with SP1, loss of either MITF or MYC did not reduce PREX1 mRNA levels; however, in the *NRAS*-mutant cell line SK-MEL-119, loss of MYC did reduce PREX1 protein and mRNA (Figure 4-1B,C). In future, to address what transcription factor regulates PREX1 expression downstream of ERK in *NRAS*- and *BRAF*-mutant melanoma, I would perform a promoter analysis using a luciferase driven reporter as described in Wong et al.[125]. Using fragments of the PREX1 reporter region, the binding sites of potential transcription factors can be identified. The individual candidate transcription factors can then be knocked down in a panel of *NRAS*- and *BRAF*-mutant melanoma lines and transcription of endogenous PREX1 can be measured by Taqman quantitative PCR. I would look for loss of transcription factor phosphorylation in the presence of ERK inhibitor.

Does PREX1 contribute to resistance to ERK MAPK cascade inhibitors?

One major issue facing efforts to successfully target the ERK MAPK cascade is inhibitor resistance, both innate and acquired. Multiple mechanisms of resistance to ERK MAPK cascade inhibitors, RAFi or MEKi, have been identified, including those that re-activate ERK or those that activate other pathways through kinome reprogramming[133]. Two identified potential mechanisms of resistance to the RAF inhibitor vemurafenib are mutations in PREX2, a related isoform to PREX1, and mutational activation of RAC1 at the P29 locus[102,188,189]. My studies confirmed that PREX1 regulates RAC1 activity and that RAC1 is a major regulator of invasion.

Under short term treatment with ERK MAPK pathway inhibitors, PREX1 expression is reduced, but the effect of long term ERK inhibition on PREX1 expression has not been explored.

In order to address the role of PREX1 in long term ERK MAPK inhibitor treatment, I would treat both *NRAS*- and *BRAF*-mutant melanoma lines with low doses of inhibitor (at the growth EC_{50}), gradually increasing inhibitor concentration over time until a resistant population of cells grew out. I would then look at PREX1 protein and transcript levels as well as RAC1-GTP levels in the resistant cell population compared to the parental cell lines. I hypothesize that PREX1 transcript and protein expression would rebound and that RAC1-GTP levels would be increased in the resistant cell population. There is also the question of whether loss of PREX1 would sensitize melanoma cell lines to ERK MAPK pathway inhibitors. As I demonstrated, loss of PREX1 alone does not have an effect on proliferation of melanoma cell lines. To address the question of whether loss of PREX1 would sensitize cells to inhibitors of the ERK MAPK cascade, I would knock down PREX1 using siRNA and then treat *NRAS*-mutant melanoma cells with either trametinib or SCH772984 and *BRAF*-mutant melanoma cells with either vemurafenib or SCH772984. I would then compare the EC_{50} and GI_{50} of growth between the mismatch (MM) and PREX1 siRNA treatment conditions and would predict that loss of PREX1 would shift both the EC_{50} and GI_{50} of melanoma cell line growth.

Do novel ERK inhibitors behave similarly to SCH772984?

Pancreatic ductal adenocarcinoma (PDAC), like melanoma, has been found to be dependent on the ERK MAPK cascade and can develop resistance to therapy.

However, only a subset of PDAC cell lines are sensitive to the ERK inhibitor SCH772984 while other cell lines display innate resistance to ERK inhibition[148]. My work has shown that one potential mechanism of resistance to SCH772984 is the p38 MAPK pathway and that treatment with the p38 inhibitor LY2228820 can sensitize PDAC cell lines to ERK inhibition. However, the status of SCH772984 as a clinical candidate ERK inhibitor going forward is uncertain, presenting a need for studying the efficacy of novel ERK inhibitors in PDAC. Currently, two additional ERK inhibitors are under clinical evaluation. It will be interesting to determine if p38 inhibition can also modulate the sensitivity of PDAC to these ERK inhibitors.

We utilized a novel ERKi whose structure and mechanism of action are distinct from those of SCH772984. Preliminary experiments with that inhibitor have shown that it exerted a similar growth suppression on plastic of MIA PaCa-2 and Pa14c compared to SCH772984 (Figure 4-2A). When expanded to a larger panel of both established and PDX PDAC cell lines, SCH772984 and the novel ERKi exhibited similar inhibition of growth on plastic, as measured by GI_{50} of growth (Figure 4-2B). Even though the growth inhibitory effects were similar between the two inhibitors, the novel ERKi and SCH7772984 may inhibit ERK activity with different dynamics and/or consequences. To address this question, I treated Pa14c cells with either the novel ERKi or SCH772984 for 4 or 24 h, because previous studies have shown a rebound of ERK phosphorylation by 24 h upon treatment with other ERKi[60,148]. Both inhibitors effectively reduced phosphorylation of RSK and reduced total levels of MYC to a similar degree with similar dynamics, while also causing a compensatory increased phosphorylation of MEK, the kinase immediately

upstream of ERK (Figure 4-2C). However, only SCH772984, which has both an ATP-competitive and an allosteric mechanism of action, blocked ERK phosphorylation by MEK, and it did so at both 4 and 24 h, whereas the novel ERKi failed to prevent ERK phosphorylation at either time point. This finding suggests that the novel ERKi is not able to forestall the paradoxical activation of ERK observed with earlier generation ERK inhibitors [59] and is less likely than SCH772984 to be effective at inhibiting growth long term.

Thus, although short term growth assays and signaling effects were similar between SCH772984 and the novel ERKi, both the duration of response and mechanisms of resistance may differ between these and other ERK inhibitors. I have identified p38 as a potential mechanism of resistance to SCH772984 and would extend the study to additional ERK inhibitors, such as the novel ERKi. First, would LY2228820 also sensitize PDAC cell lines to the novel ERKi, both on plastic and in soft agar? Second, would treatment with the novel ERKi activate the p38 MAPK pathway? I would address these questions by performing both MTT and soft agar assays as well as Western blot analysis, respectively, as described in the methods of Chapter 3. Additional mechanisms of resistance can be identified by a broader screening approach. One approach to identify potential mechanisms of resistance to the novel ERKi would be a CRISPR/Cas9-based loss-of-function genetic screen, as described in Chapter 3 for SCH772984, while a second approach would be drug sensitivity and resistance testing (DSRT), using a library of clinical inhibitors to identify synergy with ERKi[112]. The DSRT screening utilizes a library of clinical candidate or approved inhibitors for large scale combinatorial studies to

identify potential synergy between compounds in the library and a candidate inhibitor of interest, such as the novel ERKi.

What are the roles of ERK1 and ERK2 in PDAC?

I have shown that pharmacologic inhibition with three different ERK inhibitors, SCH772984, BVD-523, and a novel ERKi, reduces *KRAS*-mutant PDAC growth, and that both SCH772984 and BVD-523 can synergize with the p38 inhibitor LY2228820. However, there is an outstanding question of what the differential roles of ERK1 and ERK2 are in driving PDAC growth. ERK1 and ERK2 share high sequence identity, with 86% and 88% total sequence identity and kinase domain identity, respectively, and are thought to have some functional redundancy[142,190,191]. However, it has been demonstrated that genetic knockouts of ERK1 or ERK2 yield strikingly different results in mouse models; loss of ERK1 results in viable offspring while loss of ERK2 is embryonic lethal, indicating that ERK1 activity is not sufficient to rescue loss of ERK2[192,193]. Recent evidence has also suggested alternate roles for ERK1 and ERK2 in maintenance of ERK/MAPK-dependent tumors. Loss of either ERK1 or ERK2 was sufficient to cause reduced proliferation *in vitro* in both mesothelioma and *BRAF*-mutant melanoma, including induction of apoptosis in melanoma; however, it was loss of ERK2 that reduced tumor formation and growth *in vivo* in mesothelioma[194,195].

In order to study the roles of ERK1 and ERK2 in *KRAS*-mutant PDAC, I have employed a genetic knockdown approach to study each isoform individually. Using shRNA to selectively knockdown ERK1 (MAPK3) or ERK2 (MAPK1), with two independent shRNAs per gene, I was able to successfully knock down ERK1 and

ERK2 protein expression in a panel of PDAC cell lines, and to confirm that each shRNA was specific for its respective isoform (data not shown). With the exception of the Capan-1 cell line, loss of either ERK1 or ERK2 was sufficient to inhibit 2D clonogenic growth of PDAC cells on plastic (Figure 4-3). This finding of reduced PDAC growth upon genetic knockdown of either ERK1 or ERK2 also extended to colony formation in a 3D soft agar assay (data not shown). Collectively, my preliminary data show strong evidence for differential roles for ERK1 and ERK2 in sustaining PDAC growth, with each isoform unable to compensate for the loss of the other.

To further interrogate the role of ERK1 and ERK2 in maintenance of *KRAS*-mutant PDAC growth, I would use shRNA knockdown of expression to identify substrates regulated downstream of ERK1 or ERK2. First, a candidate approach using phosphor-specific antibodies can be used to identify ERK1 or ERK2 regulated substrates, including transcription factors (Fos^{S374}, Jun^{S63}, STAT1^{S727}, STAT3^{S727}), adhesion- and motility-related proteins (FAK^{S910}, PAK1^{T212}, Paxillin^{S383}), and ERK regulatory proteins (MKP1/2^{S359})[196-201]. To approach identification of ERK1- or ERK2-selective substrates in an unbiased fashion, I would employ a SILAC (stable isotope labeling by amino acids in cell culture) based mass spectrometry approach to identify phosphorylated proteins downstream of ERK1 and/or ERK2 (Figure 4-4). I would selectively knock down ERK1, ERK2 or both isoforms using a doxycycline (DOX) inducible shRNA vector system[202], which I have optimized for use with ERK1 and ERK2 shRNA sequences in PDAC cell lines (data not shown). Use of a DOX inducible knockdown system can allow for control of ERK knockdown in a dose- and

time-dependent manner, while a SILAC labeling approach would allow for -DOX (Light Lysine/Arginine) and +DOX (Heavy Lysine/Arginine) conditions to be directly compared in the same mass spectrometry run. Bioinformatics sequence analysis would be done using databases such as PHOSPHOSITE and SCANSITE to filter results for proteins containing either the DEF or DEJL docking domains for ERK, as well as the putative ERK phosphorylation site Pro-X-Ser/Thr-Pro. ERK1- and/or ERK2-selective substrates identified by mass spectrometry can then be validated by Western blot analysis of those phosphorylated substrates

Do p38 and ERK1/2 share common downstream substrates in PDAC and do p38α and p38β have overlapping functions?

p38, much like ERK, is the terminal node of a MAPK cascade and is represented by two isoforms, MAPK14 (p38α) and MAPK11 (p38β), that are often assumed to have redundant functions in phosphorylating and regulating their many downstream substrates[191]. The p38 inhibitor LY2228820 can inhibit both p38α and p38β to a similar degree[168], so a genetic knockdown approach would have to be employed to study the specific role of each isoform in sensitization of PDAC to ERK inhibition. First, I would use siRNA to selectively knock down MAPK14 or MAPK11 or both isoforms concurrently. I would treat the PDAC knockdown cell lines with SCH772984 to see if loss of one or both isoforms can also sensitize cells to ERK inhibitor in anchorage-dependent (MTT) and anchorage-independent (soft agar) growth assays.

My results shown in Chapter 3 demonstrate that p38 and ERK inhibitors can synergize in PDAC, potentially through a cell cycle arrest mechanism both dependent on and independent of MYC downregulation. However, the connection between the two pathways is not known beyond a potential connection to MYC, as seen in the AsPC-1 cell line. I would apply the phospho-proteomics approach described for studying the ERK1 and ERK2 isoforms to studying the effects of SCH772984, LY222820, or combination treatment in AsPC-1, HPAC, and MIA PaCa-2. I would compare the phospho-proteome profile between the three treatment conditions to identify potential shared candidate substrates that are either downregulated or upregulated in response to inhibitor treatment. Alternatively, reverse phase protein array (RPPA) can be applied for a discrete set of substrates downstream of the ERK and/or p38 MAPK cascades and other phosphorylated and total proteins of interest[203]. Collectively, these techniques would reveal a connection, or multiple downstream connections, between ERK and p38 that regulates the response to combined inhibition of both pathways. These proposed studies also have the potential to identify a biomarker that may be predictive of PDAC response to combined p38 and ERK inhibition.

Would concurrent inhibition of ERK and p38 result in tumor regression *in vivo*?

My results have shown that the p38 inhibitor LY2228820 can enhance the anti-tumor effects of the ERK inhibitor SCH772984 *in vitro*. However, does the synergy between p38 and ERK inhibitors hold true *in vivo* and does it result in a

cytotoxic or cytostatic response in PDAC tumors? SCH772984 treatment alone resulted in tumor regression in some PDAC xenografts while reducing, but not stopping, tumor growth in others[148]. I would investigate whether the addition of LY2228820 would enhance the anti-tumor effect of SCH772984 in an orthotopic xenograft model, where the final volume of tumor from each treatment group would be compared. I could also employ a subcutaneous xenograft model, where I would monitor tumor growth over time. Concomitantly, I would also investigate whether treatment with an ERK inhibitor can induce p38 pathway activity in tumors in a similar fashion as seen in cell culture models and if phosphorylation of HSP27 is an accurate marker of LY2228820 activity *in vivo*.

Next, there is the question of what role p38 activation may be playing in human tumors in response to targeted therapy of the ERK cascade. Given that ERK inhibitors are still in the early stages of clinical trials in all tumor types, and initial clinical trials in PDAC patients have recently begun (NCT02608229), it may be difficult at present to obtain the patient tissues needed to assess p38 in clinical tumor response and resistance in PDAC. However, our CRISPR results suggest that p38 may play a role in sensitizing multiple *KRAS*-mutant cancer types, including lung and colorectal cancers, to both ERK and MEK inhibitors. I hypothesize that p38 phosphorylation and pathway activation in response to RAF, MEK, or ERK inhibitors is a common mechanism shared by RAS-driven cancers dependent on the pathway and can potentially be predictive of response to therapy in the clinic.

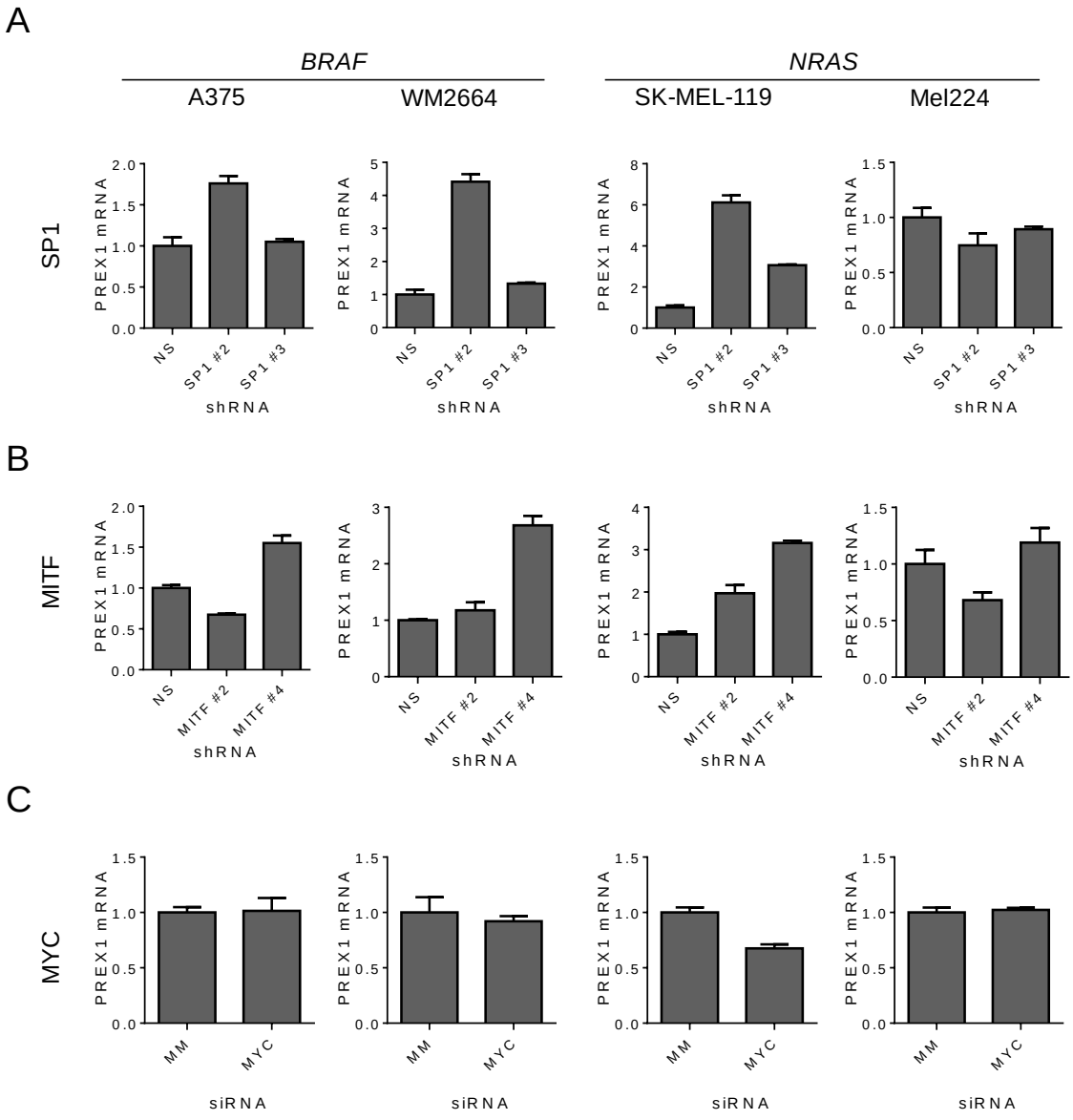


Figure 4-1. Not all transcription factors known to be regulated by ERK regulate PREX1 transcription. *BRAF*-mutant cell lines A375 and WM2664 and *NRAS*-mutant cell lines SK-MEL-119 and Mel224 were lentivirally transduced with RNAi, and PREX1 mRNA levels were measured by Taqman qPCR. Cells were transduced with (A) nonspecific (NS) or SP1 shRNA; (B) nonspecific (NS) or MITF shRNA; or (C) mismatch (MM) or MYC siRNA.

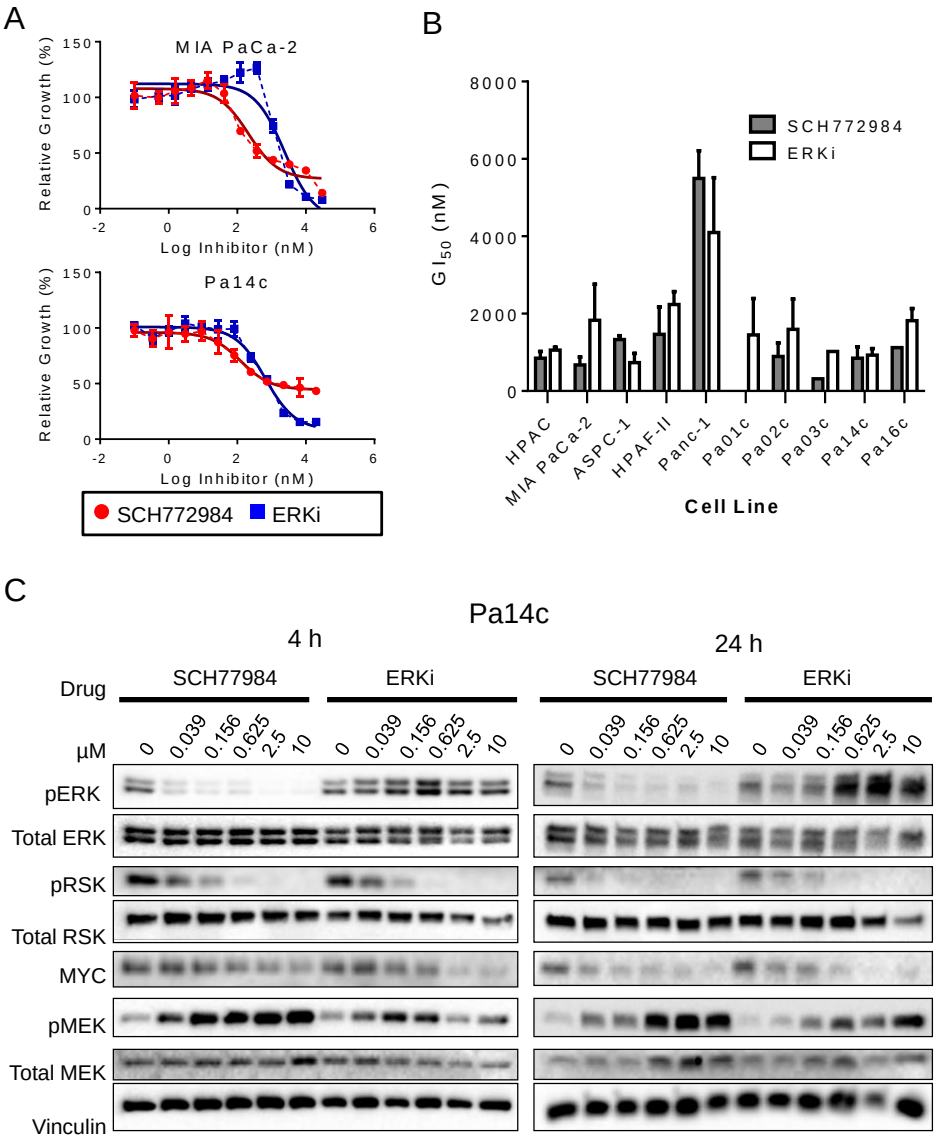


Figure 4-2. ERK inhibitors SCH772984 and novel ERKi display similar growth inhibition and signaling effects in PDAC cell lines. (A) MIA PaCa-2 and Pa14c cells were treated with SCH77284 or ERKi for 72 h and viable cells were measured by MTT. (B) Calculations of GI_{50} for SCH77294 and ERKi in a panel of 5 established and 5 patient-derived xenograft (PDX) *KRAS*-mutant PDAC cell lines. (C) Pa14c PDX cells were first treated with the indicated concentrations of SCH772984 or ERKi for 4 and 24 h, and lysates were immunoblotted for pERK, total ERK, pRSK, total RSK, MYC, pMEK and total MEK, with vinculin serving as a loading control**.**

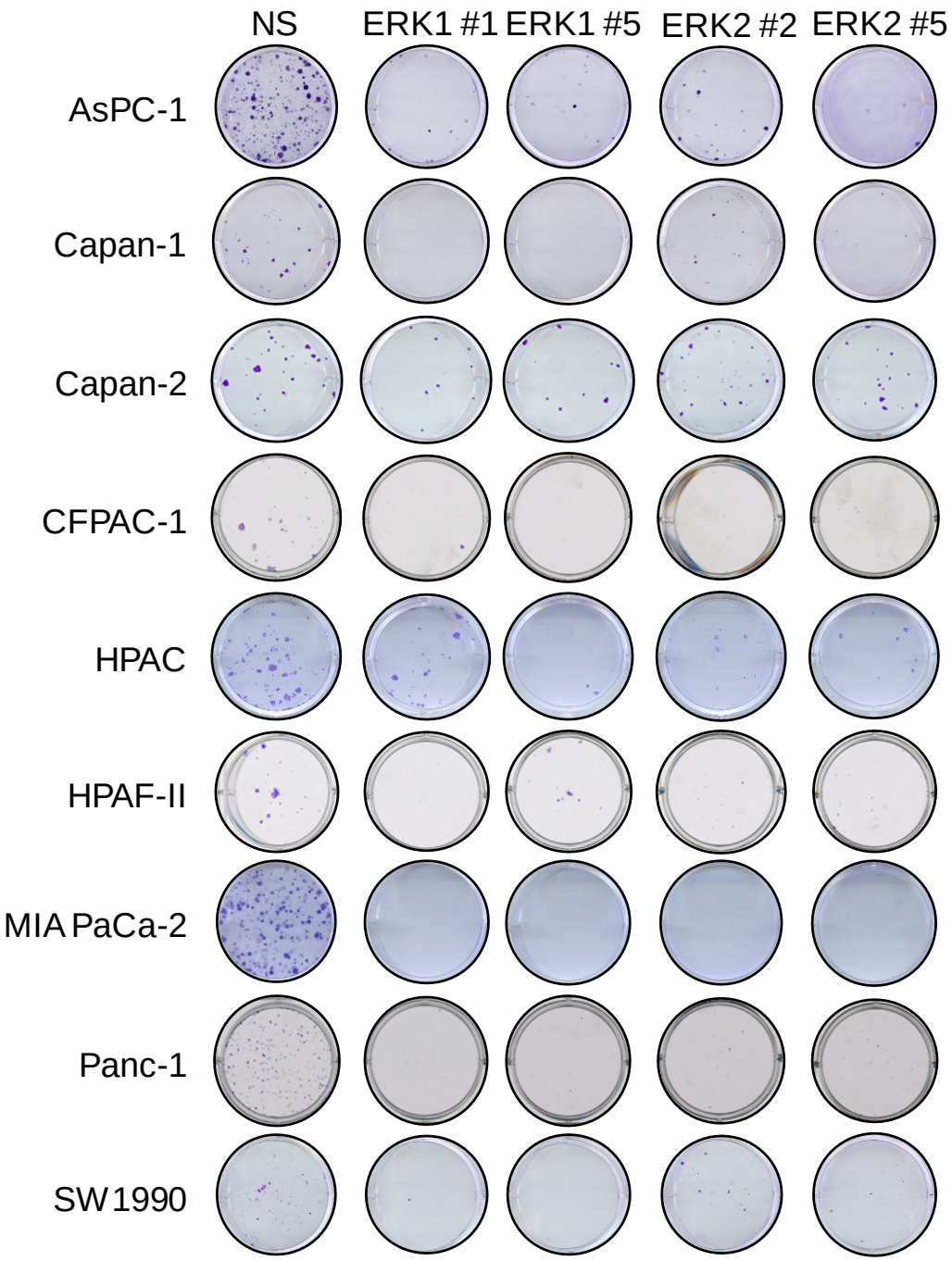


Figure 4-3. Loss of ERK1 or ERK2 reduces PDAC growth on plastic. 2D clonogenic assays, stained after 10 or 14 days, of *KRAS*-mutant PDAC cell lines after transduction with non-specific (NS), ERK1 or ERK2 shRNA. Images of each well are representative of assays run in triplicate.

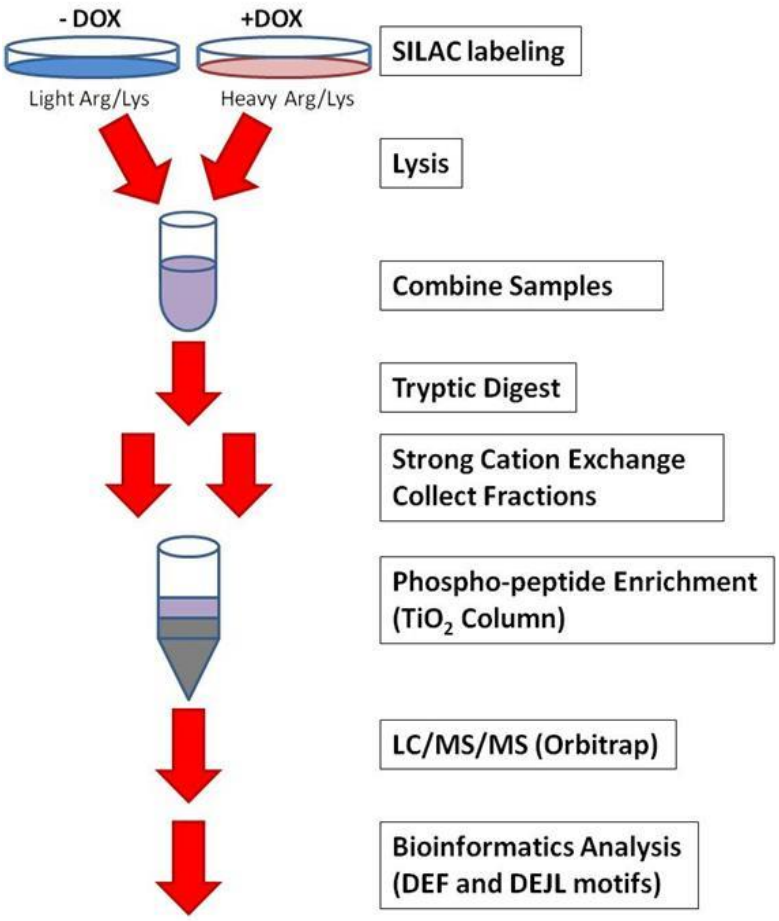


Figure 4-4. Phospho-peptide analysis workflow.

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