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Molecular Mechanisms Regulating MYC and PGC1β Expression in Colon Cancer

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MOLECULAR MECHANISMS REGULATING MYC AND PGC1β EXPRESSION IN COLON CANCER

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To my parents, Mark and Terri McCall,

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no matter what life throws in the way.

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Identification and characterization of pathways specific to tumor cell survival, but absent in normal tissues, provide opportunities to develop effective cancer therapies with reduced toxicity to the patient. Kinase suppressor of Ras 1 (KSR1) is required for the survival of colorectal cancer (CRC) cells, but dispensable in normal cells. Using KSR1 as a reference standard, we identified EPH (erythropoietin-producing hepatocellular carcinoma) receptor (EPHB4) as a KSR1 functional analog.

We show here that, like KSR1, EPHB4 is aberrantly overexpressed in human CRC cells and selectively required for their survival. Both KSR1 and EPHB4 support tumor cell survival by promoting the expression of downstream targets Myc and the transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator 1β (PGC1β). While KSR1 promotes the aberrant expression of Myc and PGC1β protein via a post-transcriptional mechanism, EPHB4 has a greater effect on Myc and PGC1β expression due to its ability to also elevate mRNA levels. Subsequent analysis of the post-transcriptional regulation demonstrates that KSR1 promotes the translation of Myc. These findings reveal novel KSR1- and EPHB4-dependent signaling pathways supporting the survival of CRC cells through regulation of Myc and PGC1β, suggesting that inhibition of these pathways should be selectively toxic to colorectal tumors.

We demonstrate that MEK inhibition reduced expression of Myc and PGC1β in CRC cells. To define the pathways that regulate expression of Myc and PGC1β, we examined the downstream effects of MEK1/2 substrates ERK1/2 and HSF1. Depletion of

HSF1 increases Myc and PGC1β expression, while ERK1/2 inhibition decreases their expression.

The data presented here define multiple mechanisms regulating Myc and PGC1β expression, suggesting that tight regulation of this pathway is critical in normal cells. Aberrant expression of Myc and PGC1β contributes to the proliferation and survival of breast and renal cell carcinomas. We show that this pathway is also critical for CRC survival and is ERK-dependent. Together, these data reveal that tumor cells in various cancers require Myc-dependent expression of PGC1β to promote cell survival, which may be exploited in the development of new cancer therapeutics.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

3D	Three-dimensional
4E-BP1	Eukaryotic initiation factor 4E binding protein 1
5' UTR	5' untranslated region
A, Ala	Alanine
ANOVA	Analysis of variance
APC	Adenomatous polyposis coli
ATP	Adenosine 5'-triphosphate
BafA1	Bafilomycin A1
BAT	Brown adipose tissue
BCA	Bicinchoninic acid
β-TrCP	Beta-transducin repeat containing E3 ubiquitin protein ligase
C, Cys	Cysteine
CA	Conserved area
CAAX	C – cysteine, A – aliphatic amino acid, X – any amino acid
CaM	Calmodulin
CaMKII	Calmodulin kinase II
cAMP	Cyclic adenosine 3',5'-monophosphate
CBP	CREB binding protein
CDK	Cyclin-dependent kinase
C/EBPβ	CCAAT/enhancer-binding protein beta
CHX	Cycloheximide
COAD	Colon adenocarcinoma
Cont	Control
CR1-3	Conserved region 1-3

CRC	Colorectal cancer
CRD	Cysteine-rich domain
CREB	cAMP-response element binding protein
CTKD	C-terminal kinase domain
D, Asp	Aspartic acid
DA	Dopamine
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E, Glu	Glutamic acid
E	Embryonic day
EC_{50}	Effective concentration which induces 50% of maximal response
ED	Euclidean distance
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eIF4A	Eukaryotic initiation factor 4A
eiF4E	Eukaryotic initiation factor 4E
EPH	Erythropoietin-producing hepatocellular carcinoma
EPHB4	EPH receptor B4
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ERRα	Estrogen-related receptor alpha
Ets	E-twenty-six
F, Phe	Phenylalanine

FA	Fatty acid
FAS	FA synthase
FBW7	F-box and WD repeat domain-containing 7
FGF	Fibroblast growth factor
FOXO1	Forkhead box O1
FST	Forced swim test
FTase	Farnesyltransferase
FTI	Farnesyltransferase inhibitor
FUSION	Functional Signature Ontology
GAB	Grb2-associated binding partner
GAP	GTPase-activating protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCN5	General control of amino acid synthesis 5
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GGTase1	Geranylgeranyltransferase 1
GPCR	G protein-coupled receptor
Grb2	Growth factor receptor-bound protein 2
GSK3	Glycogen synthase kinase 3
GTP	Guanosine 5’-triphosphate
HCEC	Human colonic epithelial cell
HCl	Hydrochloric acid
HDAC2	Histone deacetylase 2
HER2	Human epidermal growth factor receptor 2
HMGCR	HMG-CoA reductase
H-Ras	Harvey Ras

HRPT	Hypoxanthine-guanine phosphoribosyl transferase
HS	Heat shock
HSF1	Heat shock factor 1
HSR	Heat shock response
HSP	Heat shock protein
hTERT	Human telomerase reverse transcriptase
ICMT1	Isoprenylcysteine carboxymethyltransferase-1
IGF1	Insulin-like growth factor 1
IMP	Impedes mitogenic signal propagation
IRES	Internal ribosome entry site
IQGAP	IQ motif containing GTPase activating protein 1
JNK	c-Jun N-terminal kinase
K, Lys	Lysine
kDa	kilodalton
K-Ras	Kirsten Ras
KSR1/2	Kinase suppressor of Ras1/2
L, Leu	Leucine
LTP	Long-term potentiation
LXR	Liver X receptor
MARK	Microtubule affinity regulating kinase
MEF	Mouse embryonic fibroblast
MEK	Mitogen-activated protein kinase kinase
MG132	Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (Z-Leu-Leu-Leu-al)
MMTV	Mouse mammary tumor virus
MP1	MEK partner 1
MPNST	Malignant peripheral nerve sheath tumor

MS	Maternal separation
MSK	Mitogen- and stress-activated protein kinase
Mst1	Macrophage stimulating 1
mTOR	Mammalian target of rapamycin
mTORC	Mammalian target of rapamycin complex
MXI1	MAX-interacting protein 1
Myc	c-Myc
NaCl	Sodium chloride
NaF	Sodium fluoride
NCS	Neural stem cell
NEAA	Non-essential amino acids
NF1	Neurofibromin 1
N-Ras	Neuroblastoma Ras
NTKD	N-terminal kinase domain
P, Pro	Proline
PAGE	Polyacrylamide gel electrophoresis
PAK	p21-activated kinase
PARP	Poly-ADP ribose polymerase
PBS	Phosphate-buffered saline
PC	Pearson correlation
PD	Parkinson’s disease
PDCD4	Programmed cell death 4
PDGFR	Platelet-derived growth factor receptor
PGC1α/β	Peroxisome-proliferator activator gamma coactivator 1 alpha/beta
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase

PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMSF	Phenylmethanesulfonyl fluoride
polyHEMA	Poly-2-hydroxyethyl methacrylate
PPARγ	Peroxisome proliferator-activated receptor-gamma
PRC	PGC1-related coactivator
PTB	Phosphotyrosine-binding domain
PVDF	Polyvinylidene difluoride
Q, Gln	Glutamine
RT-qPCR	Quantitative reverse transcription – polymerase chain reaction
RAPTOR	Regulatory-associated protein of mTOR
RASSF	Ras-associated factor
RBD	Ras-binding domain
RCE1	Ras-converting enzyme 1
RIPA	Radio immunoprecipitation assay
RNA	Ribonucleic acid
RPM	Rotations per minute
RSEM	RNA-Seq by expectation-maximization
RSK	p90 ribosomal S6 kinase
RTK	Receptor tyrosine kinase
S, Ser	Serine
S6K	p70 S6 kinase
SCD1	Stearoyl-CoA desaturase
SD	Standard deviation
SDS	Sodium dodecyl sulfate

Sef	Similar expression to FGF
SEM	Standard error of mean
SH2	Src homology 2
Shc2	SHC (Src homology 2 domain containing) transforming protein 2
SIRT1	Sirtuin 2 ortholog 1
SOS	Son of sevenless
SREBP1	Sterol regulatory element-binding protein 1
STAT	Signal transducer and activator of transcription
T, Thr	Threonine
TAG	Triacylglycerol
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween-20
TCF	Ternary complex factor
TCGA	The Cancer Genome Atlas
TIAM	T cell invasion and metastasis-inducing 1
Tm	Melting temperature
Tris	Tris(hydroxymethyl)aminomethane
TST	Tail suspension test
VEGF	Vascular endothelial growth factor
Y, Tyr	Tyrosine
Wnt	Wingless-related integration site
XIAP	X-linked inhibitor of apoptosis
WT	Wild-type

Chapter 1: Introduction

Ras signaling

Discovery of ras oncogenes

Ras was originally identified due to the transforming properties of the rat-derived Harvey and Kirsten murine sarcoma retroviruses (1-3). The cellular homologs of the viral Harvey and Kirsten *ras* sequences were first identified in the rat genome in 1981 (4), and were subsequently identified in mouse (5) and human genomes (6). The Harvey sarcoma virus-associated oncogene was named *H-ras* and the Kirsten sarcoma virus form was termed *K-ras* in mammals. It was soon identified that human tumors often contained mutated and constitutively activated forms of Ras proteins, including cancer cells of bladder, colon, and lung origin (7-10). A third *ras*-related gene was cloned from neuroblastoma and leukemia cell lines in 1983, it was termed *N-ras* (11-14).

Meanwhile, much work was focused on determining how the oncogenes differed from the wild-type alleles. Point mutations found predominantly in codon 12, but also less commonly in codons 13 and 61, resulted in amino acid substitutions in the encoded Ras proteins (15-18). The characterization of *ras* as a true oncogene was questioned when it was discovered that *H-ras* alone could not transform freshly isolated rodent embryonic cells, but it was subsequently shown that H-Ras^{G12V} could transform primary cells that had been previously immortalized with carcinogens (19) or transfected with *myc*, SV40 large T antigen, or adenovirus *E1A* oncogene (20, 21). These findings suggested that Ras proteins can only transform cells that have undergone predisposing changes, such as acquisition of indefinite proliferation in culture (22-24). The identification of *ras* mutations in patient tumors, but not normal tissue, was an important validation that the *ras* mutations identified in cell lines were not merely artifacts of cell culture (25-27).

Approximately 30% of all human tumors screened carry a mutation in one of the canonical *ras* genes (*K-ras*, *H-ras*, *N-ras*) (28). Mutations in *ras* genes predominantly affect the *K-ras* locus, with oncogenic *ras* mutations being detected in 20-25% of all tumors samples screened, whereas the rates for *H-ras* and *N-ras* are 3% and 8%, respectively (28). Further analyses have shown that there are specific mutations that correlate with certain cancer types, for example *K-ras* mutations are present in a majority of pancreatic ductal adenocarcinomas and a large percentage of lung and colon tumors, but they occur rarely in bladder cancers where *H-ras* is the most frequently mutated isoform (28, 29). Additionally, *N-ras* mutations are frequently identified in hematopoietic tumors and malignant melanomas, whereas *K-ras* and *H-ras* mutations in melanomas are rare (28). *H-ras* mutations are the least frequent, but are prevalent in bladder cancers (28). *K-ras* has two alternative splice variants that result from differential splicing at exon 4, K-Ras4A and K-Ras4B (30, 31). Recent work in colorectal cancer has demonstrated that K-Ras4A is associated with better overall survival, while K-Ras4B is associated with significantly larger tumor size (32).

Interestingly, oncogenic hotspots are concentrated around two codons of the primary nucleotide sequence of all *ras* family members, these include codons 12 and 61. However, the frequency of mutations at each site varies among the three main *ras* family members. Approximately 99% of the detected *K-ras* mutations occur at glycine 12 (G12, 86%) and glycine 13 (G13, 13%), whereas the remaining 1% occur at glutamic acid 61 (Q61) (28). Oncogenic mutations in *N-ras* genes have the highest rate of mutations at Q61 (60%), and lower rates at codons 12 (24.4%) and 13 (12.7%) (28). Finally, *H-ras* has another pattern of mutations, with the highest percentages detected in codon 12 (54%), followed by codon 61 (34.5%), and then codon 13 (9%) (28). While early reports described *ras* amplification in some tumors and cell lines (33-35), more recent reports suggest that *ras* amplification is not a predictive marker of tumor aggressiveness (36, 37).

Activation

Heterotrimeric G proteins toggle between inactive GDP-bound and active GTP-bound states (38, 39). *H-ras* was initially described to bind guanine nucleotides, suggesting that it may possess intrinsic GTP hydrolysis (or GTPase) activity (40, 41) that turned off active signaling. In 1984, three groups reported that mutated Ras oncoproteins differed functionally from the normal counterparts in that they had impaired GTPase activity (42-44). These studies suggested that Ras proteins were consistently in an active state and, therefore, may promote continual downstream signaling. However, the extent of GTPase impairment did not always correlate with transformation indicating that it was necessary, but not sufficient, to drive aberrant Ras activation (45, 46). The first inactivators of Ras signaling were found to possess GTPase-promoting functions that preferentially acted upon normal, but not oncogenic N-Ras and H-Ras (43, 47, 48). Inactivators, such as son of sevenless (SOS) and neurofibromin-1 (NF-1), were named guanine nucleotide exchange factors (GEFs).

Oncogenic Ras mutants have impaired ability to hydrolyze GTP, either intrinsically or in response to GTPase activating proteins (GAPs). The oncogenic mutations at residues G12, G13, and Q61 are located in the N-terminal lobe of the Ras catalytic site (49). When Ras interacts with GAPs, the GAP contains an arginine finger that inserts into the Ras active site and provides a positive charge to stabilize the negative charges that accumulate during hydrolysis (50). Normally, the arginine finger interacts with G12. Thus, mutations at this residue inhibit the proper transition state complex with GAPs resulting in decreased rates of hydrolysis (51). It is thought that the increased side-chain in G13 mutations would interrupt this transition state as well. GAPs also increase hydrolysis by ordering the Ras active site, specifically placing Q61 in the active site (52). In the active site, the larger Q61 forms hydrogen bonds with both the GAP arginine finger and a water molecule (52). Substitutions at Q61 are unable to form these dual hydrogen bonds reducing overall rates of hydrolysis.

Importantly, all Ras isoforms share amino acid sequence identity in all of the regions responsible for GDP/GTP binding, GTPase activity, and effector interactions. However, another key determinant of Ras transformational activity is the post-translational lipid processing that localizes Ras to the cell membrane (53-56). The molecular mechanism of Ras lipid processing is a stepwise progression, the first of which is the addition of a farnesyl isoprenoid lipid to the C-terminal CAAX motif of Ras (Fig 1.1) (57-59). This reaction is catalyzed by the enzyme farnesyl transferase (FTase). Subsequent studies showed that this prenylation reaction is followed by proteolytic cleavage of the AAX sequence by Ras-converting enzyme-1 (RCE1) and the carboxymethylation of the terminal cysteine residue by the enzyme isoprenylcysteine carboxymethyltransferase-1 (ICMT1) (60, 61). These modifications at the CAAX motif appear to be essential for Ras association with the plasma membrane, but additional modifications are necessary for full membrane recruitment. The C-terminal lysine residues of K-Ras4B are sufficient to anchor it to the plasma membrane, but H-, N-, and K-Ras4A need an additional palmitoylation step, catalyzed by palmitoyltransferase (PTase), which adds a palmitoyl group to the C-terminus upstream of the cysteine residue (61). The modification by farnesyltransferase occurs in the endoplasmic reticulum (ER), whereas the palmitoylation step occurs after shuttling through the Golgi apparatus.

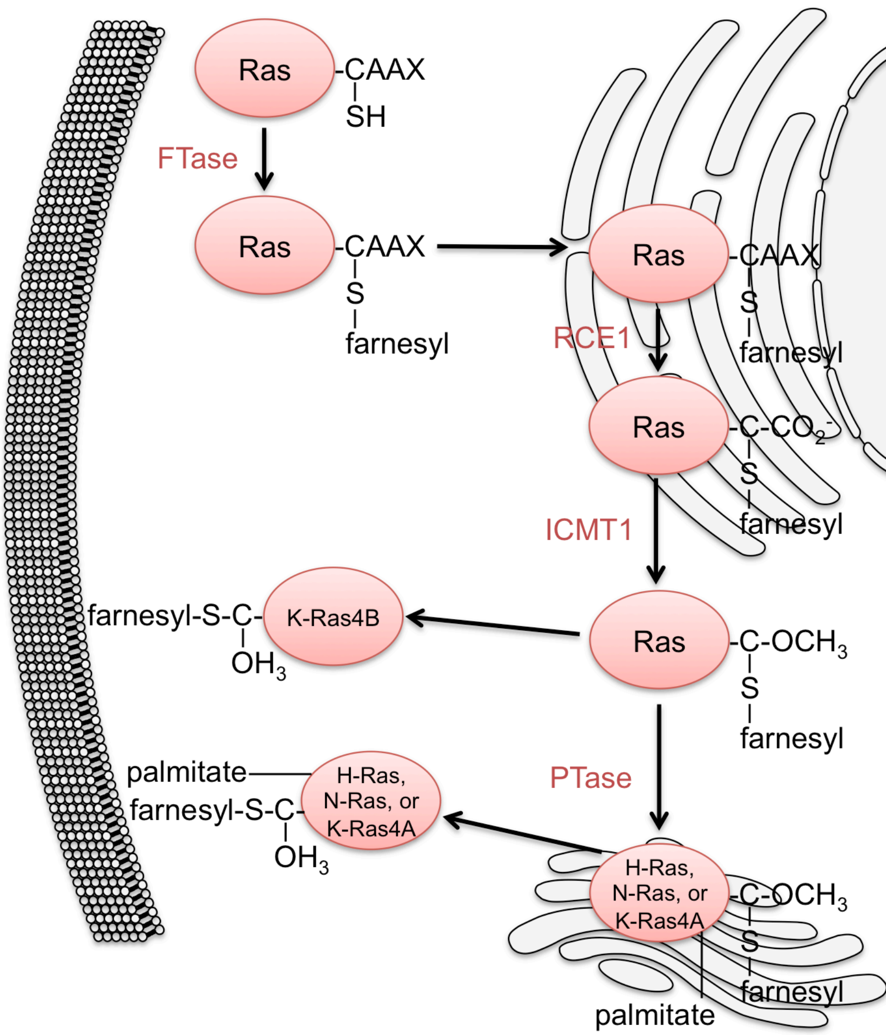


Fig 1.1 C-terminal processing of Ras.

Lipidation of the C-terminus of H-Ras, N-Ras, K-Ras4A, and K-Ras4B is necessary for membrane targeting. First, FTase catalyzes the addition of a farnesyl pyrophosphate group. The modified Ras is translocated to the endoplasmic reticulum where the AAX group is cleaved by RCE) and a methyl group is added by ICMT1. At this point, K-Ras4B can be translocated to the plasma membrane. However, H-Ras, N-Ras, and K-Ras4A are shuttled to the Golgi where PTase adds a palmitoyl group before they are transported to the plasma membrane.

Epidermal growth factor (EGF) stimulation was the first identified upstream driver of Ras activation (62). Antibodies against Ras block serum-stimulated growth in NIH-3T3 cells (63) and growth factor-induced differentiation in PC12 cells (64). Ligand stimulation of EGF receptor (EGFR) or platelet-derived growth factor receptor (PDGFR) was found to transiently induce GTP-bound Ras in mammalian cells (65, 66). It was therefore hypothesized that Ras activation was required for signaling by extracellular mitogens.

Ras isoforms are preferentially expressed and activated in different tissues (67). Additionally, it is now appreciated that there are distinct functions of Ras isoforms in development. K-Ras4B, but not K-Ras4A, is necessary for embryogenesis (68, 69). H-Ras and N-Ras are not necessary for normal development, as animals lacking these genes are viable and develop normally (70, 71). More recent work suggests that H-Ras can functionally replace K-Ras during embryogenesis, but only when driven by the *K-ras* promoter (72). This suggests that there is functional redundancy among the proteins, but that tissue-specific expression is a necessary regulator during development.

Downstream signaling

Ligand-induced receptor tyrosine kinase (RTK) dimerization promotes autophosphorylation in *trans* resulting in receptor activation (73). These phosphorylation sites serve as binding sites for proteins that contain a Src homology 2 (SH2) domain, a phosphotyrosine binding domain (PTB), or both, as is expressed in Shc (73, 74). Shc then recruits Grb2 (growth factor receptor-bound protein 2) and SOS1/2 leading to Ras activation (73). Ras-dependent signaling regulates many cellular functions including gene expression, proliferation, survival, differentiation, cell cycle entry, and cytoskeletal dynamics. Dysregulation of these cellular functions is a hallmark of cancer (75).

At least seven families of proteins have been shown to interact with Ras in a GTP-dependent fashion (76, 77) (Fig 1.2). C-RAF (RAF-1) was the first known Ras effector (78-83). RAF is the first kinase in the RAF/MEK/ERK signaling cascade (see below). The p110 catalytic subunits of phosphoinositide 3-kinase (PI3K) were recognized as the second class of validated Ras effectors (84). The p110 catalytic domain and AKT (also known as protein kinase B, a key downstream target of PI3K) were identified independently as retroviral oncogenes (77). Additionally, knock-in mice homozygous for a PI3K p110α lacking a Ras-binding domain (RBD) are resistant to lung tumors induced by oncogenic K-Ras4B (85). The presence of an RBD is a common feature of Ras effectors. Ral-specific GEFs were identified by screening for proteins with RBDs in the C-terminal ends (86-88). Initial studies in NIH-3T3 cells suggested that RalGEFs and their substrates, RalA and RalB small GTPases, played minor roles in cellular transformation (89, 90). However, studies in human cells suggested that RalGEF-Ral interactions are important in cancers of the pancreas, prostate, and bladder (91, 92).

Less well-known effectors of Ras signaling include TIAM1 (T lymphoma invasion and metastasis-inducing 1), PLCε (phospholipase Cε), and Nore1. TIAM1 is a Rac-specific GEF that was identified *in silico* by searching for novel proteins with RBDs (93). TIAM1-Rac signaling regulates the actin cytoskeleton and activates PAK (p21-activated kinases) and JNK (c-Jun N-terminal kinase). PLCε is a novel isoform of PLC that contains an RBD. It connects Ras signaling to the production of secondary messengers, diacylglycerol (DAG) and calcium (94). In a carcinogen-induced mouse model of skin cancer, TIAM1 or PLCε disruption prevented H-Ras-driven oncogenesis (95, 96).

Finally, members of the RASSF (Ras-associated domain family) family were found to contain RBDs and mediate Ras-induced apoptosis (97, 98). RASSF5 (Nore1) was first identified in a yeast two-hybrid assay as an effector of H-Ras (99). When Nore1

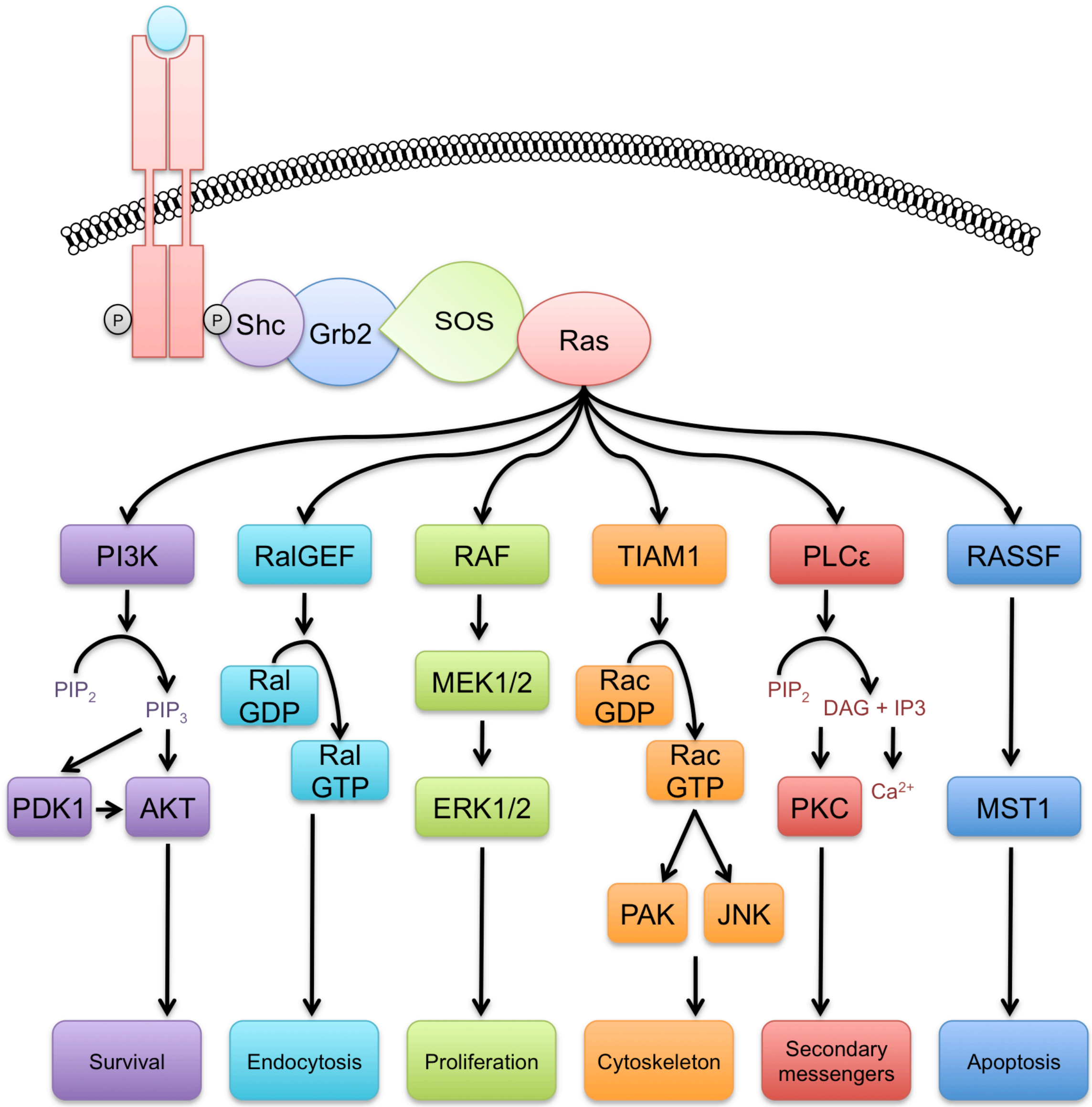


Fig 1.2 Ras signaling pathways in mammalian cells.

Phosphorylated RTKs serve as docking sites for Shc, Grb2, and SOS1/2 (RasGEF) to activate Ras. GTP-bound Ras activates several pathways, of which several are depicted here. The two best studied pathways are the PI3K-AKT and the RAF/MEK/ERK kinase cascade leading to increased survival and proliferation. RalGEFs promote the activation of small GTPases, RalA and RalB. Activation of TIAM1, PLCε, and RASSF (Nore1) contribute to cytoskeleton rearrangement, secondary messenger signaling, and apoptosis, respectively.

complexes with Mst1 (Macrophage stimulating 1), it results in a Ras-dependent, pro-apoptotic pathway (97). Subsequent studies indicated that RASSF1, RASSF2, RASSF4, and RASSF6 can also play pro-apoptotic roles (100-103). This is consistent with the observation that RASSF proteins are often downregulated in cancer cells (98).

Ras/RAF/MEK/ERK

There are three main components of the RAF/MEK/ERK signaling pathway; however, there are several isoforms of each. The RAF family consists of C-RAF, B-RAF, and A-RAF. MEK and ERK each have 2 members that regulate this pathway, MEK1/2 and ERK1/2, respectively. RAF, MEK, and ERK isoforms can each form dimers that further define the specificity of signaling (104, 105).

All three paralogs of RAF contain three conserved regions: the N-terminal CR1, which contains the RBD and cysteine-rich domain (CRD); the CR2, which contains important residues for RAF membrane recruitment during activation; and the CR3, which contains the kinase domain (106). Each RAF isoform appears to have a distinct mechanism of activation, with B-RAF considered to be more active than C-RAF or A-RAF (107-109). Mutations in B-RAF are commonly found in cancers, including melanomas, but mutations in C-RAF and A-RAF are rare (110, 111). Upon growth factor stimulation, activated Ras recruits RAF to the membrane and promotes the formation of functionally asymmetric RAF homo- and heterodimers in which one monomer, typically, B-RAF, allosterically stimulates the kinase activity of the other (106, 112, 113). In fact, kinase-dead forms of B-RAF occur in human cancers and are oncogenic (114, 115). This is due to their ability to dimerize with wild-type C-RAF and subsequently activate MEK1/2 and ERK1/2 signaling (116).

RAF phosphorylates and activates MEK1/2 at Ser217 and Ser221, which are located in the activation loop of MEK (117). MEKs can be partially activated by phosphorylation at either site, and substitution of these sites with acidic residues enhances basal activity (118). Different RAF isoforms activate MEK1 and MEK2 differentially: A-RAF is a weak activator; B-RAF preferentially activates MEK1; and C-RAF efficiently activates both MEKs (119). *mek2*$^{-/-}$ mice are viable, fertile, and show no phenotypic abnormalities (120). Conversely, *mek1* disruption is embryonic lethal in the recessive condition (121, 122). Homozygous *mek1* mutants die between embryonic day (E) 8.5 (121) and E10.5 (122) as a result of placental defects.

MEK1 can downregulate MEK2-dependent ERK signaling (121). The MEK heterodimer is negatively regulated by ERK-mediated phosphorylation of MEK1 on Thr292, a residue that is absent in MEK2 (121, 123, 124). If MEK1 is absent or unable to bind MEK2, the negative feedback phosphorylation loop in which ERK inhibits MEK1 is lost and MEK2-dependent phosphorylation and activation of ERK is prolonged (121). *mek1*$^{-/-}$ fibroblasts migrate more slowly than wild-type counterparts in response to fibronectin (122). However, *mek1* ablation enhances growth factor-induced fibroblast migration due to increased ERK activation in MEK1-deficient cells (121).

MEK1/2 catalyze the phosphorylation of ERK1/2 on Thr202/185 and Tyr204/187(125). Phosphorylation at both sites is necessary for significant kinase activity, with phosphorylation at the tyrosine preceding that at the threonine (126). ERK1/2 catalyze the phosphorylation of serine/threonine residues that occur in the sequence Ser/Thr-Pro (127), also known as proline-directed phosphorylation. Known substrates of ERK1/2 include the nuclear targets, TCF (ternary complex factor) family of transcription factors and MSK (mitogen- and stress-activated protein kinases), and the cytoplasmic target, p90 ribosomal S6 kinase (RSK) (128). ERK1 and ERK2 are 84% identical in sequence and share many, if not all, functions (129). However, the *erk1* gene is dispensable for normal development of mice, but ablation of *erk2* is embryonic lethal

(130-132) suggesting that differences in function (at least during development) are present.

EGFR is a potent activator of ERK1/2, but a weak activator of the PI3K pathway (133). However, at low levels of EGF stimulation, PI3K activity induces recruitment of Grb2-associated binding partner (GAB) to the membrane and contributes to ERK1/2 pathway activation. With prolonged EGF stimulation, the dependency on PI3K is decreased as EGFR recruits Shc-Grb2-SOS1/2 complexes that activate the Ras/ERK-dependent signaling (134). Long-lasting pulses of EGF-induced ERK1/2 activity can persist over the course of 4-5 consecutive cell divisions (135).

Scaffold proteins

Scaffold (or adaptor) proteins are used by the cell to confer spatial or temporal regulation of cellular signaling (136). There are several scaffolding proteins that interact with members of the RAF/MEK/ERK signaling cascade, and they are predicted to restrict ERK to certain subcellular compartments (137). For example, Kinase suppressor of Ras 1 and 2 (KSR1/KSR2) interact with B-RAF, C-RAF, MEK1/2, and ERK1/2 and scaffold Ras-dependent signaling effectors at the plasma membrane (see below) (138). IQGAP1 is a large, widely expressed protein that modulates actin dynamics, microtubule dynamics, cell-cell adhesion, and transcriptional regulation (139). IQGAP1 binds to B-RAF, MEK1, MEK2, ERK1, and ERK2 regulating their activation in response to EGF (140-142) and CD44 (143). While ERK is constitutively bound to IQGAP1 and binding is not sensitive to EGF stimulation, the interaction between IQGAP and MEK1 increases and the IQGAP1/MEK2 interaction decreases following EGF treatment (141). Knockout of IQGAP renders B-RAF insensitive to EGF stimulation (142).

β-arrestins desensitize and promote the internalization of G protein-coupled receptors (GPCRs) (139). Following activation, GPCRs are phosphorylated and internalized to terminate signaling. β-arrestins recruit RAF, MEK1/2, and ERK1/2 to the GPCRs enhancing the activation of ERK, and accompany the receptors to early endosomes (139). β-arrestins prevent the translocation of active ERK1/2 to the nucleus, restricting ERK to cytosolic substrates. Therefore, β-arrestins spatiotemporally regulate GPCR-induced ERK1/2 activation (139, 144, 145).

MEK partner 1 (MP1) binds only the MEK and ERK interaction and tethers MEK and ERK at the endosomes, which is critical for full ERK activation (146, 147). Several scaffolds have been characterized that specifically bind to and enhance the activation of MEK1, but not MEK2 (141, 148-150). For example, MP1 and its binding partner p14, specifically organize MEK1 and ERK1 to coordinate signaling through early and/or late endosomes (139, 146, 149).

Sef (Similar expression to FGF) is a transmembrane scaffold for MEK1/2 and ERK1/2 that anchors these effectors to the Golgi apparatus. Sef only binds to activated MEK1/2 and inhibits the dissociation of the MEK-ERK complex, preventing ERK1/2 translocation to the nucleus and promoting the ERK-dependent phosphorylation of specific cytoplasmic substrates (151).

KSR1

Deletion or loss-of-function alleles of *ksr1* suppress the rough eye and multivulval phenotypes of activated Ras in *Drosophila melanogaster* and *Caenorhabditis elegans*, respectively (152-154). KSR proteins constitute a novel family of proteins that have remarkable overall structural similarity to proteins of the RAF family (138). All members are characterized by the presence of five conserved regions (CA1-5) (152) (Fig 1.3). The CA1 is a 40 amino acid region unique to KSR proteins, and its function remains undefined. The CA2 is a proline-rich region, the CA3 is a cysteine-rich, zinc-finger domain, the CA4 is a serine/threonine-rich region, and the CA5 is a putative kinase domain (138, 152, 155). Further dissection of the KSR protein suggests that the positive effects on ERK signaling are mediated by amino acids 319-390 which correspond to the CA3 (156, 157), while the inhibitory effects appear to be mediated by the kinase-like domain (CA5) (158, 159). KSR and RAF both have kinase domains in the C-terminal half of the protein. However, the kinase-like domains of human and mouse KSR1 lack a critical lysine in the ATP-binding domain, which is responsible for hydrolysis and transfer of the γ-phosphate group of ATP, and is conserved in other kinases (160). The lack of conservation at this amino acid renders the kinase inactive. Furthermore, KSR1 does not contain conserved residues that correlate with peptidic recognition sequences that are found in both serine/threonine and tyrosine kinases (138, 154).

KSR1 lacks the RBD that is present in RAF family proteins suggesting that KSR1 does not bind Ras directly. However, KSR1 regulates Ras-mediated signaling because it functions as a molecular scaffold of the RAF/MEK/ERK kinase cascade that enhances oncogenic signaling. Both MEK1 and MEK2 bind directly to the CA5 region of KSR (157, 158). MEK is stably associated with KSR in both quiescent and growth factor-stimulated cells (161, 162). However, their dissociation may be necessary for maximal cellular transformation, as cells harboring KSR1 mutations that disrupt KSR1-MEK interactions ($KSR1^{C809Y}$) have increased colony formation in soft agar (unpublished observations, Kortum & Lewis). ERK1/2 binding is induced upon Ras activation at the FxFP motif in the CA4 domain of KSR (161, 163, 164). The KSR1 interaction with RAF is also Ras-dependent, but requires interactions with other proteins, such as MEK (155, 165).

KSR1 regulates the intensity and duration of ERK activation to modulate cellular proliferation and oncogenic potential (166). The intensity and duration of ERK activation are critical to the regulation of downstream processes (167-170). For example, in PC12 cells, EGF induces a transient activation of ERK leading to proliferation, while stimulation with nerve growth factor (NGF) leads to prolonged ERK activation and translocation to

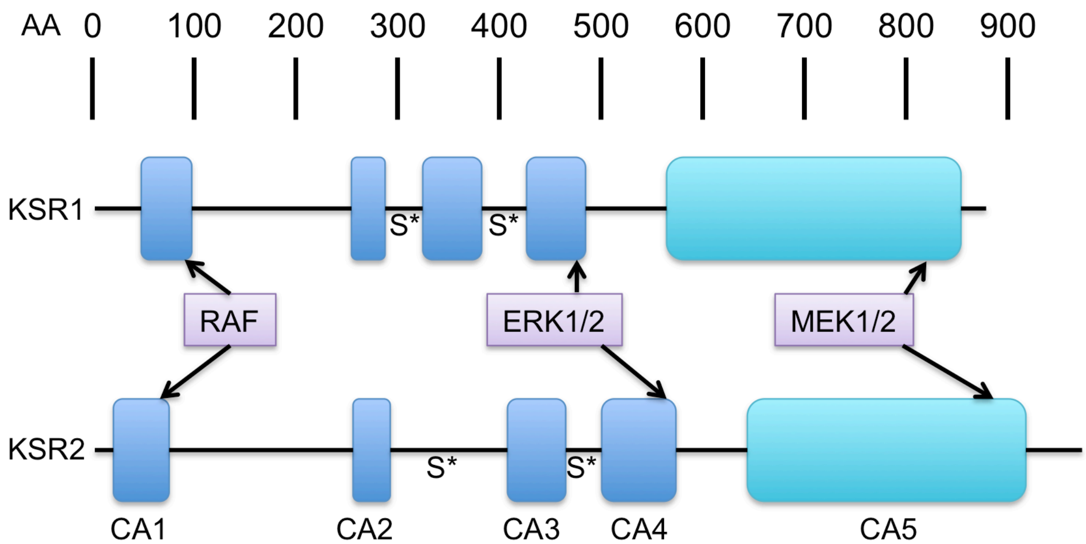


Fig 1.3 Conserved domains of KSR proteins.

Murine KSR1 (873 AA) and KSR2 (947 AA) are depicted above. All KSR proteins share five conserved areas (CAs). CA1 is a 40-residue domain unique in KSR proteins and is necessary for KSR/RAF complexes. CA2 is a proline-rich stretch. CA3 is a cysteine-rich, zinc-finger domain that promotes translocation to the plasma membrane. Phosphorylation of serine residues (S*) on each side of CA3 is required of 14-3-3 binding and sequestration in the cytoplasm. CA4 is a serine/threonine-rich region that is highly phosphorylated and required for ERK binding. CA5 is a kinase-like domain that lacks kinase catalytic function, but is necessary for KSR/MEK interactions.

the nucleus to promote differentiation (168). In MEFs, treatment with EGF again leads to transient ERK activation, whereas prolonged activation with PDGF induces phosphorylation of immediate early proteins and cell cycle progression (167, 170). Loss of KSR1 reduces growth factor-induced ERK activation in MEFs (166). Furthermore, KSR1 is necessary and sufficient for Ras^{V12}-driven transformation in MEFs, with an optimal dosing level similar to that observed with maximal ERK signaling. Cells expressing increasing levels of KSR1 have increased EGF- and PDGF-induced ERK activation. However, after reaching a maximal level of ERK activation, higher levels of KSR1 actually reduce ERK signaling to levels at or below controls (166). The mechanism by which excessive levels of KSR1 repress ERK signaling is undefined. It is predicted that when KSR1 is overexpressed it forms separate complexes with RAF, MEK, and ERK preventing the complex composition needed for maximal ERK signaling.

KSR1 also interacts with caveolin-1 and is responsible for MEK/ERK redistribution to the caveolin-1-rich fractions of the plasma membrane (171). The interaction between KSR1 and caveolin-1 is necessary for optimal ERK activation, as cells containing a KSR1 mutant unable to bind caveolin-1 are deficient in the early stages of growth factor-mediated ERK activation (171). Moreover, KSR1 modulates Ras^{V12}-induced replicative senescence in MEFs (172). Oncogenic Ras induces cell growth arrest by RAF/MEK/ERK-mediated activation of $p19^{ARF}$/p53 and INK4/Rb tumor suppressor pathways. H-Ras^{V12} fails to induce p53, $p19^{ARF}$, $p16^{INK4a}$, and $p15^{INK4b}$ expression in MEFs lacking KSR1 resulting in proliferation instead of growth arrest (172). Abolishing the interaction between KSR1 and caveolin-1 also has adverse effects on H-Ras^{V12}-induced senescence and transformation (171). Taken together, these data demonstrate that KSR1 is a potent modulator of Ras signaling.

KSR1 is phosphorylated on multiple residues that may be involved in the subcellular distribution of KSR1 resulting in significant effects on ERK signaling (173, 174). Members of the MARK family, C-TAK1 and EMK, and nm23H1 all phosphorylate KSR1 at Ser392 (175-177). C-TAK1-mediated phosphorylation of KSR1 on Ser297 and Ser392 is crucial for KSR1 to interact with the regulatory protein 14-3-3 (163, 176). In unstimulated cells, the 14-3-3 binding of KSR1 sequesters it in the cytoplasm. Growth factor stimulation and Ras activation, induces protein phosphatase 2A (PP2A)-dependent dephosphorylation of KSR1 on Ser392 releasing 14-3-3 and exposing the CA3 region, which is essential for translocation and accumulation at the plasma membrane (178). KSR1 is rapidly translocated to the plasma membrane, where it can promote the Ras/MAPK pathway activity (176, 178).

KSR1 phosphorylation at both Thr274 and Ser392 modulates the proliferative potential of EGF- and PDGF-dependent signaling (179). Expression of KSR1 mutated at Thr274 and Ser392 promotes sustained ERK1/2 activation in response to treatment with either EGF or PDGF and accelerates cell cycle progression (179). Normally, the EGF response is transient and does not promote S-phase entry. However, blocking KSR1 phosphorylation at these sites induces a PDGF-like response to EGF stimulation. It is predicted that phosphorylation at these two sites promotes the degradation of KSR1, as the mutant has an increased half-life, which results in increased proliferation and colony formation in soft agar (179).

Four sites on KSR1 (Thr260, Thr274, Ser320 and Ser443) are phosphorylated upon growth factor stimulation or expression of H-Ras^{V12} (163, 180). Mutations at these sites had no effect on Ras-dependent signaling or the ability of KSR1 to scaffold the RAF/MEK/ERK pathway (163), but docking of ERK1/2 on KSR1 accelerated the phosphorylation (181). Phosphorylation of KSR1 (and BRAF) by ERK1/2 promotes the dissociation of BRAF/KSR1 complexes and promotes the release of KSR1 from the membrane (181). This negative feedback loop can thereby modulate the duration of KSR1-dependent signaling.

The Ras effector protein IMP (impedes mitogenic signal propagation) modulates Ras-dependent signaling by inactivating KSR1 (182, 183). IMP promotes KSR1 hyperphosphorylation and localizes KSR1 to a detergent-insoluble fraction (183). IMP overexpression inhibits H-Ras- and C-RAF-mediated transformation in cells by preventing MEK1/2 activation by C-RAF. Upon H-Ras^{V12} expression or growth factor stimulation, IMP is autopolyubiquitinated and degraded, releasing KSR1 to translocate to the plasma membrane (183). Overall, these data demonstrate that KSR function is regulated via phosphorylation and compartmentalization through complex formation.

ksr1$^{-/-}$ mice are grossly normal despite suppressed ERK activation. However, they are resistant to murine polyoma middleT-driven tumor formation (184). Additionally, mice lacking *ksr1* have disorganized hair follicles (185) and have fewer and enlarged adipocytes as compared to their wild-type counterparts (186). *In vitro*, deletion of *ksr1* prevents the ERK- and RSK-dependent phosphorylation and stable expression of the transcription factor C/EBPβ, which is required for the subsequent expression of C/EBPα and PPARγ necessary for adipogenesis. Interestingly, increasing KSR1 expression to levels that promote maximum ERK activation actually inhibits the adipogenic program by promoting the phosphorylation of PPARγ at an inhibitory site (186). These data suggest that controlled expression of KSR1 ensures appropriate levels of ERK activity for progression through the adipogenesis program.

In MEFs, KSR1 enhances the glycolytic and oxidative phosphorylation potential of cells by inducing the expression of the metabolic regulators peroxisome proliferator-activated receptor gamma coactivator 1α (PGC1α) and estrogen-related receptor α (ERRα) only in the presence of activated Ras (187). This pathway is essential for the transformation of cells by oncogenic Ras. In *ksr1*$^{-/-}$ MEFs expressing H-Ras^{V12}, ectopic PGC1α is sufficient to rescue ERRα expression, metabolic capacity, and anchorage-independent growth. The ability of PGC1α to promote anchorage-independent growth requires its interaction with ERRα. Conversely, the expression of a constitutively active ERRα in *ksr1*$^{-/-}$ cells with H-Ras^{V12} is sufficient to normalize the metabolic capacity, but not rescue anchorage-independent growth (187).

PGC1α was not detected in colon tumor cell lines. However, a related gene PGC1β and the same coactivator, ERRα, were identified as downstream effectors of Ras signaling in CRC cells and tumors (188). KSR1 is overexpressed in colon tumor cells as compared to expression in immortalized, but not transformed human colonic epithelial cells (HCECs) (188). HCECs were isolated from normal human colon and immortalized with CDK4 and human telomerase reverse transcriptase (hTERT). HCECs maintain wild-type adenomatous polyposis coli (APC), K-Ras, and TP53 proteins. They also form crypt-like structures in 3D culture, but are incapable of anchorage-independent growth or tumor formation in nude mice (189). Depletion of KSR1 is selectively toxic to the cancer cells and is required for anchorage-independent growth and tumor maintenance in colon cancer cell lines (188). The downstream effectors of KSR1-dependent signaling, PGC1β and ERRα, are also upregulated in colon tumor cells. Suppression of PGC1β or ERRα expression decreases tumor colon cell viability and anchorage-independent growth, as well as delays and suppresses tumor formation in nude mice (188). Taken together, these data suggest that targeting KSR1 or its downstream effectors may be a successful approach to target colon cancer.

Targeting Ras signaling

Targeting Ras and other components of Ras-dependent signaling has been extensively explored and is an ongoing area of investigation. Here we summarize the different levels of Ras signaling that have been targeted clinically and present pre-clinical work on innovative ways being developed to target Ras directly.

For patients harboring tumors with high EGFR expression and wild-type *K-ras*, treatment with monoclonal antibodies against EGFR, such as cetuximab and panitumumab, has significant clinical benefit (190-193), whereas negligible responses are observed with patients carrying mutant *K-ras* (192, 194). Overall, tumor-free progression and overall survival is better in patients with wild-type *K-ras* (195). In colorectal cancer, resistance to EGFR inhibitors seems to develop through selection of preexisting clones that contain *K-ras* mutations (196).

The post-translational modifications of Ras have been prime targets of Ras inhibition. Farnesyltransferase inhibitors (FTIs) were developed to simulate the CAAX motif of Ras and were used as competitive inhibitors to block the post-translational processing needed for Ras activation. Mouse studies showed regression of MMTV-*H-ras*-driven mammary carcinomas with FTI treatment with no detectable systemic toxicities (197). However, unlike H-Ras, N-Ras and K-Ras are subsequently modified with an alternative prenylation catalyzed by geranylgeranyltransferase 1 (GGTase-1) making them insensitive to FTIs (198, 199).

Targeted inhibition of RCE1 and ICMT1 in NIH-3T3 cells and yeast (*ste14*) indicated that blocking proteolytic cleavage of Ras was not sufficient to inhibit its functions (200, 201). More recently, it was shown that *rce1*-deficient mice have a 50% reduction in membrane-bound K-Ras and H-Ras corresponding with decreased tumor xenograft growth of mouse skin carcinoma cells (202). Similar studies targeting ICMT1 in MEFs show partial reduction in membrane association of K-Ras with concomitant loss of tumorigenesis (203). Compounds targeting ICMT1, such as methotrexate, have some effectiveness in MEFs and DKOB8 human CRC cells in cell culture and animal models (204, 205).

While direct targeting of Ras has been difficult, studies have shown that a reduction in K-Ras expression in cancerous cells, by antisense, miRNA or siRNA oligonucelotides, halts proliferation and leads to cellular death (206, 207). A new approach involves targeting DNA secondary structures called G-quadruplexes in the promoter of *K-ras* (208). Instead of forming the traditional double-helix, negative superhelicity induced by transcription can promote local unwinding of the DNA in these G/C-rich regions allowing for rearrangement of the guanine-rich strand to form planar structures via hydrogen bonds between four guanine residues known as G-quadruplexes. Formation of G-quadruplexes in DNA has been shown to modulate transcription and in RNA modulates translation. Strategies to stabilize the G-quadruplexes in the *K-ras* promoter, thereby reducing its transcription, are currently underway (208).

Other strategies for targeting Ras focus on inhibition of the downstream signaling pathways. B-RAF-specific, ATP-competitive inhibitors (such as vemurafenib) have shown promise in the clinic in melanoma patients with mutant B-RAF (B-RAF^{V600E}) (209). However, there is a paradoxical effect in that they accelerate the growth of cells with mutant Ras (210-212). Paradoxical activation is caused by transactivation resulting from the induction of RAF homo- (C-RAF/C-RAF) and heterodimers (B-RAF/C-RAF) (112). In the presence of RAF inhibitors and activated Ras, the drug binds directly to the ATP-binding site of one kinase (the “activator” kinase) and promotes the transactivation of the other kinase in the dimer (the “receiver” kinase) (112, 116). In B-RAF^{V600E} tumors, K-Ras is not activated, as these mutations are mutually exclusive (213). Thus, transactivation is minimal and ERK signaling is inhibited (112). Additionally, RAF inhibition in the absence of activated Ras induces the dimerization of B-RAF and KSR1 to inhibit downstream ERK activation (181). This is likely due to the fact that KSR1 is not a kinase and B-RAF is bound to the inhibitor; thus, there is no “receiver” kinase to transactivate.

RAF inhibitor PLX4720 does not induce B-RAF/C-RAF dimers, but still activates MEK and ERK in transformed cells (112, 210, 214). B-RAF inhibitors PLX4720 and

GDC0879 induce the dimerization of C-RAF and KSR1. While KSR1/B-RAF complexes inhibit ERK signaling (181), KSR1/C-RAF dimerization promotes ERK signaling. These inhibitors also require KSR1 to activate MEK and ERK in Ras-transformed cells (113). RAF transactivation requires phosphorylation of an N-terminal acidic domain in the "activator," but not the "receiver" kinase. This motif is located between residues 446-449 and is constitutively phosphorylated in B-RAF, but not in C-RAF and KSR1 (116). However, point mutations in equivalent regions of KSR1 and C-RAF (KSR1$^{YLQE/DDEE}$ and C-RAF$^{SSYY/DDEE}$) in kinase-dead constructs (KSR1^{A587F} or C-RAF^{A373F}) resulted in potent ERK activation (116). Together, these data solidify the idea that RAF kinases (and KSR1) form functionally asymmetric dimers, and that this is a mechanism of resistance to RAF inhibitors in the presence of activated Ras.

Direct inhibition of MEK has not been clinically successful in achieving increasing progression-free survival. Preclinical studies identified distinct mechanisms by which cells acquire resistance to MEK inhibition, including amplification of B-RAF (215), STAT3 upregulation (216), or acquisition of mutations in the binding pocket of MEK that block inhibitor binding (217, 218). MEK inhibition also induces paradoxical activation of the ERK pathway. This is due to loss of negative feedback loops. Activated ERK can directly phosphorylate both RAF (219) and MEK1 (123, 124) suppressing RAF/MEK/ERK-dependent signaling. In the absence of ERK-dependent MEK inhibition, MEK can continue to activate ERK. This mechanism also operates in K-Ras-mutated colorectal cancer cells and induces AKT activation in response to MEK inhibition (220).

Inhibition of ERK overcomes acquired resistance to MEK inhibition (221). Several compounds have been developed that target ERK1/2 directly (221-224). FR18024 is an ATP-competitive inhibitor that has not been completely characterized (225). VTX-11e is a potent ATP-competitive ERK inhibitor with oral bioavailability (223). SCH772984 is a recently identified, highly potent and selective inhibitor of ERK1/2 (224) that induces a novel allosteric pocket adjacent to the ATP binding site contributing to high selectivity and low off-rate (226). Direct ERK inhibition is a relatively new way to target Ras signaling. Further preclinical testing needs to be completed before its clinical viability can be assessed.

Summary

It has been more than 30 years since the first gene associated with rat sarcoma virus was identified in human tumors (227). Since then, we have learned an enormous amount regarding structure, activation, and downstream signaling pathways, only some of which is summarized here. However, targeting Ras signaling in cancers remains a moving target. Each therapy has resulted in the discovery of acquired or even innate tumor resistance. This has been beneficial in further mapping of the extensive network of Ras-dependent signaling, including both positive and negative feedback loops, but is increasingly frustrating for the future of clinically relevant therapeutics targeting Ras.

Here we identify new ways to target Ras-dependent signaling in colon cancer cells and further define Ras/RAF/MEK signaling networks. The studies described in this dissertation examine the following topics:

1) Identification of KSR1 and EPHB4 as vulnerabilities in colon cancer that regulate Myc and PGC1β expression
2) Characterization of MEK-dependent signaling, including the ERK- and HSF1-dependent regulation of Myc and PGC1β expression

Chapter 2: Material and Methods

Gene expression-based high-throughput screen and functional signature ontology analysis

The gene expression-based high-throughput screen has been previously described (188, 228). The gene expression-based signature measured in the screen is based on six genes (ACSL5, BNIP3L, ALDOC, LOXL2, BNIP3, and NDRG1) that are consistently affected by KSR1 depletion as well as two housekeeping genes (PPIB and HPRT) that were included for normalization. To identify targets that are KSR1 functional analogs based on their gene expression-based signature, two similarity metrics were employed, Euclidean distance (ED) and Pearson correlation (PC). KSR1-depleted positive controls cluster with a low ED and high PC. Linear regression analysis was used to establish a cutoff (PC > 0.25 * ED + 0.5) for KSR1 similarity based on the ED and PC values of KSR1 positive controls. Targets that clustered with the positive control KSR1-depleted wells and exceeded the established cutoff based on these two metrics were designated as possible KSR1 functional analogs and candidates for further evaluation.

Cell culture

Colorectal cancer cell lines HCT116, SW480, DLD1, SK-CO-1, Caco2, and HCT15 were purchased from American Type Culture Collection (ATCC). The CBS and GEO colorectal cancer cell lines were gifts from Dr. Michael Brattain (U. Nebraska Med. Ctr.). Cells were grown in either Dulbecco's modified Eagle medium (DMEM) or Eagle's minimum essential medium with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 0.1 mM nonessential amino acids (NEAA). All colorectal cancer cells were grown at 37°C with ambient O_2 and 5% CO_2. Immortalized non-transformed human colonic epithelial cell lines (HCEC) were a gift from J. Shay (UT Southwestern) (189). HCECs were grown in medium composed of 4 parts DMEM to 1 part medium 199 (Sigma-Aldrich) with 2% cosmic calf serum (GE Healthcare), 25 ng/mL EGF, 1 µg/mL hydrocortisone, 10 µg/mL insulin, 2 µg/mL transferrin, 5 nM sodium selenite, and 50 µg/mL gentamycin sulfate. HCECs were grown in a hypoxia chamber with 2% O_2 and 5% CO_2 at 37°C.

siRNA transfections

Pooled or individual (Table 2.1) siRNAs targeting EPHB4 (M-003124-02), KSR1 (LU-003570-00-0002), Myc (L-003282), MAP2K1 (J-003571), MAP2K2 (J-003573), MAPK3 (J-003592), MAPK1 (J-003555), HSF1 (M012109), as well as a non-targeting siRNA control (D-001810-01) (DharmaconGE), were introduced into HCT116 or Caco2 cells using the Lipofectamine RNAiMAX (Invitrogen) reverse transfection protocol per manufacturer's instructions. Briefly, 125 pmol of siRNA and 5-7 µL of RNAiMax were combined in OPTI-MEM for 5 minutes. DNA:Lipofectamine complexes were overlaid with 2 mL of cells (150,000 cells/mL) in 6-well plates. Final RNAi concentrations are 50 nM. HCECs were transfected following the RNAiMax reverse transfection protocol using 2.5 µL RNAiMax transfection reagent per 6 mL of antibiotic free-medium and 150,000 cells/mL with a final RNAi concentration of 10 nM in 6-cm dishes (CorningTM, PrimariaTM). After a 72-hour transfection, cells were lysed in RIPA lysis buffer with protease and phosphatase inhibitors (described below).

Reagents

The EPHB4 receptor tyrosine kinase inhibitor (AZ12672857) was a gift from J. Kettle (AstraZeneca). The EPHB4 inhibitor was dissolved in DMSO to achieve a stock concentration of 10 mM. Z-Leu-Leu-Leu-al (MG132, C2211), cycloheximide (CHX, C7698), Bafilomycin A1 (BafA1, B1793), poly-2-hydroxyethyl methacrylate (polyHEMA, P3932), and propidium iodide (PI, P4170) were purchased from Sigma-Aldrich and were used at the concentrations specified in the figures and accompanying text.

Target	Item #	siRNA	Target Sequence
hKSR1	J-003570	6	CAUCAUAGACAGCAGUUUA
		7	GAGCAAGUCCCAUGAGUCU
		8	GGAAUGAAGCGUGUCCUGA
		9	AGAAAGAGGUGAUGAACUA
hEPHB4	D-003124	5	GGACAAACACGGACAGUAU
		6	GUACUAAGGUCUACAUCGA
		7	GGAGAGAAGCAGAAUAUUC
		8	GCCAAUAGCCACUCUAACA
hMyc	J-003282	23	ACGGAACUCUUGUGCGUAA
		24	GAACACACAACGUCUUGGA
		25	AACGUUAGCUUCACCAACA
		26	CGAUGUUGUUUCUGUGGAA
hMAP2K1 (MEK1)	J-003571	6	CCAUGCUGCUGGCGUCUAA
		7	GAGGUUCUCUGGAUCAAGU
		8	CGACGGCUCUGCAGUUAAC
		9	GCACAAGGUCCUACAUGUC
hMAP2K2 (MEK2)	J-003573	8	CGACAGCGCAUGCAGGAAC
		9	GAUCAGCAUUUGCAUGGAA
		10	GGUCCGAGGUGGAAGAAGU
		11	UCUUUGAACUCCUGGACUA
hMAPK3 (ERK1)	J-003592	7	GACCGGAUGUUAACCUUUA
		8	CCUGCGACCUUAAGAUUUG
		9	CCAAUAAACGGAUCACAGU
		10	AGACUGACCUGUACAAGUU
hMAPK1 (ERK2)	J-003555	11	UCGAGUAGCUAUCAAGAAA
		12	CACCAACCAUCGAGCAAAU
		13	GGUGUGCUCUGCUUAUGAU
		14	ACACCAACCUCUCGUACAU
hHSF1	M-012109	2	UAGCCUGCCUGGACAAGAA
		3	CCACUUGGAUGCUAUGGAC
		4	GAGUGAAGACAUAAAGAUC
		5	AGAGAGACGACACGGAGUU
hCont #1	D-001810	1	UGGUUUACAUGUCGACUAA

Table 2.1 Sequences of individual siRNA duplexes

Anchorage-independent growth on poly-2-hydroxyethyl methacrylate (polyHEMA)-coated plates

polyHEMA stock solution (10 mg/mL) was made by dissolving polyHEMA in 95% ethanol and shaking at 37°C until fully dissolved (6 hours to overnight). Black-sided, clear-bottom, 96-well plates were coated with polyHEMA by evaporating 200 µl of the 10 mg/mL stock polyHEMA solution in each well. Cells were plated in complete medium on polyHEMA-coated wells at a concentration of 2 x 10^4 cells/100 µl 48 hours post-transfection (as described above). Cell viability was measured per the manufacturer's protocol using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega). Specifically, this was done by adding 90 µl of CellTiter-Glo® reagent, shaking for two minutes to lyse the cells, incubating at room temperature for 10 minutes, and measuring luminescence (POLARstar OPTIMA).

Cell growth assay

Cells (5,000/well) were transfected on white, 96-well plates. Transfections were done as described above but at a ratio of 1:25 for all of the reagents. At 0 and 72 hours post-transfection, 10 µL of alamarBlue® (ThermoFisher Scientific) was robotically added to each well. Plates were incubated at 37°C for three hours and fluorescence was measured (POLARstar OPTIMA).

Propidium iodide staining

Cells were assayed for apoptosis using the sub-G1 peak measured following propidium iodide (PI) staining. Prior to staining, all medium in each sample well was collected and placed in a 12 x 75 mm round bottom polystyrene tube (BD Falcon, 352054). Cells were washed once with PBS, the PBS was saved, and cells were subsequently treated with 0.25% trypsin for 5-10 minutes. Saved medium was then used to resuspend the trypsin-treated cells from the corresponding wells, which were collected and placed in the polystyrene tubes. Cells were pelleted by centrifugation for 5 minutes at 2800 RPM using an Immunofuge II. The supernatant was aspirated, and the cell pellets were resuspended in 2 mL of PBS, then pelleted again by centrifugation for 3 minutes at 2800 RPM. The PBS was aspirated and the cells were fixed in 2 mL of ice cold 70% ethanol for at least one hour at -20°C. Cells were then warmed to room temperature (~15 minutes), pelleted by centrifugation for 3 minutes, then rehydrated in 2 mL of room temperature PBS and incubated at 37°C for 15 minutes. Cells were then pelleted, the PBS aspirated, and the cells were resuspended in PI stain overnight. Data was acquired using a Becton-Dickinson FACSCalibur flow cytometer and analyzed using ModFit analysis software to detect a sub-G1 peak of fluorescence.

Western blot analysis

Whole-cell lysate extracts were prepared in radioimmunoprecipitation assay (RIPA) buffer composed of 50 mM Tris-HCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% Sodium dodecyl sulfate, 150 mM NaCl, 2mM EDTA, 50 mM NaF, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 2 mM EDTA, 1 mM PMSF. Cytoplasmic and nuclear fractionations were performed using NE-PER Nuclear/Cytoplasmic Extraction Reagents (Thermo Scientific, 78835) Protein concentration was determined using the Promega BCA protein assay. Samples were diluted in 1X sample buffer (5X stock – 313 mM Tris-HCl pH 6.8, 10% SDS, 50% glycerol, 0.05% bromophenol blue) with 100 mM DTT (20X stock = 2 M). SDS-PAGE was performed, membranes were blocked in Odyssey PBS blocking buffer (LI-COR Biosciences, 927-40000), and incubated in primary antibody (listed below) overnight at 4°C. LI-COR secondary antibodies (IRDye 800CW, 680LT, or 680RD) were diluted 1:5000-1:10,000 in 0.1% TBS-Tween 20 (for nitrocellulose) or 0.1% TBST + 0.01% SDS (for PVDF). Membranes were imaged using the LI-COR Odyssey.

Antibodies

Primary antibodies were diluted as follows: EPHB4 (mAb 265, a gift from Vasgene) 1:500 and (D1C7N, 14960, Cell Signaling) 1:1000; KSR1 (H-70, Santa Cruz) 1:1000; α-tubulin (B-5-1-2, Santa Cruz) 1:2500; β-actin (C-4, Santa Cruz) 1:2000; PGC1β (provided by Dr. A. Kralli, The Scripps Research Institute) 1:5000; c-Myc (5605, Cell Signaling) 1:1000; PARP (9542, Cell Signaling) 1:1000; pERK (9106, Cell Signaling) 1:1000; ERK (9102, Cell Signaling) 1:1000; pMEK (4694, Cell Signaling) 1:1000; MEK (9122, Cell Signaling) 1:1000; pMyc T58 (ab85380, Abcam) 1:500, pMyc S62 (ab78318, Abcam) 1:500; FBW7 (CDC4 H-300, Santa Cruz) 1:1000; β-TrCP (4394, Cell Signaling) 1:1000; and HDAC2 (ab7029, Abcam) 1:5000; PDCD4 (D29C6, 9535, Cell Signaling) 1:1000); p4E-BP1 T70 (9455, Cell Signaling) 1:1000; eIF4A (C32B4, 2013, Cell Signaling) 1:1000; eIF4E (9742, Cell Signaling) 1:1000, peIF4E S209 (9741, Cell Signaling) 1:1000; 4E-BP1 (53H11, 9644, Cell Signaling) 1:1000.

RT-qPCR

RNA was harvested using 1 mL TriReagent (MRC, TR118) and stored at -80°C until extraction. RNA was extracted per manufacturer's protocol and final RNA pellets were resuspended in nuclease-free water. DNase digestion was performed (Qiagen, 79254) and RNA cleanup was completed (Qiagen, 74106). RNA was quantified using the NanoDrop 2000 (Thermo Scientific). Reverse transcription was performed using iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad, 170-8840) with 1 µg of total RNA per 20 µL reaction. RT-qPCR was performed using the primers and conditions listed in Table 2.2. All targets were amplified using SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad) with 40 cycles of a 2-step program (95°C x 5 sec, T_m x 45 sec) on an MX3000P (Stratagene). Data were normalized using two of the normalization

Table 2.2. Sequences of qPCR primers

Target	Accession #	Amplicon size (bp)	Exons	T_m (°C)	F primer 5' -> 3'	R primer 5' -> 3'
hPPARGC1B	NM_001172699	102	12-13	62	GAATATTTCAGTAAGCTGTCA	GCCCAGATACACTGACTACG
hESRRA	NC_000011.10	116	2-3	60	CACTATGGTGTGGCATCCTGT	CGTCTCCGCTTGGTGATCTC
hMyc	NM_002467.4	119	3	58	GGCTCCTGGCAAAAGGTCA	CTGCGTAGTTGTGCTGATGT
hKSR1	NM_014238.1	127	20-21	60	AGTTTCTCCAGCATGTCCATC	GAATGAAGCGTGTCCTGACT
hEPHB4	NM_004444	90	12-13	62	AGCTGGATGACTGTGAACTG	GCCCGTCATGATTCTCACA
hMAP2K1	NM_002755	103	6-7	62	CAACCGCCATCTCTACCAG	GGCACAAGGTCCTACATGTC
hMAP2K2	NM_030662	130	2-3	60	TGTACGGCGAGTTGCATT	ACCAAAGTCCAGCACAGAC
hGAPDH	NM_002046.1	111	2-3	58	GGTGAAGGTCGGAGTCAACGG	GAGGTCAATGAAGGGGTCATTG
hACTB	NM_001101.1	59	4	60	ACCGAGCGCGGCTACAG	CTTAATGTCACGCACGATTTCC
hHPRT1	NM_000194	128	6-8	60	GTATTCATTATAGTCAAGGGCATATCC	AGATGGTCAAGGTCGCAAG

genes listed here: HPRT, β-actin, and/or GAPDH. Analysis was performed according to the q-base protocol, as previously published (229).

TCGA

mRNA expression was analyzed based on the RSEM normalized RNA-Seq values of primary tumor (n=285) and normal solid tissue (n=41) samples as well as patient-matched samples (n=26) within The Cancer Genome Atlas (TCGA) Colon Adenocarcinoma (COAD) dataset. Results were analyzed for statistical significance using unpaired and paired Student's *t* tests for the unpaired and patient-matched samples, respectively.

Myc translation

Myc translation reporter constructs (pGML, phpL and phpmL) for luciferase assays were a gift from Anne Willis (Medical Research Council, Leicester, UK) (230, 231). RNAi depletions were performed in 6-well plates as described above. The following day, cells were transfected with 3 µg of the translation vector and 1 µg of pSV-β-galactosidase vector (Promega, E1081) using 10 µL of Lipofectamine 2000 (Invitrogen) per well. After 24 hours, luciferase and β-gal expression was assessed using Dual-Light® System (Applied Biosciences, T1003) according to the manufacturer's instructions. Briefly, cells were rinsed twice with PBS, lysed with Lysis Solution (100 mM potassium phosphate pH 7.8, 0.2% Triton X-100, 0.5 mM DTT), and 10 µL of each lysate was added (in triplicate) to a 96-well plate. Luminescence was measured (POLARstar OPTIMA) for 1 second per well. When quantifying basal translation from each vector, luciferase expression was normalized to β-galactosidase expression. When comparing the affect of KSR1 or EPHB4 depletion on Myc translation, luciferase signal was normalized to protein input.

Statistical analysis

P and EC_{50} values were calculated using Prism Software (GraphPad, La Jolla, CA). A *P* value of less than 0.05 was considered statistically significant. Values presented here are shown as mean +/- standard deviation (SD) unless otherwise noted. EC_{50} values were calculated in Prism using an algorithm for fitting non-linear curves with variable slopes.

Chapter 3: KSR1 and EPHB4 regulate Myc and PGC1β to promote survival of human colon tumor cells

Portions of the material covered in this chapter are the topic of a manuscript submitted for publication by McCall JL, Gehring D *et al*.

Introduction

Colorectal cancer (CRC) is the third most common cancer in the U.S. and worldwide (232). It is sporadic in nature with only 15-30% having a major hereditary component (233, 234). CRC is a heterogeneous disease with distinct molecular features of the tumor contributing to the prognosis and response to targeted therapies (235). Several critical genes and pathways are important in the initiation and progression of CRC, most notably the Wnt, Ras/MAPK, PI3K, TGFβ, p53, and DNA mismatch-repair pathways (236). Oncogenic Ras mutations commonly occur in human CRC, with approximately 43% of patients harboring activating K-Ras mutations (237). Patients carrying an oncogenic form of Ras have poorer prognoses compared to patients harboring wild-type Ras (238-240). Their poor response to therapy can be attributed to the observed attenuation in benefit from anti-epidermal growth factor receptor (EGFR) therapies (241) or resistance to RAF inhibitor therapies (242). Ras proteins are a family of small GTPases that regulate a number of cellular signaling pathways associated with the promotion of an oncogenic phenotype, particularly through the MAPK and PI3K pathways (243). The MAPK signaling pathway is composed of the downstream signaling molecules RAF, MEK, and ERK, whose subcellular locations are modulated by KSR1 (244). KSR1 is a scaffold of the RAF/MEK/ERK kinase cascade and is required for maximal MAPK-dependent signaling (180, 245). While KSR1 is required for the survival of CRC cells, it is dispensable in normal colon epithelial cells (188). *ksr1*$^{-/-}$ mice develop normally with attenuated ERK signaling and display a reduced tumor burden in an MMTV-driven mouse tumor model (185, 246). Given that KSR1 is dispensable for normal cells, but indispensable for colorectal cancer cells, we sought to detect and exploit further vulnerabilities in human colon tumor cells. To do this, we developed a gene expression-based high-throughput screen and used functional signature ontology

(FUSION) (188, 228) to identify functional analogs of KSR1. From this screen, we identified EPH (erythropoietin-producing hepatocellular carcinoma) receptor B4 (EPHB4) as a KSR1-like, cancer-specific vulnerability that may be exploited by targeted therapies.

EPH receptors are the largest family of receptor tyrosine kinases (RTKs) with important roles in tissue organization and growth during development as well as in tissue homeostasis in adults (247-249). Humans have nine EPHA and five EPHB receptors that are classified by their ability to bind their respective ligands, ephrin (EPH-receptor interacting protein) A and ephrin B on an adjacent cell. There are five type-A and three type-B ephrin ligands. Ephrin B ligands are transmembrane and receptor-ligand binding is capable of transmitting both forward (through the RTK) and reverse (via the ligand) signaling [reviewed in (250, 251)]. This bidirectional signaling results in repulsion between the two cells and is responsible for establishing boundaries between distinct cell types (252, 253). For example, EPHB4 binding to its ligand ephrin B2 contributes to the establishment of capillaries in the vasculature with EPHB4 expressed primarily in the venous endothelium and ephrin B2 in the arterial endothelium (254, 255). *ephb4* or *ephrin B2* knockout mice are embryonic lethal due to their inability to develop proper vasculature systems (256-258). In the intestine and colon, EPHB-expressing cells are present in the progenitor cells of the crypts, whereas the ephrin B ligand is present in the more differentiated cells (255, 259). The repulsion of EPH-ephrin binding leads to opposing gradients and contributes to the morphology of the intestine and colonic crypts (255, 259).

We have recently shown that tumor-specific expression of PGC1β is required for colon cancer survival (188). Previous work has shown that PGC1β is a direct downstream target of Myc (260, 261). Myc-dependent PGC1β transcription is inhibited by hypoxia in renal clear cell carcinoma due to induction of MXI1 (a repressor of Myc activity) (261), a mechanism that may be operative in the hypoxic regions of many tumor types. In breast cancer cells, HER2 and IGF1 signaling regulate PGC1β via induction of Myc mRNA expression and/or regulation of Myc protein stability. Tight regulation of Myc expression is essential for normal cell function (262, 263). Dysregulation of Myc occurs in more than half of all human tumors and often correlates with aggressive disease (264, 265), resistance to therapy (266-268), and poor prognosis (269-271). Myc activates or represses the transcription of a large number of genes involved in key cellular processes such as cell proliferation, metabolism, apoptosis, and protein synthesis (272). In cancer cells, Myc activation can be induced through constitutive activation of a pathway (i.e., Wnt activation in tumors with APC mutations) (273), or through alterations of the Myc gene (i.e., amplification and translocation) (274, 275). Defects in the APC pathway occur in many human colon carcinomas and result in enhanced TCF-dependent transcriptional activation of Myc (273). In fact, Myc is essential for the for the “crypt progenitor cell-like” phenotype of APC-deficient cells *in vivo* (259). Simultaneous deletion of APC and Myc in the murine adult small intestine rescued the phenotypes of APC deficiency; cells proliferated, differentiated, and migrated like wild-type intestinal enterocytes (276).

Additionally, Ras activation and subsequent phosphorylation events enhance Myc protein stability (277, 278). Constitutive expression may cause Myc to bind to and activate E-box-driven genes that would be regulated by other E-box transcription factors in normal nonproliferative cells, thereby increasing the downstream targets of Myc in cancer cells (279). Recent work from The Cancer Genome Atlas (TCGA) Network discovered that, in a comprehensive examination of human colon and rectal cancers of diverse anatomical origin and mutation status, changes in Myc transcriptional targets were found in nearly all of the tumors (280), suggesting an important role for Myc in CRC. While a promising target for CRC, Myc is a transcription factor and traditionally considered “undruggable” (262, 281). Although there are new strategies emerging to inhibit Myc, including interrupting key dimerization events or DNA binding (262), finding additional or alternative ways to target Myc protein expression or its downstream effectors may provide therapeutic benefits to many cancer patients.

Here we examined EPHB4 and its relationship to Myc and downstream effectors of KSR1 signaling to identify pathways on which colorectal cancer cells are uniquely dependent. We show that EPHB4 has phenotypic and molecular effects in colorectal cancer cells similar to KSR1, and that both KSR1 and EPHB4 are essential for the survival of colorectal cancer cells, but dispensable for the survival of non-transformed, immortalized human colonic epithelial cells (HCECs). Additionally, we demonstrate that both molecules support the expression of PGC1β, which is required for maintaining tumor cell viability. Finally, we show that EPHB4 supports Myc expression by elevating Myc mRNA, while KSR1 promotes the expression of PGC1β by enhancing the translation of Myc mRNA into protein.

Results

EPHB4 is identified as a functional analog of KSR1

Kinase Suppressor of Ras 1 (KSR1) regulates the oncogenic potential of activated Ras (166). Our lab recently showed that KSR1 also promotes anchorage-independent growth and tumor maintenance in human colon tumor cell lines (188). We demonstrated that KSR1 is selectively toxic to colorectal cancer (CRC) cells as compared to immortalized, non-transformed human colonic epithelial cells (HCECs). Using a gene expression signature representing depletion of KSR1, we developed a high-throughput screen termed Functional Signature Ontology (FUSION) (228) to identify functional analogs of KSR1. Details regarding the screen, gene signature, and FUSION have been described previously (188, 228). Based on unsupervised hierarchical clustering of reporter gene expression following RNAi-mediated depletions of individual genes, we found that knockdown of EPH receptor B4 (EPHB4) clustered with the RNAi-mediated KSR1 depletion (siKSR1) positive controls. We further quantified and visualized this relationship by examining and plotting Pearson correlation versus Euclidean distance similarity metrics (Fig 3.1A, Beth Clymer). Depletion of EPHB4 has a Euclidean distance of 1.44 and a Pearson correlation of 0.88, and siEPHB4 (blue) clusters with the siKSR1 (red) reference standards. Based on previous work demonstrating that gene expression-based signatures can be used to represent the functional state of a cell (188, 228, 282, 283), the similarity of siKSR1- and siEPHB4-dependent gene expression signatures suggests that EPHB4 is likely to share functional similarity with KSR1.

EPHB4 expression is elevated in a variety of human cancers including cancers of the head and neck, prostate, bladder, ovaries, large intestine, lung, brain, pancreas, and the esophagus (269, 271, 284-290). We analyzed the expression of EPHB4 in a panel of colon tumor cells as compared to its expression in HCECs. Western blotting revealed that EPHB4 protein is overexpressed in all colon tumor cell lines tested (Fig 3.1B, Drew Gehring). RT-qPCR analyses demonstrate that the abundance of protein cannot be entirely attributed to an overabundance of mRNA (Fig 3.1C). While there is a trend towards increased mRNA levels in all colon tumor cell lines as compared to HCECs, only SK-CO-1 cells show a statistically significant increase. To evaluate the relevance of these findings in human tumors, we examined EPHB4 gene expression in the colon adenocarcinoma dataset within The Cancer Genome Atlas (TCGA) and demonstrated that EPHB4 is significantly increased at the mRNA level in human colon tumor samples compared to normal solid tissue samples (Fig 3.1D, Beth Clymer). These findings were consistent both when using all available data (top) or using only the patient-matched tumor and normal samples (bottom). In fact, every patient-matched tumor demonstrated an increase in EPHB4 expression relative to the normal sample.

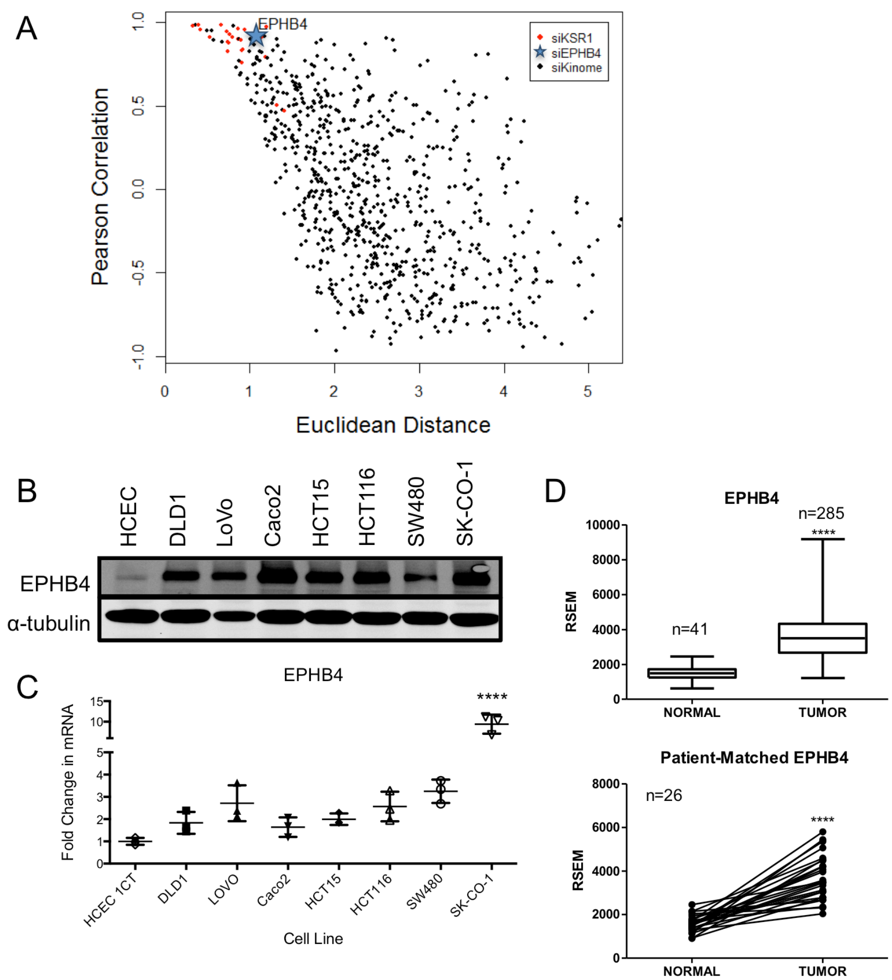


Fig 3.1 Genome-scale RNAi screen identifies EPH Receptor B4 (EPHB4) as a KSR1-like effector.

(A) Identification of EPHB4 as a KSR1 functional analog using Pearson correlation and Euclidean distance similarity metrics. (B) Western blot and (C) RT-qPCR of EPHB4 levels in a panel of colon tumor cell lines and immortalized, non-transformed HCECs. RT-qPCR data are shown as mean ± SD. ****p<0.0001 (matched, one-way ANOVA, Dunnett's post-test). (D) EPHB4 gene expression (RNA-Seq) data from TCGA for unpaired primary colon tumors and normal solid tissue samples (top) primary tumors and patient-matched normal solid tissue samples (bottom). Number (n) of samples analyzed in each is shown. (A) Student's unpaired t-test. (B) **** p < 0.0001. Student's paired t-test. **** p < 0.0001. The results published here are in whole or part based upon data generated by the TCGA Research Network: http://cancergenome.nih.gov/. (3.1A and 3.1D were performed by Beth Clymer, 3.1B was performed by Drew Gehring)

Depletion of EPHB4 is selectively toxic to colon tumor cells

Depletion of KSR1 is selectively toxic to colon tumor cells as compared to HCECs (188). To determine whether EPHB4, like KSR1, is required for tumor cell survival, we measured viability, anchorage-independent growth, and apoptosis in two colon tumor cell lines and HCECs following knockdown of KSR1 or EPHB4 by RNAi. Cell growth was measured by alamarBlue® Cell Viability Assay after 72 hours of KSR1 or EPHB4 depletion. In HCT116 cells, KSR1 and EPHB4 RNAi reduced cell growth as compared to controls by 81% and 71%, respectively (Fig 3.2), whereas, in the Caco2 cells, cell viability was decreased by 95% with KSR1 depletion and 69% with depletion of EPHB4. Cell growth was unaffected in the HCEC cell line. To measure anchorage-independent growth, cell growth was measured on a polyHEMA-coated plate (291, 292) using CellTiter-Glo® Luminescent Cell Viability Assay, as described previously (188). Following depletion of KSR1 or EPHB4, growth in anchorage-independent conditions was reduced by 57% and 53% in HCT116 cells and 74% and 51% in Caco2 cells, respectively (Fig 3.3). HCECs are unable to proliferate in an anchorage-independent environment and were not used in this experiment. Validation of target knockdown is shown by western blot in Fig 3.2 and Fig 3.3. To determine if the reduced cell viability in normal and anchorage-independent conditions is due to increased apoptosis, PARP cleavage was assessed by western blot following depletion of KSR1 and EPHB4 in HCECs, HCT116, and Caco2 cells. HCECs showed no PARP cleavage following target knockdown, whereas HCT116 and Caco2 cells demonstrated PARP cleavage upon KSR1 or EPHB4 depletion (Fig 3.4). These observations show that KSR1 and EPHB4 are selectively required for colon tumor cell survival and growth and suggest that without KSR1 or EPHB4 cells undergo apoptosis.

Downstream effectors of KSR1-dependent signaling in colon tumor cell lines include the RAF/MEK/ERK kinase cascade and PGC1 family of transcriptional regulators

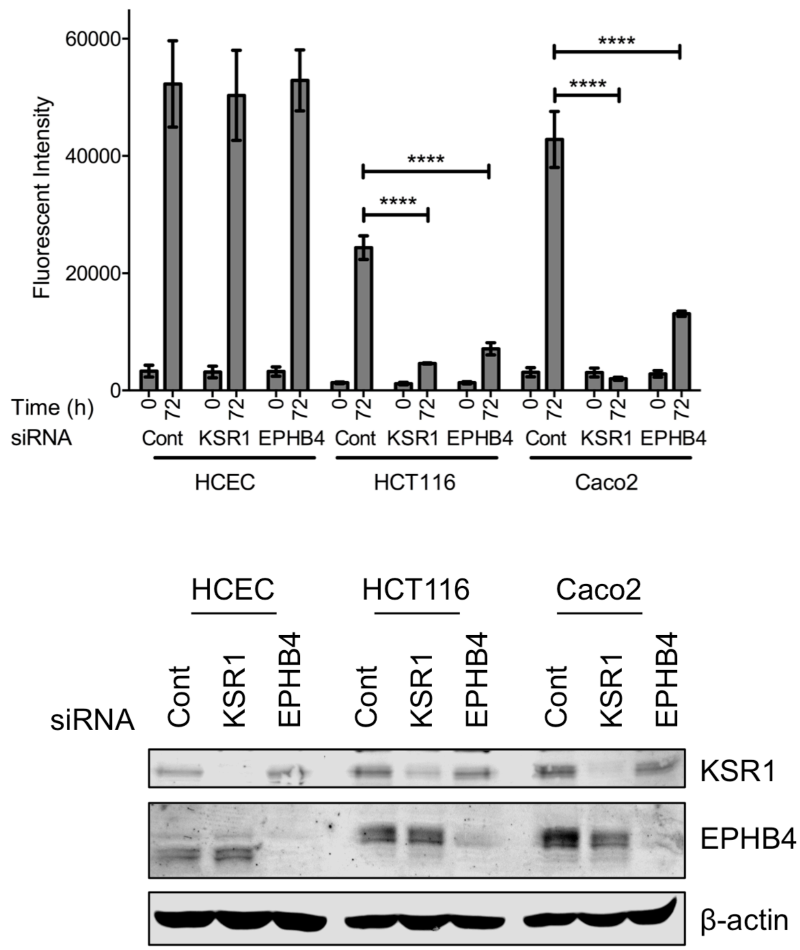


Fig 3.2 Depletion of KSR1 or EPHB4 is selectively toxic to CRC cells.
Viability of HCEC, HCT116, and Caco2 cells was measured following RNAi of KSR1 or EPHB4 by alamarBlue® assays. Data are shown as mean fluorescent intensity ± SD. ****$p<0.0001$ (matched two-way ANOVA, Dunnett's post-test for multiple comparisons). Validation of target knockdown at 72 h is shown by western blot below.

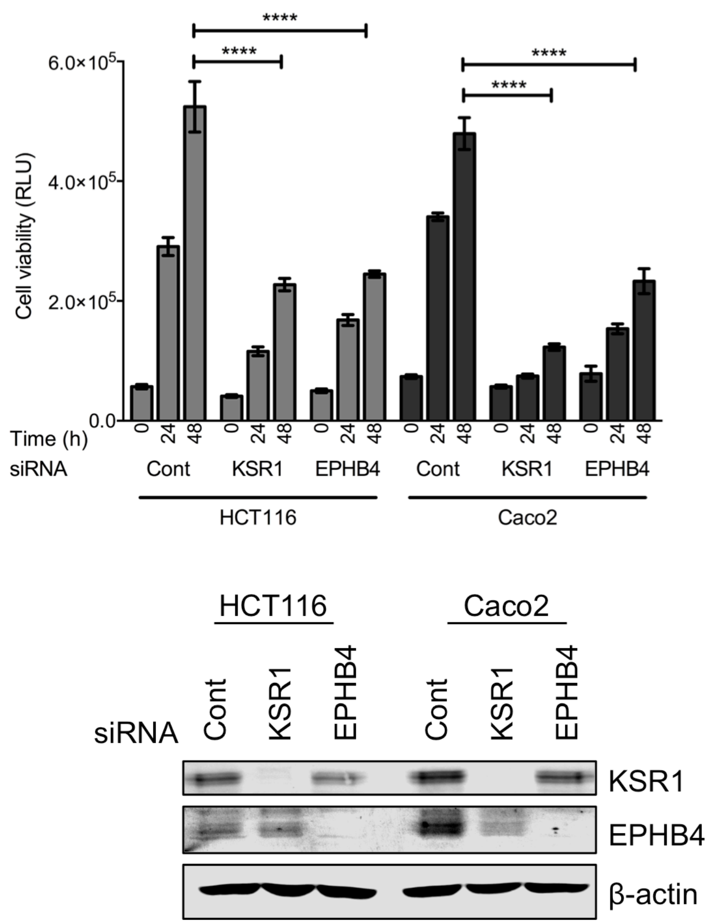


Fig 3.3 Depletion of KSR1 or EPHB4 inhibits anchorage-independent growth. Viability of HCT116 and Caco2 cells was measured following RNAi of KSR1 or EPHB4 in anchorage-independent conditions by CellTiter-Glo® assays. Data are shown as relative light units (RLU) ± SD. ****$p<0.0001$ (matched two-way ANOVA, Dunnett's post-test for multiple comparisons). Validation of target knockdown at 48 h timepoint is shown by western blot below.

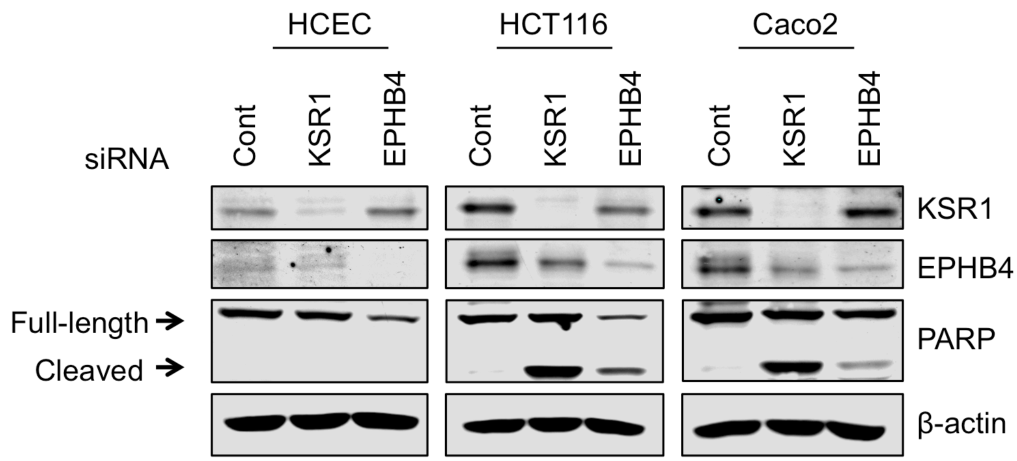


Fig 3.4 KSR1 and EPHB4 depletion selectively induces apoptosis in CRC cells. PARP cleavage was measured by western blot following depletion of KSR1 or EPHB4 for 72 h in HCEC, HCT116, and Caco2 cells.

(166, 172, 180, 187, 188). We recently identified PGC1β as a key downstream effector of KSR1 in human colon tumor cells and showed that its expression is required for colon cancer survival both *in vitro* and *in vivo* (188). To determine whether EPHB4 disrupts either of these pathways, we assessed MEK1/2 and ERK1/2 activation and total PGC1β protein levels by western blot after 72 hours of EPHB4 depletion in HCT116 and Caco2 cells. We observe that depletion of EPHB4 does not affect MEK1/2 or ERK1/2 phosphorylation. However, EPHB4 RNAi does suppress PGC1β levels (Fig 3.5). These data suggest that EPHB4 is acting as a functional analog of KSR1 to regulate PGC1β.

Inhibition of EPHB4 kinase activity is selectively toxic to colon tumor cells

Currently, there are several clinical trials in various cancers involving the pharmacological targeting of EPHB4 (250, 251). One strategy for targeting EPHB4 is inhibition of its kinase activity. To test whether this, like RNAi-mediated EPHB4 depletion, is selectively toxic to colon tumor cells, we treated HCECs and a panel of colon tumor cell lines with increasing doses of an EPHB4 kinase inhibitor, AZ12672857 (AZ2857), for 72 hours and cell viability was measured using CellTiter-Glo®. The EC_{50} for each cell line was determined from four independent experiments using a non-linear curve fit with a variable slope (Fig 3.6A, Drew Gehring). HCECs (EC_{50} = 14 μM) were less sensitive to EPHB4 inhibition than HCT116 or Caco2 cells, 3.2 μM and 2.6 μM, respectively. The EC_{50} of three additional colon tumor cell lines (SW480, DLD1, and SK-CO-1) are shown in the table of Fig 3.6A (Drew Gehring). HCECs tolerated doses up to 20 μM (highest tested) without increasing the percentage of PI-stained cells in the sub-G1 peak when measured by flow cytometry (Fig 3.6B, Drew Gehring). However, at the same dose, HCT116 and Caco2 cells had >50% sub-G1 cells after 72 hours of treatment with AZ2857. Taken together, these data indicate that inhibition of EPHB4 decreases total ATP levels as measured by the CellTiter-Glo® cell viability assay in the HCECs,

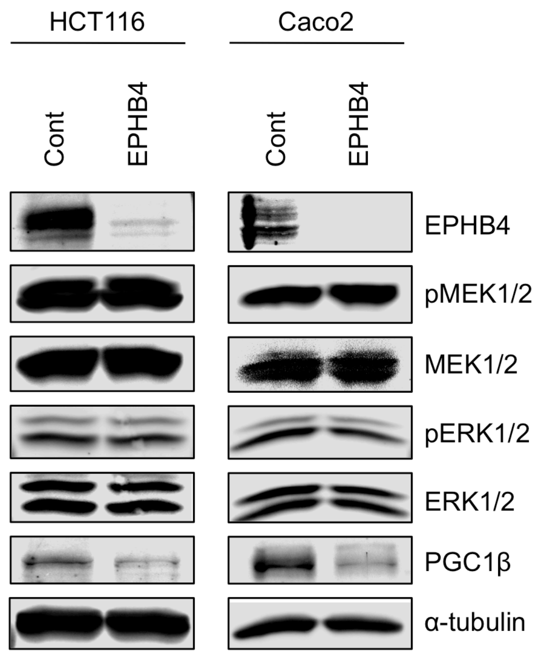
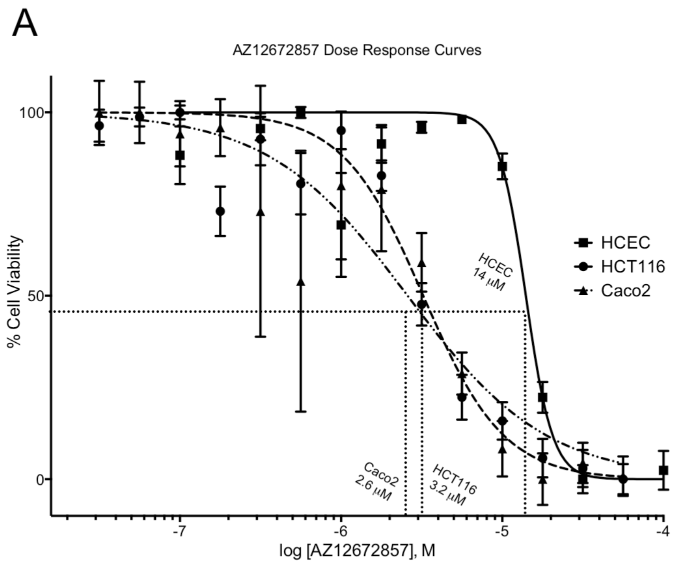


Fig 3.5 Depletion of EPHB4 decreases PGC1β expression, but not MEK and ERK activation.

EPHB4 was depleted by siRNA for 72 h in HCT116 cells. Protein expression was measured by western blot. (Experiment performed by Kurt Fisher)



Cell Line	EC_{50}, µM
HCEC	14.0
HCT116	3.2
Caco2	2.6
SW480	3.2
DLD1	1.7
SK-CO-1	2.4

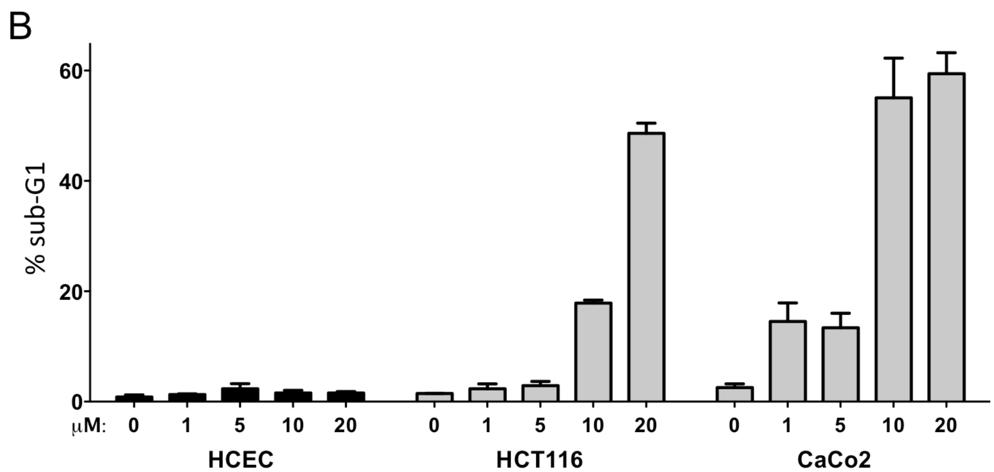


Fig 3.6 EPHB4 inhibitors are selectively toxic to colon tumor cell lines.

HCEC, Caco2, HCT116, SW480, DLD1, and SK-CO-1 cells were treated with increasing doses of AZ12672857 and cell viability was measured by CellTiter-Glo® at 72 hours. Each data point represents four independent experiments. Data are presented as mean ± SEM. Data were normalized and the EC_{50} for each cell line was calculated using an algorithm for fitting a non-linear curve with variable slope in GraphPad Prism. (B) HCEC, HCT116, and Caco2 cells were treated with increasing doses of AZ12672857. The sub-G1 peak was quantified following PI staining and analysis by flow cytometry. (Experiments performed by Drew Gehring) possibly through reduced growth or induction of senescence, but that treatment with AZ2857 does not induce apoptosis. However, in the tumor cell lines, HCT116 and Caco2, treatment with AZ2857 clearly reduces cell viability via induction of cell death.

KSR1 and EPHB4 regulate Myc and PGC1β

Previous research demonstrated that Myc regulates PGC1β transcription in renal cell carcinoma (261) and breast cancer cells (260). Therefore, we examined whether Myc regulates PGC1β in colon tumor cell lines. In HCT116 and Caco2 cells, Myc was depleted with a pool of siRNA; PGC1β protein levels were decreased with Myc knockdown as assessed by western blot (Fig 3.7A). To determine if this effect was due to a single siRNA and potentially an off-target effect, the four siRNA duplexes (duplexes 22, 23, 24, and 25) that compose the pool were assessed independently. Expression of PGC1β protein correlated with degree of Myc knockdown (Fig 3.7B). The pool of all four siRNA duplexes was used in the following experiments. Next, PGC1β mRNA levels were measured by RT-qPCR following depletion of Myc. In both HCT116 and Caco2 cells, Myc depletion significantly decreased expression of PGC1β mRNA (Fig 3.8). Expression of Myc is required for the formation of intestinal crypts, but is dispensable for homeostasis of the adult epithelium (293). To evaluate the importance of Myc expression to CRC cell viability, two tumor cell lines (HCT116 and Caco2) and one normal colon cell line (HCECs) were transfected with siMyc or a non-targeting siRNA and cell viability was assessed 72 hours post-transfection. Depletion of Myc reduced cell viability in HCT116 (60%) and Caco2 (64%) cells, but did not affect growth in the HCECs (Fig 3.9), indicating that the tumor cells are more reliant on the expression of Myc for cell growth. Validation of target knockdown is shown by western blot.

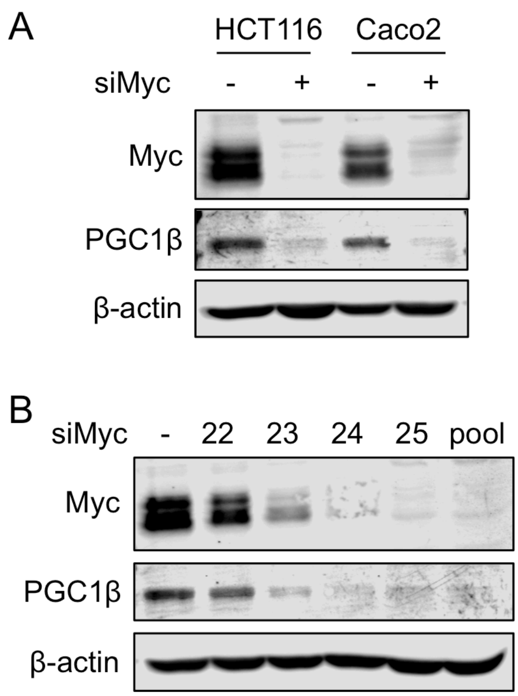


Fig 3.7 Myc regulates PGC1β in colon tumor cells.

(A) Western blot following RNAi of Myc in HCT116 (left) and Caco2 (right) cells. (B) Myc and PGC1β protein expression in HCT116 cells transfected with individual or pooled (all 4) Myc siRNA duplexes.

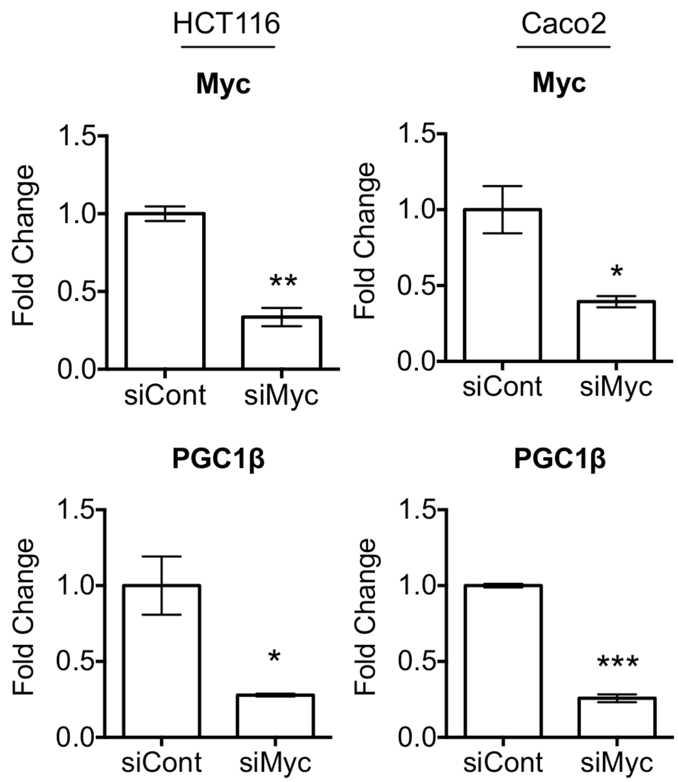


Fig 3.8 Myc depletion reduces PGC1β mRNA levels in CRC cells.

RNA levels of Myc and PGC1β were measured by RT-qPCR in HCT116 (left) and Caco2 (right) cells following RNAi of Myc. Data are shown as mean ± SD. *p<0.05, **p<0.01, ***p<0.001 (paired, two-tailed *t*-test).

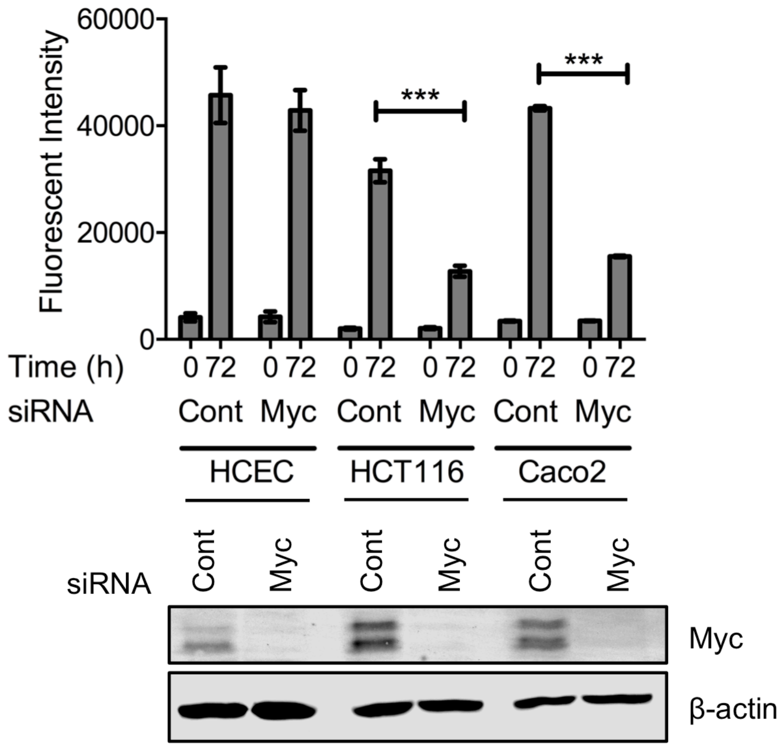


Fig 3.9 Myc depletion decreases viability in colon tumor cell lines.

Cell viability was measured by alamarBlue® following depletion of Myc. Data are shown as mean ± SD. ***p<0.001 (matched, two-way ANOVA).

To determine whether KSR1 and EPHB4 are regulating PGC1β through a Myc-dependent pathway, we assessed Myc and PGC1β protein levels following depletion of KSR1 or EPHB4 resulted in diminished expression of Myc and PGC1β (Fig 3.10A), with EPHB4 depletion having the greatest effect on Myc levels. To confirm that these data are not the result of a single siRNA or an off-target effect, we transfected the four individual siRNA duplexes for KSR1 (Fig 3.10B) and EPHB4 (Fig 3.10C) into HCT116 cells and measured target, Myc, and PGC1β protein expression by western blot 72 hours post-transfection. With the exception of KSR1 siRNA 6, all individual duplexes sufficiently depleted the expression of their target as well as Myc and PGC1β. Due to its lack of target knockdown, the KSR1 siRNA duplex 6 was not used in the siKSR1 pool in any experiment. Additionally, HCT116 and Caco2 cells were treated with increasing doses of the EPHB4 kinase inhibitor, AZ2857, for 72 hours. Western blots indicate that pharmacological inhibition of EPHB4 decreases Myc and PGC1β protein levels similar to that seen with depletion using siRNA (Fig 3.11A, Drew Gehring). HCT116 cells were treated with 10 μM AZ2857 for 0-72 hours and total levels of Myc and ERK activation were assessed by western blot. Inhibition of EPHB4 reduced Myc levels in an ERK-independent manner (Fig 3.11B).

To assess whether EPHB4 also regulates PGC1β mRNA levels, HCT116 and Caco2 cells were transfected with siRNA targeting EPHB4 (or a non-targeting siRNA) or treated with 10 μM of AZ2857 for 72 hours. Myc and PGC1β mRNA levels were measured by RT-qPCR. Data from three biological replicates (each measured in triplicate) are shown in Fig 3.12A (siRNA) and Fig 3.12B (EPHB4 inhibitor). Depletion of EPHB4 by siRNA decreased Myc and PGC1β mRNA expression by 46% and 49% in HCT116 and 70% and 26% (not significant) in Caco2 cells, respectively. Inhibition of EPHB4 with the kinase inhibitor AZ2857 consistently decreased levels of both Myc and PGC1β mRNA 70% and 45% in HCT116 and 67% and 56% in Caco2, respectively.

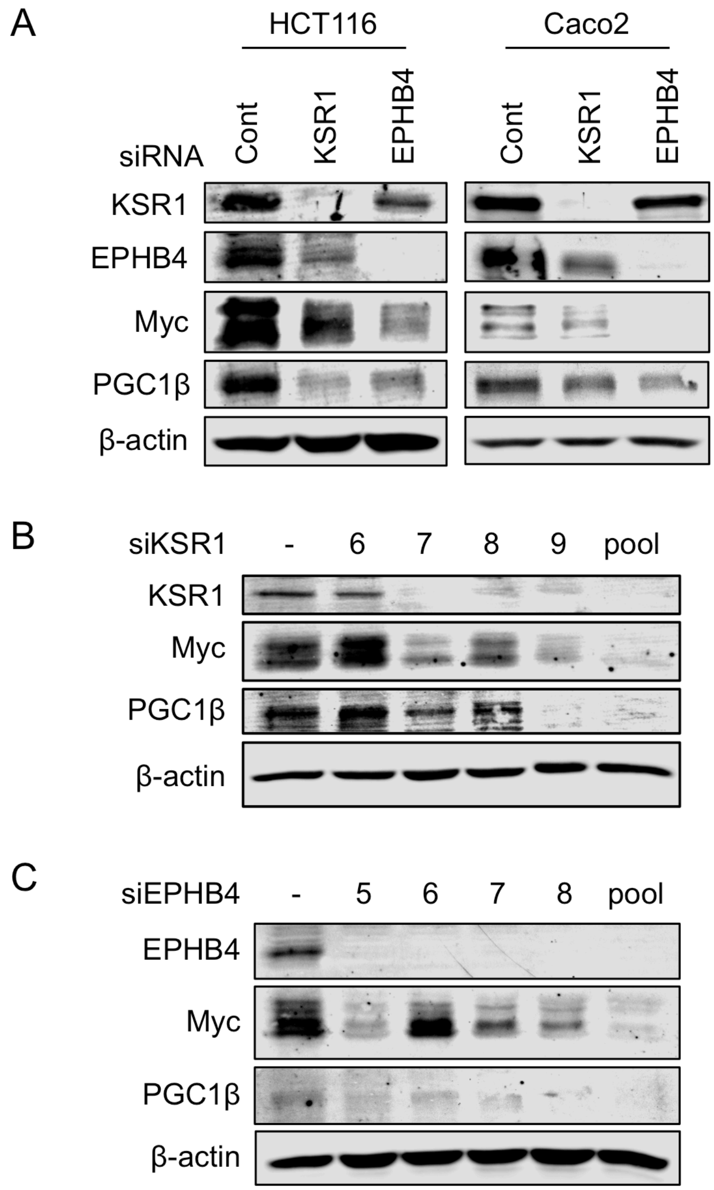


Fig 3.10 Inhibition of KSR1 or EPHB4 decreases Myc and PGC1β protein levels. Myc and PGC1β protein levels were assessed by western blotting following RNAi of KSR1 or EPHB4 in HCT116 (left) and Caco2 (right) cells. (B) Individual siRNA duplexes for KSR1 (top) and EPHB4 (bottom) were transfected into HCT116 cells and Myc and PGC1β protein expression was assessed by western blot. The KSR1 pool contains duplexes 7-9. The EPHB4 pool contains all 4 siRNA duplexes.

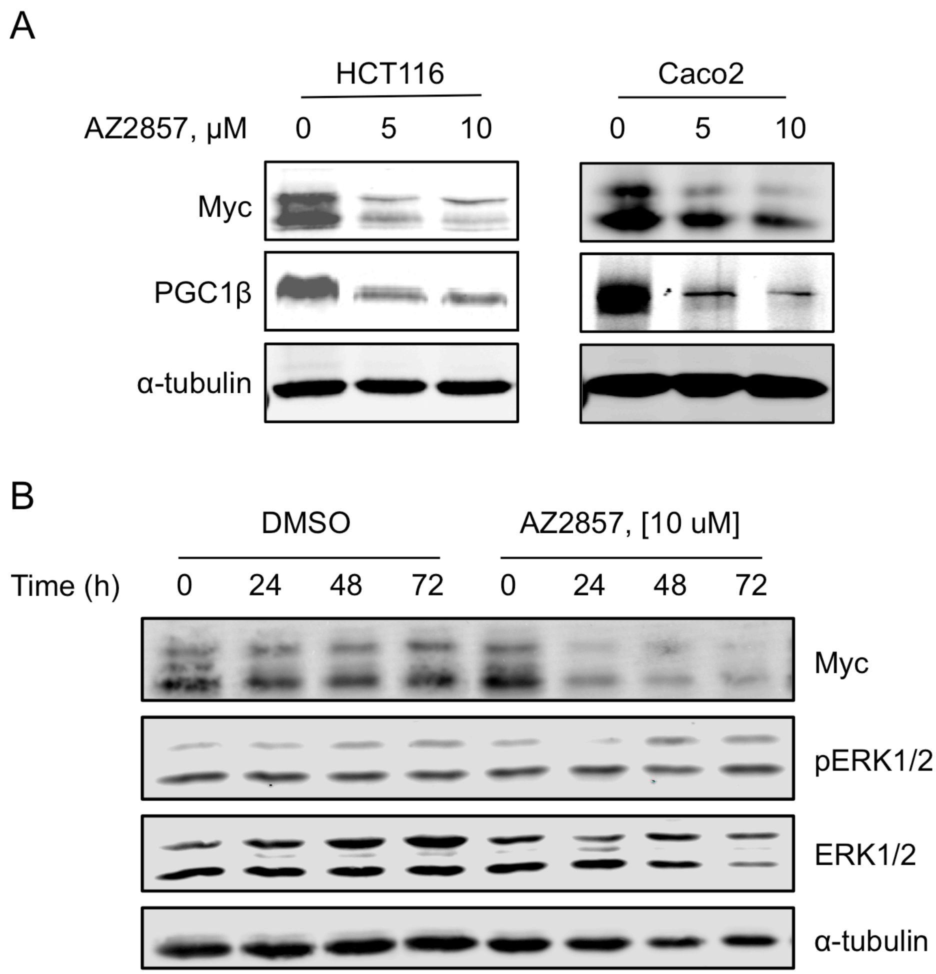


Fig 3.11 Treatment with EPHB4 kinase inhibitor decreases Myc protein levels. Myc and PGC1β protein levels were assessed by western blotting following treatment with AZ12672857 (AZ2857), an EPHB4 inhibitor in HCT116 (left) and Caco2 (right) cells. (B) Myc, phospho-ERK, and total ERK1/2 protein levels were assessed by western blotting following treatment of HCT116 with 10 µM AZ2857 for 0-72 h. (Experiment 3.11A performed by Drew Gehring)

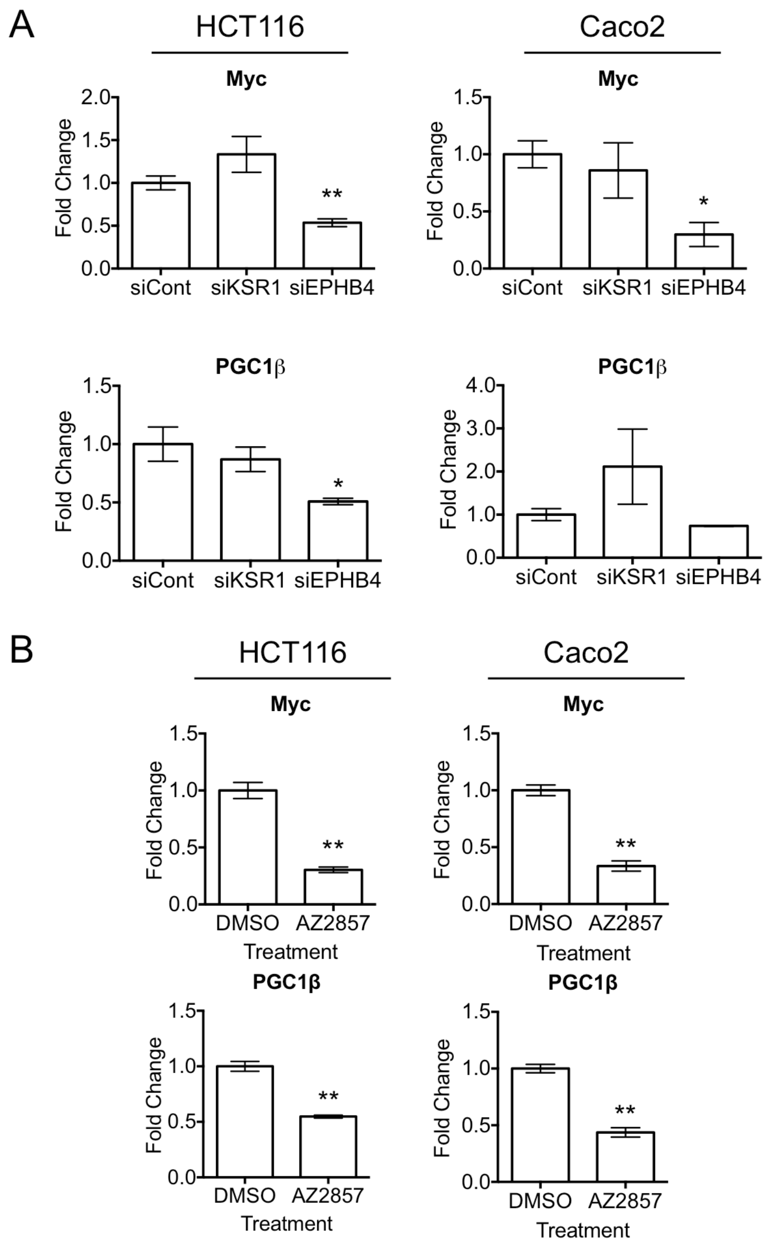


Fig 3.12 EPHB4 inhibition decreases Myc and PGC1β mRNA expression.
RNA levels of Myc (top) and PGC1β (bottom) were measured by RT-qPCR in HCT116 (left) and Caco2 (right) cells following RNAi of EPHB4 (A) or treatment with AZ2857 (B). Data are shown as mean ± SD. *p<0.05, **p<0.01 (A) repeated measures, one-way ANOVA with Dunnett's post-test for multiple comparisons; (B) paired, two-tailed *t*-test.

Depletion of KSR1 did not affect mRNA levels of Myc or PGC1β (Fig 3.12A). These observations suggest that EPHB4 may regulate PGC1β transcription in a Myc-dependent manner. Although EPHB4 was identified by FUSION using KSR1 as a reference standard, and both proteins share common downstream effectors (Myc and PGC1β), these data reveal that the mechanisms by which EPHB4 and KSR1 regulate Myc and PGC1β are not identical.

KSR1 and EPHB4 do not affect Myc stability

One mechanism by which Myc protein stability is regulated is through proteasome-mediated degradation by F-box and WD repeat domain-containing 7 (FBW7)-containing Skp1-Cul1-Fbox (SCF) ubiquitin ligase (277). FBW7 is frequently deleted or mutated in a variety of cancers including gastric (294), colon (280, 295), and breast (296). In contrast, FBW7 is rarely mutated in pancreatic cancer, but the protein is significantly downregulated by activated Ras-RAF-MEK-ERK signaling (297). ERK-dependent phosphorylation of FBW7 leads to ubiquitination and proteasome-mediated degradation by an undefined E3 ligase resulting in elevated expression of FBW7 substrates, such as Myc (297). On the basis of these observations, we tested whether depletion of KSR1 or EPHB4 increased the amount of FBW7 in the colon cancer cell line, HCT116, which expresses FBW7 (298). Since KSR1 is necessary for maximal ERK phosphorylation and subsequent activation (166), we anticipated that depletion of KSR1 would increase cellular levels of FBW7. However, as shown in Fig 3.5 and 3.11B, depletion of EPHB4 does not affect phosphorylation of MEK or ERK. Therefore, we predicted that depletion of EPHB4 would not enhance FBW7 levels in the HCT116 cells. As anticipated, FBW7 is significantly upregulated in HCT116 cells upon KSR1 depletion. However, it is also upregulated by EPHB4 depletion, although to a lesser degree than KSR1 depletion (Fig 3.13). Another SCF ubiquitin ligase, $SCF^{\beta\text{-TrCP}}$, targets an alternate

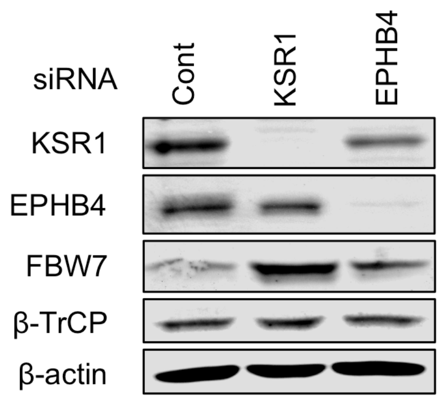


Fig 3.13 Depletion of KSR1 or EPHB4 increases FBW7 expression in HCT116 cells. Expression of the E3 ubiquitin ligases FBW7 and β-TrCP were assessed by western blot after knockdown of KSR1 or EPHB4.

phosphodegron at Ser279 and Ser283 to ubiquitinate and stabilize Myc (299). We, therefore, tested the expression of β-TrCP following KSR1 or EPHB4 depletion. Expression of β-TrCP was unchanged in either condition (Fig 3.13).

FBW7-dependent degradation of Myc is dependent on consecutive phosphorylation at Ser62, then Thr58 (277, 300). Phosphorylation at these sites exhibit opposing roles, with Ser62 promoting Myc stability and Thr58 promoting ubiquitin/proteasome-dependent degradation by FBW7 (277, 300, 301). In the absence of Ser62 phosphorylation, Myc protein is rapidly degraded by one of several FBW7-independent mechanisms (277, 302). To determine if depletion of KSR1 or EPHB4 is promoting Myc degradation by mediating phosphorylation of either of these sites, we assessed Myc phosphorylation following proteasome inhibition with MG132 using phospho-specific antibodies. The ratio of phosphorylated to total Myc after MG132 treatment was quantified in three independent experiments (Fig 3.14). While levels of Thr58 phosphorylation remained unchanged and highly variable, phosphorylation at Ser62 was consistently decreased in KSR1- and EPHB4-depleted cells indicating that KSR1- or EPHB4-dependent phosphorylation of Myc at Ser62 contributes to the stabilization of Myc.

We further assessed the role of KSR1 and EPHB4 in FBW7-dependent regulation of Myc using HCT116 cells with genetic deletion of FBW7 (298). Myc and PGC1β protein levels were assessed in the presence and absence of KSR1 or EPHB4. Knockdown of KSR1 or EPHB4 resulted in decreased Myc and PGC1β protein even in the absence of FBW7 (Fig 3.15). Taken together, these data suggest that although KSR1 and EPHB4 can augment FBW7 levels and increase phosphorylation of Myc at Ser62, these proteins primarily regulate Myc expression by an FBW7-independent mechanism and further suggest the possibility that an additional E3 ligase recognizing Myc phosphorylation at Ser62.

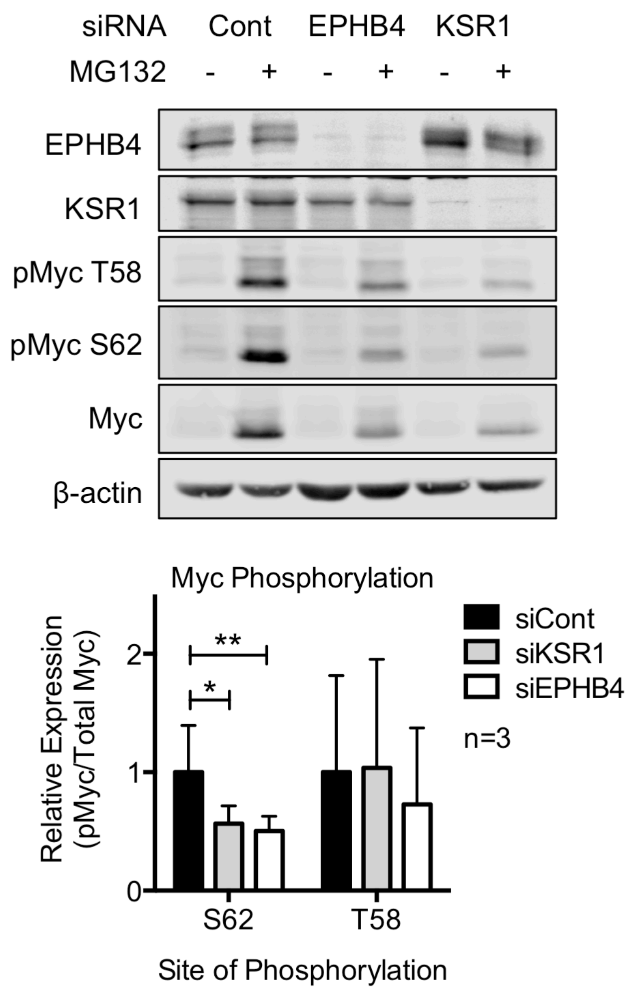


Fig 3.14 Depletion of KSR1 or EPHB4 decreases Myc phosphorylation at Ser62 in HCT116 cells.

Expression of phosphorylated and total Myc following KSR1 or EPHB4 depletion in the presence or absence of MG132 was assessed by western blot. Phospho-Myc expression was quantified and normalized to total Myc. Quantification of three replicates (mean ± SD) is shown in the graph below.

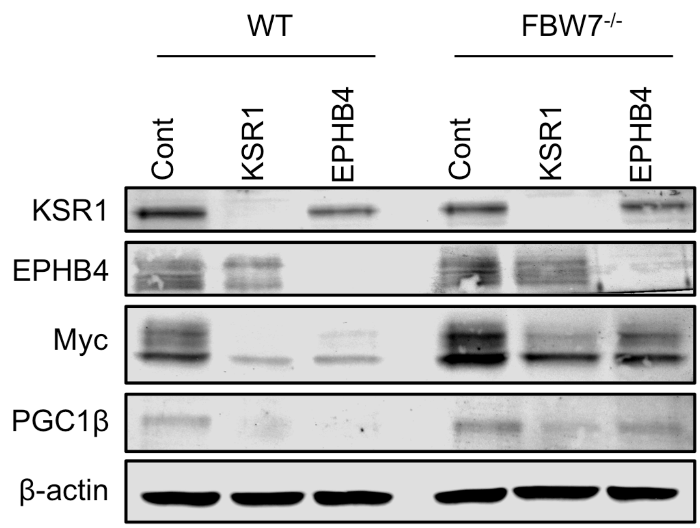


Fig 3.15 Depletion of KSR1 or EPHB4 decreases Myc stability in WT and FBW7$^{-/-}$ HCT116 cells.

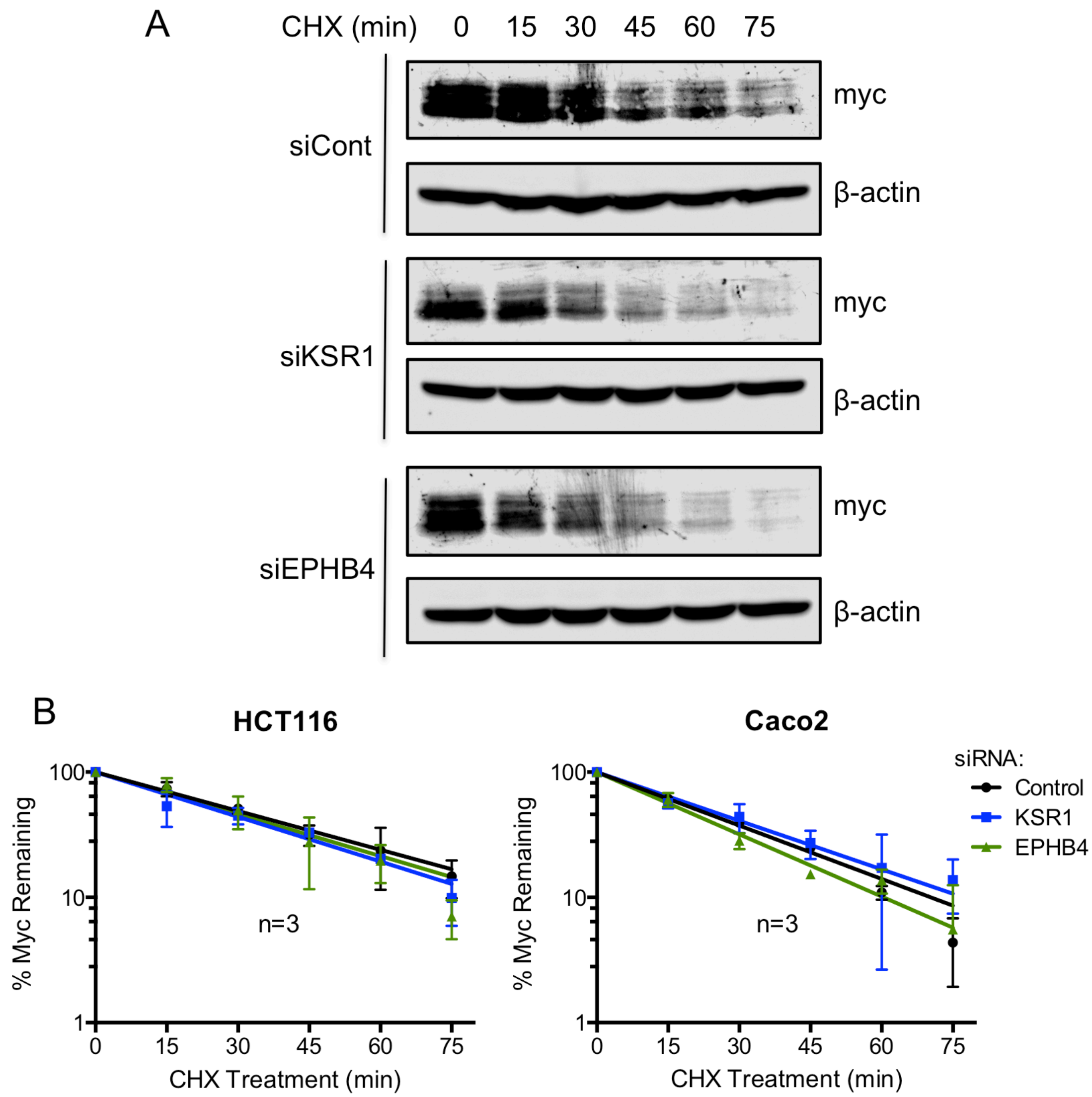
Myc and PGC1β protein expression is assessed by western blot following KSR1 or EPHB4 depletion in WT and FBW7$^{-/-}$ HCT116 cells.

To test the hypothesis that depletion of KSR1 or EPHB4 is affecting Myc levels by regulating protein stability, we examined the turnover of Myc in HCT116 cells following treatment with CHX with and without RNAi of KSR1 or EPHB4. Representative western blots of HCT116 cells from three independent experiments are shown (Fig 3.16A). Data from three independent experiments in each cell line was quantified and the half-life of Myc in each condition was calculated using GraphPad Prism software. Depletion of KSR1 or EPHB4 did not change the rate of Myc turnover (Fig 3.16B).

KSR1 promotes the translation of Myc

Regulation of protein synthesis is mediated by key inhibitors of translation, eukaryotic initiation factor 4E binding protein 1 (4E-BP1) and programmed cell death 4 (PDCD4) [reviewed in (303)]. 4E-BP1 sequesters eukaryotic initiation factor 4E (eiF4E) to inhibit translation. Phosphorylation of 4E-BP1 releases eIF4E and de-represses protein synthesis [reviewed in (303)]. Similarly, PDCD4 sequesters eukaryotic initiation factor 4A (eIF4A). Phosphorylation of PDCD4 leads to its nuclear localization or proteasome-mediated degradation (304). PDCD4 can be phosphorylated by p70 S6 kinase (S6K) or p90 ribosomal protein S6K (RSK) (304, 305). 4E-BP1 and eIF4E regulate cap-dependent translation, while PDCD4 and eIF4A regulate both cap-dependent and –independent translation [reviewed in (303)].

To determine if KSR1 or EPHB4 affects the expression of these key regulators of protein synthesis, KSR1 and EPHB4 were depleted by siRNA for 48 hours in HCT116 and Caco2 cells. In both cell lines, depletion of KSR1, but not EPHB4, decreased 4E-BP1 and eIF4E phosphorylation (Fig 3.17). This suggests that depletion of KSR1 inhibits cap-dependent translation. Additionally, depletion of KSR1, and not EPHB4, increased the total levels of PDCD4, suggesting that KSR1 can promote both cap-dependent and cap-independent translation.



HCT116

	Half Life		
	Value	+Error	-Error
Control	29.106	3.573	2.869
KSR1	25.293	3.192	2.549
EPHB4	26.944	5.808	4.058

Caco2

	Half Life		
	Value	+Error	-Error
Control	21.184	1.134	1.024
KSR1	23.270	4.030	2.993
EPHB4	18.185	1.843	1.532

Fig 3.16 Depletion of KSR1 or EPHB4 does not affect Myc stability in HCT116 or Caco2 cells.

Depletion of KSR1 or EPHB4 performed for 72 hours prior to treatment with 100 µg/mL CHX or vehicle for 0, 15, 30, 45, 60, or 75 minutes. Myc levels were assessed by western blot (A). Assay was performed three times in each cell line. Myc expression was quantified and normalized to β-actin. Myc half-life was calculated using a non-liner, one-phase decay (Y0 = 100, plateau = 0) with automatic outlier elimination in GraphPad Prism (B).

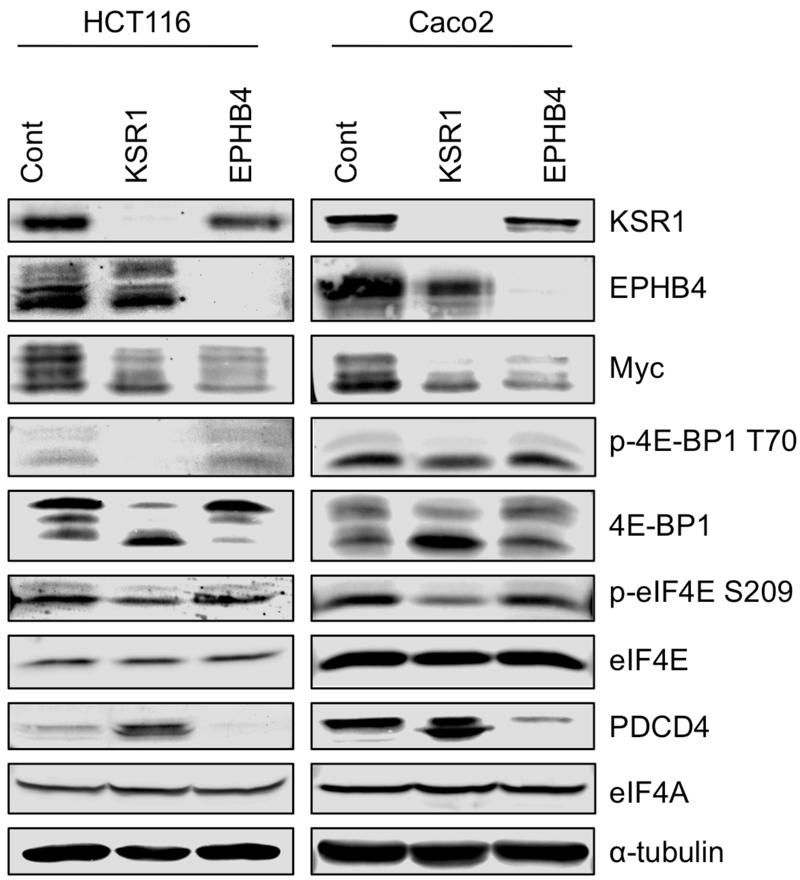


Fig 3.17 Depletion of KSR1 affects key inhibitors of protein translation.
HCT116 and Caco2 cells were transfected with siRNA targeting KSR1 or EPHB4, or a non-targeting siRNA (Cont) for 48 hours. Protein expression levels were assessed by western blot.

We further assessed the role of KSR1 and EPHB4 in Myc translation using luciferase reporter constructs containing the Myc 5' UTR. The 5' UTR of Myc mRNA contains an internal ribosome entry site (IRES) (230), and therefore Myc is translated in both a cap- and IRES-dependent manner. Translation from the IRES element depends on eIF4A (helicase), but is independent of eIF4E (cap binding protein) (306). First, we measured total translation of Myc (cap- and IRES-dependent) using the pGML reporter construct (231, 307), which contains the entire 5' UTR of Myc. Luciferase signal was normalized to total protein in each sample. Depletion of KSR1, but not EPHB4, decreased translation of Myc in HCT116 and Caco2 cells by 49% and 33%, respectively (Fig 3.18). Since KSR1 also affected the expression of PDCD4, we also measured the effect of KSR1 depletion on IRES-dependent translation of Myc. Depletion of KSR1 decreases IRES-dependent Myc translation in HCT116 and Caco2 cells by 63% and 68%, respectively (Fig 3.19).

To determine the relative contribution of IRES-dependent translation of Myc to total Myc synthesis, HCT116 and Caco2 cells were transfected with pGML (Myc 5' UTR), phpL (hairpin only), or phpmL (hairpin-Myc 5' UTR) luciferase reporter constructs with a pSV-β-galactosidase vector for 24 hours. Luciferase expression was normalized to β-galactosidase expression in each well. IRES-dependent translation comprises 32% and 14% of total Myc translation in HCT116 and Caco2 cells, respectively (Fig 3.20).

KSR1 protects EPHB4 from lysosome-dependent degradation

To assess the possible relationships between KSR1 and EPHB4, KSR1 and EPHB4 were depleted by siRNA for 72 hours in HCT116 and Caco2 cells. Levels of KSR1 and EPHB4 protein (Fig 3.21A) and mRNA (Fig 3.21B) were measured by western blot and RT-qPCR, respectively. Depletion of KSR1 results in a consistent reduction in EPHB4 protein expression, but not mRNA levels. EPHB4 knockdown does

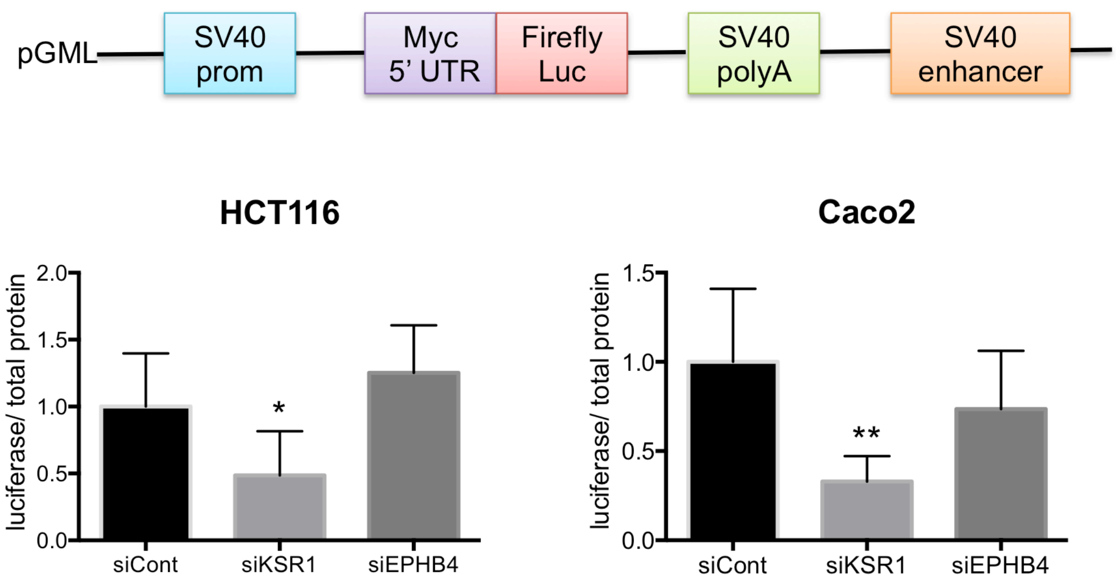


Fig 3.18 KSR1 promotes the translation of Myc protein.

HCT116 and Caco2 cells were depleted of KSR1 or EPHB4 for 24 hours and then transfected with the pGML (Myc 5' UTR) luciferase reporter construct for an additional 24 hours. Luciferase expression was normalized to total protein in each well. Data are shown as mean ± SD. *$p<0.05$, **$p<0.01$ (repeated measures, one-way ANOVA with Dunnett's post-test for multiple comparisons).

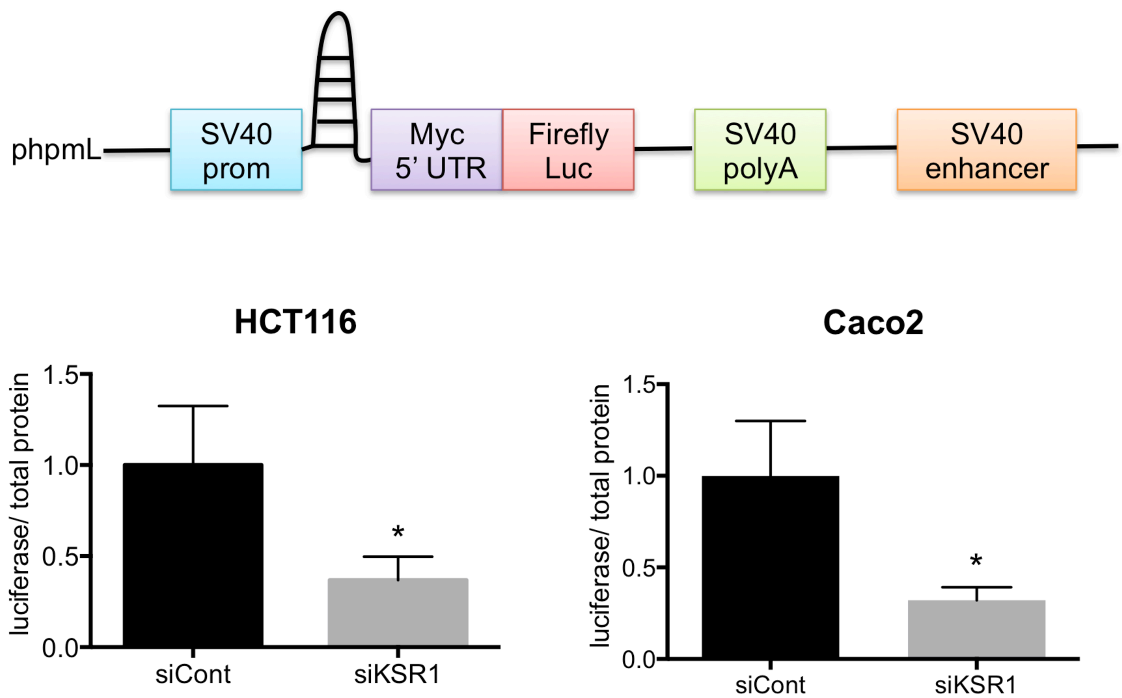


Fig 3.19 KSR1 promotes IRES-dependent translation of Myc.

HCT116 and Caco2 cells were depleted of KSR1 for 24 hours and then transfected with the phpmL (hairpin-Myc 5' UTR) luciferase reporter construct for an additional 24 hours. Luciferase expression was normalized to total protein in each well. Data are shown as mean ± SD. *$p<0.05$ (paired, tailed *t*-test).

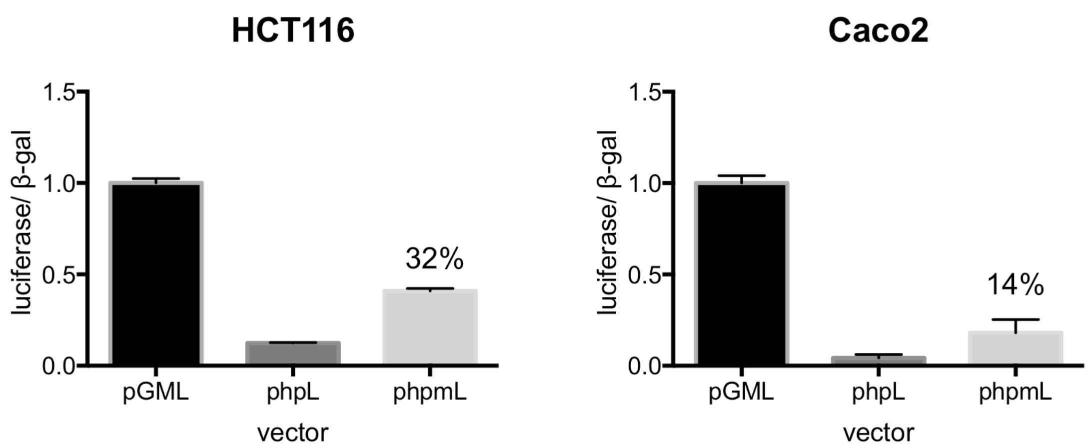


Fig 3.20 IRES-dependent translation accounts for a portion of total Myc translation.

HCT116 and Caco2 cells were transfected with pGML (Myc 5' UTR), phpL (hairpin only), or phpmL (hairpin-Myc 5' UTR) luciferase reporter constructs with a pSV-β-galactosidase vector for 24 hours. Luciferase expression was normalized to β-galactosidase expression in each well. Data are shown as technical replicates of a single biological replicate.

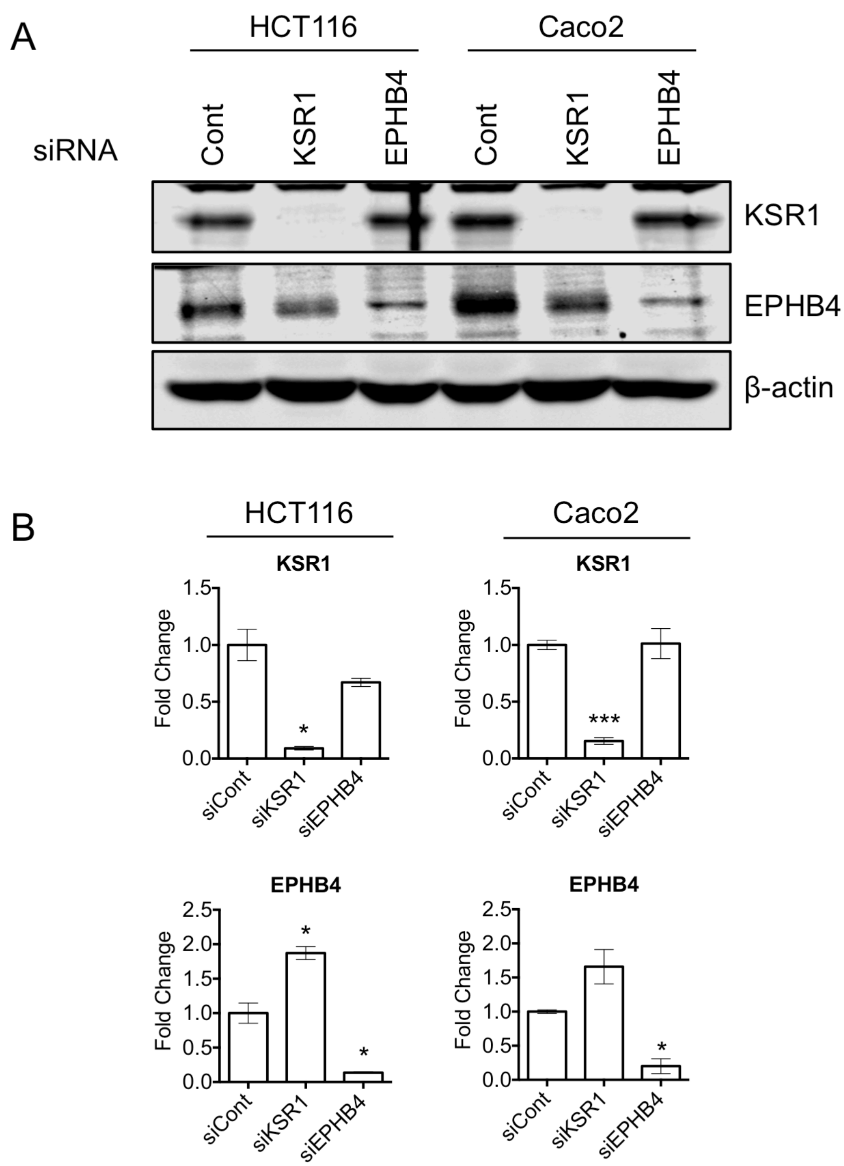


Fig 3.21 Depletion of KSR1 decreases EPHB4 protein, but not mRNA, expression. KSR1 or EPHB4 was depleted in HCT116 and Caco2 cells for 72 h. (A) KSR1 and EPHB4 protein levels were assessed by western blot. (B) KSR1 and EPHB4 mRNA levels were measured by RT-qPCR. Data are shown as mean ± SD. *p<0.05, ***p<0.001 (repeated measures, one-way ANOVA with Dunnett's post-test for multiple comparisons).

not affect KSR1 protein or mRNA expression in either cell line. This suggests that KSR1 regulates EPHB4 protein levels via a post-transcriptional mechanism.

KSR1 regulates proteins, such as Myc and PGC1β, which are degraded by the proteasome (188, 308). Therefore, we tested whether KSR1 depletion induces proteasome-mediated degradation of EPHB4. HCT116 and Caco2 cells were depleted of KSR1 for 72 hours and incubated with and without 10 μM MG132 for the final 6 hours of knockdown. Treatment with MG132 was unable to rescue the EPHB4 levels when KSR1 was depleted (Fig 3.22). Inhibition of proteasomal degradation of Myc was used as a positive control for MG132 treatment. In HCT116 cells without KSR1 depletion, MG132 treatment increases EPHB4 expression suggesting that EPHB4 degradation is partially mediated by the proteasome in these cells, but the effect of KSR1 on EPHB4 stability is independent of proteasome-mediated degradation.

A canonical method of RTK signal termination is downregulation after ligand binding (309-311). Lysosome-mediated degradation of RTKs, including EPHB1, has been well documented (312-314). We assessed whether EPHB4 degradation is mediated by the lysosome and whether KSR1 stabilizes EPHB4 expression by suppressing this degradation. KSR1 was depleted in HCT116 and Caco2 cells for 72 hours with and without treatment with 100 nM bafilomycin A1 (BafA1), an inhibitor of autophagosome-lysosome fusion, for the final 8 hours of knockdown. Treatment with BafA1 alone increased the expression of EPHB4 in both HCT116 and Caco2 cells (Fig 3.19). Additionally, when cells were depleted of KSR1, treatment with BafA1 rescues EPHB4 expression. Increased LC3BII and p62 expression are used as positive controls for BafA1 treatment. Taken together, these data indicate that KSR1 stabilizes EPHB4 by suppressing lysosome-mediated degradation.

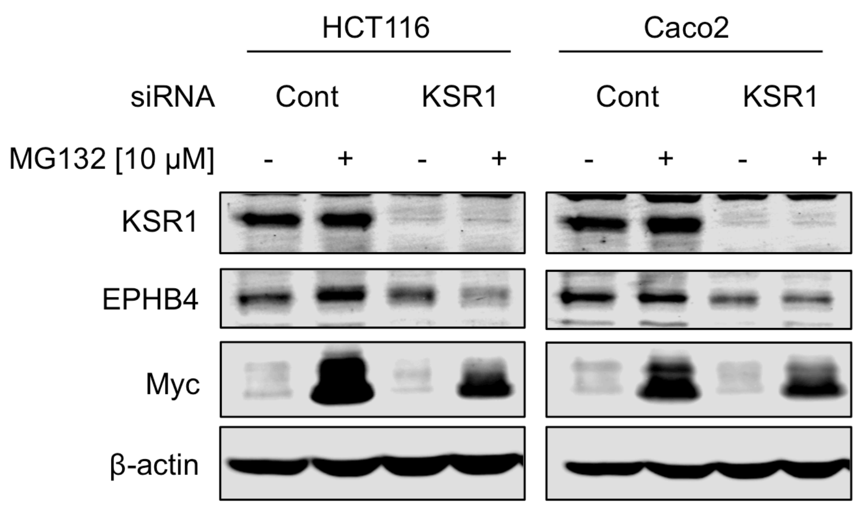


Fig 3.22 EPHB4 expression is not rescued by proteasomal inhibition with MG132. KSR1 was depleted in HCT116 and Caco2 cells for 72 h. Cells were treated with and without 10 μM MG132 for the last 8 h of knockdown. Proteins were analyzed by western blot. Myc is used as a positive control for MG132 treatment.

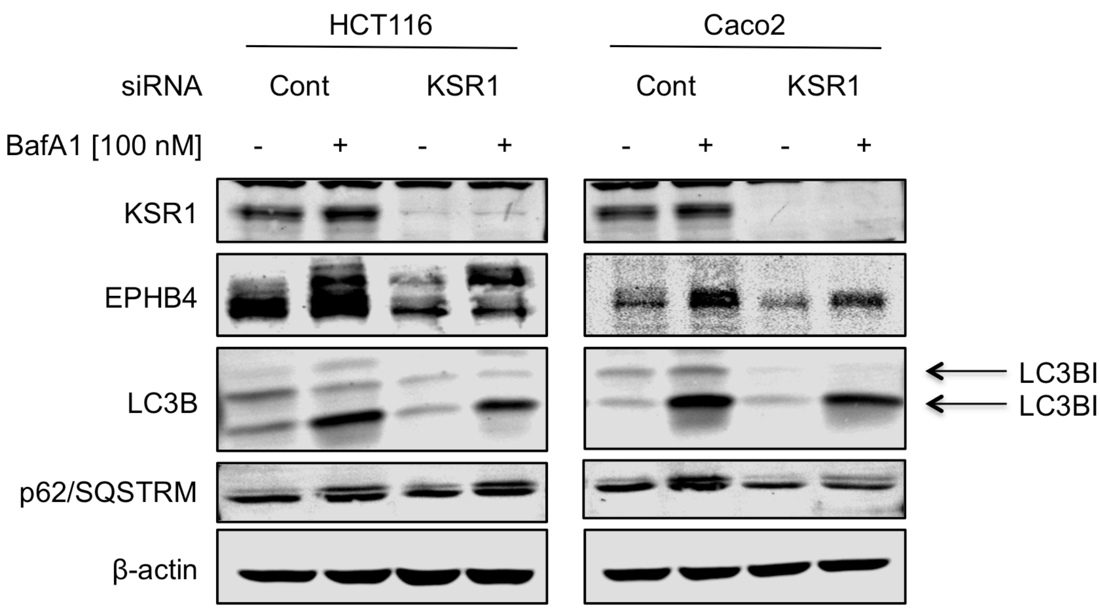


Fig 3.23 KSR1 expression protects EPHB4 from lysosome-dependent degradation. KSR1 was depleted in HCT116 and Caco2 cells for 72 h. Cells were treated with and without 100 nM Bafilomycin A1 (BafA1) for the last 8 h of knockdown. Proteins were analyzed by western blot. LC3B and p62/SQSTRM are used as positive controls for BafA1 treatment.

Discussion

Here we identify a new pathway critical for colon tumor cell survival impacted by effectors of Ras and Wnt signaling. Two proteins, KSR1 and EPHB4, are required for increased Myc protein expression in human colon tumor cells, which then promotes the expression of its downstream effector PGC1β (Fig 3.24). We recently showed that Ras-induced and KSR1-dependent PGC1β upregulation is required for colon cancer survival *in vitro* and *in vivo* (188). Here, we show that KSR1- and EPHB4-dependent mechanisms increase and stabilize Myc expression, which drives PGC1β expression in colon tumor cell lines to promote their survival.

Using KSR1 as a reference standard, we used FUSION (188, 228) to identify EPHB4 as a gene that is required for colon tumor cell survival. The mechanistic role that EPHB4 plays in cancer remains controversial. However, a preponderance of data indicates that EPHB4 is overexpressed broadly in human cancers including cancers of the head and neck, prostate, bladder, ovaries, large intestine, lung, brain, pancreas, and the esophagus (269, 271, 284-290). Further research has shown that the ablation or inhibition of EPHB4 in a number of cancer cell types reduces tumor cell viability including: prostate (315), bladder (286), ovarian (269), colon (271), lung (287), head and neck squamous cell carcinoma (316), and esophageal (290). Additionally, patient data have shown that EPHB4 levels negatively correlate with overall patient survival in ovarian cancer and glioblastoma (269, 288).

Expression of EPHB2 and EPHB4 is regulated by Wnt/β-catenin signaling in human CRC (271, 317). β-catenin's binding partners, p300 and CBP, determine which gene is transcribed, with p300 promoting EPHB2 expression and CBP promoting EPHB4 (271). EPHB2 is present in the normal colon and EPHB4 is only expressed when tumors arise (271). These data contrast with previous studies showing that EPHB4 is expressed

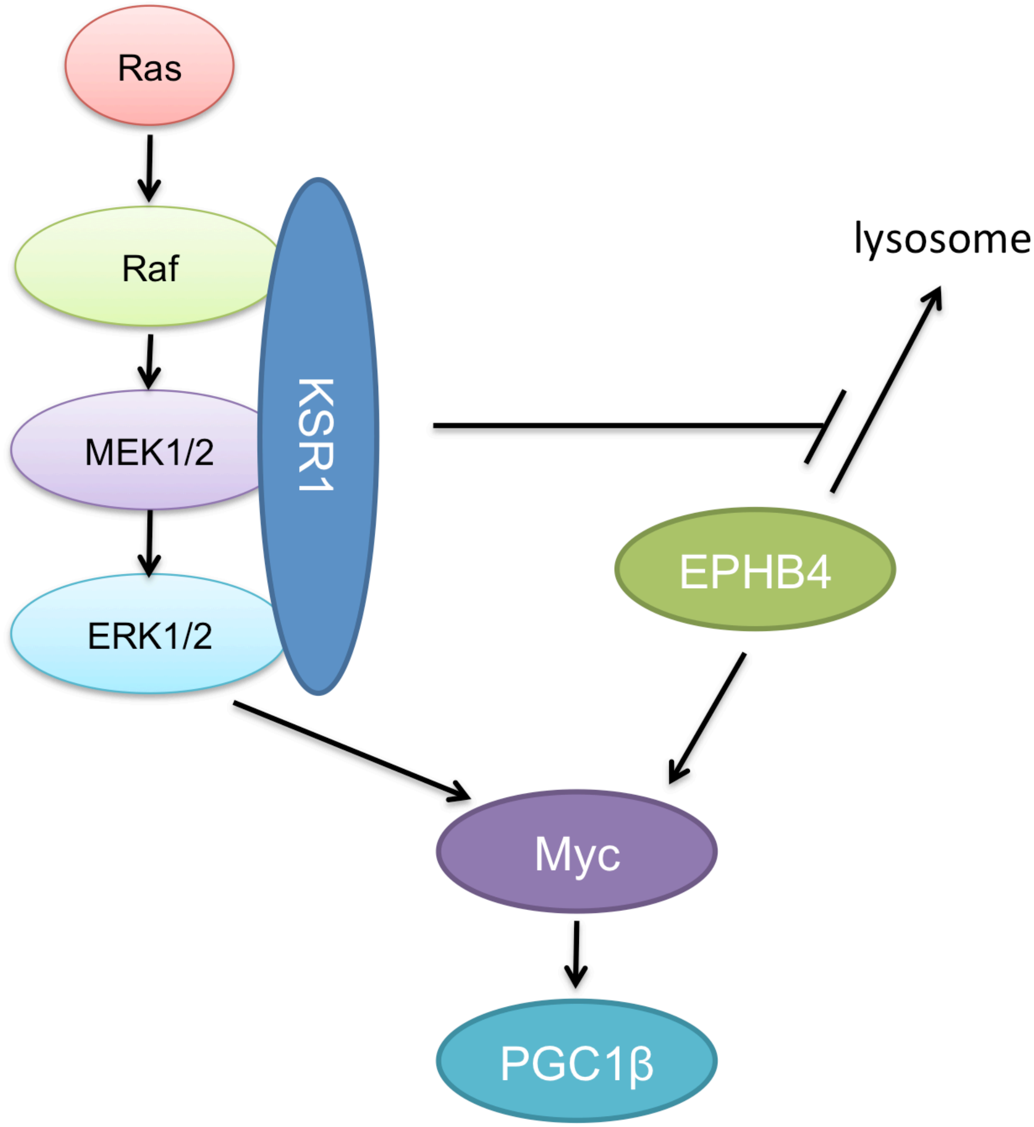


Fig 3.24 Model of MEK/ERK-dependent regulation of Myc and PGC1β expression

in human colonic crypts and early CRC lesions (318) followed by promoter hypermethylation and epigenetic silencing in more advanced stages (319). These data also indicate that EPHB4 expression is highest at the bases of crypts, suggesting that it plays an important role in maintaining the population of stem and progenitor cells located in that region of the crypt (318). The repulsive interaction that occurs when the ephrinB2 ligand-expressing cells of the upper region of the crypt come in contact with the EPHB4 receptor-expressing cells located at the base of the crypt suggests that this expression pattern may aid in compartmentalizing tumor cells and reducing the dissemination of such cells; in these studies, EPHB4 is functioning as a tumor suppressor (318, 320-322). While controversial, the majority of studies support the idea that increased EPH forward signaling promotes cell segregation and is primarily tumor-suppressive, whereas reverse signaling through the ephrin ligand is tumor-promoting driving neoangiogenesis and invasion (321, 323). However, further data suggest that ligand-independent forward signaling when EPHB4 is overexpressed promotes tumorigenesis, while ephrinB2-dependent activation is tumor suppressing (324, 325). Additionally, EPHB forward signaling can also be cross-activated by FGFR and ERBB receptors leading to non-canonical forward signaling that promotes cell proliferation (251), thereby contributing to tumorigenesis.

We show that EPHB4 is overexpressed in a panel of colon tumor cell lines and, like KSR1, EPHB4 depletion via siRNA or small-molecule inhibition is selectively toxic to colon tumor cells as compared to immortalized, but non-transformed HCECs. The mechanism of HCEC resistance to EPHB4 inhibition may result from the fact that EPHB4 expression is minimal in this cell line and the cells do not rely on its overexpression for survival. These data are consistent with previous studies showing that EPHB4 is absent in normal colon, but is expressed in all 102 human colorectal cancer sections analyzed by both immunohistochemistry and RT-qPCR (271).

Recent work from The Cancer Genome Atlas (TCGA) Network discovered that, in a comprehensive examination of human colon and rectal cancers of diverse anatomical origin and mutation status, changes in Myc transcriptional targets were found in nearly 100% of the tumors (280), suggesting an important role for Myc in CRC. While a promising target for CRC, Myc is a transcription factor and traditionally considered “undruggable” (262, 281). Although there are new strategies emerging to inhibit Myc, including interrupting key dimerization events or DNA binding (262), finding additional or alternative ways to target Myc protein expression or its downstream effectors may provide therapeutic benefits to many cancer patients.

Our studies show that EPHB4 regulates Myc expression through the promotion of mRNA levels. However, KSR1 does not share this mechanism of action, which led us to examine alternative explanations for its ability to increase Myc levels in human colon tumor cells. Further analyses of post-transcriptional mechanisms suggest that KSR1 promotes the translation of Myc. Myc mRNA can be translated into protein under conditions where initiation from the 5’ cap structure and ribosome scanning is inhibited.

Previous work has shown that, following hypoxia, PGC1β mRNA is decreased in renal clear cell carcinoma cells that is caused by an induction of MXI1, a repressor of Myc activity (261). Further work demonstrated that Myc regulates HER2- and IGF1-dependent induction of PGC1β in breast cancer cells (260). Our work indicates that Myc also mediates EPHB4 and KSR1 regulation of PGC1β expression. Combined with these reports, our work suggests that tumor cells of diverse origins find multiple ways to regulate Myc-driven PGC1β expression.

Lysosomal degradation of RTKs is well documented (326). Here we show that EPHB4 is primarily degraded via the lysosome and that KSR1 depletion promotes that degradation. Canonical lysosomal RTK degradation occurs following activation of the receptor by its respective ligand. However, ligand-independent receptor degradation has been reported (327, 328). Mechanisms involved in KSR1-dependent regulation of EPHB4 have not been explored further. However, it is anticipated that KSR1-dependent effects on Myc and PGC1β are not simply due to KSR1 stabilizing EPHB4. This prediction is based on the differential effects (transcriptional versus post-transcriptional regulation of Myc expression) observed with KSR1 and EPHB4 depletion. However, the observation that KSR1 can promote the stability of RTKs may allude to a broader mechanism by which KSR1 supports tumorigenesis.

The identification of these relationships highlights an important aspect of FUSION. Although the screen was intended to identify genes whose knockdown mimicked that of KSR1 depletion in a Ras-mutated cell line, it is designed in a way that focuses on phenotype, which does not necessarily require direct effects on KSR1-specific pathways. Therefore, we can identify critical effectors, such as EPHB4, whose inhibition has the same effect as depletion of KSR1, but whose mechanism of action is different. Additionally, although the cell line used in our screen (HCT116) has activated Ras, this study indicates that we have and can identify vulnerabilities in cancer tumor cells that are independent of constitutively activated Ras pathways. This may lead to the identification of potential targets that are applicable in a wide variety of cancer cell types.

Here we highlight the benefits of using an unbiased screen and FUSION analysis to identify potential vulnerabilities present only in cancer cells that are not found in normal cells. In fact, the identification of EPHB4 as a novel effector of Myc signaling prompted us to evaluate the relationship between KSR1 and Myc, establishing that the identification of targets using FUSION can also reveal novel information about KSR1 as well. We demonstrate novel mechanisms by which KSR1 and EPHB4 regulate PGC1β via promotion of Myc translation and mRNA expression, respectively. While the regulation of PGC1β by Myc has been previously studied in renal cell carcinoma (261) and breast cancer cells (260), we show that this pathway is present in and critical for colon cancer cell survival. Taken together, these data reveal that tumor cells in various cancers have a unique dependence on Myc-dependent expression of PGC1β for cell survival, which may be exploited in the development of new cancer therapeutics.

Chapter 4: PGC1β expression is promoted by ERK and inhibited by HSF1

Introduction

Heat shock factors (HSFs) are a small group of transcription factors that regulate the heat shock response (HSR). There is a single HSF in yeast, worms, and flies, but four HSFs (HSF1, 2, 3, 4) in mammals, of which HSF1 is the master regulator of HSR [reviewed in (329)]. HSR is a highly conserved, protective mechanism that manages environmental stresses, promotes survival, and regulates the longevity of the organism. Following exposure to certain stresses, HSF1 is activated to induce a group of proteins known as the heat shock proteins (HSPs) (330, 331). Activation of HSF1 is a multi-step process consisting of trimerization, extensive post-translational modifications (including phosphorylation, acetylation, and sumoylation), nuclear translocation, DNA-promoter binding, and induction of transcriptional targets (including *Hsps*) [reviewed in (329)]. In addition to the classical induction of the heat shock response, HSF1 has been shown to regulate up to 3% of the yeast genome including genes functioning in energy production and signal transduction (332).

HSF1 is a potent modifier of tumorigenesis and is required for tumor initiation and maintenance in a variety of cancer models (333). HSF1 is dispensable under non-stress conditions, but crucial for growth and survival in tumor cells (333). Elevated levels of HSF1 have been detected in several types of cancer including cancers of the breast, lung, and colon (333, 334), but no somatic mutations in HSF1 have been identified in human cancers thus far. Additionally, HSF1 drives a transcriptional program distinct from the HSR to upregulate cancer-specific genes and support oncogenic processes such as cell cycle regulation, signaling, metabolism, adhesion, and translation (334). The HSF1 cancer-specific transcriptome signature was found to be associated with increased metastasis and reduced survival in lung, breast, and colon cancer patients (334).

Unlike mutant Ras, overexpression of HSF1 is unable to transform immortalized MEFs (333). Conversely, MEFs lacking *hsf1* are resistant to transformation induced by oncogenic H-Ras^{V12D} or PDGF-B (333). Additionally, mice deficient in HSF1 exhibit a lower incidence of tumors and increased survival as compared to their wild-type controls both in a model of chemical skin carcinogenesis, as well as in a genetic model expressing oncogenic p53 (335). HSF1 expression in tumor cells is a prime example of "non-oncogene addiction" (336). While not every protein in a given tumor-promoting pathway can be activated to an extent that directly promotes oncogenesis, they can be rate limiting within their pathways and represent potential drug targets. These potential targets are important because they represent approaches to treat cancers that are not dependent on traditional oncogenes.

A recent publication has identified HSF1 as a new substrate of MEK1/2 (335). Using a combination of inhibitors and mutant constructs, they show that phosphorylation of HSF1 on Ser326 stabilizes HSF1 protein and is dependent on the activity of MEK1/2, but not ERK1/2, in NIH 3T3 and HEK293T cells. *In vitro* kinase activity assays were used to demonstrate that increasing doses of a MEK inhibitor (U0126) were sufficient to inhibit phosphorylation of HSF1 at Ser326, whereas increasing doses of the ERK inhibitor (FR180204) were not. Additionally, inhibition of MEK1/2 significantly decreased direct binding of HSF1 to DNA in the presence and absence of heat shock suggesting that MEK1/2 can directly regulate the HSF1-driven transcriptional program regardless of heat shock/cellular stressors.

The canonical substrate of MEK1/2 is ERK1/2. MEK1/2 catalyzes the phosphorylation of human ERK1/2 at Tyr204/187 and Thr202/185 (125). ERK1/2-dependent signaling regulates a variety of processes including cell adhesion, cell cycle progression, migration, survival, differentiation, metabolism, proliferation, and transcription (105). Human ERK1 and ERK2 are 84% identical and share many functions

(129), but are not entirely redundant (132). The *erk1* gene is dispensable for mouse development. *erk1*$^{-/-}$ mice are deficient in thymocyte maturation and, therefore, normal T-cell effector function, but are viable, fertile, and are normal in size (130, 131). Ablation of *erk2* is embryonic lethal (132).

ERK1 and ERK2 have more than 175 documented cytoplasmic and nuclear substrates (337). In the nucleus, they can target the ternary complex factor (TCF) family of transcription factors, including the E-twenty six (Ets)-domain transcription factor Elk1. These play a role in inducing the expression of immediate early genes, which encode c-Fos and c-Myc. c-Myc and c-fos induce the expression of late-response genes that promote cell survival, division, and motility (169, 338). In the cytoplasm, ERK1/2 have a variety of substrates including c-Myc (as described in Chapter 3) and the 90-kDa ribosomal S6 kinase (RSK) family of proteins (339). RSK proteins contain two kinase domains, an N-terminal kinase domain (NTKD) or C-terminal kinase domain (CTKD) within a single polypeptide chain (339). The CTKD is involved in the autophosphorylation of RSK proteins, while the NTKD is important in substrate phosphorylation. ERK1/2 phosphorylates RSK at Thr573 in the CTKD activation loop, which subsequently catalyzes the phosphorylation of Ser380 (339). Regardless of substrate, ERK1/2 catalyze the phosphorylation of serine or threonine residues in a proline-directed manner (127). The optimal primary sequence for ERK1/2 phosphorylation is Pro-X-Ser/Thr-Pro with a proline at both the -2 and +1 positions (127).

Small-molecule inhibitors of ERK1/2 have been developed with varying success. Two main strategies have been employed: ATP mimetic inhibitors that target the active kinase (type-I inhibitors) such as FR180204 and VTX-11e, and inhibitors that target and stabilize the inactive state of an enzyme (type-II inhibitors) (224). Selectivity is a challenge for type-II inhibitors as they target a more diverse range of structures. Ideally, one would use non-ATP-based allosteric inhibitors because they are usually highly selective (210, 340). Recently a highly potent and selective ERK1/2 inhibitor was identified (224). This inhibitor does not bind to the “active” or “inactive” conformation of ERK1/2, but rather induces an allosteric site adjacent to the ATP pocket in which it binds (226). This inhibitor also has a slow dissociation rate allowing its effects to remain after the drug has been washed out making it an ideal inhibitor to use both *in vitro* and *in vivo* (226).

Peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1 (PGC1) family members, PGC1α, PGC1β, and PGC1-related coactivator (PRC), are transcriptional coactivators that serve as inducible coregulators of nuclear receptors that control cellular energy via metabolic pathways (341). PGC1α was first identified in brown adipose tissue (BAT) through its functional interaction with the nuclear receptor PPARγ during thermogenesis (342). PGC1β and PRC were subsequently identified to regulate PPARγ-dependent transcription (343-345). In addition to PPARγ, PGC1 coactivators enhance the transcriptional activity of a variety of nuclear receptors, including liver X receptor (LXR) (346) and estrogen-related receptors (ERRs) (347, 348) as well as non-nuclear receptors, including forkhead box O1 (FOXO1) (349) and SREBP1 (346). PGC1α and PGC1β serve diverse functions in multiple organ systems. PGC1α and PGC1β are both highly expressed in mitochondria-enriched tissues with high energy demands, including BAT, cardiac muscle/tissue, and slow-twitch skeletal muscle (342, 344, 345). However, PGC1α is enriched in the brain and kidneys. PGC1α is a cold-inducible coactivator that is also stimulated in the skeletal muscle by exercise (350) and in the heart and liver by fasting (351, 352). PCG1β is also induced by fasting, but not cold exposure, indicating some conserved and some unique mechanisms of upstream regulation of PGC1 proteins (344, 353).

PGC1α and PGC1β are the most robust coactivators of ERRα (342, 344, 345). Under normal conditions, the PGC1/ERRα complex regulates metabolic homeostasis in tissues such as BAT and muscle. It is likely that some of the same mechanisms that regulate PGC1/ERRα in normal physiology are also involved in cancer. For instance, PGC1α is induced by hypoxia in the skeletal muscle, resulting in hypoxia-inducible factor (HIF)-independent, but ERRα-dependent expression of VEGF and increased angiogenesis (354). Additionally, the PGC1/ERRα complex is positively regulated by oncogenic pathways in cancer cells. In breast cancer, HER2 activation increases the expression of PGC1β (355) resulting in increased ERRα-dependent transcription (356). As described in Chapter 3, PGC1β is a direct transcriptional target of Myc (260, 261), which is overexpressed in a variety of cancers including colon cancer. We showed that Myc expression is regulated by KSR1 in the colon tumor cell lines. Our lab has previously published that PGC1α and PGC1β expression is independent of ERK1/2 in mouse embryonic fibroblasts (187) and colon tumor cell lines (188), respectively, but is dependent on the presence of activated Ras and KSR1 (187). With the recent identification of a new MEK1/2 substrate, HSF1, I hypothesized that the KSR1-dependent PGC1β expression in colon cancer cell lines is mediated by a MEK1/2-dependent and ERK1/2-indpendent mechanism.

Results

Depletion of KSR1 reduces HSF1 expression

With the recent identification of HSF1 as a novel MEK1/2 substrate, we set out to determine if KSR1 affected the phosphorylation of HSF1 at Ser326. We hypothesized that KSR1 regulates HSF1 phosphorylation and possibly functions as a scaffold in a proposed RAF/MEK/HSF1 cascade. To test whether KSR1 affects phosphorylation of HSF1, KSR1 was depleted in HCT116 cells for 72 hours and protein levels were assessed by western blot. KSR1 depletion reduced both phosphorylated and total levels of HSF1 (Fig 4.1). The reduction in total HSF1 protein levels may be due to a decrease

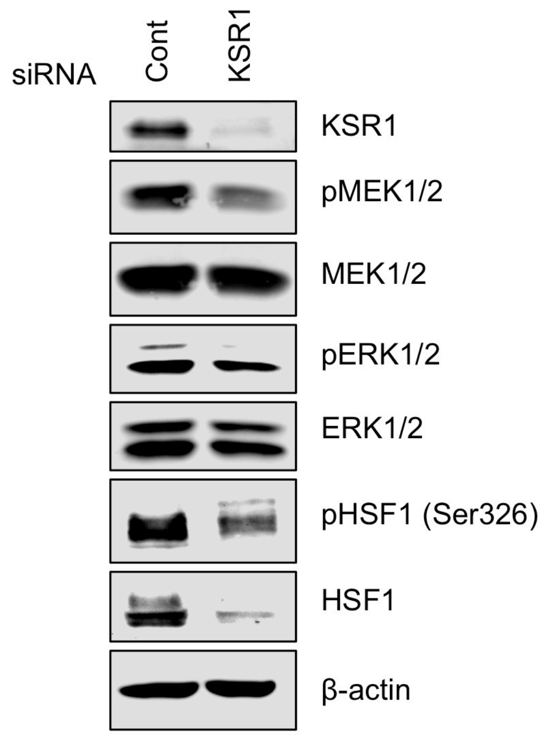


Fig 4.1 Depletion of KSR1 decreases pHSF1 (Ser326) and total HSF1 levels. HCT116 cells were transfected with siRNA targeting KSR1 or a non-targeting siRNA (Cont) for 72 hours. Protein expression levels were assessed by western blot.

in HSF1 downstream targets, particularly heat shock proteins (e.g., HSP90) that stabilize and sequester HSF1 in the cytoplasm. Additionally, depletion of KSR1 for 72 hours leads to persistent MEK inhibition and prolonged MEK inhibition has been previously demonstrated to reduce total HSF1 levels in malignant peripheral nerve sheath tumor (MPNST) cells (357).

Prolonged Depletion of MEK1/2 decreases PGC1β expression

We have previously demonstrated that 24 hours of treatment with the MEK inhibitors U0126 or PD0325901 does not decrease PGC1β expression in HCT116 cells (188), but we never studied the effects of prolonged MEK1/2 inhibition on PGC1β. To determine if PGC1β expression is dependent on the expression and activity of MEK1/2, HCT116 cells were treated with DMSO or the MEK inhibitor U0126 for 0-96 hours. Due to the short half-life of U0126 in medium, inhibitor was replenished every 24 hours. We found that cells treated with U0126 had substantially decreased levels of PGC1β protein by 24 hours (Fig 4.2). Levels of the co-activator ERRα protein were depleted in a similar manner. PGC1β levels do decrease in DMSO-treated cells at the 72-hour and 96-hour timepoints. At these timepoints, the wells are completely confluent, which may be contributing to this decrease. However, at all timepoints, the expression of PGC1β is less in MEKi-treated cells than in the controls (Fig 4.2). Previous studies suggest that prolonged MEK inhibition can decrease KSR1 levels. Therefore, to rule out that the effects on PGC1β were due to decreased KSR1 we assessed KSR1 expression at all timepoints. Phosphorylation of ERK1/2 was used to assess the degree of MEK inhibition.

Next, we determined whether inhibition of MEK decreases PGC1β and ERRα mRNA levels. As shown in Chapter 3, Myc regulates PGC1β expression in colon tumor cell lines. Therefore, we also examined whether MEK inhibition decreased Myc mRNA levels as well. HCT116 cells were treated with DMSO or 20 μM U0126 or PD98059 for

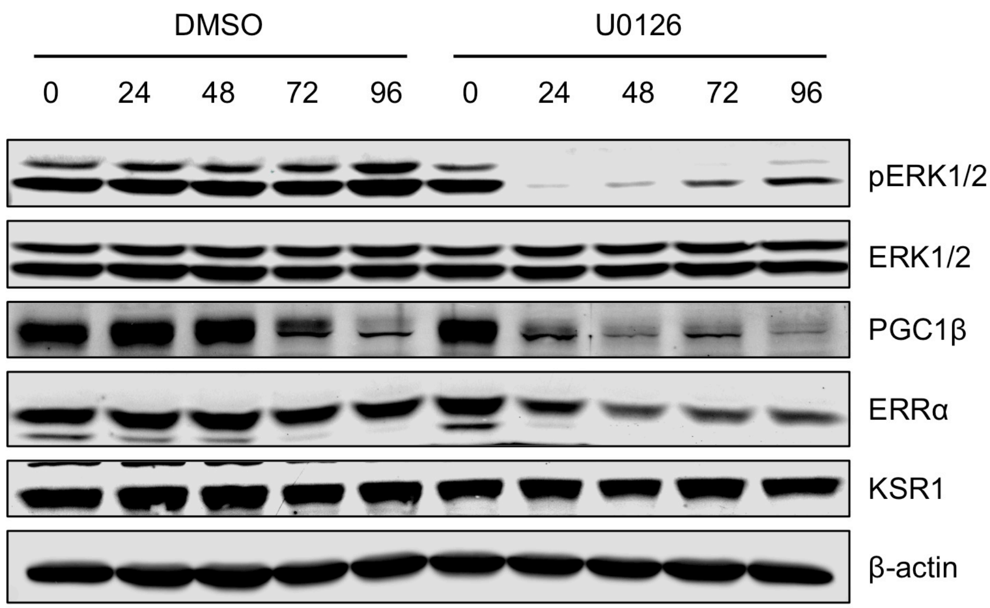


Fig 4.2 MEK inhibition suppresses PGC1β and ERRα expression.

HCT116 cells were treated with 20 μM MEK inhibitor (U0126) or vehicle for 0-96 hours. PGC1β and ERRα levels were assessed by western blot.

48 hours. Treatment with the MEK inhibitors resulted in decreased Myc and PGC1β, but not ERRα, mRNA levels (Fig 4.3A). It should be noted that although the decrease in expression is small, 19-20% for Myc and 24-25% for PGC1β, it is statistically significant. To verify that the inhibitors are working in this experiment, we treated a second set of wells simultaneously for western blot analysis. Fig 4.3B shows that inhibition of MEK decreases Myc, PGC1β, and ERRα protein expression in all replicates. It should be noted that MEK1/2 inhibition decreases protein expression (Myc = 49%, PGC1β = 41%, and ERRα = 51%) more than mRNA levels (Myc = 19%, PCG1β = 25%, and ERRα = 8% in U0126-treated cells. This suggests that MEK1/2 regulates these proteins via a post-transcriptional mechanism.

siRNA-mediated depletion of MEK1/2 showed a significant decrease in mRNA levels of Myc, but not PGC1β or ERRα (Fig 4.4). It is possible that using siRNA, the effect on PGC1β takes longer to develop than it does using MEK inhibitors and would be significant at later time points. Alternatively, this experiment may distinguish the ability of kinase inhibition to more effectively debilitate MEK signaling than siRNA, which may not be capable of targeting all MEK mRNA. Finally, these results may be indicative of off-target effects of MEK kinase inhibitors.

MEK1/2 depletion inhibits Myc, PGC1β, and ERRα expression with and without heat shock

To examine the contributions of MEK1 and MEK2 individually and elucidate the mechanism behind MEK-dependent regulation of PGC1β, HCT116 cells were transfected with siRNA targeting MEK1, MEK2, or MEK1/2 for 72 hours. Half of the cells were subjected to heat shock (HS) at 43°C for 30 minutes to induce phosphorylation of HSF1. Depletion of MEK1 or MEK2 alone did not have a significant effect on ERK1/2 or HSF1 phosphorylation. However, simultaneous RNAi-mediated knockdown of MEK1/2

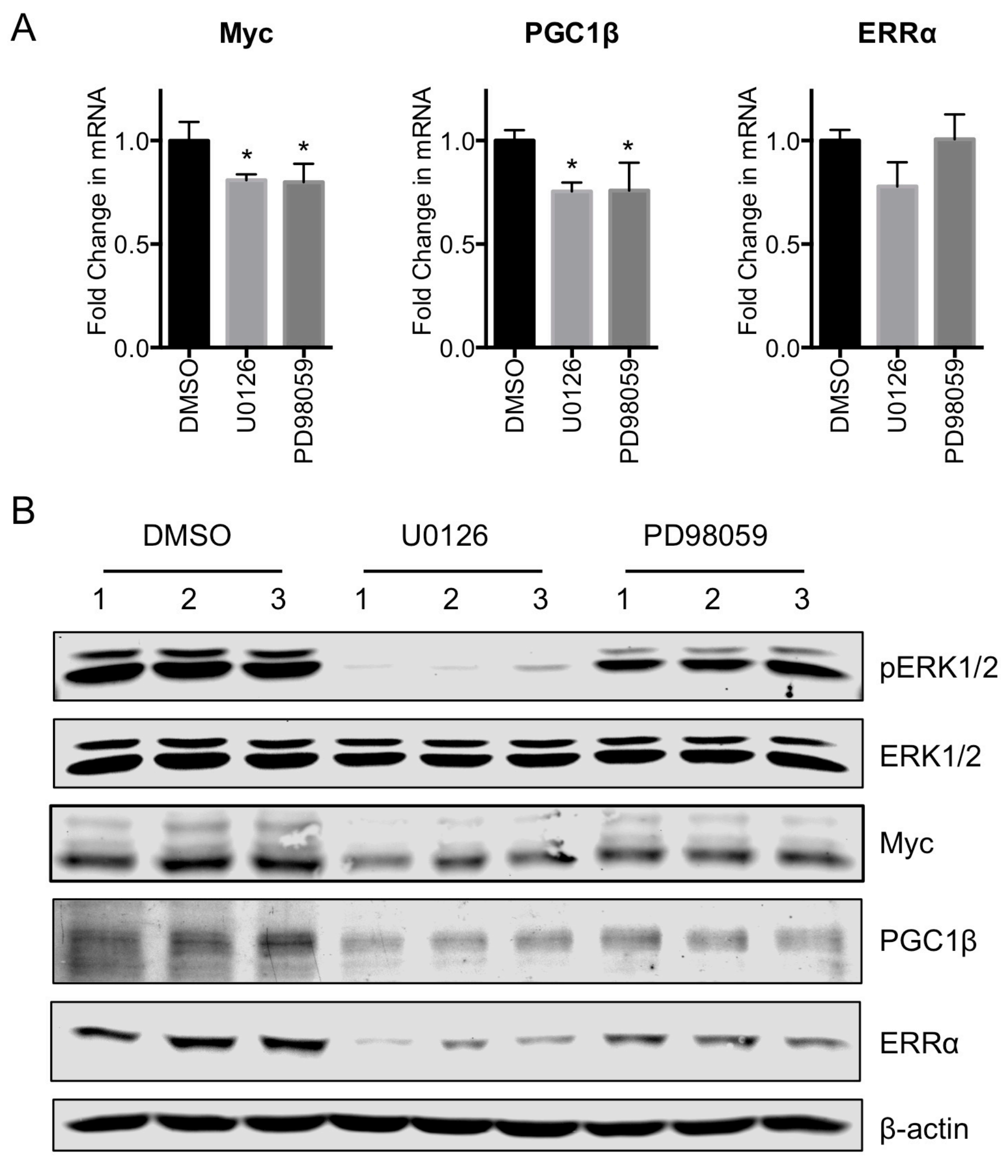


Fig 4.3 MEK inhibition suppresses mRNA levels of Myc and PGC1β, but not ERRα. (A) HCT116 cells were treated with 20 μM U0126 or PD98059 (or vehicle) for 48 hours. Myc, PGC1β, and ERRα mRNA levels were measured by RT-qPCR. Data from three biological replicates are presented as mean ± SD. Significance was measured using a one-way ANOVA with a Dunnett's multiple comparison post-test. * $p < 0.05$ (B) Duplicate wells of each replicate in A were plated for western blot analysis of Myc, PGC1β, and ERRα expression

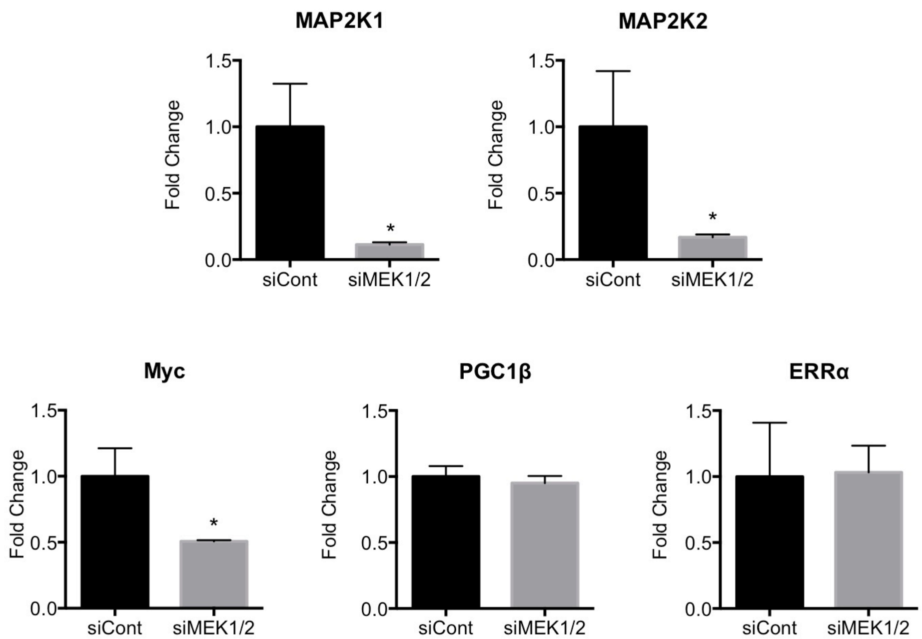


Fig 4.4 MEK depletion suppresses Myc mRNA levels.

MEK1 (MAP2K1) and MEK2 (MAP2K2) were simultaneously depleted in HCT116 for 72 hours. Myc, PGC1β, and ERRα mRNA levels were quantified using RT-qPCR. Data from three biological replicates are presented as mean ± SD. Significance was measured using a one-way, paired *t*-test. * $p < 0.05$

decreased expression of activated ERK and HSF1 in the absence of HS (Fig 4.5). Phosphorylation of HSF1 at Ser326 was stimulated by HS, even when MEK1/2 was knocked down in the HCT116 cells, indicating that the residual activated MEK1/2 is sufficient to phosphorylate HSF1 upon HS induction or that HSF1 may be phosphorylated at Ser326 by an additional kinase in the colon tumor cell lines. Depletion of MEK1 or MEK2 alone had minimal to no effect on Myc, PGC1β, or ERRα protein levels. However, depletion of MEK1/2 together decreased levels of all three. It was previously reported that sustained inhibition (96 hour) of MEK could decrease KSR1 levels (357). We, therefore, verified that the decreases observed in MYC, PGC1β, and ERRα expression were not due to downregulation of KSR1.

MEK1/2 and ERK1/2 inhibition prevents HS-induced HSF1 phosphorylation

Previous reports suggest that the MEK-dependent HSF1 phosphorylation at Ser326 is ERK-independent (335). Tang *et al*. treated HEK293T cells with 1 μM FR180204 or 100 nM SCH772984 (two ERK1/2 inhibitors) overnight and measured phosphorylated (Ser326) and total HSF1 by western blot (335). They found that treatment with ERK inhibitors increased phosphorylation of Ser326 on HSF1 over that of DMSO-treated cells. To confirm this finding in colon cancer cells, HCT116 cells were treated with 20 μM U0126 or 1 μM SCH772984 for 24 hours. Surprisingly, inhibition of MEK1/2 or ERK1/2 resulted in decreased levels of HSF1 phosphorylation (Fig 4.6). To determine if inhibition of ERK1/2 also prevented HS-induced phosphorylation of HSF1, HCT116 cells were subjected to 30 minutes of HS at 43°C after 24 hours of treatment with U0126 or SCH772984. MEK1/2 or ERK1/2 inhibition was sufficient to block HSF1 phosphorylation following HS (Fig 4.6).

HSF1 inhibits the expression of Myc and PGC1β

HCT116 and Caco2 cells were transfected with MEK1/2 siRNA for 72 hours. To

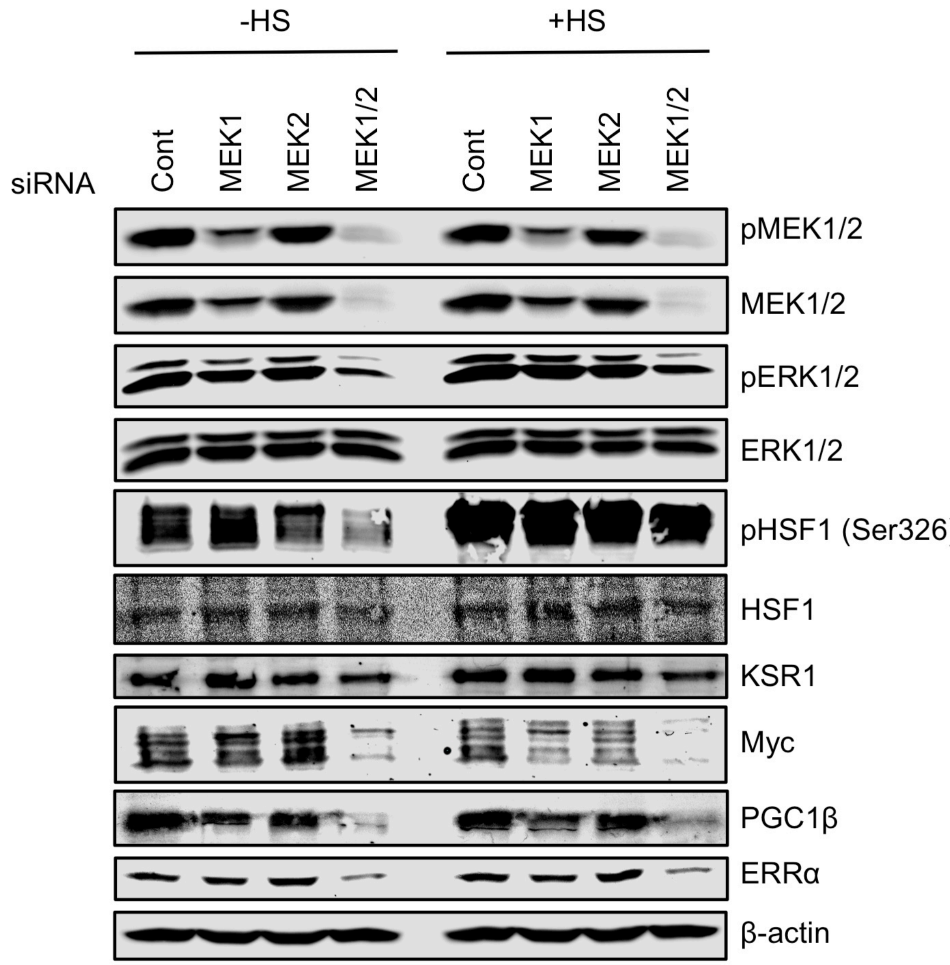


Fig 4.5 MEK siRNA decreases Myc, PGC1β, and ERRα expression.
HCT116 cells were transfected with siRNA targeting MEK1, MEK2, or MEK1/2 for 72 hours then subjected to the presence or absence of heat shock (HS) at 43°C for 30 minutes.

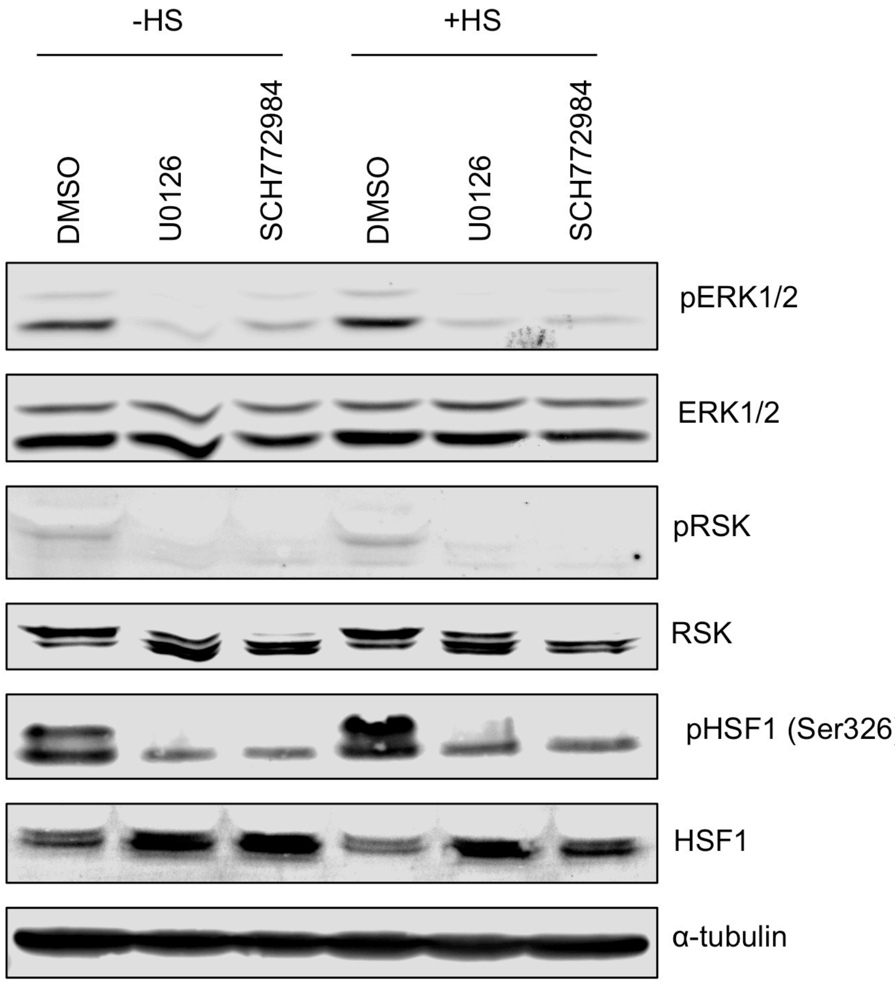


Fig 4.6 Inhibition of MEK and ERK decreases pHSF1 (Ser326) expression in colon cancer cells.

HCT116 and Caco2 cells were treated with 20 μM U0126, 1 μM SCH772984, or DMSO for 24 hours then subjected to the presence or absence of heat shock (HS) at 43°C for 30 minutes. Protein expression levels were assessed by western blot.

evaluate the nuclear translocation of HSF1, cells were harvested using nuclear/cytoplasmic fractionation. Protein expression and compartmentalization was assessed by western blot. In HCT116 and Caco2 cells, depletion of MEK1/2 reduced the amount of phosphorylated HSF1 in the nuclear compartment (Fig 4.7). A similar pattern is observed in Myc, PGC1β, and ERRα expression where reduced phosphorylated HSF1 expression in the nucleus correlates with less Myc, PGC1β, and ERRα protein. This suggests that nuclear translocation of HSF1 is necessary for sustained expression of these transcriptional regulators.

To determine whether HSF1 directly promotes the expression of Myc, PGC1β, and ERRα, HSF1 was knocked down by siRNA and protein expression was measured by western blot. HSF1 depletion surprisingly increased Myc and PGC1β expression in HCT116 and Caco2 cells (Fig 4.8). There was no effect on ERRα. Further, it was determined that 72 hours of HSF1 depletion does not affect KSR1 levels or ERK activation (Fig 4.8). Therefore, the increased Myc and PGC1β expression is not a consequence of KSR1 or ERK1/2 signaling upregulation. These data suggest that MEK1/2 regulates HSF1 phosphorylation and the expression of Myc and PGC1β through independent pathways.

Activated ERK1/2 induces the expression of Myc, PGC1β, and ERRα

Inhibition of MEK1/2 through siRNA depletion and kinase inhibitors decreases Myc, PGC1β, and ERRα expression via an HSF1-independent mechanism. Currently, HSF1 and ERK1/2 are the only documented MEK1/2 substrates. We, therefore, readdressed the possibility that MEK1/2 is regulating these proteins through an ERK-dependent pathway. ERK1/2 was depleted in HCT116 and Caco2 cells for 72 hours by siRNA and lysates were harvested using nuclear/cytoplasmic fractionation. In HCT116 and Caco2 cells, ERK1/2 depletion decreased Myc, PGC1β, and ERRα in the nucleus

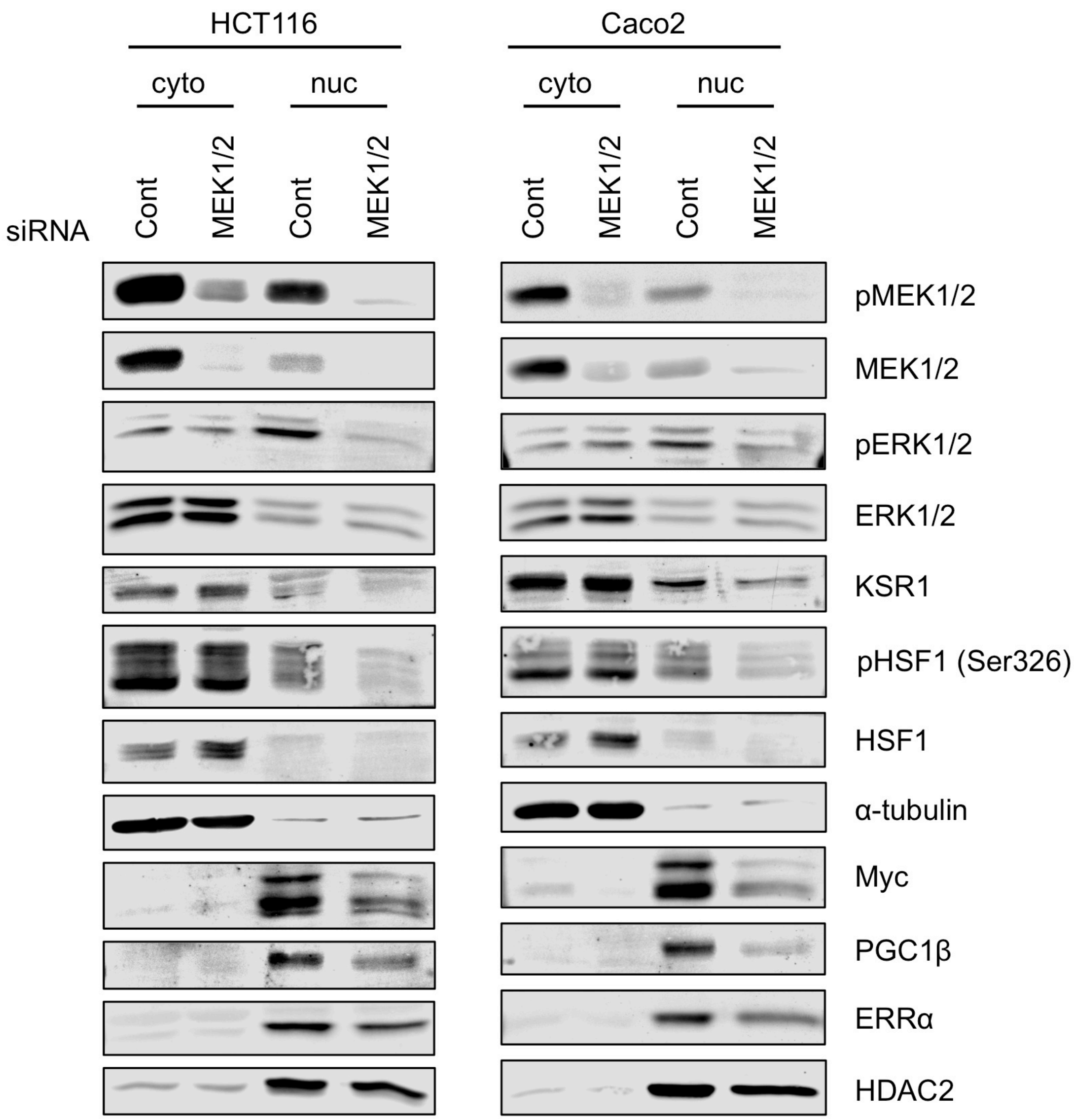


Fig 4.7 MEK depletion inhibits nuclear translocation of pHSF1 S326 in colon tumor cell lines.

HCT116 cells were transfected with siRNA targeting MEK1/2 or control for 72 hours. Nuclear and cytoplasmic fractions were isolated and protein expression levels were assessed by western blot.

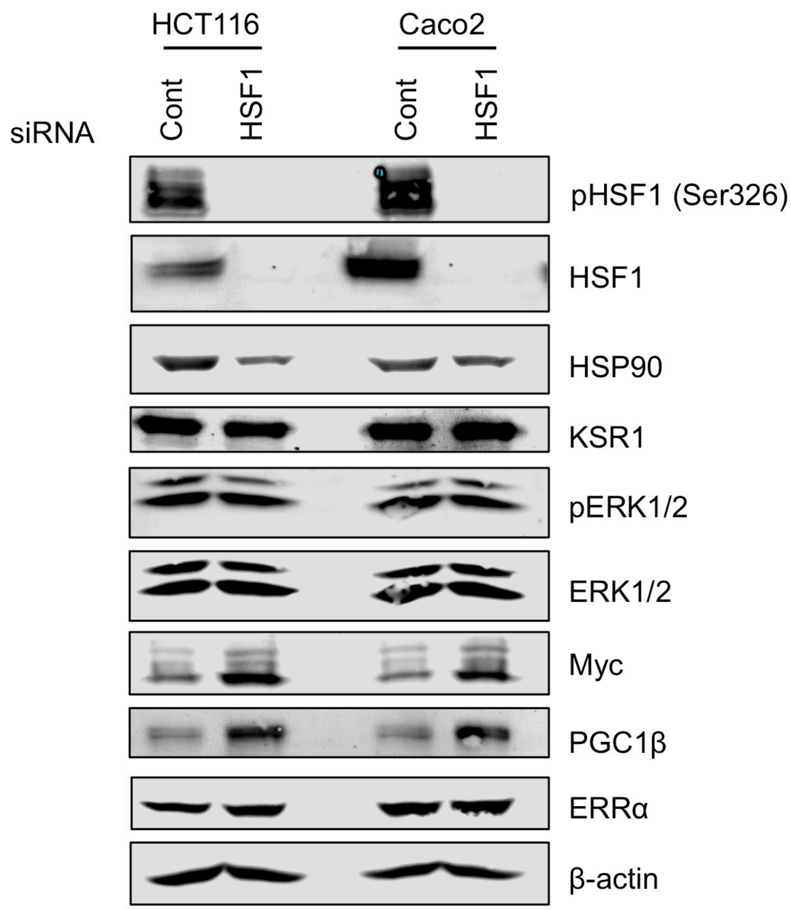


Fig 4.8 HSF1 depletion increases Myc and PGC1β protein expression in colon tumor cell lines.

HCT116 and Caco2 cells were transfected with siRNA targeting HSF1 or control for 72 hours. Protein expression levels were assessed by western blot.

(Fig 4.9). Interestingly, 72 hours of ERK1/2 depletion increased total levels of phosphorylated HSF1 (Ser326), 15% in HCT116 and 11% in Caco2, but did not affect nuclear translocation (Fig 4.9). These data are consistent with the mechanism proposed by Tang *et al*. (335), where ERK1/2 inhibition upregulates phospho-Ser326 HSF1 through inhibition or loss of a feedback loop and subsequent activation of MEK1/2.

A small-molecule inhibitor was used to assess whether ERK inhibition mimicked the results from the knockdown studies. HCT116 and Caco2 cells were treated with 1 μM SCH772984 for 0-72 hours. Protein expression of Myc, PGC1β, and ERRα was assessed by western blot. Inhibition of phospho-RSK was used as a positive control for ERK inhibition. Treatment with SCH772984 decreased levels of Myc, PGC1β, and ERRα in both cell lines by 24 hours (Fig 4.10). Taken together, these data suggest that the expression of Myc, PGC1β, and ERRα are dependent on the activity of ERK1/2.

Discussion

Here we further define the mechanisms of Myc, PGC1β, and ERRα regulation in colon tumor cells (summarized in Fig 4.11). We have identified that MEK1/2 plays a dual role in the regulation of these proteins. Through the phosphorylation of HSF1, MEK1/2 inhibits Myc, PGC1β, and ERRα expression. However, as shown in Fig 4.3 and Fig 4.4, this effect is overcome by the ERK-dependent promotion of Myc, PGC1β, and ERRα. In the absence of HS, it is apparent that the MEK/ERK axis is the primary regulator of Myc, PGC1β, and ERRα expression in colon cell lines. This may be due to ERK1/2-dependent phosphorylation of HSF1 at Ser307, which antagonizes both nuclear translocation and transcriptional activity of HSF1 in absence of stress (358). Fig 4.9 demonstrates that even with increased phosphorylation of HSF1 at Ser326 following ERK1/2 siRNA-mediated depletion, Myc, PGC1β, and ERRα protein levels are still decreased.

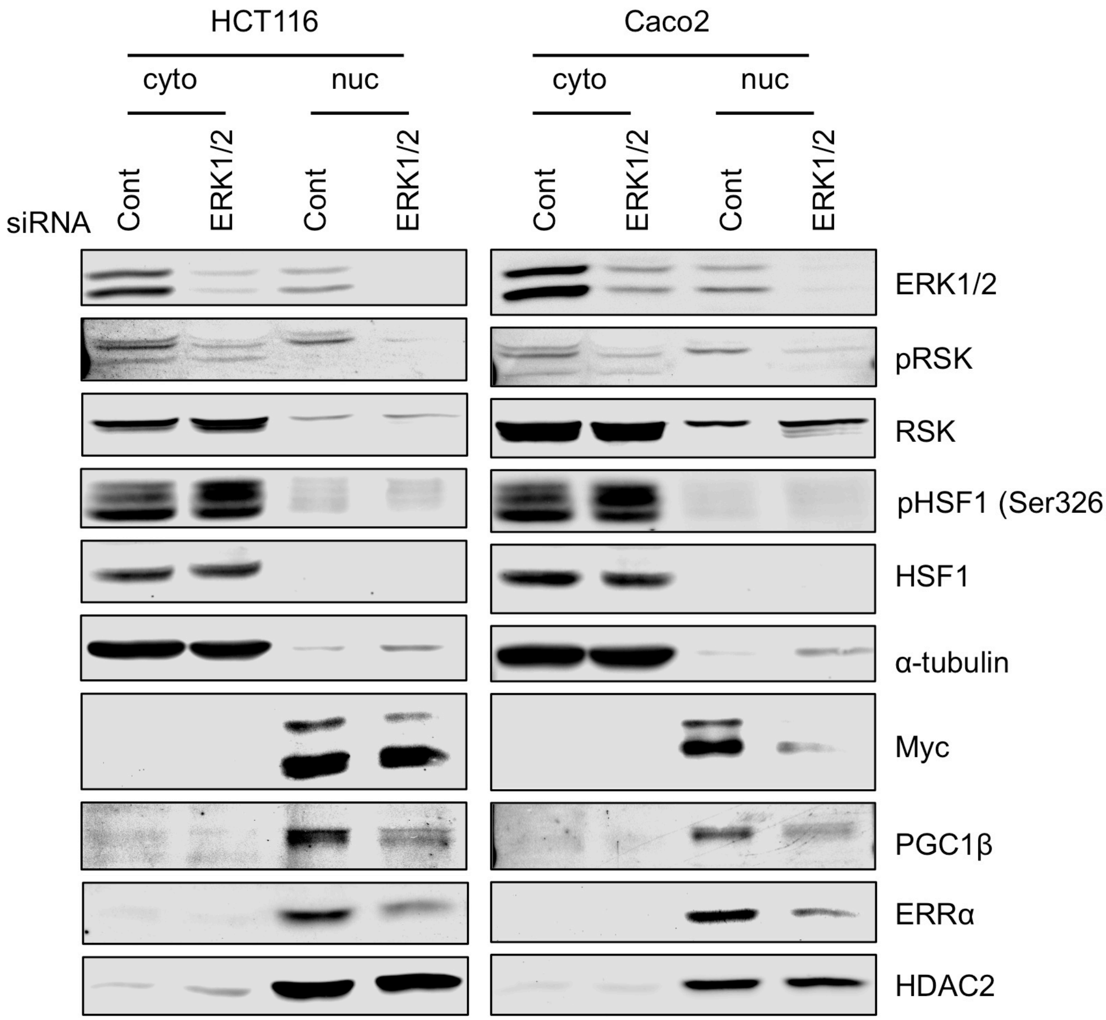


Fig 4.9 ERK1/2 depletion inhibits Myc, PGC1β, and ERRα expression, but does not affect nuclear translocation of pHSF1 S326 in colon tumor cell lines.
HCT116 and Caco2 cells were transfected with siRNA targeting ERK1/2 or control for 72 hours. Nuclear and cytoplasmic fractions were isolated and protein expression levels were assessed by western blot.

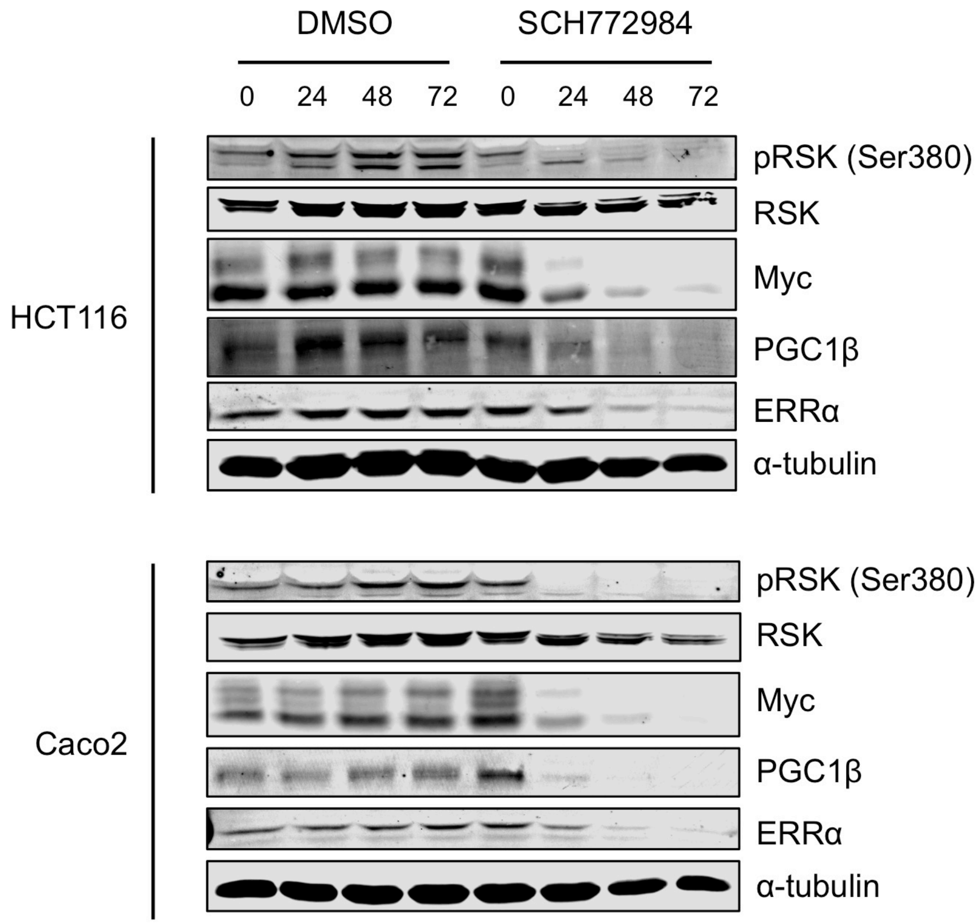


Fig 4.10 ERK inhibition decreases expression of Myc, PGC1β, and ERRα.
HCT116 (top) and Caco2 (bottom) cells were treated with 1 μM SCH772984 (ERK1/2 inhibitor) or vehicle for 0-72 hours. Myc, PGC1β, and ERRα levels were assessed by western blot.

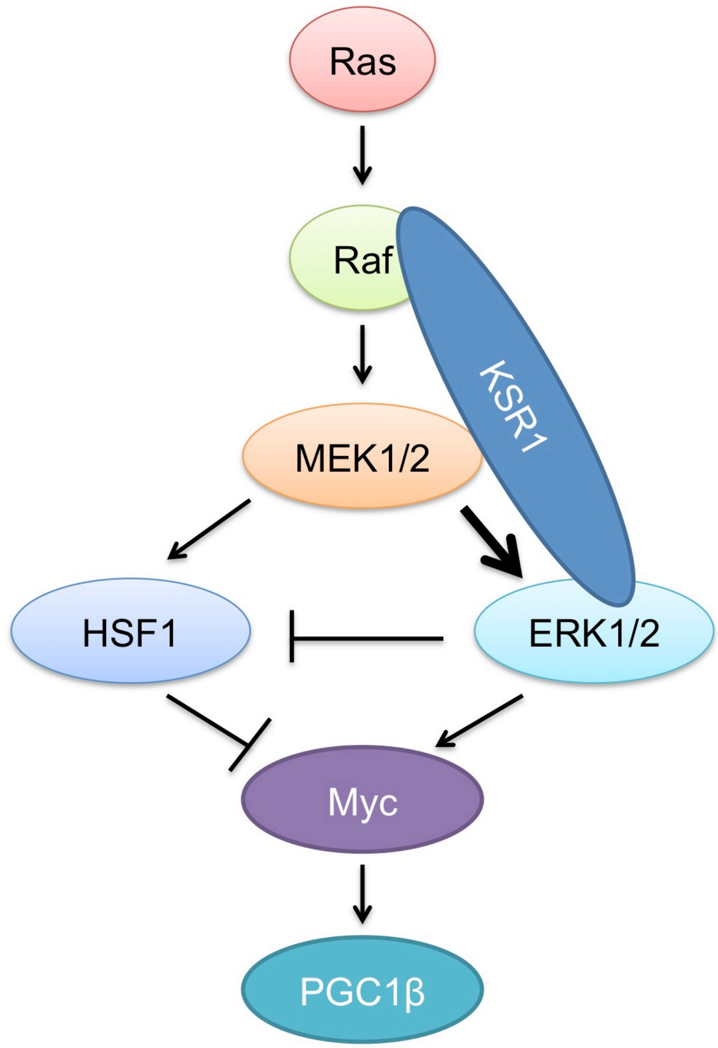


Fig 4.11 Model of MEK/ERK-dependent regulation of Myc and PGC1β expression.

The ability to induce HSF1 phosphorylation in the absence of MEK1/2 contradicts data presented by Tang and colleagues (335). They showed that inhibition of MEK in NIH-3T3 cells with 20 μM U0126 for 3 hours prior to HS (43°C x 30 minutes) was sufficient to suppress phosphorylation of HSF1 at Ser326 (335). Interestingly, they also show that HS induces phosphorylation of MEK and ERK in NIH-3T3 cells (335), which we do not observe in the HCT116 cells. This suggests that the mechanisms regulating MEK-dependent phosphorylation of HSF1 may be variable in different cell types. Additionally, while Tang *et al*. (335) provide a plethora of circumstantial evidence indicating MEK1/2 phosphorylates HSF1, they do not have a concrete experiment in which purified, active MEK1/2 directly phosphorylates HSF1 at Ser326. This should be addressed in future experiments.

Here we show that MEK1/2 and ERK1/2 inhibition for 24 hours can prevent HS-induced phosphorylation of HSF1 (Fig 4.6). However, after 72 hours of ERK1/2 depletion, HSF1 phosphorylation at Ser326 is increased. This is consistent with the idea that MEK1/2 phosphorylates HSF1 at Ser326 and MEK1/2 activity is upregulated by prolonged ERK inhibition via a negative feedback loop. The ERK1/2 activity is still repressed at 72 hours as indicated by reduced RSK phosphorylation in Figures 4.9 and 4.10. The ability of ERK1/2 inhibition to prevent HSF1 phosphorylation at Ser326 at early timepoints (24 hours), but to promote phosphorylation at late timepoints (72 hours) is puzzling. It is possible that ERK1/2 directly phosphorylates Ser326, as the sequence surrounding the phosphorylation site fits the consensus sequence for proline-directed phosphorylation (359), but taking our studies and the previous studies by Tang *et al*. (335) into account, this mechanism is unlikely. Alternatively, an ERK1/2-dependent priming phosphorylation may be necessary for phosphorylation at Ser326. It is known that sequential phosphorylation of Ser307 by ERK1/2 and Ser303 by GSK3β is necessary for repression of HSF1 transcription (360) and FBW7-dependent ubiquitination (361). A similar priming phosphorylation may be required for subsequent phosphorylation at Ser326. Finally, studies in HeLa cells indicate that mTOR complex 1 (mTORC1) can phosphorylate HSF1 at Ser326 (362). Cross-activation between the Ras-ERK and PI3K-Akt pathways occurs when ERK1/2 phosphorylates RAPTOR to promote Ras-dependent activation of mTORC1 (363). Therefore, ERK inhibition at 24 hours may prevent the mTORC1-dependent phosphorylation of HSF1 at Ser326, but prolonged ERK inhibition upregulates MEK1/2 through feedback loops and promotes MEK1/2-dependent HSF1 phosphorylation. Mechanisms of ERK-dependent HSF1 phosphorylation need to be further defined.

The upregulation of Myc and PGC1β following depletion of HSF1 may be due to HSF1-dependent promotion of HIF1α. In mammary cells, HSF1 regulates HIF1α translation via upregulation of HuR, which generally promotes mRNA stability and translation (364). HuR is overexpressed in a variety of cancers and correlates with cancer progression, including colon cancer progression (365-368). HIF1 regulates Myc by two mechanisms: 1) HIF1 binds to and activates transcription of *MXI1*, which encodes a repressor of Myc transcriptional activity, and 2) HIF1 promotes MXI1-independent, proteasome-dependent degradation of Myc (261). Alternatively, HuR has been shown to directly bind to Myc mRNA and decrease its expression (369). Further work needs to be conducted to determine if either of these mechanisms are relevant in colon tumor cell lines.

Here we present the novel finding that MEK1/2 promotes PGC1β and ERRα via an ERK-dependent mechanism (Fig 4.2 and Fig 4.3). This conclusion is supported by both direct depletion of ERK1/2 using siRNA and small-molecule inhibition experiments (Fig 4.9 and Fig 4.10). As demonstrated here, the two MEK1/2 substrates may have opposing roles on common downstream targets, such as HSF1-dependent inhibition and ERK1/2-dependent promotion of Myc and PGC1β expression (Fig 4.8 and Fig 4.9). With the identification of an additional MEK1/2 substrate, HSF1, moving forward it will be essential to distinguish between MEK1/2- and ERK1/2-dependent mechanisms.

Chapter 5: Conclusions

The experiments in this dissertation sought to identify additional vulnerabilities in cancer cells that, like KSR1 depletion, may be targets for cancer therapeutics and further define the molecular mechanisms that regulate key proteins in colon cancer survival. In the course of these studies, we identified several mechanisms by which cells regulate Myc and PGC1β expression in human CRC. We demonstrate that depletion of KSR1 or EPHB4 negatively regulates the protein levels, whereas depletion of HSF1 actually increases Myc and PGC1β protein expression. We further show that KSR1 and EPHB4 decrease Myc expression via different mechanisms. KSR1 depletion does not affect Myc and PGC1β mRNA expression, whereas depletion of EPHB4 decreases mRNA levels of both. In support of separate mechanisms of Myc and PGC1β regulation, the effects of KSR1 on Myc and PGC1β protein levels are ERK-dependent. However, EPHB4 depletion does not affect MEK or ERK phosphorylation in these cell lines. The data presented here expand our understanding of mechanisms that regulate Myc and PGC1β expression and highlight vulnerabilities in colon tumor cells that may be exploited using targeted therapies.

Is KSR1- or EPHB4-mediated expression of Myc and PGC1β dependent on activation of the RAS/RAF/MEK pathway?

The results reported here are consistent between HCT116 and Caco2 cells. HCT116 cells are heterozygous for K-Ras^{G13D}, while Caco2 cells are K-Ras^{WT}. Importantly, Caco2 cells express moderate levels of EGFR (370) and respond initially to anti-EGFR therapies with decreased proliferation (371) before acquiring MET/Src-dependent resistance (372). Signaling via c-MET can activate Ras and promote Ras-dependent signaling in the presence of EGFR inhibition (373). Therefore, Caco2 cells may depend on Ras-dependent signaling for survival even in the absence of mutated and constitutively activated Ras.

In addition to Ras activation, defects in the APC pathway occur in 80-90% of human colon carcinomas, resulting in enhanced TCF-dependent transcriptional activation of Myc and EPHB4 (271, 273). Cells with APC mutations lose the ability to regulate β-catenin signaling. Alternatively, cells can acquire a mutation in *CTNNB1* (gene encoding β-catenin). These mutations activate β-catenin-dependent signaling by decreasing their negative regulation (374). Both HCT116 and Caco2 cells harbor mutations in β-catenin leading to constitutive activation. HCT116 cells have WT APC and heterozygous β-catenin$^{\Delta Ser45}$ (374). The Ser45 residue is phosphorylated by CK1α as a priming site for GSK3β-dependent phosphorylation at Thr41, Ser37, and Ser33 and subsequent degradation by E3 ubiquitin ligases (375). Therefore, β-catenin protein in HCT116 cells is resistant to GSK3β-dependent regulation. β-catenin with the Ser45 residue deleted acts as a dominant negative, increasing β-catenin/TCF-mediated transcriptional activity (374). Caco2 cells are an example of cells that have mutant forms of both APC and β-catenin. However, the mutation in β-catenin is different than that found in HCT116 cells. Caco2 cells have a heterozygous G to C missense transversion that results in a glycine to alanine mutation at residue 245 (374). This mutation is directly N-terminal to a serine residue that is phosphorylated by CDK5 (376). Therefore, it is predicted that the G245A mutation increases the accessibility of Ser246 for phosphorylation and β-catenin activation. These mutations in β-catenin may be a mechanism by which HCT116 and Caco2 cells upregulate EPHB4 expression.

Recent work has identified pathway cross-talk in which activated K-Ras4B promotes tumorigenicity by inhibiting non-canonical Wnt/Ca^{2+}-signaling in pancreatic cells (377). The non-canonical Wnt/Ca^{2+} pathway involves activation of calmodulin (CaM) kinase II (CaMKII) and the transcription factor NF-AT as well as the inhibition of β-catenin/TCF signaling by blocking the interaction between β-catenin and TCF4. KRas4B, but not H-Ras, N-Ras, or K-Ras4A, binds to and sequesters CaM, thereby preventing CaMKII activation. In colon tumor cells lines, knockdown of K-Ras significantly represses β-catenin/TCF/LEF transcriptional activity and proliferation in SW480 (mutant for APC, wild-type β-catenin), but not HCT15 (mutant APC) or HCT116 (wild-type APC) cells with activating β-catenin mutations (377). However, Wnt/Ca^{2+}/CaMKII-dependent signaling also inhibits sphere formation in 3D culture. Inhibition of Ras downregulates CaMKII activity and prevented sphere formation in the three cell lines (377). Therefore, even in the presence of activated β-catenin, activated Ras can regulate the non-canonical Wnt signaling pathway to modulate tumorigenesis.

In the present study, we did not directly examine the effects of KSR1 or EPHB4 depletion on canonical or non-canonical Wnt/β-catenin signaling. However, when we observed that KSR1 depletion decreased EPHB4 protein levels, we did test whether this effect was due to regulation of mRNA levels or protein stability. We show that KSR1 depletion does not affect EPHB4 mRNA expression (Fig 3.21) suggesting that KSR1 is not regulating the Wnt/β-catenin-dependent transcriptional activation of EPHB4. Additionally, Wnt/β-catenin-signaling induces transcription of Myc. Taken together, it is unlikely that KSR1 is acting through Wnt/β-catenin canonical signaling to promote the upregulation of Myc and EPHB4 expression.

The mechanisms by which KSR1 and EPHB4 regulate Myc and PGC1β expression in colon tumor cell lines need to be further defined. Previous work demonstrates that inhibition of EPHB4 using a monoclonal antibody reduces proliferation and increases apoptosis in HT-29 (K-Ras^{WT}, B-RAF^{V600E}) xenografts (378). Together with our studies showing that EPHB4 depletion is toxic to colon tumor cell lines harboring mutant and wild-type K-Ras in the absence of activated B-RAF, these data suggest that

EPHB4 inhibition may be a viable therapeutic strategy selectively targeting colon tumor cells regardless of mutations in the Ras/RAF/MEK signaling pathway.

Regulation of Myc in colon tumor cell lines

Dysregulation of Myc occurs in more than half of all human tumors and often correlates with aggressive disease (264, 265), resistance to therapy (266-268), and poor prognosis (269-271). Myc activates or represses the transcription of a large number of genes involved in key cellular processes such as cell growth, metabolism, apoptosis, and protein synthesis (272). The Cancer Genome Atlas (TCGA) Network found that, in a comprehensive examination of human colon and rectal cancers of diverse anatomical origin and mutation status, changes in Myc transcriptional targets were found in nearly 100% of the tumors (280), suggesting an important role for Myc in CRC. Therefore, finding novel ways to target Myc may clinically benefit patients with colorectal cancer.

Ras activation and subsequent phosphorylation events enhance Myc protein stability (277, 278). ERK1/2-, CDK-, or JNK1/2-dependent phosphorylation of Ser62 stabilizes Myc expression, but also primes Myc for GSK3β-dependent phosphorylation at Thr58. Following phosphorylation at Thr58, Ser62 is dephosphorylated by PP2A and ubiquitinated for proteasome-mediated degradation by SCF^{FBW7} E3 ligase (277, 278). In the absence of the stabilizing phosphorylation at Ser62, Myc is rapidly degraded by alternative mechanisms (277). Here we show that while depletion of KSR1 and EPHB4 decrease phosphorylation of Myc at Ser62 (Fig 3.14); however, inhibition of phosphorylation is incomplete and Myc protein stability is unaffected by the absence of KSR1 or EPHB4 (Fig 3.16).

Protein synthesis is promoted by mTORC1 and the downstream S6K due to their ability to phosphorylate and thereby inactivate 4E-BP1 and PDCD4 proteins, which inhibit the translation initiation complex (379, 380). As a consequence, inhibition of mTORC1 blocks Myc expression in myeloma cells, and targeting protein translation with silvestrol limits the growth of Myc-driven hematopoietic tumors (381). However, treatment with a dual mTOR/PI3K inhibitor (BEZ235) failed to suppress the translation of Myc, and often times enhanced Myc expression, in colon tumor cell lines (382). Furthermore, treatment with BEZ235 enhanced phosphorylation of ERK1/2 in K-Ras wild-type and K-Ras mutant colorectal cancer lines (382). The ERK1/2 substrate RSK also phosphorylates 4E-BP1 and PDCD4 to promote protein translation (304, 383). Due to the role of KSR1 as a scaffold of the RAF/MEK/ERK kinase cascade, we predict that the KSR1-depedent promotion of Myc translation is due activation of RSK.

Myc translation can be initiated via a cap-dependent and -independent (IRES-dependent) mechanisms (230, 306). Our data suggest that KSR1 can promote both mechanisms of Myc protein synthesis (Fig 3.18 and 3.19). However, depletion of KSR1 also suppresses the phosphorylation of 4E-BP1 and PDCD4, key inhibitors of global translation. The idea that KSR1 affects global protein synthesis should be addressed in future studies. Tumors can develop an enhanced ability to promote cap-dependent protein synthesis by overexpressing eIF4E or loss of 4E-BP, [reviewed in (384)]. However, during apoptosis, growth arrest, mitosis, hypoxia, or amino acid starvation, cap-dependent translation is suppressed and IRES-mediated translation is induced (307, 385). In addition to Myc, IRES-dependent translation of mRNAs encoding HIF1α, VEGFA, Bcl2, X-linked inhibitor of apoptosis (XIAP), and p120Catenin has been reported [reviewed in (384)]. It is important to note that we have only tested the ability of KSR1 to promote IRES-mediated translation of Myc. However, if KSR1 promotes global IRES-mediated translation, there may be a KSR1-dependent mechanism by which cancer cells survive during times of stress.

The action of HSF1 is multifaceted. It promotes cell proliferation and survival in response to diverse oncogenic stimuli, enhances ERK activation in response to serum starvation, modulates protein translation, and supports glucose uptake and glycolysis (333). While HSF1 is not necessary for survival in normal conditions, it is required during the heat shock response and the proteotoxic stress induced by oncogenesis (357). Due to the role of HSF1 in proteostasis and heat shock response, it was anticipated that upregulation of Myc and PGC1β by HSF1 would promote survival in the presence of stress. In contrast to EPHB4 and KSR1, the presence of HSF1 actually decreases the expression of Myc and PGC1β protein (Fig 4.8).

Recent reports demonstrate that HSF1 plays an essential role in the development of lymphomas in p53-deficient mice and the development of carcinomas in a Ras tumor model (333, 386). This role in early-stage tumorigenesis is likely due to a role for HSF1 in evasion of oncogene-induced senescence (387). Tumor maintenance is also dependent on HSF1-mediated expression of HuR, which can stabilize and promote HIF1α translation (387). Upregulation of HuR controls mRNA stability and/or translation of many proteins involved in cancer, including proteins involved in angiogenesis (e.g., HIF-1, HIF-2, and vascular endothelial growth factor [VEGF]), cell survival (e.g., p53 and Sirt1), proliferation (e.g., cyclins, Cdc2, and p21), and others (365, 368). The HuR-dependent reduction in Myc mRNA expression (364) or stability (369) may be overcome by the ERK-dependent promotion of protein stability or translation in CRC cells.

Regulation of PGC1β in colon tumor cell lines

We demonstrate here that the expression of PGC1β is regulated by a variety of mechanisms including KSR1- and EPHB4-dependent upregulation and HSF1-dependent suppression. However, with the exception of the induction of apoptosis, we do not examine the downstream effects of PGC1β depletion in colon tumor cells. PGC1s are a small family of transcriptional coactivators that augment the responses of transcription factors and play a critical role in the control of metabolism (388). PGC1 coactivators directly interact with nuclear receptors, including PPARα and PPARγ, estrogen-related receptors (ERRs), liver X receptors (LXR), hepatocyte nuclear factor 4α (HNF-4α), and non-receptor transcription factors and regulatory elements including cAMP response element-binding protein (CREB), the lipogenic transcription factor sterol regulatory element binding protein 1c (SREBP-1c), and forkhead box O1 (FOXO1) (388). Therefore, further examination of PGC1β-mediated signaling in both normal and tumor cells is warranted.

Lipogenic nuclear receptors LXRα and LXRβ are nutrient-responsive receptors that heterodimerize with RXR to influence gene expression promoting fatty acid (FA) biosynthesis and triacylglycerol (TAG) secretion (388). LXR increases the synthesis of FA and TAG by upregulating sterol regulatory element binding protein 1c (SREBP-1c) (389). PGC1β plays a critical role in stimulating the expression of genes that regulate hepatic lipogenesis and TAG secretion (388). Adenoviral-mediated overexpression of hepatic PGC1β in rats induces increased TAG synthesis and VLDL secretion leading to hypertriglyceridemia and hypercholesterolemia (347). PGC1β induces lipogenesis in the liver by coactivating both LXR and SREBP-1 to promote expression of FA synthase (FAS), stearoyl-CoA desaturase (SCD1), and HMG-CoA reductase (HMGCR) (347). Both PGC1β and SREBP-1c, but not PGC1α, are induced in the liver in response to an acute high fat diet (24-48 h) (347).

Previous work in our lab shows that KSR1 regulates adipogenesis by coordinating ERK- and RSK-dependent phosphorylation and stabilization of CCAAT/enhancer-binding protein beta (C/EBPβ) (186). C/EBPα, C/EBPδ, and C/EBPβ transcription factors promote PPARγ expression (390). C/EBP transcription factors also regulate SREBP-1c gene expression during adipogenesis (391). Therefore, KSR1 may contribute to lipid accumulation by both promoting the expression of nuclear receptors as well as stabilizing the co-activator PGC1β.

PGC1β induces angiogenesis in skeletal muscle (392). Angiogenesis can occur under pathological conditions, such as tumor growth, and physiological conditions, such as embryonic development and exercise. It is triggered by the secretion of soluble factors, including VEGF, PDGF, angiopoietin (ANGPT), and FGF, from tissue (393). Under hypoxic conditions, hypoxia-inducible factor 1α (HIF-1α) is stabilized and free to dimerize with HIF-1β to activate proangiogenic genes such as VEGF (394). However, PGC1β induces the expression of VEGF in cell culture and *in vivo* via an ERRα-dependent and HIF-1α-independent mechanism. EPHB4 signaling is a potent regulator of VEGF-dependent angiogenesis (395-398). Several studies observed that EPHB4-ephrinB2 reverse signaling regulates VEGF-dependent pathways by specifically preventing VEGFR internalization required for activation of such pathways (396, 397). Alternatively, others have reported that EPHB4-ephrinB2 forward signaling is required for angiogenesis, as a small molecule inhibitor of EPHB4 kinase activity suppresses VEGF-driven angiogenesis *in vivo* (398). Future work should address whether EPHB4 forward signaling-dependent potentiation of angiogenesis is due to its ability to promote PGC1β expression.

We show that inhibition of Myc suppresses PGC1β mRNA expression in colon tumor cell lines (Fig 3.8). However, additional experiments indicate that in the presence of cycloheximide, PGC1β protein expression can be rescued by the addition of MG132 for 2 hours (McCall, data not shown). This suggests that PGC1β expression is not entirely dependent on the transcriptional regulation by Myc. PGC1 proteins are modified by post-transcriptional modifications, primarily reversible acetylation, phosphorylation, and methylation (388). Both PGC1α and PGC1β complex with the acetyltransferase GCN5 (general control of amino acid synthesis 5); GCN5 then acetylates several lysine residues on PGC1 proteins to inhibit their transcriptional activity (399, 400). This is opposed by SIRT1 (sirtuin 2 ortholog 1)-dependent deacetylation and activation (400, 401). Interactions between KSR proteins and SIRT1 have been identified (Fernandez and Lewis, unpublished), but KSR1-dependent acetylation of PGC1 proteins has not been examined thus far.

Phosphorylation of PGC1β is relatively unstudied. Dr. McDonnell's group at Duke University has identified several serines on PGC1β that are predicted to be phosphorylated, and they modified those serines to alanines to inhibit phosphorylation (personal communication). We examined the expression of four of these constructs (S256A, S384A, S524A, and S638A) with and without the addition of the proteasomal inhibitor, MG132. Interestingly, PGC1β^{S638A} had the lowest expression without MG132, but relatively equal expression as the other constructs in the presence of MG132 (Das and Lewis, unpublished). Further examination of this site indicates that there is a proline at the +1 position (LSLP**s**PEGLSLK). Therefore, the potential that ERK1/2, or alternatively MEK1/2, phosphorylates PGC1β at this site should be examined. The lysine at 645 is the equivalent of the GCN5-dependent acetylation site on PGC1 proteins in mice (400). The relationship, if present, between Ser638 phosphorylation and K645 acetylation should be examined. Furthermore, we have previously published that PGC1β is ubiquitinated in colon tumor cell lines (188). Therefore, determining the residue(s) modified by ubiquitination would be beneficial in further defining the mechanisms regulating PGC1β protein expression.

Summary

Cells are regulated by a vast network of signaling pathways that maintain cell homeostasis. Alterations in these networks can lead to the promotion of tumor development. The studies described here identify vulnerabilities in colon tumor cells that can be exploited for therapeutic intervention. Currently, there are several inhibitors of EPHB4 signaling, including monoclonal antibodies and kinase inhibitors that are being developed as cancer therapeutics. But thus far targeting KSR1 directly has been elusive. However, depletion of EPHB4 or KSR1 in colon tumor cell lines results in the decreased expression of common effectors, Myc and PGC1β. The results of these experiments further demonstrate that Myc and PGC1β expression are regulated by multiple mechanisms in colon tumor cells. The data show that depletion of KSR1 or EPHB4 decreases Myc and PGC1β levels, while depletion of HSF1 increases their expression. While previous work demonstrated ERK-dependent regulation of Myc stability, these data are the first to show that KSR1-dependent regulation of PGC1β is mediated by ERK activation. Therefore, in addition to EPHB4 inhibitors, the use of ERK inhibitors may prove to be a viable option for the treatment of patients with colon tumors.

In future studies, it will be important to determine if Myc-dependent expression of PGC1β enhances the oxidative and glycolytic capacity of the colon tumor cells. If so, the extent to which this mechanism is common among tumor types that demonstrate elevated levels of protein translation should be assessed. Furthermore, it should be determined whether the Myc-mediated increase in PGC1-dependent metabolic capacity is restricted to cells with increased translation of Myc mRNA or if it is also present in cells with increased Myc expression due to alternative mechanisms (*e.g.* gene amplification). Finally, the extent to which this mechanism represents a unique vulnerability to tumor cells should be examined.

Appendix A: Characterizing the roles of KSR1 and KSR2 in mouse behavior and lessons on littermate controls

Rationale

Anxiety (including panic disorder, generalized anxiety disorder, and social phobia) is a common non-motor symptom in 25-49% of patients with Parkinson's disease (PD) (402, 403), a rate higher than that reported in healthy or comparably disabled elderly controls, indicating that anxiety is associated with the disease mechanism rather than purely social distress due to motor impairment. Anxiety is considered a normal adaptive response that detects and prepares an individual against an imminent or potential threat; however, increased anxiety has a negative impact on health-related quality of life (404).

The physical symptoms of PD mainly result from progressive and profound loss of dopaminergic neurons that project to the striatum, but are also associated with losses in the pathways that project to the amygdala and hippocampus (405). The amygdala is considered the key structure responsible for the generation of emotional behaviors (406) including fear-related behaviors (407, 408). In fact, levels of dopamine (DA) in the amygdala are increased in response to aversive events (409). Furthermore, DA signaling in the amygdala and striatum are required for learning and maintaining conditioned avoidance responses (410).

Anecdotal observations from Lewis lab members suggested that mice lacking *ksr2* are abnormally calm when handled. Previous work in other labs has demonstrated that *ksr1* is required for some forms of long-term associative memory formation (411). KSR proteins are highly expressed in the brain (411, 412), including in the hippocampus and the amygdala (411), but little is known about their functions. KSR1 is expressed in both the cell bodies and dendrites of the neurons in the CA3 region of the hippocampus, but not glial cells (411). KSR2 is also expressed in the hippocampal neurons (Guo & Lewis, unpublished). However, brains of *ksr* knockout mice do not show any gross alterations in brain morphology compared to wild-type mice (411) (Costanzo-Garvey & Lewis, unpublished). It has been reported that in the hippocampus, KSR1 is important for protein kinase C (PKC)-dependent ERK signaling, but does not mediate the cyclic AMP (cAMP)/protein kinase A (PKA)-dependent pathway (411). KSR1 is also important for long-term associative memory formation (411) and both PKA and MAPK/ERK signaling are required for long-term potentiation (LTP) in the hippocampus (413-415) and amygdala (416). Due to the high level of KSR1 expression in the amygdala, we sought to determine whether KSR proteins functioned in the brain to regulate anxiety- and depression-related behaviors.

Methods

Open Field

Mice are placed in a 49 cm x 49 cm x 38 cm white box and allowed to explore for 20 minutes. Locomotor paths are monitored using a video tracking system, Ethovision (Noldus, Leesburg, VA, USA), and analyzed for time in the center (defined as 7 cm from each wall), total distance traveled, as well as total distance traveled in the center. This test measures both anxiety (increased time spent in the center) and habituation (persistent exploration).

Elevated Zero

A zero maze (34 cm ID and 46 cm OD, braced on 4 legs, 40 cm tall) is used according to procedures previously described (417). Briefly, mice are placed in the center of one of the closed quadrants and are allowed to move freely in the maze for 6 minutes. Latency to enter an open quadrant, time spent in open quadrants, and number of zone transitions (all four paws transferring from the open arm to closed arm or *vice versa*) are scored by two blinded observers. The elevated zero maze complements the open field test, but is a better measure of pure anxiety (418). Mice are naturally exploratory, but tend to avoid open and potentially dangerous areas [reviewed in (418)]. Therefore, mice prefer to remain in the closed arms of the maze. Mice who venture out more often (quantified as zone transitions) and spend more time in the open arms are classified as anxiolytic.

Forced Swim Test (FST)

A 1 L beaker is filled halfway with warm water. The test mouse is then placed into the beaker. The water is deep enough that the animal has to either swim or float to remain above the surface (it cannot stand), but the walls are too high to permit escape. The trial is videotaped for 6 minutes. At the end of this period, the mouse is removed from the beaker, dried with a paper towel, and returned to its home cage. The beaker is emptied and refilled in preparation for testing the next mouse. Primary outcome is the amount of time actively resisting (either swimming or attempting to climb) compared to the amount of time spent floating. Antidepressant phenotypes are characterized by greater amounts of time floating rather than swimming/climbing.

Tail Suspension

A thin aluminum rod (the crossbar) is mounted using standard clamps across two lab stands that are parallel to the table surface, and approximately 0.3 m above the surface. The mouse is removed from the home cage, and is gently fastened to this crossbar using adhesive masking tape. The mouse is then videotaped for 6 minutes. At the end of this period, the mouse is removed from the bar, the tape removed from the tail, and the animal returned to its home cage. The bar and adjoining areas are cleaned with chlorohexidine in preparation for testing the next mouse. Primary outcome is the amount of time spent actively resisting this position compared to the amount of time dangling without any visible muscular exertion. Antidepressant phenotypes are characterized by greater amounts of time dangling rather than resisting.

Stereotypy

The open field arena was “binned” into nine regions that varied in size, but contained similar numbers of data points per bin. The bins were constrained in that they were set as rectangles that shared a common row, but not column. Raw locomotor (x,y) coordinate data from the video tracking system were imported into MATLAB (MathWorks Inc, Natick, MA, USA). Data were binned using a MATLAB algorithm provided by Dr. Bonasera, as previously described (419). Patterns were required to be longer than two bins and not overlap in time to proceed with analyses. A commercially available program (Theme, Noldus) was used to evaluate pattern structure. Within a given trial, we calculated what percentage of total trial time the animal is engaged in route-tracing behavior. We also determined the maximum length of patterns from each trial.

Statistics

All statistics were performed using Prism Software (GraphPad). All data are presented as mean ± SEM. Data presented as genotypes over time were analyzed with a two-way ANOVA with Bonferoni’s post-test for multiple comparisons. Figures with two columns were analyzed using a two-tailed, student’s *t*-test. All others were analyzed with a one-way ANOVA with Bonferoni’s post-test for multiple comparisons. A *p* value of less than 0.05 was considered statistically significant.

Results and Discussion

Pilot study with non-littermate WT controls indicates that KSR1 is important in mouse anxiety

Age-matched *ksr1*$^{-/-}$ male mice (5-6 weeks in age) were placed in an open field arena (one at a time) and allowed to freely roam for 20 minutes. This test measures anxiety, exploration, and habituation behaviors. Sessions were recorded with an overhead camera and route mapping was performed using Ethovision software. A wild-type mouse will typically spend time in the corners and around the edges of the arena, displaying thigmotactic behaviors. A mouse that enters the center of the arena more often is considered anxiolytic or less anxious.

Examples of wild-type and *ksr1*$^{-/-}$ mice route maps are shown in Fig A.1A. In the open field test, *ksr1*$^{-/-}$ mice travel further (Fig A.1B) and spend significantly more time in the center than the wild-type controls (Fig A.1C). Since *ksr1*$^{-/-}$ mice exhibit increased locomotive activity as compared to WT controls, we quantified the stereotypic behaviors (specifically route-tracing) displayed by each phenotype. Stereotypies are defined as motor actions of unknown functional purpose that are repeatedly performed in a near-identical manner (420, 421). These behaviors may include patterns (route-tracing), repetitive head movements, and syntactic grooming (420). Increased stereotypic behaviors are a behavioral correlate of aberrant striatal function (422-424). Particularly, increased DA receptor stimulation at synapses within the striatum can induce increases in stereotypy (419, 425). To quantify the patterns of routes taken by each mouse, we used t-pattern sequential analyses (419, 426). This is a mathematical approach that determines whether a sequence of events occurs within a specified time interval at a rate greater than that expected by chance. *ksr1*$^{-/-}$ mice spend a larger percentage of their time performing stereotypic behaviors (Fig A.1C), but do not have longer patterns than their wild-type counterparts (Fig A.1D).

To determine if this anxiolytic phenotype is also observed in the elevated zero maze, *ksr1*$^{-/-}$ and wild-type mice were placed in the closed arm of the elevated zero maze and allowed to freely explore for six minutes. In this test, mice are given a choice to spend time in the unprotected, open arm, or the protected, closed arm. Mice generally avoid open areas, especially when brightly lit. Therefore, while it is common for a wild-type mouse to transition to the other closed arm, they usually run quickly to get out of the

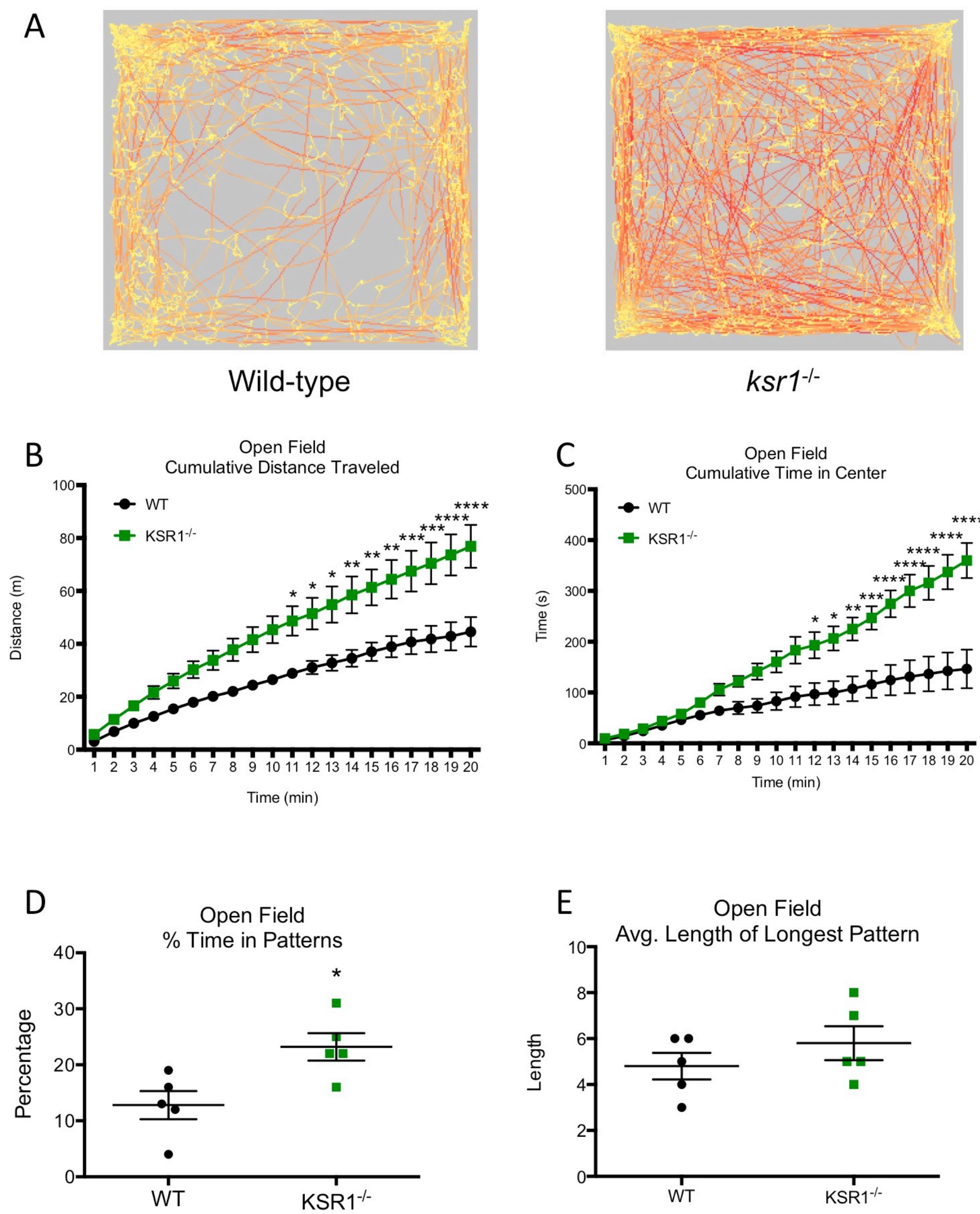


Fig A.1 Age-matched *ksr1$^{-/-}$* mice display an anxiolytic phenotype as measured in the open field arena.

Age-matched WT and *ksr1$^{-/-}$* mice (5-6 weeks, male) were tested using the open field test. (A) Example of data analyzed for each genotype. (B) Cumulative distance traveled. (C) Cumulative time spent in the center of the arena. (D) Percentage of time spent in stereotypic (repetitive) patterns. (E) Average length in longest pattern.

open space. Again, all trials were recorded using an overhead video camera system and route-mapping for each trial was performed using Ethovision software (Noldus).

An example of the arena and maps for each genotype are shown in Figure A.2A. *ksr1*$^{-/-}$ mice exhibited an abnormal locomotive pattern during the elevated zero maze (Fig A.2C). They also spend significantly more time in the open arm of the elevated zero maze than wild-type mice (Fig A.2D). Taken together, these data suggest that *ksr1*$^{-/-}$ mice have decreased anxiety.

Age-matched *ksr1*$^{-/-}$ female mice (5-6 weeks of age) also exhibit a significant anxiolytic phenotype in the elevated zero maze, but fail to recapitulate the phenotype seen in the male mice in the open field test (data not shown).

Age-matched ksr2$^{-/-}$ *mice trend towards decreased anxiolytic phenotypes*

Given our anecdotal evidence that *ksr2*$^{-/-}$ mice appear to be calmer when handled than wild-type mice, we tested the effect of genotype on anxiety-related mouse correlates using the open field arena and elevated zero maze. Male and female *ksr2*$^{-/-}$ and wild-type mice were tested. Only male data are presented here. *ksr2*$^{-/-}$ mice have slightly more total movement in the open field than wild-type mice (Fig A.3A). They also spend slightly more time in the center (Fig A.3B). However, the route-tracing stereotypic phenotypes observed in mice lacking *ksr1* are not present in *ksr2*$^{-/-}$ mice (Fig A.3C-D). We further tested these mice in the elevated zero maze. Again, *ksr2*$^{-/-}$ male mice have a trend of increased locomotion (Fig A.3E) and increased time spent in the open arms of the elevated zero maze (Fig A.3F), but these results are not significant. *ksr2*$^{-/-}$ female mice did not exhibit these trends.

Taken together, male mice lacking *ksr2* have a trend towards anxiolytic phenotypes, but the results were not significant for the open field and elevated zero

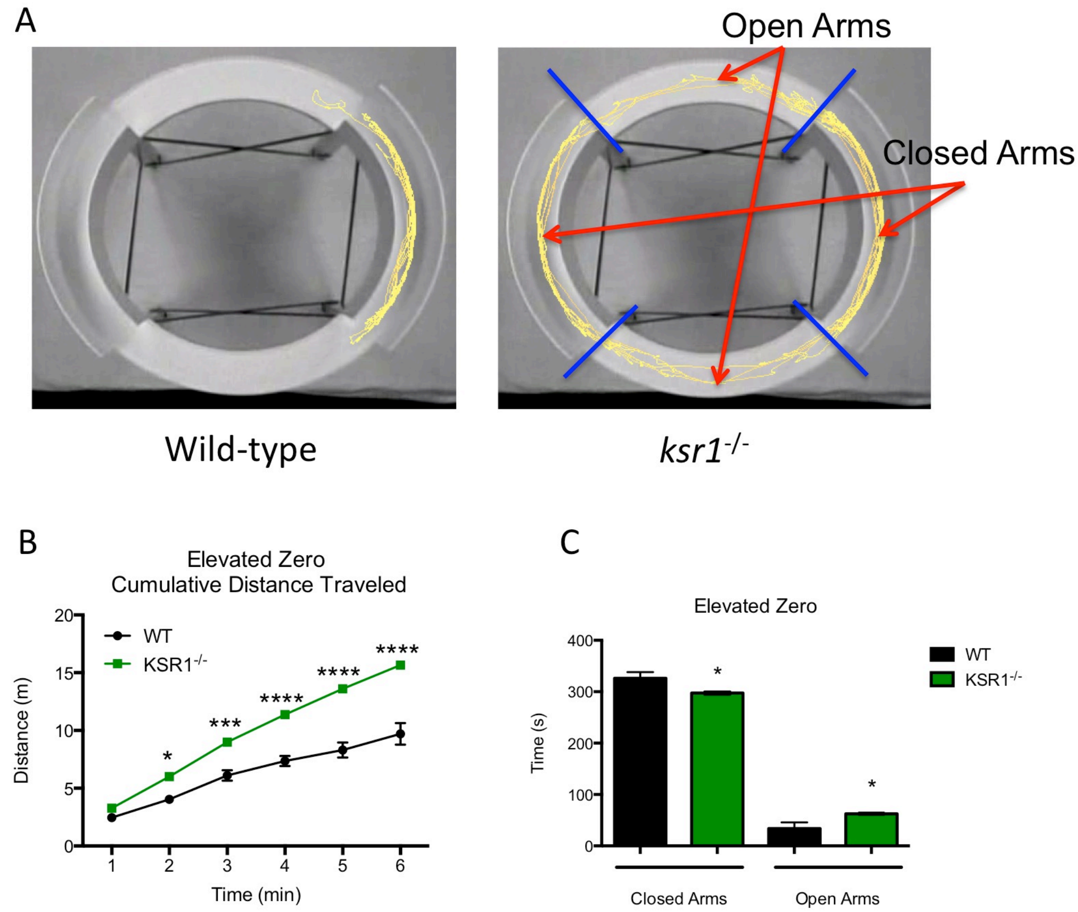


Fig A.2 Age-matched *ksr1*$^{-/-}$ mice display an anxiolytic phenotype as measured in the elevated zero maze.

Age-matched WT and *ksr1*$^{-/-}$ mice (5-6 weeks, male) were tested using the elevated zero maze. (A) Example of data analyzed for each genotype. (B) Cumulative distance traveled. (C) Cumulative time spent in each arm of the maze.

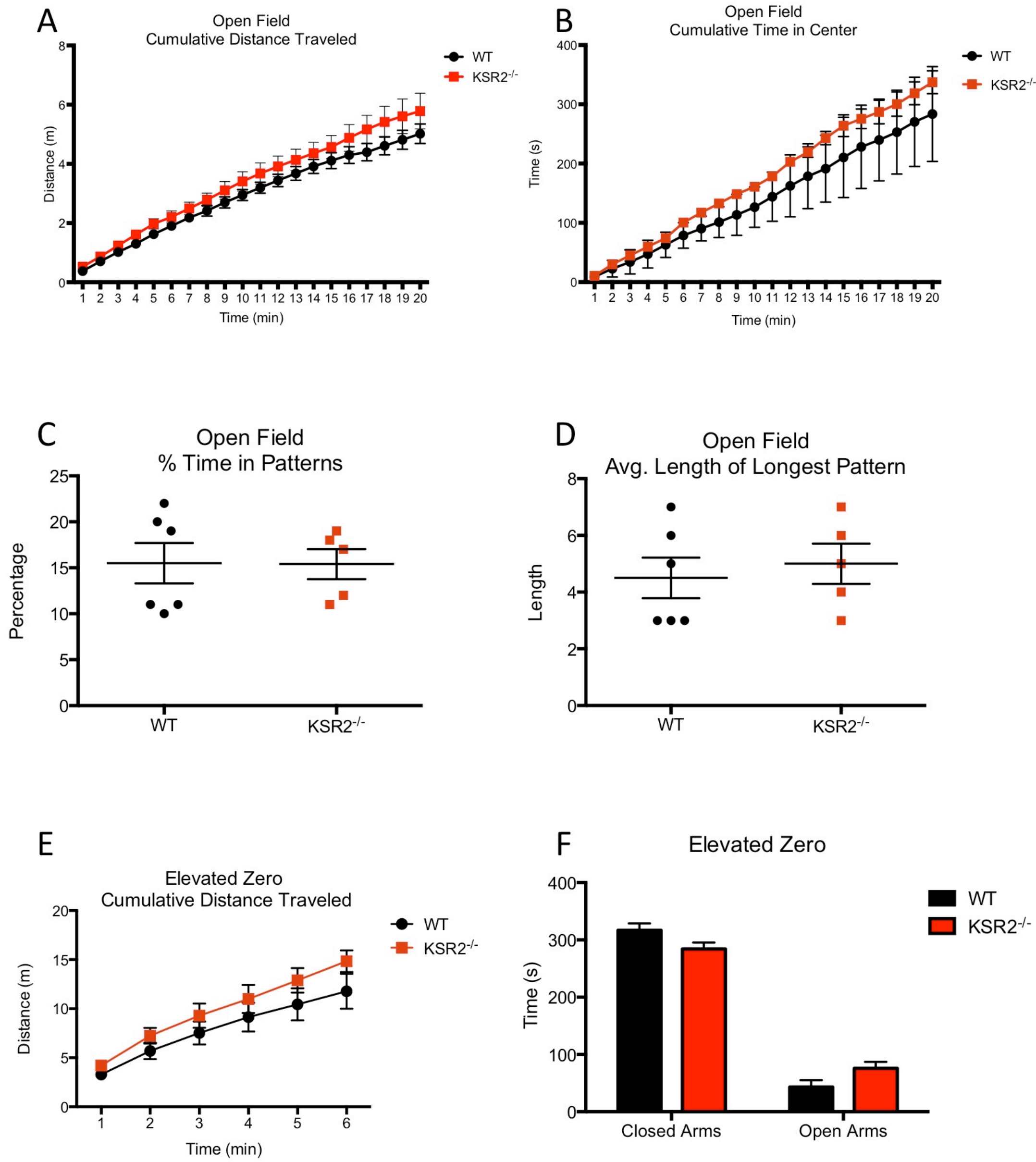


Fig A.3 Age-matched *ksr2*$^{-/-}$ mice do not display anxiolytic phenotypes.
Age-matched WT and *ksr2*$^{-/-}$ mice (5-6 weeks, male) were tested using the open field test (A-D) and the elevated zero maze (E-F).

maze. However, power calculations indicate that significance may be obtained using additional mice (12 and 7 for open field and elevated zero maze, respectively).

Littermate-controlled studies indicate that KSR1 does not affect mouse anxiety

Several confounding factors can affect the results of behavioral tests (427-429). Therefore, it is recommended that all studies are performed with littermates. Additionally, the estrus cycle can affect results; so male mice are preferentially used in these assays (430). To address these issues, we repeated the assays using a littermate-controlled cohort of wild-type, *ksr1*$^{+/-}$, and *ksr1*$^{-/-}$ male mice (5-6 weeks in age). This group failed to recapitulate any of the original phenotype observed in the pilot study (Fig A.4). This may be due to several reasons: 1) the pilot study used age-matched controls, whereas the current study used littermate controls; 2) the pilot and current studies were performed in different seasons, which may affect mouse behaviors. The first point is very important because maternal behavior during the neonatal period has been shown to regulate anxiety in adult mice (431); therefore, it is essential to use matching littermate, wild-type controls in these analyses.

Interestingly, data from age-matched and littermate-matched *ksr1*$^{-/-}$ mice are nearly identical in all reported measurements. However, the wild-type mice are significantly different. Of major note is that in the pilot study, these genotypes not only came from different litters, but were also derived from parents with different genotypes. The *ksr1*$^{-/-}$ mice are fertile and at the time of the study the colony was maintained using null x null crosses. The wild-type mice were obtained from wild-type x wild-type crosses or from the *ksr2* colony. *ksr2*$^{-/-}$ mice are infertile and their colony is maintained *ksr2*$^{+/-}$ X *ksr2*$^{+/-}$ breeding. Additionally, *ksr2*$^{-/-}$ mice often do not survive until weaning. In pilot studies, we used the "extra," age-matched wild-type mice from the *ksr2*$^{+/-}$ X *ksr2*$^{+/-}$ cross

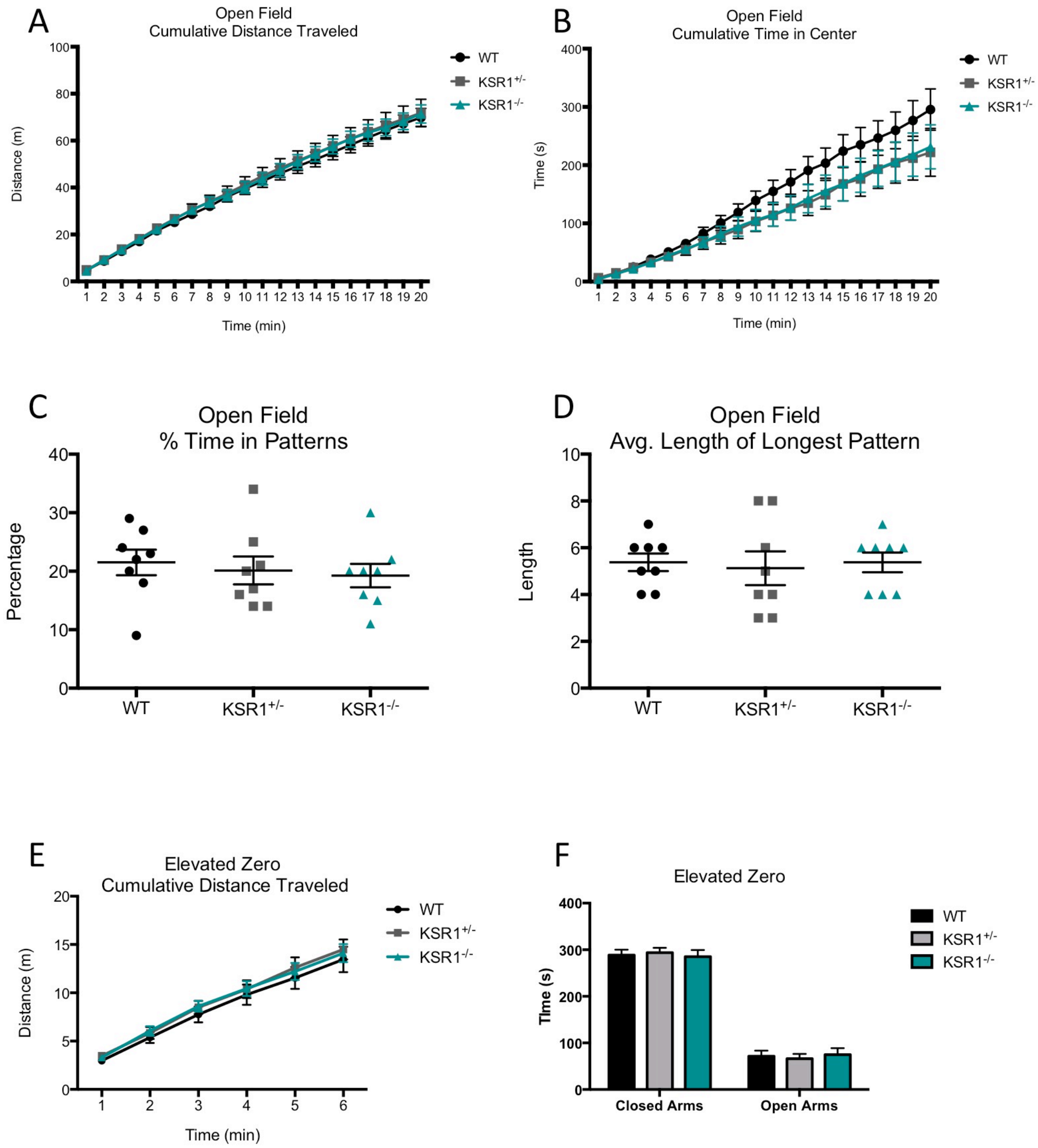


Fig A.4 Littermate-controlled *ksr1*$^{-/-}$ mice do not display anxiolytic phenotypes. Age-matched WT, *ksr1*$^{+/-}$, and *ksr1*$^{-/-}$ mice (5-6 weeks, male) were tested using the open field test (A-D) and the elevated zero maze (E-F).

to pair for comparison to *ksr1*$^{-/-}$ mice.

Furthermore, each colony is numbered differently for genotyping in the second week of life. Mice in the *ksr1* colony and wild-type crosses are healthy and large. They are marked using ear clipping. Knockout mice in the *ksr2* colony are weak and very small. We have an issue with ear clippings tearing and generally being too large for their ear size. Therefore, we have an exemption from IACUC to mark these mice using toe clippings. Recent studies indicate that toe clipping does not adversely affect the behavior studies we performed. Thus, it is anticipated that the clipping method is not the primary cause for the significant differences observed in the wild-type groups (432, 433).

It is likely that maternal care is drastically different in these three breeding schemes and may be contributing to the differences we observed in the wild-type mice. Animal studies support the idea that physical and emotional neglect and parental loss during childhood can lead to negative consequences in adulthood, such as an increased likelihood to develop depression and anxiety disorders (434, 435). In rats, males that undergo maternal separation (MS) for 3-4.5 hours a day for the first 2 or 3 weeks of life exhibit an increase in fearfulness in adulthood (436, 437). Males that undergo repeated separation show greater hypothalamic-pituitary-adrenal activity both basally and in response to an acute stressor upon reaching adulthood (437, 438). In C57BL/6 mice, behaviors of males and females can be altered by MS (431). Specifically, they show that maternally separated males showed higher levels of anxiety and fear behavior in adulthood, as measured by the open field test and elevated plus maze, compared to control males. Conversely, they found that maternally separated females exhibit less anxiety and fear behavior in adulthood, but only during diestrus (431), suggesting that estrus cycle should be monitored when conducting behavioral studies. Thus, it appears that early life stressors in the form of maternal separation can have a lasting influence on the physiology and behavior of offspring for both rats and mice.

Future work addressing the differences in maternal care among the colonies would be relevant to this work, but was not pursued. However, this work did shift the way the breeding colonies are managed. We now breed *ksr1*$^{-/-}$ mice from *ksr1*$^{+/-}$ X *ksr1*$^{+/-}$ crosses for all studies. Furthermore, we have started producing inducible *ksr2*$^{fl/fl}$ mice. Under the traditional breeding scheme, obtaining *ksr2*$^{-/-}$ and wild-type male littermates to use in the behavioral studies was essentially impossible as mice lacking *ksr2* expression often do not survive past weaning. Using an inducible knockout mouse model, we can properly measure the contribution of KSR2 to anxiolytic phenotypes using littermate controls.

At the time of these studies, we also conducted assays to measure a potential depression-related phenotype in the littermate-controlled *ksr1*$^{-/-}$ mice, including the tail suspension test (TST) and Porsolt's forced swim test (FST). These tests were performed on the same mice as the open field and elevated zero maze. Videos of these tests were recorded and need to be analyzed by two independent, blinded reviewers for time spent immobile. However, due to the innate agility of the C57BL/6 strain, the tail suspension test may not be an appropriate test to measure depression (439). If the data from the FST are indicative of a depressive phenotype, we may need to utilize a learned helplessness paradigm to prevent lost data due to tail climbing.

Literature Cited

1. **Harvey JJ.** 1964. An Unidentified Virus Which Causes the Rapid Production of Tumours in Mice. Nature **204:**1104-1105.
2. **Kirsten WH, Mayer LA.** 1967. Morphologic responses to a murine erythroblastosis virus. J Natl Cancer Inst **39:**311-335.
3. **Scolnick EM, Parks WP.** 1974. Harvey sarcoma virus: a second murine type C sarcoma virus with rat genetic information. J Virol **13:**1211-1219.
4. **DeFeo D, Gonda MA, Young HA, Chang EH, Lowy DR, Scolnick EM, Ellis RW.** 1981. Analysis of two divergent rat genomic clones homologous to the transforming gene of Harvey murine sarcoma virus. Proc Natl Acad Sci U S A **78:**3328-3332.
5. **Ellis RW, DeFeo D, Furth ME, Scolnick EM.** 1982. Mouse cells contain two distinct ras gene mRNA species that can be translated into a p21 onc protein. Mol Cell Biol **2:**1339-1345.
6. **Chang EH, Gonda MA, Ellis RW, Scolnick EM, Lowy DR.** 1982. Human genome contains four genes homologous to transforming genes of Harvey and Kirsten murine sarcoma viruses. Proc Natl Acad Sci U S A **79:**4848-4852.
7. **Parada LF, Tabin CJ, Shih C, Weinberg RA.** 1982. Human EJ bladder carcinoma oncogene is homologue of Harvey sarcoma virus ras gene. Nature **297:**474-478.
8. **Der CJ, Krontiris TG, Cooper GM.** 1982. Transforming genes of human bladder and lung carcinoma cell lines are homologous to the ras genes of Harvey and Kirsten sarcoma viruses. Proc Natl Acad Sci U S A **79:**3637-3640.
9. **Santos E, Tronick SR, Aaronson SA, Pulciani S, Barbacid M.** 1982. T24 human bladder carcinoma oncogene is an activated form of the normal human homologue of BALB- and Harvey-MSV transforming genes. Nature **298:**343-347.
10. **Perucho M, Goldfarb M, Shimizu K, Lama C, Fogh J, Wigler M.** 1981. Human-tumor-derived cell lines contain common and different transforming genes. Cell **27:**467-476.
11. **Shimizu K, Goldfarb M, Suard Y, Perucho M, Li Y, Kamata T, Feramisco J, Stavnezer E, Fogh J, Wigler MH.** 1983. Three human transforming genes are related to the viral ras oncogenes. Proc Natl Acad Sci U S A **80:**2112-2116.

12. **Hall A, Marshall CJ, Spurr NK, Weiss RA.** 1983. Identification of transforming gene in two human sarcoma cell lines as a new member of the ras gene family located on chromosome 1. Nature **303:**396-400.

13. **Taparowsky E, Shimizu K, Goldfarb M, Wigler M.** 1983. Structure and activation of the human N-ras gene. Cell **34:**581-586.

14. **Murray MJ, Cunningham JM, Parada LF, Dautry F, Lebowitz P, Weinberg RA.** 1983. The HL-60 transforming sequence: a ras oncogene coexisting with altered myc genes in hematopoietic tumors. Cell **33:**749-757.

15. **Reddy EP, Reynolds RK, Santos E, Barbacid M.** 1982. A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene. Nature **300:**149-152.

16. **Tabin CJ, Bradley SM, Bargmann CI, Weinberg RA, Papageorge AG, Scolnick EM, Dhar R, Lowy DR, Chang EH.** 1982. Mechanism of activation of a human oncogene. Nature **300:**143-149.

17. **Taparowsky E, Suard Y, Fasano O, Shimizu K, Goldfarb M, Wigler M.** 1982. Activation of the T24 bladder carcinoma transforming gene is linked to a single amino acid change. Nature **300:**762-765.

18. **Capon DJ, Chen EY, Levinson AD, Seeburg PH, Goeddel DV.** 1983. Complete nucleotide sequences of the T24 human bladder carcinoma oncogene and its normal homologue. Nature **302:**33-37.

19. **Newbold RF, Overell RW.** 1983. Fibroblast immortality is a prerequisite for transformation by EJ c-Ha-ras oncogene. Nature **304:**648-651.

20. **Land H, Parada LF, Weinberg RA.** 1983. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature **304:**596-602.

21. **Ruley HE.** 1983. Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature **304:**602-606.

22. **Rhim JS, Jay G, Arnstein P, Price FM, Sanford KK, Aaronson SA.** 1985. Neoplastic transformation of human epidermal keratinocytes by AD12-SV40 and Kirsten sarcoma viruses. Science **227:**1250-1252.

23. **Yoakum GH, Lechner JF, Gabrielson EW, Korba BE, Malan-Shibley L, Willey JC, Valerio MG, Shamsuddin AM, Trump BF, Harris CC.** 1985. Transformation of human bronchial epithelial cells transfected by Harvey ras oncogene. Science **227:**1174-1179.

24. **Yancopoulos GD, Nisen PD, Tesfaye A, Kohl NE, Goldfarb MP, Alt FW.** 1985. N-myc can cooperate with ras to transform normal cells in culture. Proc Natl Acad Sci U S A **82:**5455-5459.

25. **Nakano H, Yamamoto F, Neville C, Evans D, Mizuno T, Perucho M.** 1984. Isolation of transforming sequences of two human lung carcinomas: structural and functional analysis of the activated c-K-ras oncogenes. Proc Natl Acad Sci U S A **81:**71-75.

26. **Santos E, Martin-Zanca D, Reddy EP, Pierotti MA, Della Porta G, Barbacid M.** 1984. Malignant activation of a K-ras oncogene in lung carcinoma but not in normal tissue of the same patient. Science **223:**661-664.

27. **Feig LA, Bast RC, Jr., Knapp RC, Cooper GM.** 1984. Somatic activation of rasK gene in a human ovarian carcinoma. Science **223:**698-701.

28. **Forbes SA, Bindal N, Bamford S, Cole C, Kok CY, Beare D, Jia M, Shepherd R, Leung K, Menzies A, Teague JW, Campbell PJ, Stratton MR, Futreal PA.** 2011. COSMIC: mining complete cancer genomes in the Catalogue of Somatic Mutations in Cancer. Nucleic Acids Res **39:**D945-950.

29. **Shimizu N, Ohtsubo M, Minoshima S.** 2007. MutationView/KMcancerDB: a database for cancer gene mutations. Cancer Sci **98:**259-267.

30. **Capon DJ, Seeburg PH, McGrath JP, Hayflick JS, Edman U, Levinson AD, Goeddel DV.** 1983. Activation of Ki-ras2 gene in human colon and lung carcinomas by two different point mutations. Nature **304:**507-513.

31. **Welman A, Burger MM, Hagmann J.** 2000. Structure and function of the C-terminal hypervariable region of K-Ras4B in plasma membrane targetting and transformation. Oncogene **19:**4582-4591.

32. **Abubaker J, Bavi P, Al-Haqawi W, Sultana M, Al-Harbi S, Al-Sanea N, Abduljabbar A, Ashari LH, Alhomoud S, Al-Dayel F, Uddin S, Al-Kuraya KS.** 2009. Prognostic significance of alterations in KRAS isoforms KRAS-4A/4B and KRAS mutations in colorectal carcinoma. J Pathol **219:**435-445.

33. **Heighway J, Hasleton PS.** 1986. c-Ki-ras amplification in human lung cancer. Br J Cancer **53:**285-287.

34. **Taya Y, Hosogai K, Hirohashi S, Shimosato Y, Tsuchiya R, Tsuchida N, Fushimi M, Sekiya T, Nishimura S.** 1984. A novel combination of K-ras and myc amplification accompanied by point mutational activation of K-ras in a human lung cancer. EMBO J **3:**2943-2946.

35. **Graham KA, Richardson CL, Minden MD, Trent JM, Buick RN.** 1985. Varying degrees of amplification of the N-ras oncogene in the human breast cancer cell line MCF-7. Cancer Res **45:**2201-2205.

36. **Akkiprik M, Celikel CA, Dusunceli F, Sonmez O, Gulluoglu BM, Sav A, Ozer A.** 2008. Relationship between overexpression of ras p21 oncoprotein and K-ras codon 12 and 13 mutations in Turkish colorectal cancer patients. Turk J Gastroenterol **19:**22-27.

37. **Wang Q, Chaerkady R, Wu J, Hwang HJ, Papadopoulos N, Kopelovich L, Maitra A, Matthaei H, Eshleman JR, Hruban RH, Kinzler KW, Pandey A, Vogelstein B.** 2011. Mutant proteins as cancer-specific biomarkers. Proc Natl Acad Sci U S A **108:**2444-2449.

38. **McCormick F.** 1989. ras GTPase activating protein: signal transmitter and signal terminator. Cell **56:**5-8.

39. **Bourne HR, Sanders DA, McCormick F.** 1990. The GTPase superfamily: a conserved switch for diverse cell functions. Nature **348:**125-132.

40. **Papageorge A, Lowy D, Scolnick EM.** 1982. Comparative biochemical properties of p21 ras molecules coded for by viral and cellular ras genes. J Virol **44:**509-519.

41. **Shih TY, Papageorge AG, Stokes PE, Weeks MO, Scolnick EM.** 1980. Guanine nucleotide-binding and autophosphorylating activities associated with the p21src protein of Harvey murine sarcoma virus. Nature **287:**686-691.

42. **McGrath JP, Capon DJ, Goeddel DV, Levinson AD.** 1984. Comparative biochemical properties of normal and activated human ras p21 protein. Nature **310:**644-649.

43. **Gibbs JB, Sigal IS, Poe M, Scolnick EM.** 1984. Intrinsic GTPase activity distinguishes normal and oncogenic ras p21 molecules. Proc Natl Acad Sci U S A **81:**5704-5708.

44. **Sweet RW, Yokoyama S, Kamata T, Feramisco JR, Rosenberg M, Gross M.** 1984. The product of ras is a GTPase and the T24 oncogenic mutant is deficient in this activity. Nature **311:**273-275.

45. **Der CJ, Finkel T, Cooper GM.** 1986. Biological and biochemical properties of human rasH genes mutated at codon 61. Cell **44:**167-176.

46. **Trahey M, McCormick F.** 1987. A cytoplasmic protein stimulates normal N-ras p21 GTPase, but does not affect oncogenic mutants. Science **238:**542-545.

47. **Vogel US, Dixon RA, Schaber MD, Diehl RE, Marshall MS, Scolnick EM, Sigal IS, Gibbs JB.** 1988. Cloning of bovine GAP and its interaction with oncogenic ras p21. Nature **335:**90-93.

48. **Trahey M, Wong G, Halenbeck R, Rubinfeld B, Martin GA, Ladner M, Long CM, Crosier WJ, Watt K, Koths K, et al.** 1988. Molecular cloning of two types of GAP complementary DNA from human placenta. Science **242:**1697-1700.

49. **Gorfe AA, Grant BJ, McCammon JA.** 2008. Mapping the nucleotide and isoform-dependent structural and dynamical features of Ras proteins. Structure **16:**885-896.

50. **Maegley KA, Admiraal SJ, Herschlag D.** 1996. Ras-catalyzed hydrolysis of GTP: a new perspective from model studies. Proc Natl Acad Sci U S A **93:**8160-8166.

51. **Gremer L, Gilsbach B, Ahmadian MR, Wittinghofer A.** 2008. Fluoride complexes of oncogenic Ras mutants to study the Ras-RasGap interaction. Biol Chem **389:**1163-1171.

52. **Prior IA, Lewis PD, Mattos C.** 2012. A comprehensive survey of Ras mutations in cancer. Cancer Res **72:**2457-2467.

53. **Shih TY, Weeks MO, Gruss P, Dhar R, Oroszlan S, Scolnick EM.** 1982. Identification of a precursor in the biosynthesis of the p21 transforming protein of harvey murine sarcoma virus. J Virol **42:**253-261.

54. **Sefton BM, Trowbridge IS, Cooper JA, Scolnick EM.** 1982. The transforming proteins of Rous sarcoma virus, Harvey sarcoma virus and Abelson virus contain tightly bound lipid. Cell **31:**465-474.

55. **Willumsen BM, Christensen A, Hubbert NL, Papageorge AG, Lowy DR.** 1984. The p21 ras C-terminus is required for transformation and membrane association. Nature **310:**583-586.

56. **Srivastava SK, Lacal JC, Reynolds SH, Aaronson SA.** 1985. Antibody of predetermined specificity to a carboxy-terminal region of H-ras gene products inhibits their guanine nucleotide-binding function. Mol Cell Biol **5:**3316-3319.

57. **Casey PJ, Solski PA, Der CJ, Buss JE.** 1989. p21ras is modified by a farnesyl isoprenoid. Proc Natl Acad Sci U S A **86:**8323-8327.

58. **Reiss Y, Goldstein JL, Seabra MC, Casey PJ, Brown MS.** 1990. Inhibition of purified p21ras farnesyl:protein transferase by Cys-AAX tetrapeptides. Cell **62:**81-88.

59. **Schaber MD, O'Hara MB, Garsky VM, Mosser SC, Bergstrom JD, Moores SL, Marshall MS, Friedman PA, Dixon RA, Gibbs JB.** 1990. Polyisoprenylation of Ras in vitro by a farnesyl-protein transferase. J Biol Chem **265:**14701-14704.
60. **Schafer WR, Trueblood CE, Yang CC, Mayer MP, Rosenberg S, Poulter CD, Kim SH, Rine J.** 1990. Enzymatic coupling of cholesterol intermediates to a mating pheromone precursor and to the ras protein. Science **249:**1133-1139.
61. **Hancock JF, Paterson H, Marshall CJ.** 1990. A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize p21ras to the plasma membrane. Cell **63:**133-139.
62. **Kamata T, Feramisco JR.** 1984. Epidermal growth factor stimulates guanine nucleotide binding activity and phosphorylation of ras oncogene proteins. Nature **310:**147-150.
63. **Mulcahy LS, Smith MR, Stacey DW.** 1985. Requirement for ras proto-oncogene function during serum-stimulated growth of NIH 3T3 cells. Nature **313:**241-243.
64. **Hagag N, Halegoua S, Viola M.** 1986. Inhibition of growth factor-induced differentiation of PC12 cells by microinjection of antibody to ras p21. Nature **319:**680-682.
65. **Satoh T, Endo M, Nakafuku M, Nakamura S, Kaziro Y.** 1990. Platelet-derived growth factor stimulates formation of active p21ras.GTP complex in Swiss mouse 3T3 cells. Proc Natl Acad Sci U S A **87:**5993-5997.
66. **Gibbs JB, Marshall MS, Scolnick EM, Dixon RA, Vogel US.** 1990. Modulation of guanine nucleotides bound to Ras in NIH3T3 cells by oncogenes, growth factors, and the GTPase activating protein (GAP). J Biol Chem **265:**20437-20442.
67. **Leon J, Guerrero I, Pellicer A.** 1987. Differential expression of the ras gene family in mice. Mol Cell Biol **7:**1535-1540.
68. **Johnson L, Greenbaum D, Cichowski K, Mercer K, Murphy E, Schmitt E, Bronson RT, Umanoff H, Edelmann W, Kucherlapati R, Jacks T.** 1997. K-ras is an essential gene in the mouse with partial functional overlap with N-ras. Genes Dev **11:**2468-2481.
69. **Koera K, Nakamura K, Nakao K, Miyoshi J, Toyoshima K, Hatta T, Otani H, Aiba A, Katsuki M.** 1997. K-ras is essential for the development of the mouse embryo. Oncogene **15:**1151-1159.

70. **Esteban LM, Vicario-Abejon C, Fernandez-Salguero P, Fernandez-Medarde A, Swaminathan N, Yienger K, Lopez E, Malumbres M, McKay R, Ward JM, Pellicer A, Santos E.** 2001. Targeted genomic disruption of H-ras and N-ras, individually or in combination, reveals the dispensability of both loci for mouse growth and development. Mol Cell Biol **21:**1444-1452.

71. **Umanoff H, Edelmann W, Pellicer A, Kucherlapati R.** 1995. The murine N-ras gene is not essential for growth and development. Proc Natl Acad Sci U S A **92:**1709-1713.

72. **Potenza N, Vecchione C, Notte A, De Rienzo A, Rosica A, Bauer L, Affuso A, De Felice M, Russo T, Poulet R, Cifelli G, De Vita G, Lembo G, Di Lauro R.** 2005. Replacement of K-Ras with H-Ras supports normal embryonic development despite inducing cardiovascular pathology in adult mice. EMBO Rep **6:**432-437.

73. **Lemmon MA, Schlessinger J.** 2010. Cell signaling by receptor tyrosine kinases. Cell **141:**1117-1134.

74. **Margolis B, Borg JP, Straight S, Meyer D.** 1999. The function of PTB domain proteins. Kidney Int **56:**1230-1237.

75. **Hanahan D, Weinberg RA.** 2011. Hallmarks of cancer: the next generation. Cell **144:**646-674.

76. **Ahearn IM, Haigis K, Bar-Sagi D, Philips MR.** 2012. Regulating the regulator: post-translational modification of RAS. Nat Rev Mol Cell Biol **13:**39-51.

77. **Cox AD, Der CJ.** 2010. Ras history: The saga continues. Small GTPases **1:**2-27.

78. **Han M, Golden A, Han Y, Sternberg PW.** 1993. C. elegans lin-45 raf gene participates in let-60 ras-stimulated vulval differentiation. Nature **363:**133-140.

79. **Van Aelst L, Barr M, Marcus S, Polverino A, Wigler M.** 1993. Complex formation between RAS and RAF and other protein kinases. Proc Natl Acad Sci U S A **90:**6213-6217.

80. **Warne PH, Viciana PR, Downward J.** 1993. Direct interaction of Ras and the amino-terminal region of Raf-1 in vitro. Nature **364:**352-355.

81. **Zhang XF, Settleman J, Kyriakis JM, Takeuchi-Suzuki E, Elledge SJ, Marshall MS, Bruder JT, Rapp UR, Avruch J.** 1993. Normal and oncogenic p21ras proteins bind to the amino-terminal regulatory domain of c-Raf-1. Nature **364:**308-313.

82. **Moodie SA, Willumsen BM, Weber MJ, Wolfman A.** 1993. Complexes of Ras.GTP with Raf-1 and mitogen-activated protein kinase kinase. Science **260:**1658-1661.

83. **Dickson B, Sprenger F, Morrison D, Hafen E.** 1992. Raf functions downstream of Ras1 in the Sevenless signal transduction pathway. Nature **360:**600-603.

84. **Rodriguez-Viciana P, Warne PH, Dhand R, Vanhaesebroeck B, Gout I, Fry MJ, Waterfield MD, Downward J.** 1994. Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature **370:**527-532.

85. **Gupta S, Ramjaun AR, Haiko P, Wang Y, Warne PH, Nicke B, Nye E, Stamp G, Alitalo K, Downward J.** 2007. Binding of ras to phosphoinositide 3-kinase p110alpha is required for ras-driven tumorigenesis in mice. Cell **129:**957-968.

86. **Spaargaren M, Bischoff JR.** 1994. Identification of the guanine nucleotide dissociation stimulator for Ral as a putative effector molecule of R-ras, H-ras, K-ras, and Rap. Proc Natl Acad Sci U S A **91:**12609-12613.

87. **Kikuchi A, Demo SD, Ye ZH, Chen YW, Williams LT.** 1994. ralGDS family members interact with the effector loop of ras p21. Mol Cell Biol **14:**7483-7491.

88. **Hofer F, Fields S, Schneider C, Martin GS.** 1994. Activated Ras interacts with the Ral guanine nucleotide dissociation stimulator. Proc Natl Acad Sci U S A **91:**11089-11093.

89. **Urano T, Emkey R, Feig LA.** 1996. Ral-GTPases mediate a distinct downstream signaling pathway from Ras that facilitates cellular transformation. EMBO J **15:**810-816.

90. **White MA, Vale T, Camonis JH, Schaefer E, Wigler MH.** 1996. A role for the Ral guanine nucleotide dissociation stimulator in mediating Ras-induced transformation. J Biol Chem **271:**16439-16442.

91. **Hamad NM, Elconin JH, Karnoub AE, Bai W, Rich JN, Abraham RT, Der CJ, Counter CM.** 2002. Distinct requirements for Ras oncogenesis in human versus mouse cells. Genes Dev **16:**2045-2057.

92. **Bodemann BO, White MA.** 2008. Ral GTPases and cancer: linchpin support of the tumorigenic platform. Nat Rev Cancer **8:**133-140.

93. **Lambert JM, Lambert QT, Reuther GW, Malliri A, Siderovski DP, Sondek J, Collard JG, Der CJ.** 2002. Tiam1 mediates Ras activation of Rac by a PI(3)K-independent mechanism. Nat Cell Biol **4:**621-625.

94. **Kelley GG, Reks SE, Ondrako JM, Smrcka AV.** 2001. Phospholipase C(epsilon): a novel Ras effector. EMBO J **20:**743-754.

95. **Malliri A, van der Kammen RA, Clark K, van der Valk M, Michiels F, Collard JG.** 2002. Mice deficient in the Rac activator Tiam1 are resistant to Ras-induced skin tumours. Nature **417:**867-871.

96. **Bai Y, Edamatsu H, Maeda S, Saito H, Suzuki N, Satoh T, Kataoka T.** 2004. Crucial role of phospholipase Cepsilon in chemical carcinogen-induced skin tumor development. Cancer Res **64:**8808-8810.

97. **Khokhlatchev A, Rabizadeh S, Xavier R, Nedwidek M, Chen T, Zhang XF, Seed B, Avruch J.** 2002. Identification of a novel Ras-regulated proapoptotic pathway. Curr Biol **12:**253-265.

98. **Richter AM, Pfeifer GP, Dammann RH.** 2009. The RASSF proteins in cancer; from epigenetic silencing to functional characterization. Biochim Biophys Acta **1796:**114-128.

99. **Vavvas D, Li X, Avruch J, Zhang XF.** 1998. Identification of Nore1 as a potential Ras effector. J Biol Chem **273:**5439-5442.

100. **Vos MD, Ellis CA, Bell A, Birrer MJ, Clark GJ.** 2000. Ras uses the novel tumor suppressor RASSF1 as an effector to mediate apoptosis. J Biol Chem **275:**35669-35672.

101. **Vos MD, Ellis CA, Elam C, Ulku AS, Taylor BJ, Clark GJ.** 2003. RASSF2 is a novel K-Ras-specific effector and potential tumor suppressor. J Biol Chem **278:**28045-28051.

102. **Eckfeld K, Hesson L, Vos MD, Bieche I, Latif F, Clark GJ.** 2004. RASSF4/AD037 is a potential ras effector/tumor suppressor of the RASSF family. Cancer Res **64:**8688-8693.

103. **Allen NP, Donninger H, Vos MD, Eckfeld K, Hesson L, Gordon L, Birrer MJ, Latif F, Clark GJ.** 2007. RASSF6 is a novel member of the RASSF family of tumor suppressors. Oncogene **26:**6203-6211.

104. **Leicht DT, Balan V, Kaplun A, Singh-Gupta V, Kaplun L, Dobson M, Tzivion G.** 2007. Raf kinases: function, regulation and role in human cancer. Biochim Biophys Acta **1773:**1196-1212.

105. **Shaul YD, Seger R.** 2007. The MEK/ERK cascade: from signaling specificity to diverse functions. Biochim Biophys Acta **1773:**1213-1226.

106. **Desideri E, Cavallo AL, Baccarini M.** 2015. Alike but Different: RAF Paralogs and Their Signaling Outputs. Cell **161:**967-970.

107. **Baljuls A, Mueller T, Drexler HC, Hekman M, Rapp UR.** 2007. Unique N-region determines low basal activity and limited inducibility of A-RAF kinase: the role of N-region in the evolutionary divergence of RAF kinase function in vertebrates. J Biol Chem **282:**26575-26590.

108. **Galabova-Kovacs G, Kolbus A, Matzen D, Meissl K, Piazzolla D, Rubiolo C, Steinitz K, Baccarini M.** 2006. ERK and beyond: insights from B-Raf and Raf-1 conditional knockouts. Cell Cycle **5:**1514-1518.

109. **Wimmer R, Baccarini M.** 2010. Partner exchange: protein-protein interactions in the Raf pathway. Trends Biochem Sci **35:**660-668.

110. **Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, Mould C, Parker A, Stevens C, Watt S, Hooper S, Wilson R, Jayatilake H, Gusterson BA, Cooper C, Shipley J, Hargrave D, Pritchard-Jones K, Maitland N, Chenevix-Trench G, Riggins GJ, Bigner DD, Palmieri G, Cossu A, Flanagan A, Nicholson A, Ho JW, Leung SY, Yuen ST, Weber BL, Seigler HF, Darrow TL, Paterson H, Marais R, Marshall CJ, Wooster R, et al.** 2002. Mutations of the BRAF gene in human cancer. Nature **417:**949-954.

111. **Emuss V, Garnett M, Mason C, Marais R.** 2005. Mutations of C-RAF are rare in human cancer because C-RAF has a low basal kinase activity compared with B-RAF. Cancer Res **65:**9719-9726.

112. **Poulikakos PI, Zhang C, Bollag G, Shokat KM, Rosen N.** 2010. RAF inhibitors transactivate RAF dimers and ERK signalling in cells with wild-type BRAF. Nature **464:**427-430.

113. **Hu J, Yu H, Kornev AP, Zhao J, Filbert EL, Taylor SS, Shaw AS.** 2011. Mutation that blocks ATP binding creates a pseudokinase stabilizing the scaffolding function of kinase suppressor of Ras, CRAF and BRAF. Proc Natl Acad Sci U S A **108:**6067-6072.

114. **Garnett MJ, Rana S, Paterson H, Barford D, Marais R.** 2005. Wild-type and mutant B-RAF activate C-RAF through distinct mechanisms involving heterodimerization. Mol Cell **20:**963-969.

115. **Wan PT, Garnett MJ, Roe SM, Lee S, Niculescu-Duvaz D, Good VM, Jones CM, Marshall CJ, Springer CJ, Barford D, Marais R, Cancer Genome P.** 2004. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. Cell **116:**855-867.

116. **Hu J, Stites EC, Yu H, Germino EA, Meharena HS, Stork PJ, Kornev AP, Taylor SS, Shaw AS.** 2013. Allosteric activation of functionally asymmetric RAF kinase dimers. Cell **154:**1036-1046.

117. **Alessi DR, Saito Y, Campbell DG, Cohen P, Sithanandam G, Rapp U, Ashworth A, Marshall CJ, Cowley S.** 1994. Identification of the sites in MAP kinase kinase-1 phosphorylated by p74raf-1. EMBO J **13:**1610-1619.

118. **Mansour SJ, Matten WT, Hermann AS, Candia JM, Rong S, Fukasawa K, Vande Woude GF, Ahn NG.** 1994. Transformation of mammalian cells by constitutively active MAP kinase kinase. Science **265:**966-970.

119. **Kolch W.** 2000. Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. Biochem J **351 Pt 2:**289-305.

120. **Belanger LF, Roy S, Tremblay M, Brott B, Steff AM, Mourad W, Hugo P, Erikson R, Charron J.** 2003. Mek2 is dispensable for mouse growth and development. Mol Cell Biol **23:**4778-4787.

121. **Catalanotti F, Reyes G, Jesenberger V, Galabova-Kovacs G, de Matos Simoes R, Carugo O, Baccarini M.** 2009. A Mek1-Mek2 heterodimer determines the strength and duration of the Erk signal. Nat Struct Mol Biol **16:**294-303.

122. **Giroux S, Tremblay M, Bernard D, Cardin-Girard JF, Aubry S, Larouche L, Rousseau S, Huot J, Landry J, Jeannotte L, Charron J.** 1999. Embryonic death of Mek1-deficient mice reveals a role for this kinase in angiogenesis in the labyrinthine region of the placenta. Curr Biol **9:**369-372.

123. **Brunet A, Pages G, Pouyssegur J.** 1994. Growth factor-stimulated MAP kinase induces rapid retrophosphorylation and inhibition of MAP kinase kinase (MEK1). FEBS Lett **346:**299-303.

124. **Xu B, Wilsbacher JL, Collisson T, Cobb MH.** 1999. The N-terminal ERK-binding site of MEK1 is required for efficient feedback phosphorylation by ERK2 in vitro and ERK activation in vivo. J Biol Chem **274:**34029-34035.

125. **Seger R, Ahn NG, Posada J, Munar ES, Jensen AM, Cooper JA, Cobb MH, Krebs EG.** 1992. Purification and characterization of mitogen-activated protein kinase activator(s) from epidermal growth factor-stimulated A431 cells. J Biol Chem **267:**14373-14381.

126. **Ferrell JE, Jr., Bhatt RR.** 1997. Mechanistic studies of the dual phosphorylation of mitogen-activated protein kinase. J Biol Chem **272:**19008-19016.

127. **Davis RJ.** 1993. The mitogen-activated protein kinase signal transduction pathway. J Biol Chem **268:**14553-14556.

128. **Roskoski R, Jr.** 2012. ERK1/2 MAP kinases: structure, function, and regulation. Pharmacol Res **66:**105-143.

129. **Lloyd AC.** 2006. Distinct functions for ERKs? J Biol **5:**13.

130. **Nekrasova T, Shive C, Gao Y, Kawamura K, Guardia R, Landreth G, Forsthuber TG.** 2005. ERK1-deficient mice show normal T cell effector function and are highly susceptible to experimental autoimmune encephalomyelitis. J Immunol **175:**2374-2380.

131. **Pages G, Guerin S, Grall D, Bonino F, Smith A, Anjuere F, Auberger P, Pouyssegur J.** 1999. Defective thymocyte maturation in p44 MAP kinase (Erk 1) knockout mice. Science **286:**1374-1377.

132. **Yao Y, Li W, Wu J, Germann UA, Su MS, Kuida K, Boucher DM.** 2003. Extracellular signal-regulated kinase 2 is necessary for mesoderm differentiation. Proc Natl Acad Sci U S A **100:**12759-12764.

133. **Birtwistle MR, Hatakeyama M, Yumoto N, Ogunnaike BA, Hoek JB, Kholodenko BN.** 2007. Ligand-dependent responses of the ErbB signaling network: experimental and modeling analyses. Mol Syst Biol **3:**144.

134. **Borisov N, Aksamitiene E, Kiyatkin A, Legewie S, Berkhout J, Maiwald T, Kaimachnikov NP, Timmer J, Hoek JB, Kholodenko BN.** 2009. Systems-level interactions between insulin-EGF networks amplify mitogenic signaling. Mol Syst Biol **5:**256.

135. **Albeck JG, Mills GB, Brugge JS.** 2013. Frequency-modulated pulses of ERK activity transmit quantitative proliferation signals. Mol Cell **49:**249-261.

136. **Morrison DK, Davis RJ.** 2003. Regulation of MAP kinase signaling modules by scaffold proteins in mammals. Annu Rev Cell Dev Biol **19:**91-118.

137. **Omerovic J, Prior IA.** 2009. Compartmentalized signalling: Ras proteins and signalling nanoclusters. FEBS J **276:**1817-1825.

138. **Morrison DK.** 2001. KSR: a MAPK scaffold of the Ras pathway? J Cell Sci **114:**1609-1612.

139. **Brown MD, Sacks DB.** 2009. Protein scaffolds in MAP kinase signalling. Cell Signal **21:**462-469.

140. **Roy M, Li Z, Sacks DB.** 2004. IQGAP1 binds ERK2 and modulates its activity. J Biol Chem **279:**17329-17337.

141. **Roy M, Li Z, Sacks DB.** 2005. IQGAP1 is a scaffold for mitogen-activated protein kinase signaling. Mol Cell Biol **25:**7940-7952.

142. **Ren JG, Li Z, Sacks DB.** 2007. IQGAP1 modulates activation of B-Raf. Proc Natl Acad Sci U S A **104:**10465-10469.

143. **Bourguignon LY, Gilad E, Rothman K, Peyrollier K.** 2005. Hyaluronan-CD44 interaction with IQGAP1 promotes Cdc42 and ERK signaling, leading to actin binding, Elk-1/estrogen receptor transcriptional activation, and ovarian cancer progression. J Biol Chem **280:**11961-11972.

144. **Dhanasekaran DN, Kashef K, Lee CM, Xu H, Reddy EP.** 2007. Scaffold proteins of MAP-kinase modules. Oncogene **26:**3185-3202.

145. **Kolch W.** 2005. Coordinating ERK/MAPK signalling through scaffolds and inhibitors. Nat Rev Mol Cell Biol **6:**827-837.

146. **Teis D, Wunderlich W, Huber LA.** 2002. Localization of the MP1-MAPK scaffold complex to endosomes is mediated by p14 and required for signal transduction. Dev Cell **3:**803-814.

147. **Wunderlich W, Fialka I, Teis D, Alpi A, Pfeifer A, Parton RG, Lottspeich F, Huber LA.** 2001. A novel 14-kilodalton protein interacts with the mitogen-activated protein kinase scaffold mp1 on a late endosomal/lysosomal compartment. J Cell Biol **152:**765-776.

148. **Orton RJ, Sturm OE, Vyshemirsky V, Calder M, Gilbert DR, Kolch W.** 2005. Computational modelling of the receptor-tyrosine-kinase-activated MAPK pathway. Biochem J **392:**249-261.

149. **Schaeffer HJ, Catling AD, Eblen ST, Collier LS, Krauss A, Weber MJ.** 1998. MP1: a MEK binding partner that enhances enzymatic activation of the MAP kinase cascade. Science **281:**1668-1671.

150. **Yin G, Haendeler J, Yan C, Berk BC.** 2004. GIT1 functions as a scaffold for MEK1-extracellular signal-regulated kinase 1 and 2 activation by angiotensin II and epidermal growth factor. Mol Cell Biol **24:**875-885.

151. **Torii S, Kusakabe M, Yamamoto T, Maekawa M, Nishida E.** 2004. Sef is a spatial regulator for Ras/MAP kinase signaling. Dev Cell **7:**33-44.

152. **Therrien M, Chang HC, Solomon NM, Karim FD, Wassarman DA, Rubin GM.** 1995. KSR, a novel protein kinase required for RAS signal transduction. Cell **83:**879-888.

153. **Sundaram M, Han M.** 1995. The C. elegans ksr-1 gene encodes a novel Raf-related kinase involved in Ras-mediated signal transduction. Cell **83:**889-901.

154. **Kornfeld K, Hom DB, Horvitz HR.** 1995. The ksr-1 gene encodes a novel protein kinase involved in Ras-mediated signaling in C. elegans. Cell **83:**903-913.

155. **Therrien M, Michaud NR, Rubin GM, Morrison DK.** 1996. KSR modulates signal propagation within the MAPK cascade. Genes Dev **10:**2684-2695.

156. **Michaud NR, Therrien M, Cacace A, Edsall LC, Spiegel S, Rubin GM, Morrison DK.** 1997. KSR stimulates Raf-1 activity in a kinase-independent manner. Proc Natl Acad Sci U S A **94:**12792-12796.

157. **Denouel-Galy A, Douville EM, Warne PH, Papin C, Laugier D, Calothy G, Downward J, Eychene A.** 1998. Murine Ksr interacts with MEK and inhibits Ras-induced transformation. Curr Biol **8:**46-55.

158. **Yu W, Fantl WJ, Harrowe G, Williams LT.** 1998. Regulation of the MAP kinase pathway by mammalian Ksr through direct interaction with MEK and ERK. Curr Biol **8:**56-64.

159. **Joneson T, Fulton JA, Volle DJ, Chaika OV, Bar-Sagi D, Lewis RE.** 1998. Kinase suppressor of Ras inhibits the activation of extracellular ligand-regulated (ERK) mitogen-activated protein (MAP) kinase by growth factors, activated Ras, and Ras effectors. J Biol Chem **273:**7743-7748.

160. **Hanks SK, Hunter T.** 1995. Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. FASEB J **9:**576-596.

161. **Muller J, Cacace AM, Lyons WE, McGill CB, Morrison DK.** 2000. Identification of B-KSR1, a novel brain-specific isoform of KSR1 that functions in neuronal signaling. Mol Cell Biol **20:**5529-5539.

162. **Stewart S, Sundaram M, Zhang Y, Lee J, Han M, Guan KL.** 1999. Kinase suppressor of Ras forms a multiprotein signaling complex and modulates MEK localization. Mol Cell Biol **19:**5523-5534.

163. **Cacace AM, Michaud NR, Therrien M, Mathes K, Copeland T, Rubin GM, Morrison DK.** 1999. Identification of constitutive and ras-inducible phosphorylation sites of KSR: implications for 14-3-3 binding, mitogen-activated protein kinase binding, and KSR overexpression. Mol Cell Biol **19:**229-240.

164. **Jacobs D, Glossip D, Xing H, Muslin AJ, Kornfeld K.** 1999. Multiple docking sites on substrate proteins form a modular system that mediates recognition by ERK MAP kinase. Genes Dev **13:**163-175.

165. **Xing H, Kornfeld K, Muslin AJ.** 1997. The protein kinase KSR interacts with 14-3-3 protein and Raf. Curr Biol **7:**294-300.

166. **Kortum RL, Lewis RE.** 2004. The molecular scaffold KSR1 regulates the proliferative and oncogenic potential of cells. Mol Cell Biol **24:**4407-4416.

167. **Jones SM, Kazlauskas A.** 2001. Growth-factor-dependent mitogenesis requires two distinct phases of signalling. Nat Cell Biol **3:**165-172.

168. **Marshall CJ.** 1995. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell **80:**179-185.

169. **Murphy LO, Blenis J.** 2006. MAPK signal specificity: the right place at the right time. Trends Biochem Sci **31:**268-275.

170. **Murphy LO, Smith S, Chen RH, Fingar DC, Blenis J.** 2002. Molecular interpretation of ERK signal duration by immediate early gene products. Nat Cell Biol **4:**556-564.

171. **Kortum RL, Fernandez MR, Costanzo-Garvey DL, Johnson HJ, Fisher KW, Volle DJ, Lewis RE.** 2014. Caveolin-1 is required for kinase suppressor of Ras 1 (KSR1)-mediated extracellular signal-regulated kinase 1/2 activation, H-RasV12-induced senescence, and transformation. Mol Cell Biol **34:**3461-3472.

172. **Kortum RL, Johnson HJ, Costanzo DL, Volle DJ, Razidlo GL, Fusello AM, Shaw AS, Lewis RE.** 2006. The molecular scaffold kinase suppressor of Ras 1 is a modifier of RasV12-induced and replicative senescence. Mol Cell Biol **26:**2202-2214.

173. **Volle DJ, Fulton JA, Chaika OV, McDermott K, Huang H, Steinke LA, Lewis RE.** 1999. Phosphorylation of the kinase suppressor of ras by associated kinases. Biochemistry **38:**5130-5137.

174. **Brennan JA, Volle DJ, Chaika OV, Lewis RE.** 2002. Phosphorylation regulates the nucleocytoplasmic distribution of kinase suppressor of Ras. J Biol Chem **277:**5369-5377.

175. **Klutho PJ, Costanzo-Garvey DL, Lewis RE.** 2011. Regulation of glucose homeostasis by KSR1 and MARK2. PLoS One **6:**e29304.

176. **Muller J, Ory S, Copeland T, Piwnica-Worms H, Morrison DK.** 2001. C-TAK1 regulates Ras signaling by phosphorylating the MAPK scaffold, KSR1. Mol Cell **8:**983-993.

177. **Hartsough MT, Morrison DK, Salerno M, Palmieri D, Ouatas T, Mair M, Patrick J, Steeg PS.** 2002. Nm23-H1 metastasis suppressor phosphorylation of kinase suppressor of Ras via a histidine protein kinase pathway. J Biol Chem **277:**32389-32399.

178. **Ory S, Zhou M, Conrads TP, Veenstra TD, Morrison DK.** 2003. Protein phosphatase 2A positively regulates Ras signaling by dephosphorylating KSR1 and Raf-1 on critical 14-3-3 binding sites. Curr Biol **13:**1356-1364.

179. **Razidlo GL, Kortum RL, Haferbier JL, Lewis RE.** 2004. Phosphorylation regulates KSR1 stability, ERK activation, and cell proliferation. J Biol Chem **279:**47808-47814.

180. **McKay MM, Ritt DA, Morrison DK.** 2009. Signaling dynamics of the KSR1 scaffold complex. Proc Natl Acad Sci U S A **106:**11022-11027.

181. **McKay MM, Ritt DA, Morrison DK.** 2011. RAF inhibitor-induced KSR1/B-RAF binding and its effects on ERK cascade signaling. Curr Biol **21:**563-568.

182. **Chen C, Lewis RE, White MA.** 2008. IMP modulates KSR1-dependent multivalent complex formation to specify ERK1/2 pathway activation and response thresholds. J Biol Chem **283:**12789-12796.

183. **Matheny SA, Chen C, Kortum RL, Razidlo GL, Lewis RE, White MA.** 2004. Ras regulates assembly of mitogenic signalling complexes through the effector protein IMP. Nature **427:**256-260.

184. **Nguyen A, Burack WR, Stock JL, Kortum R, Chaika OV, Afkarian M, Muller WJ, Murphy KM, Morrison DK, Lewis RE, McNeish J, Shaw AS.** 2002. Kinase suppressor of Ras (KSR) is a scaffold which facilitates mitogen-activated protein kinase activation in vivo. Mol Cell Biol **22:**3035-3045.

185. **Lozano J, Xing R, Cai Z, Jensen HL, Trempus C, Mark W, Cannon R, Kolesnick R.** 2003. Deficiency of kinase suppressor of Ras1 prevents oncogenic ras signaling in mice. Cancer Res **63:**4232-4238.

186. **Kortum RL, Costanzo DL, Haferbier J, Schreiner SJ, Razidlo GL, Wu MH, Volle DJ, Mori T, Sakaue H, Chaika NV, Chaika OV, Lewis RE.** 2005. The molecular scaffold kinase suppressor of Ras 1 (KSR1) regulates adipogenesis. Mol Cell Biol **25:**7592-7604.

187. **Fisher KW, Das B, Kortum RL, Chaika OV, Lewis RE.** 2011. Kinase suppressor of ras 1 (KSR1) regulates PGC1alpha and estrogen-related receptor alpha to promote oncogenic Ras-dependent anchorage-independent growth. Mol Cell Biol **31:**2453-2461.

188. **Fisher KW, Das B, Kim HS, Clymer BK, Gehring D, Smith DR, Costanzo-Garvey DL, Fernandez MR, Brattain MG, Kelly DL, MacMillan J, White MA, Lewis RE.** 2015. AMPK Promotes Aberrant PGC1beta Expression To Support Human Colon Tumor Cell Survival. Mol Cell Biol **35:**3866-3879.

189. **Roig AI, Eskiocak U, Hight SK, Kim SB, Delgado O, Souza RF, Spechler SJ, Wright WE, Shay JW.** 2010. Immortalized epithelial cells derived from human colon biopsies express stem cell markers and differentiate in vitro. Gastroenterology **138:**1012-1021 e1011-1015.

190. **Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, Juan T, Sikorski R, Suggs S, Radinsky R, Patterson SD, Chang DD.** 2008. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. J Clin Oncol **26:**1626-1634.

191. **Lievre A, Bachet JB, Boige V, Cayre A, Le Corre D, Buc E, Ychou M, Bouche O, Landi B, Louvet C, Andre T, Bibeau F, Diebold MD, Rougier P, Ducreux M, Tomasic G, Emile JF, Penault-Llorca F, Laurent-Puig P.** 2008. KRAS mutations as an independent prognostic factor in patients with advanced colorectal cancer treated with cetuximab. J Clin Oncol **26:**374-379.

192. **Raponi M, Winkler H, Dracopoli NC.** 2008. KRAS mutations predict response to EGFR inhibitors. Curr Opin Pharmacol **8:**413-418.

193. **Eberhard DA, Johnson BE, Amler LC, Goddard AD, Heldens SL, Herbst RS, Ince WL, Janne PA, Januario T, Johnson DH, Klein P, Miller VA, Ostland MA, Ramies DA, Sebisanovic D, Stinson JA, Zhang YR, Seshagiri S, Hillan KJ.** 2005. Mutations in the epidermal growth factor receptor and in KRAS are

predictive and prognostic indicators in patients with non-small-cell lung cancer treated with chemotherapy alone and in combination with erlotinib. J Clin Oncol **23:**5900-5909.

194. **Saif MW, Oettle H, Vervenne WL, Thomas JP, Spitzer G, Visseren-Grul C, Enas N, Richards DA.** 2009. Randomized double-blind phase II trial comparing gemcitabine plus LY293111 versus gemcitabine plus placebo in advanced adenocarcinoma of the pancreas. Cancer J **15:**339-343.

195. **Deschoolmeester V, Boeckx C, Baay M, Weyler J, Wuyts W, Van Marck E, Peeters M, Lardon F, Vermorken JB.** 2010. KRAS mutation detection and prognostic potential in sporadic colorectal cancer using high-resolution melting analysis. Br J Cancer **103:**1627-1636.

196. **Diaz LA, Jr., Williams RT, Wu J, Kinde I, Hecht JR, Berlin J, Allen B, Bozic I, Reiter JG, Nowak MA, Kinzler KW, Oliner KS, Vogelstein B.** 2012. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. Nature **486:**537-540.

197. **Kohl NE, Omer CA, Conner MW, Anthony NJ, Davide JP, deSolms SJ, Giuliani EA, Gomez RP, Graham SL, Hamilton K, et al.** 1995. Inhibition of farnesyltransferase induces regression of mammary and salivary carcinomas in ras transgenic mice. Nat Med **1:**792-797.

198. **Rowell CA, Kowalczyk JJ, Lewis MD, Garcia AM.** 1997. Direct demonstration of geranylgeranylation and farnesylation of Ki-Ras in vivo. J Biol Chem **272:**14093-14097.

199. **Whyte DB, Kirschmeier P, Hockenberry TN, Nunez-Oliva I, James L, Catino JJ, Bishop WR, Pai JK.** 1997. K- and N-Ras are geranylgeranylated in cells treated with farnesyl protein transferase inhibitors. J Biol Chem **272:**14459-14464.

200. **Hrycyna CA, Sapperstein SK, Clarke S, Michaelis S.** 1991. The Saccharomyces cerevisiae STE14 gene encodes a methyltransferase that mediates C-terminal methylation of a-factor and RAS proteins. EMBO J **10:**1699-1709.

201. **Kato K, Cox AD, Hisaka MM, Graham SM, Buss JE, Der CJ.** 1992. Isoprenoid addition to Ras protein is the critical modification for its membrane association and transforming activity. Proc Natl Acad Sci U S A **89:**6403-6407.

202. **Bergo MO, Ambroziak P, Gregory C, George A, Otto JC, Kim E, Nagase H, Casey PJ, Balmain A, Young SG.** 2002. Absence of the CAAX endoprotease Rce1: effects on cell growth and transformation. Mol Cell Biol **22:**171-181.

203. **Bergo MO, Gavino BJ, Hong C, Beigneux AP, McMahon M, Casey PJ, Young SG.** 2004. Inactivation of Icmt inhibits transformation by oncogenic K-Ras and B-Raf. J Clin Invest **113:**539-550.

204. **Winter-Vann AM, Kamen BA, Bergo MO, Young SG, Melnyk S, James SJ, Casey PJ.** 2003. Targeting Ras signaling through inhibition of carboxyl methylation: an unexpected property of methotrexate. Proc Natl Acad Sci U S A **100:**6529-6534.

205. **Winter-Vann AM, Baron RA, Wong W, dela Cruz J, York JD, Gooden DM, Bergo MO, Young SG, Toone EJ, Casey PJ.** 2005. A small-molecule inhibitor of isoprenylcysteine carboxyl methyltransferase with antitumor activity in cancer cells. Proc Natl Acad Sci U S A **102:**4336-4341.

206. **Duursma AM, Agami R.** 2003. Ras interference as cancer therapy. Semin Cancer Biol **13:**267-273.

207. **Wickstrom E.** 2001. Oligonucleotide treatment of ras-induced tumors in nude mice. Mol Biotechnol **18:**35-55.

208. **Morgan RK, Batra H, Gaerig VC, Hockings J, Brooks TA.** 2016. Identification and characterization of a new G-quadruplex forming region within the kRAS promoter as a transcriptional regulator. Biochim Biophys Acta **1859:**235-245.

209. **Puzanov I, Amaravadi RK, McArthur GA, Flaherty KT, Chapman PB, Sosman JA, Ribas A, Shackleton M, Hwu P, Chmielowski B, Nolop KB, Lin PS, Kim KB.** 2015. Long-term outcome in BRAF(V600E) melanoma patients treated with vemurafenib: Patterns of disease progression and clinical management of limited progression. Eur J Cancer **51:**1435-1443.

210. **Hatzivassiliou G, Haling JR, Chen H, Song K, Price S, Heald R, Hewitt JF, Zak M, Peck A, Orr C, Merchant M, Hoeflich KP, Chan J, Luoh SM, Anderson DJ, Ludlam MJ, Wiesmann C, Ultsch M, Friedman LS, Malek S, Belvin M.** 2013. Mechanism of MEK inhibition determines efficacy in mutant KRAS- versus BRAF-driven cancers. Nature **501:**232-236.

211. **Heidorn SJ, Milagre C, Whittaker S, Nourry A, Niculescu-Duvas I, Dhomen N, Hussain J, Reis-Filho JS, Springer CJ, Pritchard C, Marais R.** 2010.

Kinase-dead BRAF and oncogenic RAS cooperate to drive tumor progression through CRAF. Cell **140:**209-221.

212. **Poulikakos PI, Persaud Y, Janakiraman M, Kong X, Ng C, Moriceau G, Shi H, Atefi M, Titz B, Gabay MT, Salton M, Dahlman KB, Tadi M, Wargo JA, Flaherty KT, Kelley MC, Misteli T, Chapman PB, Sosman JA, Graeber TG, Ribas A, Lo RS, Rosen N, Solit DB.** 2011. RAF inhibitor resistance is mediated by dimerization of aberrantly spliced BRAF(V600E). Nature **480:**387-390.

213. **Arcila M, Lau C, Nafa K, Ladanyi M.** 2011. Detection of KRAS and BRAF mutations in colorectal carcinoma roles for high-sensitivity locked nucleic acid-PCR sequencing and broad-spectrum mass spectrometry genotyping. J Mol Diagn **13:**64-73.

214. **Tsai J, Lee JT, Wang W, Zhang J, Cho H, Mamo S, Bremer R, Gillette S, Kong J, Haass NK, Sproesser K, Li L, Smalley KS, Fong D, Zhu YL, Marimuthu A, Nguyen H, Lam B, Liu J, Cheung I, Rice J, Suzuki Y, Luu C, Settachatgul C, Shellooe R, Cantwell J, Kim SH, Schlessinger J, Zhang KY, West BL, Powell B, Habets G, Zhang C, Ibrahim PN, Hirth P, Artis DR, Herlyn M, Bollag G.** 2008. Discovery of a selective inhibitor of oncogenic B-Raf kinase with potent antimelanoma activity. Proc Natl Acad Sci U S A **105:**3041-3046.

215. **Corcoran RB, Dias-Santagata D, Bergethon K, Iafrate AJ, Settleman J, Engelman JA.** 2010. BRAF gene amplification can promote acquired resistance to MEK inhibitors in cancer cells harboring the BRAF V600E mutation. Sci Signal **3:**ra84.

216. **Dai B, Meng J, Peyton M, Girard L, Bornmann WG, Ji L, Minna JD, Fang B, Roth JA.** 2011. STAT3 mediates resistance to MEK inhibitor through microRNA miR-17. Cancer Res **71:**3658-3668.

217. **Wang H, Daouti S, Li WH, Wen Y, Rizzo C, Higgins B, Packman K, Rosen N, Boylan JF, Heimbrook D, Niu H.** 2011. Identification of the MEK1(F129L) activating mutation as a potential mechanism of acquired resistance to MEK inhibition in human cancers carrying the B-RafV600E mutation. Cancer Res **71:**5535-5545.

218. **Emery CM, Vijayendran KG, Zipser MC, Sawyer AM, Niu L, Kim JJ, Hatton C, Chopra R, Oberholzer PA, Karpova MB, MacConaill LE, Zhang J, Gray NS, Sellers WR, Dummer R, Garraway LA.** 2009. MEK1 mutations confer

resistance to MEK and B-RAF inhibition. Proc Natl Acad Sci U S A **106:**20411-20416.

219. **Dougherty MK, Muller J, Ritt DA, Zhou M, Zhou XZ, Copeland TD, Conrads TP, Veenstra TD, Lu KP, Morrison DK.** 2005. Regulation of Raf-1 by direct feedback phosphorylation. Mol Cell **17:**215-224.

220. **Klinger B, Sieber A, Fritsche-Guenther R, Witzel F, Berry L, Schumacher D, Yan Y, Durek P, Merchant M, Schafer R, Sers C, Bluthgen N.** 2013. Network quantification of EGFR signaling unveils potential for targeted combination therapy. Mol Syst Biol **9:**673.

221. **Hatzivassiliou G, Liu B, O'Brien C, Spoerke JM, Hoeflich KP, Haverty PM, Soriano R, Forrest WF, Heldens S, Chen H, Toy K, Ha C, Zhou W, Song K, Friedman LS, Amler LC, Hampton GM, Moffat J, Belvin M, Lackner MR.** 2012. ERK inhibition overcomes acquired resistance to MEK inhibitors. Mol Cancer Ther **11:**1143-1154.

222. **Aronov AM, Baker C, Bemis GW, Cao J, Chen G, Ford PJ, Germann UA, Green J, Hale MR, Jacobs M, Janetka JW, Maltais F, Martinez-Botella G, Namchuk MN, Straub J, Tang Q, Xie X.** 2007. Flipped out: structure-guided design of selective pyrazolylpyrrole ERK inhibitors. J Med Chem **50:**1280-1287.

223. **Aronov AM, Tang Q, Martinez-Botella G, Bemis GW, Cao J, Chen G, Ewing NP, Ford PJ, Germann UA, Green J, Hale MR, Jacobs M, Janetka JW, Maltais F, Markland W, Namchuk MN, Nanthakumar S, Poondru S, Straub J, ter Haar E, Xie X.** 2009. Structure-guided design of potent and selective pyrimidylpyrrole inhibitors of extracellular signal-regulated kinase (ERK) using conformational control. J Med Chem **52:**6362-6368.

224. **Morris EJ, Jha S, Restaino CR, Dayananth P, Zhu H, Cooper A, Carr D, Deng Y, Jin W, Black S, Long B, Liu J, Dinunzio E, Windsor W, Zhang R, Zhao S, Angagaw MH, Pinheiro EM, Desai J, Xiao L, Shipps G, Hruza A, Wang J, Kelly J, Paliwal S, Gao X, Babu BS, Zhu L, Daublain P, Zhang L, Lutterbach BA, Pelletier MR, Philippar U, Siliphaivanh P, Witter D, Kirschmeier P, Bishop WR, Hicklin D, Gilliland DG, Jayaraman L, Zawel L, Fawell S, Samatar AA.** 2013. Discovery of a novel ERK inhibitor with activity in models of acquired resistance to BRAF and MEK inhibitors. Cancer Discov **3:**742-750.

225. **Ohori M, Kinoshita T, Okubo M, Sato K, Yamazaki A, Arakawa H, Nishimura S, Inamura N, Nakajima H, Neya M, Miyake H, Fujii T.** 2005. Identification of a selective ERK inhibitor and structural determination of the inhibitor-ERK2 complex. Biochem Biophys Res Commun **336:**357-363.

226. **Chaikuad A, Tacconi EM, Zimmer J, Liang Y, Gray NS, Tarsounas M, Knapp S.** 2014. A unique inhibitor binding site in ERK1/2 is associated with slow binding kinetics. Nat Chem Biol **10:**853-860.

227. **Malumbres M, Barbacid M.** 2003. RAS oncogenes: the first 30 years. Nat Rev Cancer **3:**459-465.

228. **Potts MB, Kim HS, Fisher KW, Hu Y, Carrasco YP, Bulut GB, Ou YH, Herrera-Herrera ML, Cubillos F, Mendiratta S, Xiao G, Hofree M, Ideker T, Xie Y, Huang LJ, Lewis RE, MacMillan JB, White MA.** 2013. Using functional signature ontology (FUSION) to identify mechanisms of action for natural products. Sci Signal **6:**ra90.

229. **Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J.** 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol **8:**R19.

230. **Stoneley M, Paulin FE, Le Quesne JP, Chappell SA, Willis AE.** 1998. C-Myc 5' untranslated region contains an internal ribosome entry segment. Oncogene **16:**423-428.

231. **Stoneley M, Subkhankulova T, Le Quesne JP, Coldwell MJ, Jopling CL, Belsham GJ, Willis AE.** 2000. Analysis of the c-myc IRES; a potential role for cell-type specific trans-acting factors and the nuclear compartment. Nucleic Acids Res **28:**687-694.

232. **American Cancer Society.** 2015. Global Cancer Facts & Figures 3rd Edition. American Cancer Society, Atlanta, GA.

233. **Taylor DP, Burt RW, Williams MS, Haug PJ, Cannon-Albright LA.** 2010. Population-based family history-specific risks for colorectal cancer: a constellation approach. Gastroenterology **138:**877-885.

234. **Kerber RA, Neklason DW, Samowitz WS, Burt RW.** 2005. Frequency of familial colon cancer and hereditary nonpolyposis colorectal cancer (Lynch syndrome) in a large population database. Fam Cancer **4:**239-244.

235. **Prenen H, Vecchione L, Van Cutsem E.** 2013. Role of targeted agents in metastatic colorectal cancer. Target Oncol **8:**83-96.

236. **Fearon ER.** 2011. Molecular genetics of colorectal cancer. Annu Rev Pathol **6:**479-507.

237. **Anonymous.** 2012. Comprehensive molecular characterization of human colon and rectal cancer. Nature **487:**330-337.

238. **Adjei AA.** 2001. Blocking oncogenic Ras signaling for cancer therapy. J Natl Cancer Inst **93:**1062-1074.

239. **Ahnen DJ, Feigl P, Quan G, Fenoglio-Preiser C, Lovato LC, Bunn PA, Jr., Stemmerman G, Wells JD, Macdonald JS, Meyskens FL, Jr.** 1998. Ki-ras mutation and p53 overexpression predict the clinical behavior of colorectal cancer: a Southwest Oncology Group study. Cancer Res **58:**1149-1158.

240. **Samowitz WS, Curtin K, Schaffer D, Robertson M, Leppert M, Slattery ML.** 2000. Relationship of Ki-ras mutations in colon cancers to tumor location, stage, and survival: a population-based study. Cancer Epidemiol Biomarkers Prev **9:**1193-1197.

241. **Lievre A, Bachet JB, Le Corre D, Boige V, Landi B, Emile JF, Cote JF, Tomasic G, Penna C, Ducreux M, Rougier P, Penault-Llorca F, Laurent-Puig P.** 2006. KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. Cancer Res **66:**3992-3995.

242. **Lito P, Rosen N, Solit DB.** 2013. Tumor adaptation and resistance to RAF inhibitors. Nat Med **19:**1401-1409.

243. **Downward J.** 2003. Targeting RAS signalling pathways in cancer therapy. Nat Rev Cancer **3:**11-22.

244. **Roy F, Therrien M.** 2002. MAP kinase module: the Ksr connection. Curr Biol **12:**R325-327.

245. **Kortum RL, Lewis RE.** 2004. The Molecular Scaffold KSR1 Regulates the Proliferative and Oncogenic Potential of Cells. Molecular and Cellular Biology **24:**4407-4416.

246. **Nguyen A, Burack WR, Stock JL, Kortum R, Chaika OV, Afkarian M, Muller WJ, Murphy KM, Morrison DK, Lewis RE, McNeish J, Shaw AS.** 2002. Kinase Suppressor of Ras (KSR) Is a Scaffold Which Facilitates Mitogen-Activated Protein Kinase Activation In Vivo. Molecular and Cellular Biology **22:**3035-3045.

247. **Pasquale EB.** 2005. Eph receptor signalling casts a wide net on cell behaviour. Nat Rev Mol Cell Biol **6:**462-475.

248. **Pasquale EB.** 2008. Eph-ephrin bidirectional signaling in physiology and disease. Cell **133:**38-52.

249. **Batlle E, Wilkinson DG.** 2012. Molecular mechanisms of cell segregation and boundary formation in development and tumorigenesis. Cold Spring Harb Perspect Biol **4:**a008227.

250. **Barquilla A, Pasquale EB.** 2015. Eph receptors and ephrins: therapeutic opportunities. Annu Rev Pharmacol Toxicol **55:**465-487.

251. **Boyd AW, Bartlett PF, Lackmann M.** 2014. Therapeutic targeting of EPH receptors and their ligands. Nat Rev Drug Discov **13:**39-62.

252. **Pasquale EB.** 2010. Eph receptors and ephrins in cancer: bidirectional signalling and beyond. Nat Rev Cancer **10:**165-180.

253. **Jorgensen C, Sherman A, Chen GI, Pasculescu A, Poliakov A, Hsiung M, Larsen B, Wilkinson DG, Linding R, Pawson T.** 2009. Cell-specific information processing in segregating populations of Eph receptor ephrin-expressing cells. Science **326:**1502-1509.

254. **Kuijper S, Turner CJ, Adams RH.** 2007. Regulation of angiogenesis by Eph-ephrin interactions. Trends Cardiovasc Med **17:**145-151.

255. **Heroult M, Schaffner F, Augustin HG.** 2006. Eph receptor and ephrin ligand-mediated interactions during angiogenesis and tumor progression. Exp Cell Res **312:**642-650.

256. **Adams RH, Wilkinson GA, Weiss C, Diella F, Gale NW, Deutsch U, Risau W, Klein R.** 1999. Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. Genes Dev **13:**295-306.

257. **Gerety SS, Anderson DJ.** 2002. Cardiovascular ephrinB2 function is essential for embryonic angiogenesis. Development **129:**1397-1410.

258. **Gerety SS, Wang HU, Chen ZF, Anderson DJ.** 1999. Symmetrical mutant phenotypes of the receptor EphB4 and its specific transmembrane ligand ephrin-B2 in cardiovascular development. Mol Cell **4:**403-414.

259. **van de Wetering M, Sancho E, Verweij C, de Lau W, Oving I, Hurlstone A, van der Horn K, Batlle E, Coudreuse D, Haramis AP, Tjon-Pon-Fong M, Moerer P, van den Born M, Soete G, Pals S, Eilers M, Medema R, Clevers H.** 2002. The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell **111:**241-250.

260. **Chang CY, Kazmin D, Jasper JS, Kunder R, Zuercher WJ, McDonnell DP.** 2011. The metabolic regulator ERRalpha, a downstream target of HER2/IGF-1R, as a therapeutic target in breast cancer. Cancer Cell **20:**500-510.

261. **Zhang H, Gao P, Fukuda R, Kumar G, Krishnamachary B, Zeller KI, Dang CV, Semenza GL.** 2007. HIF-1 inhibits mitochondrial biogenesis and cellular respiration in VHL-deficient renal cell carcinoma by repression of C-MYC activity. Cancer Cell **11:**407-420.

262. **Dang CV.** 2012. MYC on the path to cancer. Cell **149:**22-35.

263. **Levens D.** 2010. You Don't Muck with MYC. Genes Cancer **1:**547-554.

264. **Gurel B, Iwata T, Koh CM, Jenkins RB, Lan F, Van Dang C, Hicks JL, Morgan J, Cornish TC, Sutcliffe S, Isaacs WB, Luo J, De Marzo AM.** 2008. Nuclear MYC protein overexpression is an early alteration in human prostate carcinogenesis. Mod Pathol **21:**1156-1167.

265. **Palaskas N, Larson SM, Schultz N, Komisopoulou E, Wong J, Rohle D, Campos C, Yannuzzi N, Osborne JR, Linkov I, Kastenhuber ER, Taschereau R, Plaisier SB, Tran C, Heguy A, Wu H, Sander C, Phelps ME, Brennan C, Port E, Huse JT, Graeber TG, Mellinghoff IK.** 2011. 18F-fluorodeoxy-glucose positron emission tomography marks MYC-overexpressing human basal-like breast cancers. Cancer Res **71:**5164-5174.

266. **Xie C, Pan Y, Hao F, Gao Y, Liu Z, Zhang X, Xie L, Jiang G, Li Q, Wang E.** 2014. C-Myc participates in beta-catenin-mediated drug resistance in A549/DDP lung adenocarcinoma cells. APMIS **122:**1251-1258.

267. **Ilic N, Utermark T, Widlund HR, Roberts TM.** 2011. PI3K-targeted therapy can be evaded by gene amplification along the MYC-eukaryotic translation initiation factor 4E (eIF4E) axis. Proc Natl Acad Sci U S A **108:**E699-708.

268. **Gogolin S, Dreidax D, Becker G, Ehemann V, Schwab M, Westermann F.** 2010. MYCN/MYC-mediated drug resistance mechanisms in neuroblastoma. Int J Clin Pharmacol Ther **48:**489-491.

269. **Kumar SR, Masood R, Spannuth WA, Singh J, Scehnet J, Kleiber G, Jennings N, Deavers M, Krasnoperov V, Dubeau L, Weaver FA, Sood AK, Gill PS.** 2007. The receptor tyrosine kinase EphB4 is overexpressed in ovarian cancer, provides survival signals and predicts poor outcome. Br J Cancer **96:**1083-1091.

270. **Wu Q, Suo Z, Risberg B, Karlsson MG, Villman K, Nesland JM.** 2004. Expression of Ephb2 and Ephb4 in breast carcinoma. Pathol Oncol Res **10:**26-33.

271. **Kumar SR, Scehnet JS, Ley EJ, Singh J, Krasnoperov V, Liu R, Manchanda PK, Ladner RD, Hawes D, Weaver FA, Beart RW, Singh G, Nguyen C, Kahn M, Gill PS.** 2009. Preferential induction of EphB4 over EphB2 and its implication in colorectal cancer progression. Cancer Res **69:**3736-3745.

272. **Meyer N, Penn LZ.** 2008. Reflecting on 25 years with MYC. Nat Rev Cancer **8:**976-990.

273. **He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW.** 1998. Identification of c-MYC as a target of the APC pathway. Science **281:**1509-1512.

274. **Beroukhim R, Mermel CH, Porter D, Wei G, Raychaudhuri S, Donovan J, Barretina J, Boehm JS, Dobson J, Urashima M, Mc Henry KT, Pinchback RM, Ligon AH, Cho YJ, Haery L, Greulich H, Reich M, Winckler W, Lawrence MS, Weir BA, Tanaka KE, Chiang DY, Bass AJ, Loo A, Hoffman C, Prensner J, Liefeld T, Gao Q, Yecies D, Signoretti S, Maher E, Kaye FJ, Sasaki H, Tepper JE, Fletcher JA, Tabernero J, Baselga J, Tsao MS, Demichelis F, Rubin MA, Janne PA, Daly MJ, Nucera C, Levine RL, Ebert BL, Gabriel S, Rustgi AK, Antonescu CR, Ladanyi M, Letai A, et al.** 2010. The landscape of somatic copy-number alteration across human cancers. Nature **463:**899-905.

275. **Shou Y, Martelli ML, Gabrea A, Qi Y, Brents LA, Roschke A, Dewald G, Kirsch IR, Bergsagel PL, Kuehl WM.** 2000. Diverse karyotypic abnormalities of the c-myc locus associated with c-myc dysregulation and tumor progression in multiple myeloma. Proc Natl Acad Sci U S A **97:**228-233.

276. **Sansom OJ, Meniel VS, Muncan V, Phesse TJ, Wilkins JA, Reed KR, Vass JK, Athineos D, Clevers H, Clarke AR.** 2007. Myc deletion rescues Apc deficiency in the small intestine. Nature **446:**676-679.

277. **Sears R, Nuckolls F, Haura E, Taya Y, Tamai K, Nevins JR.** 2000. Multiple Ras-dependent phosphorylation pathways regulate Myc protein stability. Genes Dev **14:**2501-2514.

278. **Sears R, Leone G, DeGregori J, Nevins JR.** 1999. Ras enhances Myc protein stability. Mol Cell **3:**169-179.

279. **Altman BJ, Hsieh AL, Sengupta A, Krishnanaiah SY, Stine ZE, Walton ZE, Gouw AM, Venkataraman A, Li B, Goraksha-Hicks P, Diskin SJ, Bellovin DI, Simon MC, Rathmell JC, Lazar MA, Maris JM, Felsher DW, Hogenesch JB, Weljie AM, Dang CV.** 2015. MYC Disrupts the Circadian Clock and Metabolism in Cancer Cells. Cell Metab **22:**1009-1019.

280. **Cancer Genome Atlas N.** 2012. Comprehensive molecular characterization of human colon and rectal cancer. Nature **487:**330-337.

281. **Vita M, Henriksson M.** 2006. The Myc oncoprotein as a therapeutic target for human cancer. Semin Cancer Biol **16:**318-330.

282. **Stegmaier K, Ross KN, Colavito SA, O'Malley S, Stockwell BR, Golub TR.** 2004. Gene expression-based high-throughput screening(GE-HTS) and application to leukemia differentiation. Nat Genet **36:**257-263.

283. **Antipova AA, Stockwell BR, Golub TR.** 2008. Gene expression-based screening for inhibitors of PDGFR signaling. Genome Biol **9:**R47.

284. **Sinha UK, Kundra A, Scalia P, Smith DL, Parsa B, Masood R, Gill PS.** 2003. Expression of EphB4 in head and neck squamous cell carcinoma. Ear Nose Throat J **82:**866, 869-870, 887.

285. **Xia G, Kumar SR, Masood R.** 2005. EphB4 Expression and Biological Significance in Prostate Cancer. Cancer Research **65:**4623-4632.

286. **Xia G, Kumar SR, Stein JP, Singh J, Krasnoperov V, Zhu S, Hassanieh L, Smith DL, Buscarini M, Broek D, Quinn DI, Weaver FA, Gill PS.** 2006. EphB4 receptor tyrosine kinase is expressed in bladder cancer and provides signals for cell survival. Oncogene **25:**769-780.

287. **Ferguson BD, Liu R, Rolle CE, Tan YH, Krasnoperov V, Kanteti R, Tretiakova MS, Cervantes GM, Hasina R, Hseu RD, Iafrate AJ, Karrison T, Ferguson MK, Husain AN, Faoro L, Vokes EE, Gill PS, Salgia R.** 2013. The EphB4 receptor tyrosine kinase promotes lung cancer growth: a potential novel therapeutic target. PLoS One **8:**e67668.

288. **Tu Y, He S, Fu J, Li G, Xu R, Lu H, Deng J.** 2012. Expression of EphrinB2 and EphB4 in glioma tissues correlated to the progression of glioma and the prognosis of glioblastoma patients. Clin Transl Oncol **14:**214-220.

289. **Li M, Zhao ZW, Zhang Y, Xin Y.** 2011. Over-expression of Ephb4 is associated with carcinogenesis of gastric cancer. Dig Dis Sci **56:**698-706.

290. **Hasina R, Mollberg N, Kawada I, Mutreja K, Kanade G, Yala S, Surati M, Liu R, Li X, Zhou Y, Ferguson BD, Nallasura V, Cohen KS, Hyjek E, Mueller J, Kanteti R, El Hashani E, Kane D, Shimada Y, Lingen MW, Husain AN, Posner MC, Waxman I, Villaflor VM, Ferguson MK, Varticovski L, Vokes EE, Gill P, Salgia R.** 2013. Critical role for the receptor tyrosine kinase EPHB4 in esophageal cancers. Cancer Res **73:**184-194.

291. **Kawada M, Fukazawa H, Mizuno S, Uehara Y.** 1997. Inhibition of anchorage-independent growth of ras-transformed cells on polyHEMA surface by antisense oligodeoxynucleotides directed against K-ras. Biochem Biophys Res Commun **231:**735-737.

292. **Xu LH, Yang X, Bradham CA, Brenner DA, Baldwin AS, Jr., Craven RJ, Cance WG.** 2000. The focal adhesion kinase suppresses transformation-associated, anchorage-independent apoptosis in human breast cancer cells. Involvement of death receptor-related signaling pathways. J Biol Chem **275:**30597-30604.

293. **Bettess MD, Dubois N, Murphy MJ, Dubey C, Roger C, Robine S, Trumpp A.** 2005. c-Myc is required for the formation of intestinal crypts but dispensable for homeostasis of the adult intestinal epithelium. Mol Cell Biol **25:**7868-7878.

294. **Yokobori T, Mimori K, Iwatsuki M, Ishii H, Onoyama I, Fukagawa T, Kuwano H, Nakayama KI, Mori M.** 2009. p53-Altered FBXW7 expression determines poor prognosis in gastric cancer cases. Cancer Res **69:**3788-3794.

295. **Iwatsuki M, Mimori K, Ishii H, Yokobori T, Takatsuno Y, Sato T, Toh H, Onoyama I, Nakayama KI, Baba H, Mori M.** 2010. Loss of FBXW7, a cell cycle regulating gene, in colorectal cancer: clinical significance. Int J Cancer **126:**1828-1837.

296. **Ibusuki M, Yamamoto Y, Shinriki S, Ando Y, Iwase H.** 2011. Reduced expression of ubiquitin ligase FBXW7 mRNA is associated with poor prognosis in breast cancer patients. Cancer Sci **102:**439-445.

297. **Ji S, Qin Y, Shi S, Liu X, Hu H, Zhou H, Gao J, Zhang B, Xu W, Liu J, Liang D, Liu L, Liu C, Long J, Zhou H, Chiao PJ, Xu J, Ni Q, Gao D, Yu X.** 2015. ERK kinase phosphorylates and destabilizes the tumor suppressor FBW7 in pancreatic cancer. Cell Res **25:**561-573.

298. **Rajagopalan H, Jallepalli PV, Rago C, Velculescu VE, Kinzler KW, Vogelstein B, Lengauer C.** 2004. Inactivation of hCDC4 can cause chromosomal instability. Nature **428:**77-81.

299. **Popov N, Schulein C, Jaenicke LA, Eilers M.** 2010. Ubiquitylation of the amino terminus of Myc by SCF(beta-TrCP) antagonizes SCF(Fbw7)-mediated turnover. Nat Cell Biol **12:**973-981.

300. **Lutterbach B, Hann SR.** 1994. Hierarchical phosphorylation at N-terminal transformation-sensitive sites in c-Myc protein is regulated by mitogens and in mitosis. Mol Cell Biol **14:**5510-5522.

301. **Alvarez E, Northwood IC, Gonzalez FA, Latour DA, Seth A, Abate C, Curran T, Davis RJ.** 1991. Pro-Leu-Ser/Thr-Pro is a consensus primary sequence for substrate protein phosphorylation. Characterization of the phosphorylation of c-myc and c-jun proteins by an epidermal growth factor receptor threonine 669 protein kinase. J Biol Chem **266:**15277-15285.

302. **Hann SR.** 2006. Role of post-translational modifications in regulating c-Myc proteolysis, transcriptional activity and biological function. Semin Cancer Biol **16:**288-302.

303. **Ruggero D.** 2013. Translational control in cancer etiology. Cold Spring Harb Perspect Biol **5**.

304. **Galan JA, Geraghty KM, Lavoie G, Kanshin E, Tcherkezian J, Calabrese V, Jeschke GR, Turk BE, Ballif BA, Blenis J, Thibault P, Roux PP.** 2014. Phosphoproteomic analysis identifies the tumor suppressor PDCD4 as a RSK substrate negatively regulated by 14-3-3. Proc Natl Acad Sci U S A **111:**E2918-2927.

305. **Kroczynska B, Sharma B, Eklund EA, Fish EN, Platanias LC.** 2012. Regulatory effects of programmed cell death 4 (PDCD4) protein in interferon (IFN)-stimulated gene expression and generation of type I IFN responses. Mol Cell Biol **32:**2809-2822.

306. **Spriggs KA, Cobbold LC, Jopling CL, Cooper RE, Wilson LA, Stoneley M, Coldwell MJ, Poncet D, Shen YC, Morley SJ, Bushell M, Willis AE.** 2009. Canonical initiation factor requirements of the Myc family of internal ribosome entry segments. Mol Cell Biol **29:**1565-1574.

307. **Stoneley M, Chappell SA, Jopling CL, Dickens M, MacFarlane M, Willis AE.** 2000. c-Myc protein synthesis is initiated from the internal ribosome entry segment during apoptosis. Mol Cell Biol **20:**1162-1169.

308. **Gregory MA, Hann SR.** 2000. c-Myc proteolysis by the ubiquitin-proteasome pathway: stabilization of c-Myc in Burkitt's lymphoma cells. Mol Cell Biol **20:**2423-2435.

309. **Hammond DE, Urbe S, Vande Woude GF, Clague MJ.** 2001. Down-regulation of MET, the receptor for hepatocyte growth factor. Oncogene **20:**2761-2770.

310. **Shtiegman K, Yarden Y.** 2003. The role of ubiquitylation in signaling by growth factors: implications to cancer. Semin Cancer Biol **13:**29-40.

311. **Sorkin A, Westermark B, Heldin CH, Claesson-Welsh L.** 1991. Effect of receptor kinase inactivation on the rate of internalization and degradation of PDGF and the PDGF beta-receptor. J Cell Biol **112:**469-478.

312. **Carpenter G, Cohen S.** 1976. 125I-labeled human epidermal growth factor. Binding, internalization, and degradation in human fibroblasts. J Cell Biol **71:**159-171.

313. **King AC, Hernaez-Davis L, Cuatrecasas P.** 1980. Lysomotropic amines cause intracellular accumulation of receptors for epidermal growth factor. Proc Natl Acad Sci U S A **77:**3283-3287.

314. **Fasen K, Cerretti DP, Huynh-Do U.** 2008. Ligand binding induces Cbl-dependent EphB1 receptor degradation through the lysosomal pathway. Traffic **9:**251-266.

315. **Xia G, Kumar SR, Masood R, Zhu S, Reddy R, Krasnoperov V, Quinn DI, Henshall SM, Sutherland RL, Pinski JK, Daneshmand S, Buscarini M, Stein JP, Zhong C, Broek D, Roy-Burman P, Gill PS.** 2005. EphB4 expression and biological significance in prostate cancer. Cancer Res **65:**4623-4632.

316. **Masood R, Kumar SR, Sinha UK, Crowe DL, Krasnoperov V, Reddy RK, Zozulya S, Singh J, Xia G, Broek D, Schonthal AH, Gill PS.** 2006. EphB4 provides survival advantage to squamous cell carcinoma of the head and neck. Int J Cancer **119:**1236-1248.

317. **Batlle E, Henderson JT, Beghtel H, van den Born MM, Sancho E, Huls G, Meeldijk J, Robertson J, van de Wetering M, Pawson T, Clevers H.** 2002. Beta-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. Cell **111:**251-263.

318. **Batlle E, Bacani J, Begthel H, Jonkheer S, Gregorieff A, van de Born M, Malats N, Sancho E, Boon E, Pawson T, Gallinger S, Pals S, Clevers H.** 2005. EphB receptor activity suppresses colorectal cancer progression. Nature **435:**1126-1130.

319. **Davalos V, Dopeso H, Castano J, Wilson AJ, Vilardell F, Romero-Gimenez J, Espin E, Armengol M, Capella G, Mariadason JM, Aaltonen LA, Schwartz S, Jr., Arango D.** 2006. EPHB4 and survival of colorectal cancer patients. Cancer Res **66:**8943-8948.

320. **Noren NK, Foos G, Hauser CA, Pasquale EB.** 2006. The EphB4 receptor suppresses breast cancer cell tumorigenicity through an Abl-Crk pathway. Nat Cell Biol **8:**815-825.

321. **Noren NK, Pasquale EB.** 2007. Paradoxes of the EphB4 receptor in cancer. Cancer Res **67:**3994-3997.

322. **Clevers H, Batlle E.** 2006. EphB/EphrinB receptors and Wnt signaling in colorectal cancer. Cancer Res **66:**2-5.

323. **Nievergall E, Lackmann M, Janes PW.** 2012. Eph-dependent cell-cell adhesion and segregation in development and cancer. Cell Mol Life Sci **69:**1813-1842.

324. **Rutkowski R, Mertens-Walker I, Lisle JE, Herington AC, Stephenson SA.** 2012. Evidence for a dual function of EphB4 as tumor promoter and suppressor regulated by the absence or presence of the ephrin-B2 ligand. Int J Cancer **131:**E614-624.

325. **Stephenson SA, Douglas EL, Mertens-Walker I, Lisle JE, Maharaj MS, Herington AC.** 2015. Anti-tumour effects of antibodies targeting the extracellular cysteine-rich region of the receptor tyrosine kinase EphB4. Oncotarget **6:**7554-7569.

326. **Goh LK, Sorkin A.** 2013. Endocytosis of receptor tyrosine kinases. Cold Spring Harb Perspect Biol **5:**a017459.

327. **Katz M, Shtiegman K, Tal-Or P, Yakir L, Mosesson Y, Harari D, Machluf Y, Asao H, Jovin T, Sugamura K, Yarden Y.** 2002. Ligand-independent degradation of epidermal growth factor receptor involves receptor ubiquitylation and Hgs, an adaptor whose ubiquitin-interacting motif targets ubiquitylation by Nedd4. Traffic **3:**740-751.

328. **Liu J, Plotnikov A, Banerjee A, Suresh Kumar KG, Ragimbeau J, Marijanovic Z, Baker DP, Pellegrini S, Fuchs SY.** 2008. Ligand-independent

pathway that controls stability of interferon alpha receptor. Biochem Biophys Res Commun **367:**388-393.

329. **Richter K, Haslbeck M, Buchner J.** 2010. The heat shock response: life on the verge of death. Mol Cell **40:**253-266.
330. **Lindquist S.** 1986. The heat-shock response. Annu Rev Biochem **55:**1151-1191.
331. **Morimoto RI, Kline MP, Bimston DN, Cotto JJ.** 1997. The heat-shock response: regulation and function of heat-shock proteins and molecular chaperones. Essays Biochem **32:**17-29.
332. **Hahn JS, Hu Z, Thiele DJ, Iyer VR.** 2004. Genome-wide analysis of the biology of stress responses through heat shock transcription factor. Mol Cell Biol **24:**5249-5256.
333. **Dai C, Whitesell L, Rogers AB, Lindquist S.** 2007. Heat shock factor 1 is a powerful multifaceted modifier of carcinogenesis. Cell **130:**1005-1018.
334. **Mendillo ML, Santagata S, Koeva M, Bell GW, Hu R, Tamimi RM, Fraenkel E, Ince TA, Whitesell L, Lindquist S.** 2012. HSF1 drives a transcriptional program distinct from heat shock to support highly malignant human cancers. Cell **150:**549-562.
335. **Tang Z, Dai S, He Y, Doty RA, Shultz LD, Sampson SB, Dai C.** 2015. MEK guards proteome stability and inhibits tumor-suppressive amyloidogenesis via HSF1. Cell **160:**729-744.
336. **Solimini NL, Luo J, Elledge SJ.** 2007. Non-oncogene addiction and the stress phenotype of cancer cells. Cell **130:**986-988.
337. **Yoon S, Seger R.** 2006. The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions. Growth Factors **24:**21-44.
338. **Hollenhorst PC, McIntosh LP, Graves BJ.** 2011. Genomic and biochemical insights into the specificity of ETS transcription factors. Annu Rev Biochem **80:**437-471.
339. **Anjum R, Blenis J.** 2008. The RSK family of kinases: emerging roles in cellular signalling. Nat Rev Mol Cell Biol **9:**747-758.
340. **Zhang J, Adrian FJ, Jahnke W, Cowan-Jacob SW, Li AG, Iacob RE, Sim T, Powers J, Dierks C, Sun F, Guo GR, Ding Q, Okram B, Choi Y, Wojciechowski A, Deng X, Liu G, Fendrich G, Strauss A, Vajpai N, Grzesiek S, Tuntland T, Liu Y, Bursulaya B, Azam M, Manley PW, Engen JR, Daley

GQ, Warmuth M, Gray NS. 2010. Targeting Bcr-Abl by combining allosteric with ATP-binding-site inhibitors. Nature **463:**501-506.

341. **Finck BN, Kelly DP.** 2006. PGC-1 coactivators: inducible regulators of energy metabolism in health and disease. J Clin Invest **116:**615-622.
342. **Puigserver P, Wu Z, Park CW, Graves R, Wright M, Spiegelman BM.** 1998. A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. Cell **92:**829-839.
343. **Andersson U, Scarpulla RC.** 2001. Pgc-1-related coactivator, a novel, serum-inducible coactivator of nuclear respiratory factor 1-dependent transcription in mammalian cells. Mol Cell Biol **21:**3738-3749.
344. **Lin J, Puigserver P, Donovan J, Tarr P, Spiegelman BM.** 2002. Peroxisome proliferator-activated receptor gamma coactivator 1beta (PGC-1beta), a novel PGC-1-related transcription coactivator associated with host cell factor. J Biol Chem **277:**1645-1648.
345. **Kressler D, Schreiber SN, Knutti D, Kralli A.** 2002. The PGC-1-related protein PERC is a selective coactivator of estrogen receptor alpha. J Biol Chem **277:**13918-13925.
346. **Huss JM, Kopp RP, Kelly DP.** 2002. Peroxisome proliferator-activated receptor coactivator-1alpha (PGC-1alpha) coactivates the cardiac-enriched nuclear receptors estrogen-related receptor-alpha and -gamma. Identification of novel leucine-rich interaction motif within PGC-1alpha. J Biol Chem **277:**40265-40274.
347. **Lin J, Yang R, Tarr PT, Wu PH, Handschin C, Li S, Yang W, Pei L, Uldry M, Tontonoz P, Newgard CB, Spiegelman BM.** 2005. Hyperlipidemic effects of dietary saturated fats mediated through PGC-1beta coactivation of SREBP. Cell **120:**261-273.
348. **Schreiber SN, Knutti D, Brogli K, Uhlmann T, Kralli A.** 2003. The transcriptional coactivator PGC-1 regulates the expression and activity of the orphan nuclear receptor estrogen-related receptor alpha (ERRalpha). J Biol Chem **278:**9013-9018.
349. **Puigserver P, Rhee J, Donovan J, Walkey CJ, Yoon JC, Oriente F, Kitamura Y, Altomonte J, Dong H, Accili D, Spiegelman BM.** 2003. Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1alpha interaction. Nature **423:**550-555.

350. **Baar K, Wende AR, Jones TE, Marison M, Nolte LA, Chen M, Kelly DP, Holloszy JO.** 2002. Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1. FASEB J **16:**1879-1886.

351. **Rhee J, Inoue Y, Yoon JC, Puigserver P, Fan M, Gonzalez FJ, Spiegelman BM.** 2003. Regulation of hepatic fasting response by PPARgamma coactivator-1alpha (PGC-1): requirement for hepatocyte nuclear factor 4alpha in gluconeogenesis. Proc Natl Acad Sci U S A **100:**4012-4017.

352. **Lehman JJ, Barger PM, Kovacs A, Saffitz JE, Medeiros DM, Kelly DP.** 2000. Peroxisome proliferator-activated receptor gamma coactivator-1 promotes cardiac mitochondrial biogenesis. J Clin Invest **106:**847-856.

353. **Lin J, Tarr PT, Yang R, Rhee J, Puigserver P, Newgard CB, Spiegelman BM.** 2003. PGC-1beta in the regulation of hepatic glucose and energy metabolism. J Biol Chem **278:**30843-30848.

354. **Arany Z, Foo SY, Ma Y, Ruas JL, Bommi-Reddy A, Girnun G, Cooper M, Laznik D, Chinsomboon J, Rangwala SM, Baek KH, Rosenzweig A, Spiegelman BM.** 2008. HIF-independent regulation of VEGF and angiogenesis by the transcriptional coactivator PGC-1alpha. Nature **451:**1008-1012.

355. **Eichner LJ, Perry MC, Dufour CR, Bertos N, Park M, St-Pierre J, Giguere V.** 2010. miR-378(*) mediates metabolic shift in breast cancer cells via the PGC-1beta/ERRgamma transcriptional pathway. Cell Metab **12:**352-361.

356. **Ariazi EA, Kraus RJ, Farrell ML, Jordan VC, Mertz JE.** 2007. Estrogen-related receptor alpha1 transcriptional activities are regulated in part via the ErbB2/HER2 signaling pathway. Mol Cancer Res **5:**71-85.

357. **Dai C, Santagata S, Tang Z, Shi J, Cao J, Kwon H, Bronson RT, Whitesell L, Lindquist S.** 2012. Loss of tumor suppressor NF1 activates HSF1 to promote carcinogenesis. J Clin Invest **122:**3742-3754.

358. **Wang X, Grammatikakis N, Siganou A, Stevenson MA, Calderwood SK.** 2004. Interactions between extracellular signal-regulated protein kinase 1, 14-3-3epsilon, and heat shock factor 1 during stress. J Biol Chem **279:**49460-49469.

359. **Hutti JE, Jarrell ET, Chang JD, Abbott DW, Storz P, Toker A, Cantley LC, Turk BE.** 2004. A rapid method for determining protein kinase phosphorylation specificity. Nat Methods **1:**27-29.

360. **Chu B, Soncin F, Price BD, Stevenson MA, Calderwood SK.** 1996. Sequential phosphorylation by mitogen-activated protein kinase and glycogen synthase

kinase 3 represses transcriptional activation by heat shock factor-1. J Biol Chem **271:**30847-30857.

361. **Kourtis N, Moubarak RS, Aranda-Orgilles B, Lui K, Aydin IT, Trimarchi T, Darvishian F, Salvaggio C, Zhong J, Bhatt K, Chen EI, Celebi JT, Lazaris C, Tsirigos A, Osman I, Hernando E, Aifantis I.** 2015. FBXW7 modulates cellular stress response and metastatic potential through HSF1 post-translational modification. Nat Cell Biol **17:**322-332.
362. **Chou SD, Prince T, Gong J, Calderwood SK.** 2012. mTOR is essential for the proteotoxic stress response, HSF1 activation and heat shock protein synthesis. PLoS One **7:**e39679.
363. **Carriere A, Romeo Y, Acosta-Jaquez HA, Moreau J, Bonneil E, Thibault P, Fingar DC, Roux PP.** 2011. ERK1/2 phosphorylate Raptor to promote Ras-dependent activation of mTOR complex 1 (mTORC1). J Biol Chem **286:**567-577.
364. **Gabai VL, Meng L, Kim G, Mills TA, Benjamin IJ, Sherman MY.** 2012. Heat shock transcription factor Hsf1 is involved in tumor progression via regulation of hypoxia-inducible factor 1 and RNA-binding protein HuR. Mol Cell Biol **32:**929-940.
365. **Abdelmohsen K, Lal A, Kim HH, Gorospe M.** 2007. Posttranscriptional orchestration of an anti-apoptotic program by HuR. Cell Cycle **6:**1288-1292.
366. **Danilin S, Sourbier C, Thomas L, Lindner V, Rothhut S, Dormoy V, Helwig JJ, Jacqmin D, Lang H, Massfelder T.** 2010. Role of the RNA-binding protein HuR in human renal cell carcinoma. Carcinogenesis **31:**1018-1026.
367. **Lopez de Silanes I, Fan J, Yang X, Zonderman AB, Potapova O, Pizer ES, Gorospe M.** 2003. Role of the RNA-binding protein HuR in colon carcinogenesis. Oncogene **22:**7146-7154.
368. **Lopez de Silanes I, Lal A, Gorospe M.** 2005. HuR: post-transcriptional paths to malignancy. RNA Biol **2:**11-13.
369. **Lafon I, Carballes F, Brewer G, Poiret M, Morello D.** 1998. Developmental expression of AUF1 and HuR, two c-myc mRNA binding proteins. Oncogene **16:**3413-3421.
370. **Keese M, Magdeburg RJ, Herzog T, Hasenberg T, Offterdinger M, Pepperkok R, Sturm JW, Bastiaens PI.** 2005. Imaging epidermal growth factor receptor phosphorylation in human colorectal cancer cells and human tissues. J Biol Chem **280:**27826-27831.

371. **Skvortsov S, Sarg B, Loeffler-Ragg J, Skvortsova I, Lindner H, Werner Ott H, Lukas P, Illmensee K, Zwierzina H.** 2004. Different proteome pattern of epidermal growth factor receptor-positive colorectal cancer cell lines that are responsive and nonresponsive to C225 antibody treatment. Mol Cancer Ther **3:**1551-1558.

372. **Song N, Liu S, Zhang J, Liu J, Xu L, Liu Y, Qu X.** 2014. Cetuximab-induced MET activation acts as a novel resistance mechanism in colon cancer cells. Int J Mol Sci **15:**5838-5851.

373. **Organ SL, Tsao MS.** 2011. An overview of the c-MET signaling pathway. Ther Adv Med Oncol **3:**S7-S19.

374. **Ilyas M, Tomlinson IP, Rowan A, Pignatelli M, Bodmer WF.** 1997. Beta-catenin mutations in cell lines established from human colorectal cancers. Proc Natl Acad Sci U S A **94:**10330-10334.

375. **Liu C, Li Y, Semenov M, Han C, Baeg GH, Tan Y, Zhang Z, Lin X, He X.** 2002. Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. Cell **108:**837-847.

376. **Munoz JP, Huichalaf CH, Orellana D, Maccioni RB.** 2007. cdk5 modulates beta- and delta-catenin/Pin1 interactions in neuronal cells. J Cell Biochem **100:**738-749.

377. **Wang MT, Holderfield M, Galeas J, Delrosario R, To MD, Balmain A, McCormick F.** 2015. K-Ras Promotes Tumorigenicity through Suppression of Non-canonical Wnt Signaling. Cell **163:**1237-1251.

378. **Krasnoperov V, Kumar SR, Ley E, Li X, Scehnet J, Liu R, Zozulya S, Gill PS.** 2010. Novel EphB4 monoclonal antibodies modulate angiogenesis and inhibit tumor growth. Am J Pathol **176:**2029-2038.

379. **Gingras AC, Gygi SP, Raught B, Polakiewicz RD, Abraham RT, Hoekstra MF, Aebersold R, Sonenberg N.** 1999. Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. Genes Dev **13:**1422-1437.

380. **Dorrello NV, Peschiaroli A, Guardavaccaro D, Colburn NH, Sherman NE, Pagano M.** 2006. S6K1- and betaTRCP-mediated degradation of PDCD4 promotes protein translation and cell growth. Science **314:**467-471.

381. **Schatz JH, Oricchio E, Wolfe AL, Jiang M, Linkov I, Maragulia J, Shi W, Zhang Z, Rajasekhar VK, Pagano NC, Porco JA, Jr., Teruya-Feldstein J, Rosen N, Zelenetz AD, Pelletier J, Wendel HG.** 2011. Targeting cap-

dependent translation blocks converging survival signals by AKT and PIM kinases in lymphoma. J Exp Med **208:**1799-1807.

382. **Wiegering A, Uthe FW, Jamieson T, Ruoss Y, Huttenrauch M, Kuspert M, Pfann C, Nixon C, Herold S, Walz S, Taranets L, Germer CT, Rosenwald A, Sansom OJ, Eilers M.** 2015. Targeting Translation Initiation Bypasses Signaling Crosstalk Mechanisms That Maintain High MYC Levels in Colorectal Cancer. Cancer Discov **5:**768-781.
383. **Kawasome H, Papst P, Webb S, Keller GM, Johnson GL, Gelfand EW, Terada N.** 1998. Targeted disruption of p70(s6k) defines its role in protein synthesis and rapamycin sensitivity. Proc Natl Acad Sci U S A **95:**5033-5038.
384. **Silvera D, Formenti SC, Schneider RJ.** 2010. Translational control in cancer. Nat Rev Cancer **10:**254-266.
385. **Holcik M.** 2004. Targeting translation for treatment of cancer--a novel role for IRES? Curr Cancer Drug Targets **4:**299-311.
386. **Min JN, Huang L, Zimonjic DB, Moskophidis D, Mivechi NF.** 2007. Selective suppression of lymphomas by functional loss of Hsf1 in a p53-deficient mouse model for spontaneous tumors. Oncogene **26:**5086-5097.
387. **Meng L, Gabai VL, Sherman MY.** 2010. Heat-shock transcription factor HSF1 has a critical role in human epidermal growth factor receptor-2-induced cellular transformation and tumorigenesis. Oncogene **29:**5204-5213.
388. **Sugden MC, Caton PW, Holness MJ.** 2010. PPAR control: it's SIRTainly as easy as PGC. J Endocrinol **204:**93-104.
389. **Repa JJ, Liang G, Ou J, Bashmakov Y, Lobaccaro JM, Shimomura I, Shan B, Brown MS, Goldstein JL, Mangelsdorf DJ.** 2000. Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. Genes Dev **14:**2819-2830.
390. **Rosen ED, Hsu CH, Wang X, Sakai S, Freeman MW, Gonzalez FJ, Spiegelman BM.** 2002. C/EBPalpha induces adipogenesis through PPARgamma: a unified pathway. Genes Dev **16:**22-26.
391. **Payne VA, Au WS, Lowe CE, Rahman SM, Friedman JE, O'Rahilly S, Rochford JJ.** 2010. C/EBP transcription factors regulate SREBP1c gene expression during adipogenesis. Biochem J **425:**215-223.
392. **Rowe GC, Jang C, Patten IS, Arany Z.** 2011. PGC-1beta regulates angiogenesis in skeletal muscle. Am J Physiol Endocrinol Metab **301:**E155-163.

393. **Jain RK.** 2003. Molecular regulation of vessel maturation. Nat Med **9:**685-693.

394. **Semenza GL.** 2004. Hydroxylation of HIF-1: oxygen sensing at the molecular level. Physiology (Bethesda) **19:**176-182.

395. **Noren NK, Lu M, Freeman AL, Koolpe M, Pasquale EB.** 2004. Interplay between EphB4 on tumor cells and vascular ephrin-B2 regulates tumor growth. Proc Natl Acad Sci U S A **101:**5583-5588.

396. **Sawamiphak S, Seidel S, Essmann CL, Wilkinson GA, Pitulescu ME, Acker T, Acker-Palmer A.** 2010. Ephrin-B2 regulates VEGFR2 function in developmental and tumour angiogenesis. Nature **465:**487-491.

397. **Wang Y, Nakayama M, Pitulescu ME, Schmidt TS, Bochenek ML, Sakakibara A, Adams S, Davy A, Deutsch U, Luthi U, Barberis A, Benjamin LE, Makinen T, Nobes CD, Adams RH.** 2010. Ephrin-B2 controls VEGF-induced angiogenesis and lymphangiogenesis. Nature **465:**483-486.

398. **Martiny-Baron G, Holzer P, Billy E, Schnell C, Brueggen J, Ferretti M, Schmiedeberg N, Wood JM, Furet P, Imbach P.** 2010. The small molecule specific EphB4 kinase inhibitor NVP-BHG712 inhibits VEGF driven angiogenesis. Angiogenesis **13:**259-267.

399. **Lerin C, Rodgers JT, Kalume DE, Kim SH, Pandey A, Puigserver P.** 2006. GCN5 acetyltransferase complex controls glucose metabolism through transcriptional repression of PGC-1alpha. Cell Metab **3:**429-438.

400. **Kelly TJ, Lerin C, Haas W, Gygi SP, Puigserver P.** 2009. GCN5-mediated transcriptional control of the metabolic coactivator PGC-1beta through lysine acetylation. J Biol Chem **284:**19945-19952.

401. **Rodgers JT, Lerin C, Haas W, Gygi SP, Spiegelman BM, Puigserver P.** 2005. Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. Nature **434:**113-118.

402. **Dissanayaka NN, Sellbach A, Matheson S, O'Sullivan JD, Silburn PA, Byrne GJ, Marsh R, Mellick GD.** 2010. Anxiety disorders in Parkinson's disease: prevalence and risk factors. Mov Disord **25:**838-845.

403. **Riedel O, Klotsche J, Spottke A, Deuschl G, Forstl H, Henn F, Heuser I, Oertel W, Reichmann H, Riederer P, Trenkwalder C, Dodel R, Wittchen HU.** 2010. Frequency of dementia, depression, and other neuropsychiatric symptoms in 1,449 outpatients with Parkinson's disease. J Neurol **257:**1073-1082.

404. **Pontone GM, Williams JR, Anderson KE, Chase G, Goldstein SR, Grill S, Hirsch ES, Lehmann S, Little JT, Margolis RL, Rabins PV, Weiss HD, Marsh L.** 2011. Anxiety and self-perceived health status in Parkinson's disease. Parkinsonism Relat Disord **17:**249-254.

405. **Oades RD, Halliday GM.** 1987. Ventral tegmental (A10) system: neurobiology. 1. Anatomy and connectivity. Brain Res **434:**117-165.

406. **LeDoux JE.** 2000. Emotion circuits in the brain. Annu Rev Neurosci **23:**155-184.

407. **Ehrlich I, Humeau Y, Grenier F, Ciocchi S, Herry C, Luthi A.** 2009. Amygdala inhibitory circuits and the control of fear memory. Neuron **62:**757-771.

408. **Seymour B, Dolan R.** 2008. Emotion, decision making, and the amygdala. Neuron **58:**662-671.

409. **Kalivas PW, Duffy P.** 1995. Selective activation of dopamine transmission in the shell of the nucleus accumbens by stress. Brain Res **675:**325-328.

410. **Darvas M, Fadok JP, Palmiter RD.** 2011. Requirement of dopamine signaling in the amygdala and striatum for learning and maintenance of a conditioned avoidance response. Learn Mem **18:**136-143.

411. **Shalin SC, Hernandez CM, Dougherty MK, Morrison DK, Sweatt JD.** 2006. Kinase suppressor of Ras1 compartmentalizes hippocampal signal transduction and subserves synaptic plasticity and memory formation. Neuron **50:**765-779.

412. **Costanzo-Garvey DL, Pfluger PT, Dougherty MK, Stock JL, Boehm M, Chaika O, Fernandez MR, Fisher K, Kortum RL, Hong EG, Jun JY, Ko HJ, Schreiner A, Volle DJ, Treece T, Swift AL, Winer M, Chen D, Wu M, Leon LR, Shaw AS, McNeish J, Kim JK, Morrison DK, Tschop MH, Lewis RE.** 2009. KSR2 is an essential regulator of AMP kinase, energy expenditure, and insulin sensitivity. Cell Metab **10:**366-378.

413. **English JD, Sweatt JD.** 1996. Activation of p42 mitogen-activated protein kinase in hippocampal long term potentiation. J Biol Chem **271:**24329-24332.

414. **English JD, Sweatt JD.** 1997. A requirement for the mitogen-activated protein kinase cascade in hippocampal long term potentiation. J Biol Chem **272:**19103-19106.

415. **Roberson ED, Sweatt JD.** 1996. Transient activation of cyclic AMP-dependent protein kinase during hippocampal long-term potentiation. J Biol Chem **271:**30436-30441.

416. **Huang YY, Martin KC, Kandel ER.** 2000. Both protein kinase A and mitogen-activated protein kinase are required in the amygdala for the macromolecular synthesis-dependent late phase of long-term potentiation. J Neurosci **20:**6317-6325.

417. **Heisler LK, Chu HM, Brennan TJ, Danao JA, Bajwa P, Parsons LH, Tecott LH.** 1998. Elevated anxiety and antidepressant-like responses in serotonin 5-HT1A receptor mutant mice. Proc Natl Acad Sci U S A **95:**15049-15054.

418. **Cryan JF, Holmes A.** 2005. The ascent of mouse: advances in modelling human depression and anxiety. Nat Rev Drug Discov **4:**775-790.

419. **Bonasera SJ, Schenk AK, Luxenberg EJ, Tecott LH.** 2008. A novel method for automatic quantification of psychostimulant-evoked route-tracing stereotypy: application to Mus musculus. Psychopharmacology (Berl) **196:**591-602.

420. **Garner JP.** 2005. Stereotypies and other abnormal repetitive behaviors: potential impact on validity, reliability, and replicability of scientific outcomes. ILAR J **46:**106-117.

421. **Garner JP, Mason GJ.** 2002. Evidence for a relationship between cage stereotypies and behavioural disinhibition in laboratory rodents. Behav Brain Res **136:**83-92.

422. **Joyce JN, van Hartesveldt C.** 1984. Rotation and postural deviation elicited by microinjections of dopamine into medial and lateral regions of dorsal striatum. Pharmacol Biochem Behav **21:**979-981.

423. **Saka E, Goodrich C, Harlan P, Madras BK, Graybiel AM.** 2004. Repetitive behaviors in monkeys are linked to specific striatal activation patterns. J Neurosci **24:**7557-7565.

424. **Szostak C, Jakubovic A, Phillips AG, Fibiger HC.** 1989. Neurochemical correlates of conditioned circling within localized regions of the striatum. Exp Brain Res **75:**430-440.

425. **Abdallah L, Bonasera SJ, Hopf FW, O'Dell L, Giorgetti M, Jongsma M, Carra S, Pierucci M, Di Giovanni G, Esposito E, Parsons LH, Bonci A, Tecott LH.** 2009. Impact of serotonin 2C receptor null mutation on physiology and behavior associated with nigrostriatal dopamine pathway function. J Neurosci **29:**8156-8165.

426. **Magnusson MS.** 2000. Discovering hidden time patterns in behavior: T-patterns and their detection. Behav Res Methods Instrum Comput **32:**93-110.

427. **Priebe K, Romeo RD, Francis DD, Sisti HM, Mueller A, McEwen BS, Brake WG.** 2005. Maternal influences on adult stress and anxiety-like behavior in C57BL/6J and BALB/cJ mice: a cross-fostering study. Dev Psychobiol **47:**398-407.

428. **Grimm MS, Emerman JT, Weinberg J.** 1996. Effects of social housing condition and behavior on growth of the Shionogi mouse mammary carcinoma. Physiol Behav **59:**633-642.

429. **Tanaka T.** 1998. Effects of litter size on behavioral development in mice. Reprod Toxicol **12:**613-617.

430. **Meziane H, Ouagazzal AM, Aubert L, Wietrzych M, Krezel W.** 2007. Estrous cycle effects on behavior of C57BL/6J and BALB/cByJ female mice: implications for phenotyping strategies. Genes Brain Behav **6:**192-200.

431. **Romeo RD, Mueller A, Sisti HM, Ogawa S, McEwen BS, Brake WG.** 2003. Anxiety and fear behaviors in adult male and female C57BL/6 mice are modulated by maternal separation. Horm Behav **43:**561-567.

432. **Schaefer DC, Asner IN, Seifert B, Burki K, Cinelli P.** 2010. Analysis of physiological and behavioural parameters in mice after toe clipping as newborns. Lab Anim **44:**7-13.

433. **Paluch LR, Lieggi CC, Dumont M, Monette S, Riedel ER, Lipman NS.** 2014. Developmental and behavioral effects of toe clipping on neonatal and preweanling mice with and without vapocoolant anesthesia. J Am Assoc Lab Anim Sci **53:**132-140.

434. **Agid O, Shapira B, Zislin J, Ritsner M, Hanin B, Murad H, Troudart T, Bloch M, Heresco-Levy U, Lerer B.** 1999. Environment and vulnerability to major psychiatric illness: a case control study of early parental loss in major depression, bipolar disorder and schizophrenia. Mol Psychiatry **4:**163-172.

435. **Heim C, Nemeroff CB.** 2001. The role of childhood trauma in the neurobiology of mood and anxiety disorders: preclinical and clinical studies. Biol Psychiatry **49:**1023-1039.

436. **Ogawa T, Mikuni M, Kuroda Y, Muneoka K, Mori KJ, Takahashi K.** 1994. Periodic maternal deprivation alters stress response in adult offspring: potentiates the negative feedback regulation of restraint stress-induced adrenocortical response and reduces the frequencies of open field-induced behaviors. Pharmacol Biochem Behav **49:**961-967.

437. **Liu D, Caldji C, Sharma S, Plotsky PM, Meaney MJ.** 2000. Influence of neonatal rearing conditions on stress-induced adrenocorticotropin responses and norepinepherine release in the hypothalamic paraventricular nucleus. J Neuroendocrinol **12:**5-12.

438. **Plotsky PM, Meaney MJ.** 1993. Early, postnatal experience alters hypothalamic corticotropin-releasing factor (CRF) mRNA, median eminence CRF content and stress-induced release in adult rats. Brain Res Mol Brain Res **18:**195-200.

439. **Mayorga AJ, Lucki I.** 2001. Limitations on the use of the C57BL/6 mouse in the tail suspension test. Psychopharmacology (Berl) **155:**110-112.