

TARGETING THE RAS-RAL EFFECTOR PATHWAY FOR CANCER TREATMENT

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ABSTRACT

LEANNA R. GENTRY: Targeting the Ras-Ral effector pathway for cancer treatment
(Under the direction of Channing J. Der)

The *RAS* oncogene is the most frequently mutated gene in human cancers, and this activated Ras oncoprotein has been shown to be required for both cancer initiation and maintenance. Great strides have been made in understanding Ras signaling in cancer since the discovery of its involvement in human cancers in 1982, with numerous Ras effector pathways and modes of Ras regulation having been identified as contributing to Ras-driven oncogenesis. However, there has been limited success in developing strategies for therapeutically targeting Ras-driven oncogenesis. One effort that has gained popularity in recent years is the inhibition of Ras effector signaling. The Ral (Ras-like) small GTPases, discovered shortly after Ras in an attempt to identify *RAS*-related genes, are activated downstream of Ras by Ral guanine nucleotide exchange factors (RalGEFs). The Ral family members have since emerged as critical regulators of key cellular processes and, importantly, have been characterized as playing a role in tumorigenesis and invasion of multiple cancer types. Interestingly, divergent roles for RalA and RalB are often observed in within a cancer. Due to the high affinity of Ral for GTP, which activates Ral upon binding, the Ral GTPase family cannot be targeted directly. Therefore, indirect inhibition of Ral must be considered for targeting Ral-dependent phenotypes in Ras-driven cancers. This could be achieved through inhibition of Ral association with the plasma membrane, which is thought to be required for its activation and subsequent signaling. Alternatively, downstream effectors of Ral with validated roles in cancer could be inhibited.

Posttranslational processing of the CAAX motif located on the C-termini of Ral GTPases, among other proteins, has been considered essential for their proper subcellular localization,

activation, and function. The first and essential step of this process is prenylation by GGTase. Prenylation signals for further CAAX processing by the enzymes RCE1 and ICMT, which are under consideration as therapeutic targets. We determined that the modifications regulated by these enzymes have distinct roles and consequences for Ral GTPases. We found that both RalA and RalB require RCE1 for association with the plasma membrane, and that the absence of RCE1 caused a sustained activation of both RalA and RalB. In contrast, ICMT deficiency disrupted plasma membrane localization of RalB but not RalA, whereas RalA depended on ICMT for efficient localization to recycling endosomes. Furthermore, ICMT deficiency caused increased stability of RalB protein but not RalA. Lastly, we found that palmitoylation was critical for proper subcellular localization of RalB but not RalA. In summary, we identified isoform-specific consequences of CAAX modifications that could be contributing to the divergent localization and activities of the Ral proteins.

In order to address inhibiting Ral effectors, we sought to determine the effect of inhibiting TBK1, a kinase that is a validated effector of RalB, in pancreatic ductal adenocarcinoma, a disease characterized by greater than 90% of cases containing a K-Ras mutation. We found that a novel small molecule inhibitor of TBK1, while effective at inhibiting signaling, had a minimal effect on pancreatic cancer cell proliferation *in vitro* and *in vivo*. However, when combined with inhibition of ERK1/2, we found a synergistic proliferation defect and induction of apoptosis. This suggests combination approaches with TBK1 inhibitors may provide therapeutic benefit in the treatment of K-Ras-driven pancreatic cancer. Overall, this work provides further insight into strategies for targeting Ral for the treatment of cancer.

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LIST OF ABBREVIATIONS

Akt	Protein kinase B
Bcl-XL	B-cell lymphoma extra large
CAAX	Cysteine-aliphatic-aliphatic-terminal amino acid
CHX	Cycloheximide
EGF	Epidermal growth factor
EH	Eps homology
ER	Endoplasmic reticulum
ERK1/2	Mitogen activated protein kinase kinase kinase 1/2
Exo84	Exocyst complex component 8
FTase	Farnesyl transferase
FTI	Farnesyl transferase inhibitor
GAP	GTPase activating protein
GDP	Guanosine diphosphate
GDS	Guanine nucleotide dissociation stimulator
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GGTase	Geranylgeranyl transferase

GGTI	Geranylgeranyl transferase inhibitor
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
HVR	Hypervariable region
ICMT	Isoprenylcysteine carboxylmethyltransferase
IRF3	Interferon regulatory factor 3
LPS	Lipopolysaccharide
mCh	Red mCherry fluorescent protein
MEF	Mouse embryonic fibroblast
MEK	Mitogen-activated protein kinase kinase
mTOR	Mammalian target of rapamycin
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
PARP	Poly (ADP-ribose) polymerase
PDAC	Pancreatic ductal adenocarcinoma
PH	Pleckstrin homology domain
PI3K	Phosphoinositide 3-kinase
Rab	Ras-like proteins in brain
RAF	Rapidly accelerated fibrosarcoma

Ral	Ras-like protein
RalBP1	Ral binding protein 1
Ras	Rat sarcoma viral oncogene homolog
RBD	Ral-binding domain
RCE1	Ras converting endopeptidase 1
REM	Ras exchanger motif
Rgl	RalGDS-like
Rho	Ras homologous protein
RhoGDI	Rho GDP dissociation inhibitor
Sec5	Exocyst complex component 2
TBK1	Tank binding kinase 1
TLR	Toll-like receptor
TSC	Tuberousclerosis complex
WT	Wild-type
SI/II	Switch I/II

Chapter 1: Introduction¹

Ral GTPase family. Identified initially as Ras-like (Ral) proteins, the Ral small GTPases are members of the Ras branch of the Ras superfamily of small GTPases [1]. *RALA* was identified initially using oligonucleotide probes to identify *RAS*-related genes in a cDNA library established from immortalized simian B-lymphocytes [2]. Three years later, using the simian *RALA* cDNA as a probe, human *RALA* and a related *RALB* gene were identified from a human pheochromocytoma cDNA library [3]. Subsequently, single *RAL* orthologs were identified in *C. elegans* (*RAL-1*) [4] and *Drosophila* (*RalA*) [5] (Fig. 1-1). Interestingly, although there are well-conserved *RAS* orthologs in yeast, no *RAL* orthologs are present in *S. cerevisiae* or *S. pombe*.

The three human *RAS* genes (*HRAS*, *KRAS* and *NRAS*) comprise one of the most frequently mutated gene families in human cancers [6]. Consequently, they have been the subject of intense research scrutiny and cancer drug discovery. Initially, the discovery of Ral proteins simply added to a rapidly growing roster of proteins that now comprise a large superfamily of >150 Ras-related small GTPases [1]. However, with discoveries that Ral GTPases are key regulators of vesicular trafficking and are effectors of Ras oncoprotein-driven growth transformation, Ral proteins stepped into the spotlight in 2003 to bask in their “15 minutes of fame” [7]. Since those initial findings, more discoveries on the role of Ral in normal and cancer cell physiology have ensured that their “fame” will last considerably more than 15

¹ This chapter is adapted from a previously published work: Gentry LR, Martin TD, Reiner DJ, Der CJ. “Ral small GTPase signaling and oncogenesis: More than just 15 minutes of fame.” *Biochim Biophys Acta* 2014 1843 (12) 2976-2988

minutes. In this review, we summarize our current knowledge on Ral GTPases and we highlight recent findings in Ral function.

Ral protein structure. The highly related human RalA and RalB isoforms share 82% overall amino acid sequence identity (Fig. 1-1A) and are members of the Ras branch of the Ras superfamily (Fig. 1-1B). They share 46-51% sequence identity and domain architecture with Ras proteins [8]. However, Ral proteins contain an N-terminal 11 amino acid extension not found in Ras, accounting for the 11 residue shift in numbering compared with Ras residue numbering (Fig. 1-1C). This is followed by the G domain, involved in GTP binding and hydrolysis, and the C-terminal membrane targeting sequence. The majority of sequence divergence occurs within the C-terminal hypervariable regions (50% shared identity) (Fig. 1-1C).

Like Ras, Ral proteins cycle between inactive GDP-bound and active GTP-bound states (Fig. 1-2A). RalA and RalB share complete sequence identity in the switch I (SI) and II (SII) sequences that change conformation during GDP-GTP cycling [8] (Fig. 1-2B). As described below, SI and SII are involved in recognition by both regulators and effectors. The conservation of SI and SII sequences in *Drosophila* and *C. elegans* Ral proteins support their interaction with conserved regulators and effectors.

Similar to Ras, the intrinsic GDP-GTP exchange and GTP hydrolysis activities of Ral GTPases are very weak, with each activity accelerated by Ral-selective guanine nucleotide exchange factors (RalGEFs) and GTPase activating proteins (RalGAPs), respectively (Fig. 1-2A). RalGEFs stimulate guanine nucleotide exchange. With intracellular levels of GTP approximately 10-fold higher than GDP, RalGEF stimulation favors formation of Ral-GTP. Ral GTPase-activating proteins (RalGAPs) catalyze the hydrolysis of the bound GTP, returning Ral to an inactive conformation. When bound to GTP, RalA and RalB can interact with the same array of downstream effector proteins and mediate numerous cellular processes.

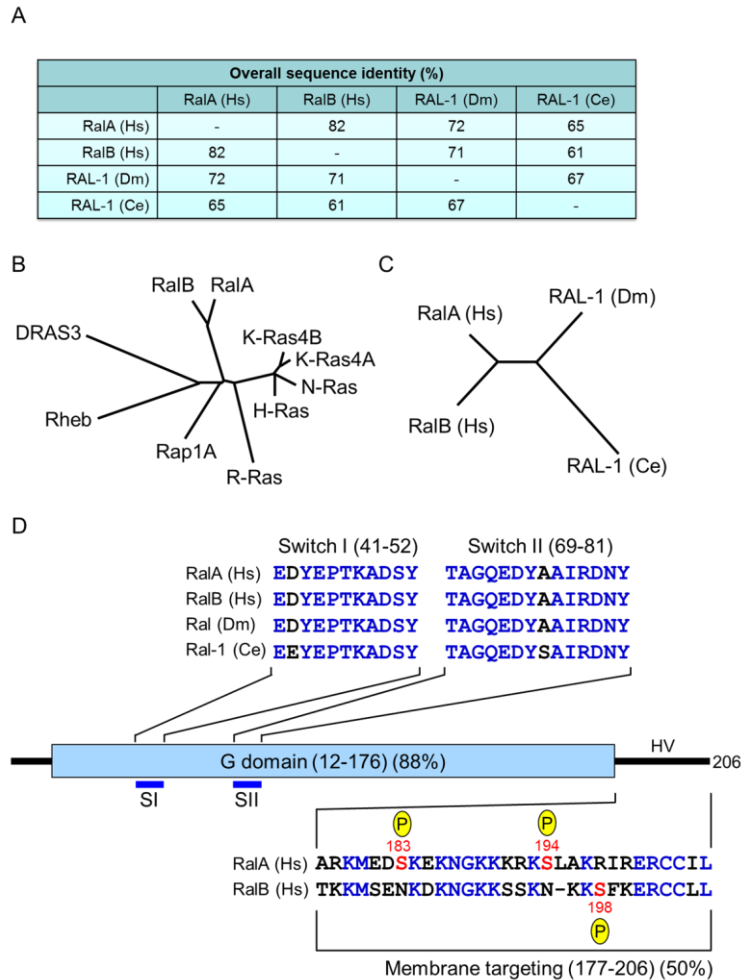


Figure 1-1. Evolutionary conservation of Ral small GTPases. A. Human and invertebrate Ral orthologs exhibit strong sequence identity. The RalA and RalB isoforms are found in all vertebrate species. There is one Ral ortholog in *C. elegans* (Ce) and *D. melanogaster* (Dm). Overall sequence identity was determined by CLUSTALW multiple sequence alignment. B. Ral GTPases are members of the Ras branch of the Ras superfamily. Shown here is a comparison with the four Ras proteins and representative members of the Ras family. The dendrogram was generated by CLUSTALW multiple sequence alignment. C. Dendrogram showing sequence relationship of human and invertebrate Ral proteins. D. Ral domain structure. Human RalA and RalB G domains (12–176) shares 88% sequence identity and contain the SI and SII domains that change in conformation during GDP–GTP cycling and are involved in interaction with regulators and effectors. The switch regions are conserved between human Ral proteins and *Drosophila* Ral and differ by a single residue in each switch in *C. elegans* Ral (identical residues indicated in blue text). The hypervariable (HV) C-terminus (50% identity) consists of the membrane targeting region and contains key post-translational phosphorylation sites that regulate Ral subcellular localization and effector interaction. Multiple sequence alignment was done by ClustalW analyses and domain topology by SMART analyses. Numbers correspond to the human Ral amino acid sequences

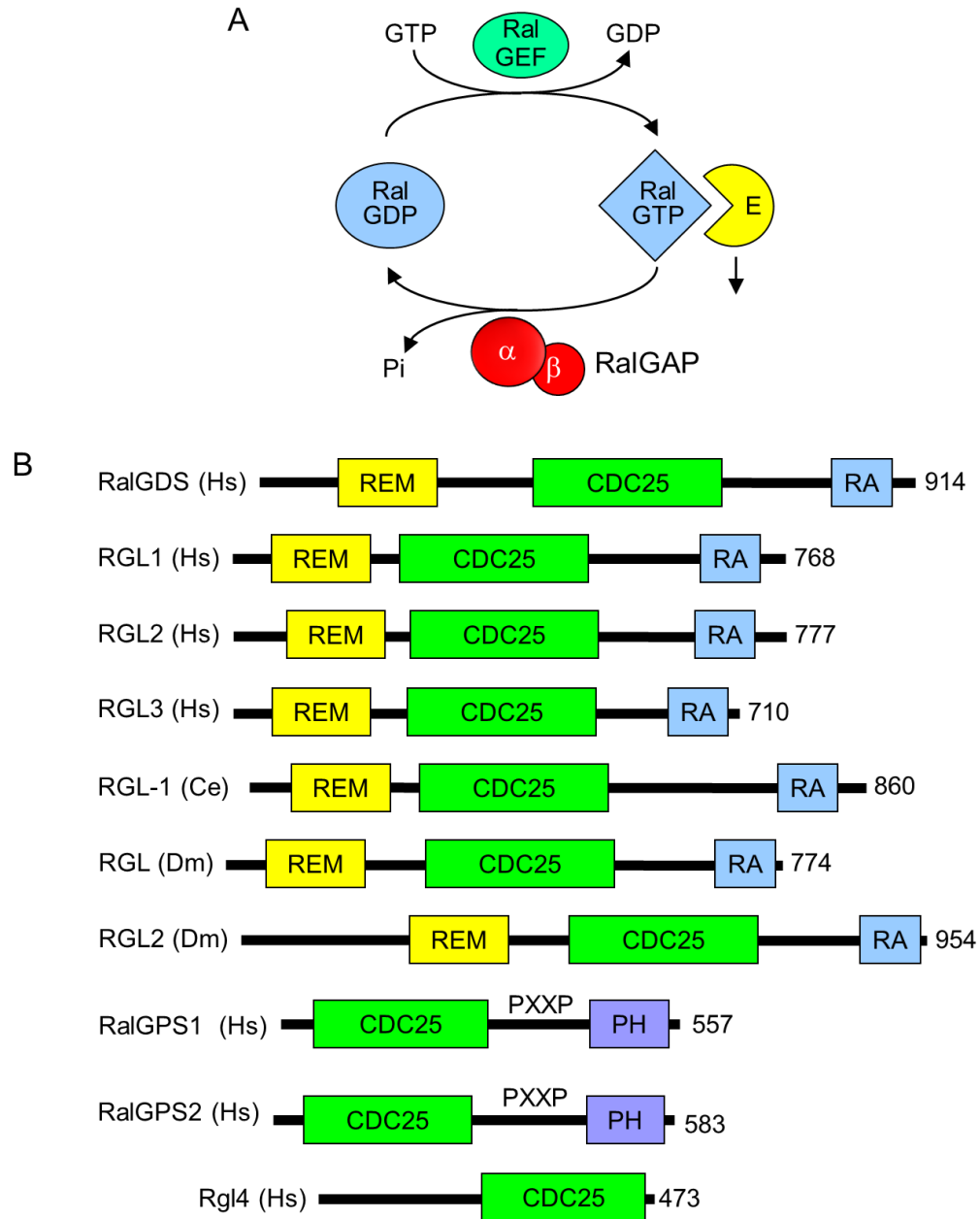


Figure 1-2, A and B. Regulators of the Ral GDP-GTP cycle. A. Regulation of Ral GDP-GTP cycling. Ral-selective GEFs and GAPs accelerate the low intrinsic exchange and GTP hydrolysis activities to promote formation of active GTP-bound and inactive GDP-bound Ral. B. The RalGEFs are highly conserved across species. All RalGEFs contain a CDC25 homology domain, which is responsible for catalytic activity. There are four human isoforms of RalGEF that contain Ras-association (RA) domain. These isoforms also contain a Ras exchanger motif (REM) that likely stabilizes the CDC25 homology domain and is essential for RalGEF catalytic activity. There is one homolog in *C. elegans* and two in *Drosophila*. The RalGEF homolog in *C. elegans* is most similar to RalGDS. The RalGPS RalGEFs lack a REM domain and do not associate with Ras, but instead contain a pleckstrin homology (PH) domain. RGL4 contains a CDC25 homology domain, but lacks a REM, RA or PH domain.

Ral GEFs. The first RalGEF identified, Ral guanine nucleotide dissociation stimulator (RalGDS) (Fig. 1-2B), was found by yeast two-hybrid screens performed in the early 1990s to identify Ras effectors [9-11]. RalGDS was found to catalyze nucleotide exchange on both RalA and RalB but not on other small GTPases including members of the Ras, Rho, and Rab families. Subsequent yeast two-hybrid library screening studies using H-Ras, R-Ras, TC21/R-Ras2, and Rit as baits identified three additional RalGEF proteins that were named Rgl (RalGDS-like), Rgl2/Rlf, and Rgl3 [12-14] [15]. These RalGEFs contain a common domain architecture including an N-terminal Ras exchanger motif (REM) domain followed by a CDC25 homology domain (RasGEF) and a C-terminal Ras-association (RA) domain (Fig. 1-2A) [16]. The CDC25 homology domain shares sequence identity with the catalytic domains of RasGEFs [17]. In addition to the three Ras isoforms, other Ras family small GTPases can also bind and activate the RA domain-containing RalGEFs [18].

RalGPS1 and RalGPS2 (Ral GEF with PH domain and SH3-binding motif) comprise a second distinct family of RalGEFs [19-21] (Fig. 1-2A). These two related proteins (63% identity) contain an N-terminal CDC25 homology RasGEF but lack a REM and RA domain. Instead, they contain a C-terminal pleckstrin homology (PH) domain. Additionally, they possess a central proline-rich sequence with PxxP motifs recognized by Src homology 3 (SH3) domain-containing proteins.

The absence of an RA domain uncouples these RalGEFs from direct association with Ras family small GTPases. Instead, the PH domain has been shown to be sufficient for membrane targeting and necessary for Ral activation [19]. The regulation of these RalGEFs is poorly understood, but some evidence suggests that RalGPS2 plays a role in regulating the actin cytoskeleton [21]. Interestingly, members of both RalGEF subclasses have been implicated in cytokinesis [22].

Another RalGEF, now designated RGL4, was identified originally as a RalGDS-related (Rgr) oncogene in a DMBA (7,12-dimethylbenz[*a*]anthracene)-induced rabbit squamous cell carcinoma [23]. However, while RGL4 does contain a CDC25 homology domain, it lacks a well-defined RA or PH

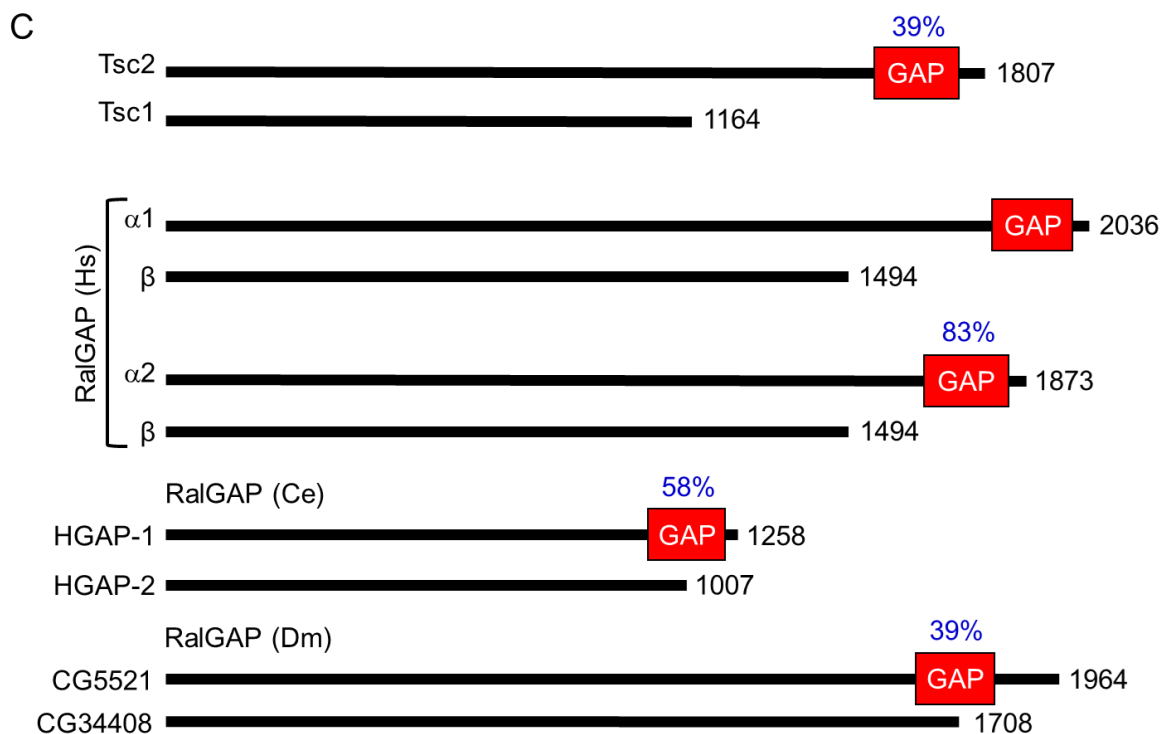
domain (Fig. 1-2B). Furthermore, while the other RalGEFs described above are highly selective activators of Ral, RGR has also been described to activate other Ras family small GTPases [24].

RalGAPs. Although the existence of RalGAPs was first reported in 1991 [25], only recently has the molecular identification of RalGAPs been achieved (Fig. 1-2C). Work done by Feig and colleagues in the early 1990s detected and characterized RalGAP activity in brain and testes cytosolic extracts, and the putative RalGAP activity was distinct in size from Ras or Rho GAPs [25]. Subsequently, using a GTPase-deficient, persistently GTP-bound mutant of RalA for affinity chromatography, two distinct RalGAP complexes were identified in brain cytosol [26]. Each heterodimeric complex consists of a shared regulatory RalGAP β subunit and one of two related catalytic RalGAP α 1 and α 2 subunits (53% overall sequence identity) (Figs. 1-2A and 1-2C). Independently, Saltiel and colleagues used a similar RalA affinity purification approach and identified RalGAP α 2 (RGC2) and RalGAP β (RGC1) as components of a Ral-selective GAP [27].

RalGAP α 1 (GARNL1/TULIP1) and RalGAP α 2 (AS250; Akt substrate of 250 kDa) were identified previously as proteins with sequence identity with the GAP catalytic domain of TSC2 (also known as tuberlin) [28, 29] (Fig. 1-2B) and distinct from the RasGAP catalytic domain [17]. RalGAP α 2 was also identified to form a complex with RalGAP β (KIAA1219). TSC2 is the catalytic subunit of a GAP selective for the Rheb small GTPases, another member of the Ras branch of the Ras superfamily [1]. However, TSC2 alone is not sufficient for RhebGAP activity and requires heterodimer formation with TSC1 (also known as hamartin). Hence, the active RalGAP α/β complexes share both sequence and structural similarities with the heterodimeric tuberous-sclerosis (TSC) complex [30]. Although RalGAP β lacks sequence similarity with Tsc1, it serves an analogous role in stabilizing RalGAP α and is required for RalGAP activity. RalGAP β is expressed ubiquitously, whereas more variable expression profiles are seen for the two RalGAP α subunits. RalGAPs are conserved in evolution, with orthologs of both subunits found in *C. elegans* and *Drosophila* (Fig. 1-2B) [26, 31]. The RalGAP α GAP catalytic domains of *C.*

C. elegans (HGAP-1) and *Drosophila* (CG5521) share 37-39% and 58-59% sequence identity, respectively, with the GAP catalytic domains of their human counterparts.

RalGAP accelerates the GTPase activity of both RalA and RalB but not for other small GTPases tested (H-Ras, Rap1, Rheb, RhoA, Ran and Rab27) [26, 27, 31]. The RalGAP α subunits share 53% overall sequence identity and 83% sequence identity in their GAP domains (Figs. 1-2C and 1-2D). Additionally, RalGAP α 2 is subject to insulin-stimulated phosphorylation by the AKT serine/threonine kinase, analogous to a similar mechanism of AKT regulation of TSC2 [32]. As with TSC2, AKT phosphorylation of RalGAP α 2 impaired the ability of the RalGAP complex to catalyze RalA GTP hydrolysis. This is not due to altered intrinsic GAP activity but to a reduced RalA interaction with RalGAP α 2.



D

GAP catalytic domain sequence identity (%)					
	Hs			Dm	Ce
	TSC2	RalGAP α 1	RalGAP α 2	CG5521	HGAP-1
TSC2 (Hs)		26	27	26	17
RalGAP α 1 (Hs)	26		83	58	39
RalGAP α 2 (Hs)	27	83		59	37
CG5521 (Dm)	26	58	59		38
HGAP-1 (Ce)	17	39	37	38	

Figure 1-2, C and D. Regulators of the Ral GDP-GTP cycle. C. The RalGAPs are heterodimeric complexes formed by either a RalGAP α 1 or RalGAP α 2 catalytic subunit with the regulatory RalGAP β subunit. The RalGAP β subunit serves to regulate the catalytic activity of the RalGAP α subunits, similar to TSC1 regulation of TSC2. Percentages indicate sequence identity with the RalGAP α 1 catalytic domain. Orthologs of the human RalGAP α and RalGAP β subunits are present in *C. elegans* and *Drosophila*. Multiple sequence alignment and sequence identity were determined by ClustalW analyses and domain topology by SMART analyses.

Ral effectors. Like Ras and other small GTPases, Ral interacts with a number of effector proteins when bound to GTP (Fig. 1-3). However, unlike with Ras, the Ral binding domains (RBD) lack primary sequence identity. The best characterized Ral effectors are RalBP1/RLIP76 and the Sec5 and Exo84 subunits of the octameric exocyst complex. The evolutionarily conserved exocyst complex mediates the tethering of post-Golgi secretory vesicles to the plasma membrane prior to exocytic fusion [33]. The exocyst subunits may also exist as monomers or subcomplexes, and can possess non-exocyst functions. Ral interaction with each subunit occurs in distinct subcellular locations, interacting with Sec5 at the plasma membrane and with Exo84 with intracellular vesicles [34, 35]. Although RalA and RalB can interact with the same set of effectors *in vitro*, as described below, the distinct biological functions of RalA and RalB are mediated by differences in subcellular localization, leading to their interaction with distinct subsets of effectors.

The structures of Ral in complex with the RBDs of these three effectors have been determined. Whereas the Sec5 RBD interacts with SI alone [36], Exo84 [37] and RalBP1 [38] RBD interaction involves both SI and SII [39]. Consistent with 100% conservation of SI and SII residues, and residues involved with effector binding, where studied, RalA and RalB interact with the same set of effectors *in vitro*.

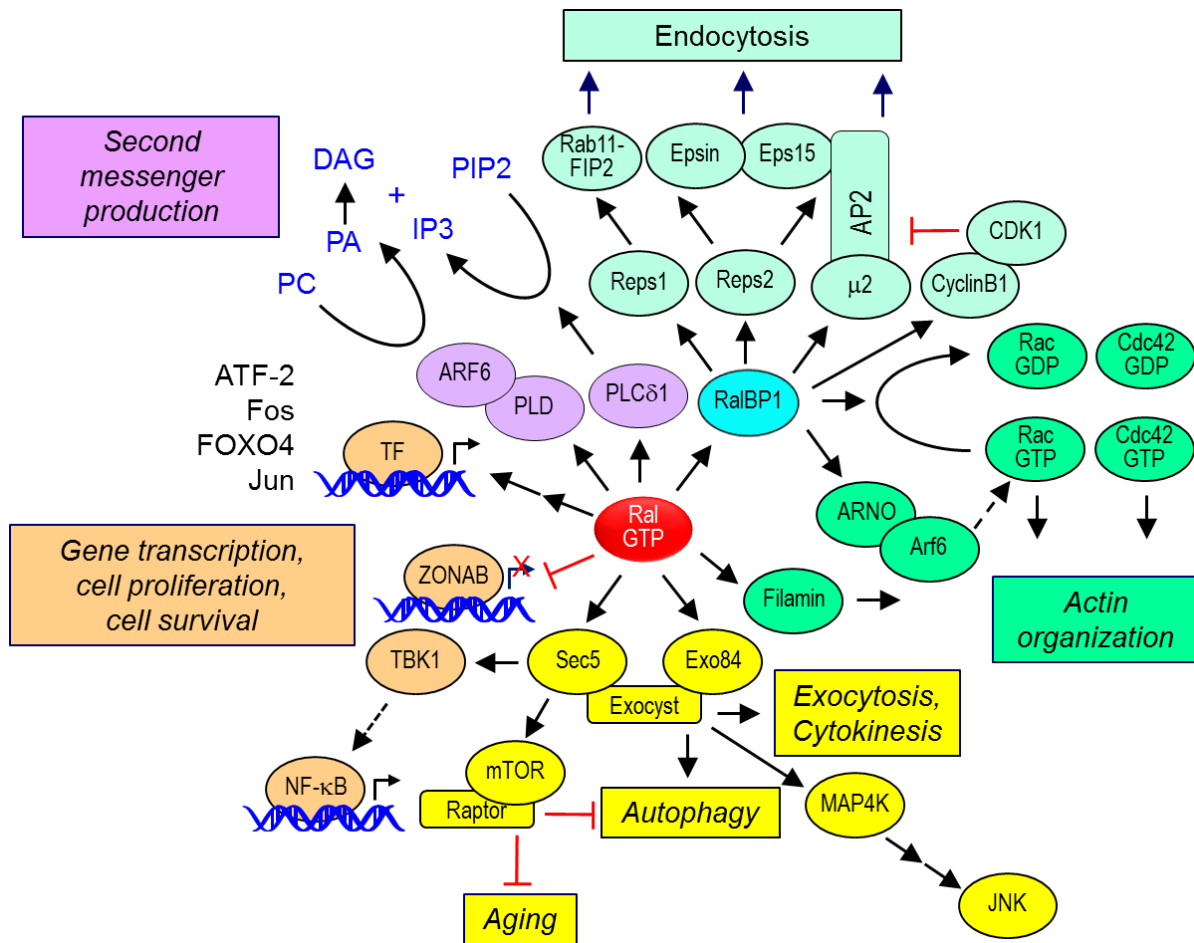


Figure 1-3. Ral effectors and effector functions. Active Ral can bind to a variety of downstream effectors and modulate numerous cellular activities. RalBP1 acts as a RhoGAP as well as a scaffold for other proteins that regulate endocytosis and other cellular processes. Ral association with Sec5 or Exo84 can regulate exocyst-dependent and -independent processes. Other effector processes include regulation of cell cycle progression through PLC1-dependent cytokinesis and cytoskeletal changes through filamin A, IP3 signaling through PLCδ1, and gene transcription through ZONAB. Ral activation also stimulates signaling pathways that lead to the activation of various transcription factors (TF), stimulating gene expression.

RalBP1. The first Ral effector described, RalBP1 (Ral binding protein 1; also called RLIP76 or RIP1), was identified in screens for proteins that bound preferentially to activated RalA [40-42]. RalBP1 orthologs are found in *Drosophila* and *C. elegans*. RalBP1 contains a RhoGAP catalytic domain that has activity for the Cdc42 and Rac small GTPases, members of the Rho branch of the Ras superfamily [1]. Cdc42 stimulates filopodia formation whereas Rac stimulates lamellipodia formation. Thus, RalBP1 provides a link between Ral and modulation of the actin cytoskeleton changes that drive these cellular activities [40].

In addition to its RhoGAP domain, RalBP1 has additional functions (Fig. 1-3). Two ATP binding motifs have been identified in RalBP1 and shown to be important for transport function involving glutathione conjugates of electrophilic compounds [43, 44]. This transport function may facilitate the cellular export of chemotherapeutic drugs and radiation-induced oxidative damage byproducts [45]. RalBP1 overexpression has been found in a spectrum of human cancers, and suppression of RalBP1 expression can impair tumorigenic growth in vivo [46]. However, phenotypes attributed to RalBP1 do not necessarily implicate their role in Ral signaling [47].

RalBP1 also functions as a scaffold and interacts with a spectrum of functionally distinct proteins that regulate endocytosis and signal transduction (Fig. 1-3). The AP2 adaptor complex, a regulator of clathrin-mediated endocytosis from the plasma membrane, associates with the N-terminal region of RalBP1 [48]. The Eps homology (EH) domain-containing proteins Reps1 and Reps2 (POB1) were identified as proteins that interacted with the C-terminus of RalBP1 [49, 50]. These proteins are known to be important for receptor tyrosine kinase-regulated endocytosis, with Reps1 interacting with Rab11-FIP2 and Reps2 binding Epsin and Eps15 [51, 52].

Another protein that associates with the RalBP1 C-terminus is cyclin B1 [53]. In turn, the RalBP1-bound cyclin B1 complexes with Cdk1, with Cdk1 phosphorylation of Epsin preventing endocytosis during mitosis. This activity was shown to be mediated by RalA activation.

RalBP1 has been implicated as a key effector for several Ral-driven processes. In these studies, the typical approach has been the utilization of mutants of Ral that are selectively impaired in effector interaction. The D49N substitution impairs RalBP1 but not Sec5 or Exo84 effector binding, whereas the D49E mutation has the opposite consequence [40, 54, 55]. For example, shRNA silencing analyses determined that RalB but not RalA was required for invadopodia formation in pancreatic cancer cell lines [56]. RalB D49E but not D49N could rescue loss of endogenous RalB and restore invadopodia formation, indicating that RalBP1 was a critical effector for this RalB activity. This RalBP1 function was GAP-independent but abolished by mutation of the ATP binding motifs [56].

RalA was shown to utilize RalBP1 to regulate mitochondrial fission at mitosis [57]. Mitochondria exist as dynamic interconnected networks that are maintained through a balance of fusion and fission. Fission facilitates equal distribution of mitochondria to daughter cells during mitosis. Fission is controlled by the GTPase Drp1 on the outer mitochondrial membrane. RalA was found to recruit RalBP1 to mitochondria, where RalBP1 acts as a scaffold to facilitate cyclin B/Cdk1 phosphorylation of Drp1 to promote mitochondrial fission. Suppression of either RalA or RalBP1 expression caused a loss of mitochondrial fission at mitosis.

Recently, RalBP1 was shown to be necessary and sufficient for RalA-driven mislocalization of the cyclin-dependent kinase inhibitor p27 KIP1, leading to inhibition of TGF- β -mediated growth arrest in epithelial cells [58]. This function appeared to require an intact RhoGAP domain.

Sec5 and Exo84 subunits of the exocyst. The best-characterized Ral effectors are two components of the exocyst complex, Sec5 and Exo84 [54, 55, 59]. The association of Ral with both Sec5 and Exo84 has been found to be important in exocytosis. Ral regulates the subcellular localization of the exocyst through mediating Sec5-paxillin association and the assembly of the octameric exocyst complex by

interacting with Sec5 and Exo84 [55, 60]. Ral interaction with Sec5 may also regulate exocyst-independent functions.

Recent evidence suggests that Ral engages exocyst subunits to perform a variety of cellular processes independent of their roles in exocytosis. White and colleagues found that the association of RalB with Sec5 is critical in the innate immune response [61]. RalB binding to Sec5 leads to an interaction of Sec5 with TBK1, a protein kinase known to regulate NF- κ B signaling. Intriguingly, TBK1 has recently been identified in siRNA screens as a synthetic lethal partner of activated K-Ras [62], although a subsequent study failed to support this relationship [63]. Recently, a mechanism where the integrin α v β 3 recruited a K-Ras-RalB complex to the plasma membrane to activate TBK1 and NF- κ B signaling was identified (Fig. 1-3) [64]. This signaling mechanism regulated tumor initiation and growth. Chapter 3 contains a study of TBK1 inhibition in Ras-driven pancreatic ductal adenocarcinoma (PDAC).

The association of RalB with the exocyst has also been shown to regulate macroautophagy [34]. When cells are grown in nutrient-rich conditions, RalB engages Sec5. Upon nutrient starvation, RalB then engages Exo84 and the exocyst, leading to an upregulation of autophagosome formation. This process is mediated through the assembly of the ULK1 serine/threonine kinase and Beclin1-VPS34 complexes on the exocyst. Autophagy has emerged as a key component of Ras-driven transformation in a variety of cell types, perhaps highlighting an underlying importance of Ras-RalGEF signaling in tumor cell autophagy.

Other effectors. One lesser-characterized Ral effector is phospholipase D1 (PLD1) [65, 66]. However, unlike other effectors, the association with Ral is not GTP-dependent and instead the association is with the N-terminal 11 amino acid extension (Fig. 1-1D). PLD1 is best known for its role in converting phosphatidylcholine to phosphatidic acid and choline in response to G-protein coupled receptor (GPCR) stimulation. Recent evidence shows that RalA is necessary for the PLD1-mediated stimulation of mTORC1 signaling [67]. Furthermore, the RalA-PLD1 interaction has been shown to

promote proper p27 localization, thus allowing for proper TGF- β signaling [58]. The interaction of both RalA and RalB with PLD1 has been shown to be critical for HeLa cell cytokinesis [22].

Filamin is an important component of the actin cytoskeleton and is involved in actin crosslinking and lamellipodia formation. The association of RalA with filamin was found to be important for filopodia formation in Swiss-3T3 cells [68]. Additionally, RalA did not induce filopodia in a human melanoma cell line that lacks expression of filamin.

Lastly, active RalA has been shown to engage the transcription factor ZONAB (zonula occludens 1-associated nucleic acid binding protein) in a cell density dependent manner in MDCK cells [69]. At high cell densities, RalA engages ZONAB, unlocking the transcription of ZONAB targets, but it is unclear which genes are turned on [69]. While a direct role for Ral association with these lesser-studied effectors has not been found in Ral-driven cancers, their important roles in mitosis, motility, and gene regulation make them intriguing targets as Ral studies progress.

Post-translational modification and regulation of Ral function. RalA and RalB exhibit the most significant sequence divergence in their C-terminal membrane targeting sequences (50% identity) (Fig. 1-1C). This sequence divergence results in their distinct subcellular localization that contributes to the functional differences described for RalA and RalB by regulating effector utilization in vivo [56, 70-73]. Both isoforms can be found at the plasma membrane as well as in endomembranes, with cell type differences seen. In this section we summarize the role of posttranslational modifications that regulate Ral subcellular localization.

Ral CAAX modifications. Like the majority of Ras family small GTPases, RalA and RalB terminate in a CAAX (C = cysteine, A = aliphatic amino acid; X = terminal amino acid) tetrapeptide motif (Fig. 1-4). The CAAX motif signals for a series of posttranslational modifications that increase hydrophobicity and promote membrane anchoring, where the terminal X residue determines protein

prenyltransferase specificity [74]. When X = S, A, Q, and M, the protein is preferentially recognized by farnesyltransferase (FTase)-catalyzed addition of a C15 farnesyl isoprenoid lipid; when X = L or I, it signals for geranylgeranyltransferase type I (GGTase-I)-catalyzed addition of a C20 geranylgeranyl isoprenoid.

For Ral, the initial step is catalyzed by covalent addition of geranylgeranyl to the cysteine residue of the CAAX motif by cytosolic GGTase-I [75]. This is followed by endoproteolytic removal of the AAX residues, catalyzed by endoplasmic reticulum-associated Ras converting enzyme 1 (RCE1), and subsequent carboxymethylation of the now terminal prenylated cysteine residue, catalyzed by isoprenylcysteine carboxyl methyltransferase (ICMT).

The CAAX-signaled modifications are critical for both RalA and RalB function. Mutation of the cysteine residue to prevent all CAAX-signaled modifications disrupts Ral membrane association and function [75]. Similarly, treatment with a pharmacologic inhibitor of GGTase-I also disrupted Ral membrane association and signaling. Since inhibition of the GGTase-I modification prevents all subsequent modifications, the role of the Rce1 and ICMT catalyzed modifications in Ral function remain to be addressed.

Recently, the Ral CAAX motifs were identified as members of a distinct subset of CA₁A₂X motifs where the A₁ residue is a second cysteine residue (CCAX). CCAX motifs can undergo an alternative modification pathway (Fig. 1-4). As shown for a Rho family small GTPase (Cdc42), this motif can signal for dual lipid modification: prenylation followed by covalent addition of a palmitate fatty acid [76]. For Cdc42, after the initial GGTase-I catalyzed prenylation step, a Golgi-associated protein acetyltransferase (PAT) catalyzes covalent addition of palmitate to the adjacent cysteine residue rather than the conventional modification by Rce1 and ICMT. For Cdc42, this alternative modification prevented its recognition by RhoGDI, a protein that masks the prenyl lipid and disrupts membrane association, resulting in a cytosolic pool of Cdc42. Since there is no known RalGDI, the consequences of

this palmitate modification on Ral subcellular localization and membrane association, and function, have not been determined previously. Chapter 2 explores the consequence of RCE1 and ICMT processing as well as palmitoylation of the Ral CAAX motif on Ral localization and function.

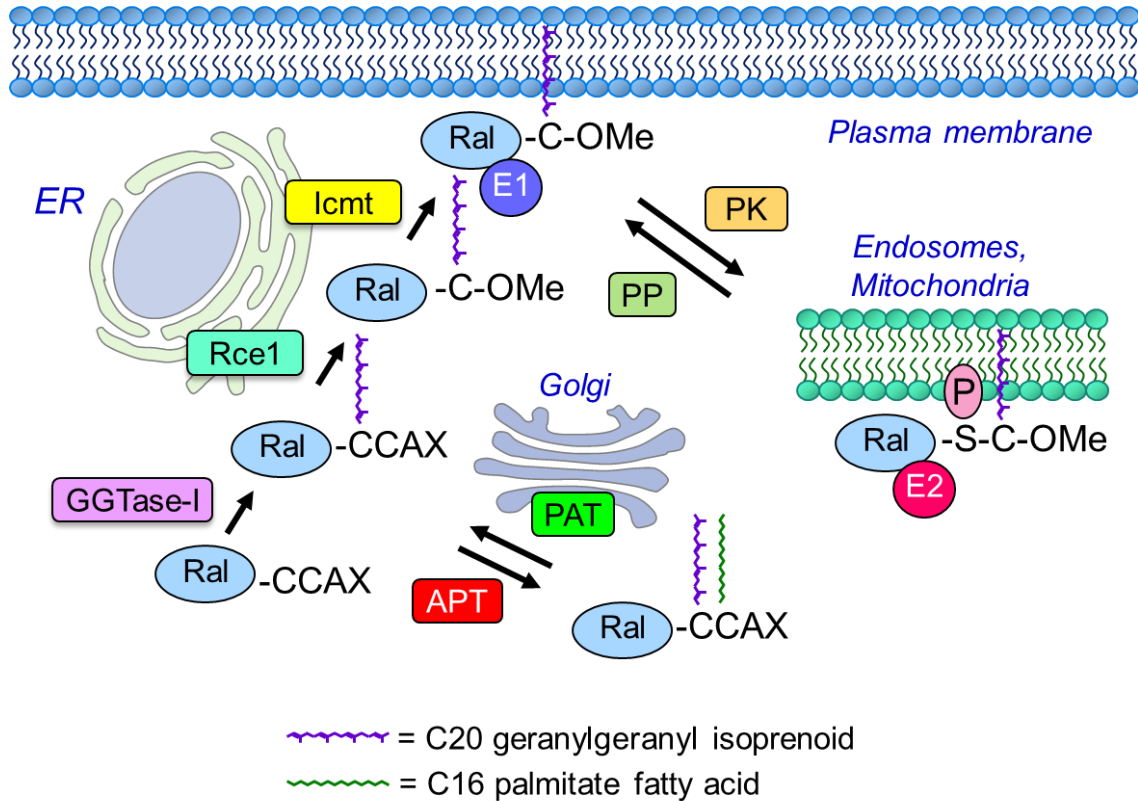


Figure 1-4. Regulation of Ral subcellular localization and membrane association. CAAX motif-signaled posttranslational modifications. Ral is geranylgeranylated by GGTase-I on the first cysteine residue of the CAAX (RalA: CCIL; RalB: CCLL). By the canonical CAAX processing pathway, Ral is then modified at the endoplasmic reticulum (ER) by Ras converting enzyme (Rce1) which cleaves between the two cysteine residues. Isoprenyl cysteine carboxyl methyltransferase (ICMT) then catalyzes methylation of the isoprenylated free cysteine residue, facilitating recruitment to the plasma membrane. A second non-canonical pathway has been described by which Ral is palmitoylated at the cysteine residue at the A1 position by Golgi-associated protein acetyltransferase (PAT) after geranylgeranylation. Palmitate addition is reversible and depalmitoylation is catalyzed by acylprotein thioesterase (APT). Whether this double lipid modified form is associated with a different membrane compartment has not been determined. Ral subcellular localization is also regulated by a dynamic and reversible protein kinase (PK)-mediated phosphorylation and protein phosphatase (PP)-mediated dephosphorylation cycle. RalA and RalB possess distinct C-terminal phosphorylation sites for different protein kinases (PK). Phosphorylation causes dissociation from the plasma membrane and translocation to specific endomembrane compartments, resulting in a switch in effector (E) interaction.

Phosphorylation regulation of subcellular localization and effector interaction.

An emerging

theme in the regulation of small GTPases is reversible post-translational modifications that dynamically regulate subcellular localization, thereby influencing effector interaction and biological activity [77]. In particular, recent studies have highlighted protein kinase-mediated phosphorylation of small GTPases in their C-terminal membrane-targeting regions. For example, K-Ras4B phosphorylation by protein kinase C (PKC) on S181 in its C-terminal membrane targeting sequence altered K-Ras4B subcellular localization [78]. Nonphosphorylated K-Ras4B was plasma membrane associated, whereas S181 phosphorylation K-Ras4B caused translocation to mitochondrial and endoplasmic reticulum (ER) membranes. S181 is positioned within a polybasic amino acid stretch in K-Ras4B that serves as a second signal that together with the CAAX modifications promote full plasma membrane association. The negative charge caused by phosphorylation reduces the positive charge of the polybasic stretch. The ER-associated K-Ras4B then associated with inositol trisphosphate receptors (InsP3) on the ER in a Bcl-xL-dependent fashion, blocking the ability of Bcl-xL to potentiate the InsP3 regulated flux of calcium from ER to mitochondria that is required for respiration, inhibition of autophagy, and cell survival [79].

The Ral proteins are also regulated by similar mechanisms, with distinct protein kinases phosphorylating serine residues distinct for the C-termini of RalA and RalB (Fig. 1-4). Aurora-A kinase and protein kinase A (PKA) have been found to phosphorylate RalA on S194 [80, 81] and protein phosphatase 2A dephosphorylates RalA at S194 as well as S183 [82]. Counter and colleagues showed that phosphorylation of RalA on S194 was critical for RalA to promote the anchorage-independent growth in vitro and tumorigenic growth in nude mice of pancreatic ductal adenocarcinoma (PDAC) cell lines [71]. This phosphorylation event dramatically altered RalA subcellular localization from the plasma membrane to internal membranes, where it had an enhanced interaction with RalBP1. More recently, Aurora-A phosphorylation of RalA has been found to promote RalA translocation to the outer face of mitochondria, where it then recruits RalBP1 to stimulate mitochondrial fission [57].

Studies by our lab and others have found that RalB is similarly regulated by PKC α phosphorylation of S198 in the C-terminal membrane targeting sequence [72, 81]. In one study, it was found that S198 phosphorylation caused RalB translocation from the plasma membrane to endocytic vesicles [72]. Associated with this change in subcellular localization was a switch in effector utilization. Whereas unphosphorylated RalA preferentially associated with Sec5, S198 phosphorylation caused preferential association with RalBP1. Phosphorylation of RalB S198 was necessary for proper exocytic vesicle trafficking and fusion at the plasma membrane, with delivery of surface α -5 integrin being regulated by dynamic RalB phosphorylation. Independently, Theodorescu and colleagues found that phosphorylation of RalB S198 was critical in regulating the ability of RalB to promote the metastatic growth of bladder cancer cells in a nude mouse model [81].

Ubiquitination. In the past few years, regulation of small GTPases by ubiquitination has gained recognition [77]. For example, monoubiquitination of K-Ras on K147 reduces GAP sensitivity, thus allowing K-Ras to remain active and signaling in the absence of upstream input [83]. Ubiquitination of the Ral proteins has also been shown to influence their activity and function. Regulation of the ubiquitination of RalA modulated RalA activity as well as lipid raft exposure [84]. Furthermore, ubiquitination of RalB promoted binding to Sec5 to regulate innate immunity, whereas deubiquitination allowed for binding to Exo84 and subsequent induction of autophagy [85].

Divergent roles of Ral in cancer. Since RalGEFs participate in downstream signaling from activated Ras proteins, it was initially speculated that Ral protein activation may contribute to Ras-driven cellular transformation. However, when explored initially in NIH 3T3 mouse fibroblasts, a critical and significant role for Ral GTPases in Ras-driven cancer seemed unlikely [86, 87]. However, when Counter and colleagues explored the role of Ral in Ras-mediated growth transformation of immortalized human astrocytes, fibroblast or epithelial cells, a more significant role for Ral GTPases as effectors of Ras in

human cancer was observed, suggesting species differences in the effectors that are important in Ras oncogene function [88].

That Ral GTPases serve critical roles in human cancer cell growth gained greater traction when White and colleagues found that RalB was critical for tumor but not normal cells for survival, while RalA was necessary for the anchorage-independent growth of cancer cells [89]. Importantly, this also marked the first time RalA and RalB were found to have non-overlapping functions. Since these key studies, a major theme of Ral proteins is their significant and often divergent roles in numerous cancer types. In the following section we review some of the key findings made with regards to the role of the two Ral isoforms as drivers in different human cancers. Since the RA domain-containing RalGEFs can be activated by other Ras family small GTPases, as well as by non-Ras mechanisms, and since some RalGEFs are regulated by non-Ras mechanisms, an involvement of Ral in cancers where RAS mutations are not common is not surprising.

Bladder carcinoma. Evaluation of a panel of human bladder cancer cell lines found preferentially increased levels of activated RalA and RalB in *RAS*-mutant [90] or invasive cell lines [91]. Using RNAi or ectopic expression of activated Ral mutants, Theodorescu and colleagues found that RalA and RalB played antagonistic roles in the migratory activity of the *KRAS*-mutant UM-UC-3 bladder cancer cell line, with RalA suppressing and RalB enhancing motility [92].

Activating *RAS* mutations occur in a low percentage (~10%) of bladder cancers. Therefore, a Ras-RalGEF mechanism may be less relevant for Ral activation in this cancer type. Consistent with this possibility, a recent study found RalGAP α 2 expression in normal bladder urothelium, but reduced expression associated with advanced clinical stage and poor patient survival [91]. Furthermore, genetic depletion of *Ralgap α 2* in mice did not cause any apparent abnormalities but did enhance the invasive phenotype of chemically-induced bladder tumors. Thus, loss of RalGAP function may be an important mechanism for Ral activation in bladder cancer.

Colorectal carcinoma. Oncogenic *KRAS* and *NRAS* mutations occur in 45% and 8%, respectively, of colorectal cancer (CRC) tumors. Ral signaling has been shown to be a critical regulator of the anchorage-independent growth properties of CRC tumor cells [93]. Martin et al found that RNAi-mediated suppression of RalA resulted in a decrease in soft agar colony growth while loss of RalB had the opposite effect, leading to an enhancement of anchorage-independent growth. They found that RalA and RalB modulated this phenotype by utilizing both common and distinct effector proteins. Using Ral effector binding mutants that are selectively uncoupled from Exo84, Sec5, or RalBP1, they showed that RalA required Exo84 and RalBP1 binding to promote the anchorage-independent growth of CRC cells. Conversely, RalB required Sec5 and RalBP1 to suppress soft agar colony formation. Intriguingly, loss of one Ral isoform was found to increase the activation of the other isoform suggesting compensatory crosstalk between RalA and RalB. What specifically mediates this crosstalk between RalA and RalB is unknown, but it could be through either enhanced RalGEF accessibility for the remaining Ral protein or a downregulation of RalGAP activity upon single Ral isoform depletion. Depletion of RalB has also been shown to cause apoptosis in colorectal cancer cells [61].

Hepatocellular carcinoma. *RAS* mutations are rare (>2%) in hepatocellular carcinoma (HCC). RalA was found to be significantly overactivated in hepatocellular carcinoma (HCC) cells and tissues compared to nonmalignant samples. Suppression of RalA expression caused a significant decrease in the viability and invasiveness of HCC cells. A role for RalB was not addressed. Finally, in a transgenic mouse model for HCC (farnesoid X receptor–deficiency induced) elevated RalA-GTP was detected in the liver tumors [94].

Lung adenocarcinoma. *KRAS* mutations are found in 30% of lung adenocarcinomas and several studies have addressed the role of Ral in lung cancer. In one study, variable levels of RalA-GTP, independent of *KRAS* mutation status, were detected in a panel of lung adenocarcinoma or squamous carcinoma cell lines [95]. shRNA suppression of RalA expression in the *KRAS* mutant A549 lung

adenocarcinoma cell line reduced the proliferation and invasion in vitro. In a second more comprehensive study, immunohistochemistry analyses of non-small cell lung cancers (NSCLC), it was found that high RalA and RalB protein expression was associated with poor survival. The levels of activated RalA but not RalB were higher in *KRAS*-mutant NSCLC cell lines [96]. Depletion of *RALA* or *RALB* or both reduced anchorage-dependent and –independent growth for either *KRAS* mutant and wild type cell lines. Depletion of *RALA*, *RALB*, or both also impaired the tumorigenic growth of *KRAS*-mutant NSCLC cells. Interesting, very limited analyses in this and another study suggested mutation-selective involvement of Ral in NSCLC, where *KRAS* G12C mutant NSCLC cell lines showed greater activation and/or dependence on Ral for growth [97].

In contrast to human lung tumor cell line studies, RalA and RalB were found to exhibit redundant functions when assessed in mouse development and in a *Kras* G12D-driven mouse model of lung adenocarcinoma [73]. *Ralb* deficient mice were viable with no overt phenotype whereas a *Rala* deficiency caused embryonic lethality that was further exacerbated by a combined *Ralb* deficiency. Neither a *Rala* nor a *Ralb* deficiency impaired *Kras*-driven lung tumor development. However, a combined loss of both *Rala* and *Ralb* significantly reduced lung tumor development. These results suggest redundancy in RalA and RalB function for tumor development. One possible explanation for this different conclusion may be that the human lung tumor cell line studies addressed the role of Ral in tumor maintenance whereas the mouse study addressed the role of Ral in tumor initiation and progression.

Malignant peripheral nerve sheath tumors. Loss of the NF1 RasGAP, rather than RAS mutational activation, is seen in neurofibromatosis type 1 and malignant peripheral nerve sheath tumors (MPNST) [98]. When compared with a nontransformed mouse Schwann cell line, RalA-GTP levels were elevated in a panel of MPNST cell lines established from tumors that arose from a *NF1*- and *Tp53*-deficient genetically-engineered mouse model. When evaluated in one cell line, RalA suppression impaired proliferation and invasion in vitro and tumorigenic growth in vivo [99]. RalA activation was also seen in

human MPNST cell lines and tissue, and restoration of NF1 GAP activity reduced RalA activity, indicating that this was associated with Ras activation.

Melanoma. *RAS* mutations, predominantly *NRAS*, occur in 28% of skin cutaneous melanomas. With *BRAF* mutations seen in 60% of melanomas in a non-overlapping frequency with *RAS* mutations, activation of the canonical Raf-MEK-ERK mitogen-activated protein kinase (MAPK) pathway alone may seem to be sufficient for Ras-driven melanoma growth. However, analysis of Ral activation in a panel of human melanoma cells showed a consistently high level of total and activated RalA, but not RalB, activation that was independent of *NRAS* or *BRAF* mutation status [100]. Additionally, RalA and to a lesser degree RalB are necessary for the tumorigenic growth of melanomas, also regardless of *BRAF* and *NRAS* mutation status.

Studies using tumor suppressor Arf-deficient immortalized mouse melanocytes to investigate the contributions of Ras downstream signaling to melanomagenesis also indicated a role for Ral signaling [101]. Ectopic expression of the RalGEF Rgl2 engineered to contain a membrane localization sequence (to mimic Ras activation of RalGEF) was sufficient to promote the anchorage-independent growth and Matrigel invasion of these melanocytes similar to that caused by oncogenic N-Ras. Surprisingly, in contrast, activated *BRAF* V600E did not enhance proliferation or invasion. Finally, ectopic expression of a dominant negative mutant of RalB that blocks RalGEF function partially impaired the growth of *NRAS*-transformed melanocytes. Thus, together with the findings of Zipfel et al. [100], Ral GTPases can act as drivers of melanoma cancer growth in both *RAS* wild type and mutant cancer cells.

Ovarian carcinoma. One study has now revealed that Ral signaling has a role in ovarian cancer. Specifically, higher levels of RalA activity was found in human tumor samples compared to benign samples. Furthermore, depletion of RalA decreased proliferation and invasion of ovarian cancer cell line OVCAR-5 in vitro and decreased tumor genesis [102].

Pancreatic ductal adenocarcinoma. A significant requirement for activated Ral signaling in pancreatic adenocarcinoma (PDAC) cell line tumorigenic and invasive growth has been established. Human PDAC has a high frequency of activating *KRAS* mutations and Ral activation is seen in both human tissue samples and tumor cell lines [103-105]. Interestingly, activation of RalA was found at a higher frequency than the activation of either ERK or AKT in PDAC cells, suggesting a critical role for the RalGEF-Ral pathway downstream of oncogenic K-Ras.

Depletion of RalA and RalB via RNAi has elucidated roles for RalA in anchorage-independent and tumorigenic growth and RalB in invasive and metastatic growth of PDAC cells [105]. PDAC cells with stable RNAi depletion of RalA results in reduced subcutaneous tumor formation upon injection into immune compromised mice. These same cells expressing RalB RNAi do not form lung metastases post-injection into the tail-vein of nude mice. In addition to playing a role in tumor initiation, RalA has also been shown to be necessary for PDAC tumor maintenance. The use of inducible RNAi to stably deplete RalA from established primary tumors resulted in regression of the tumor, indicating a necessity for persistent RalA signaling in established PDAC tumors.

There is also recent evidence that active K-Ras signaling to RalB but not RalA plays a critical role in the formation of invadopodia in PDAC cells [56]. Invadopodia are actin-rich membrane protrusions that are known to be involved in the secretion of matrix metalloproteases (MMP) during tumor cell invasion. RalB requires the ability to interact with RalBP1 to mediate this process and RalBP1 itself is necessary for the formation of invadopodia in PDAC cells. Surprisingly, the RhoGAP activity of RalBP1 is not necessary for invadopodia formation while the ATPase activity is required. Why the ATPase activity is necessary for RalBP1 to mediate invadopodia formation is unclear.

RalGEFs have also been found to play a role in PDAC. Rgl2 is overexpressed in PDAC patient tumors and has been shown to be necessary for both the anchorage-independent and invasive growth of PDAC tumor cells [106]. RNAi-mediated depletion of Rgl2 results in a significant decrease in both RalA

and RalB activation. Interestingly, expression of constitutively active RalA could not rescue soft agar growth after the loss of Rgl2 indicating that Rgl2 may have non-Ral regulatory functions or that the RalA interaction with Rgl2 is critically important for the regulation of anchorage-independent growth. Rgl2 was found to be co-localized with RalB but not RalA at the leading edge of migrating CFPac-1 PDAC cells. Loss of Rgl2 results in a loss of RalB from the leading edge, perhaps giving insight into how the migratory and invasive activity of PDAC cells relies on Rgl2/RalB signaling.

Prostate carcinoma. Increased RalA-GTP levels were observed in the *RAS* wild type human prostate carcinoma cell line PC3. Suppression of RalA did not impair tumor formation but did abolish bone metastasis [107]. In contrast, suppression of RalB expression did not impair metastasis.

Squamous cell carcinoma. Squamous cell carcinoma is the second most common type of skin cancer. Using an in vitro model of Ras-induced human squamous cell carcinoma (SCC) of the skin, it was found that RalA suppressed rather than promoted progression [108]. Suppression of RalA but not RalB stimulated the progression of *HRAS*-transformed human keratinocytes to a more invasive state.

In contrast to the in vitro observations, different roles for Ral were observed in a mouse model of carcinogen-induced SCC [73]. Single application of the mutagen DMBA, followed by repeated applications of phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA) causes *Hras* mutation and the development of benign papillomas, with a subset progressing to SCC. Neither a *Rala* nor a *Ralb* deficiency impaired papilloma development. As described above for *Kras*-driven lung adenocarcinoma formation, only combined loss of both *Rala* and *Ralb* significantly reduced papilloma development. Genetic ablation of *Ralgds* in this same carcinogenesis model also significantly reduced tumor incidence, size, and progression [109].

Conclusions and Future Prospects. Ral GTPase signaling has emerged as being critically important in both normal and neoplastic cell physiology. Over the last two and a half decades we have learned a great deal about how Ral proteins regulate many biological processes. From these studies one of the most

striking observations has been the very distinct functions observed for RalA and RalB despite similar structural and biochemical properties and shared effector utilization. Do these distinct functions simply reflect their spatially distinct interactions with the same set of effectors or are there Ral isoform selective effectors that remain to be discovered? Only recently have RalGAPs been discovered and much more remains to be learned regarding their roles in regulation of Ral activity and signaling. With increasing evidence for key roles for Ral GTPases as drivers in cancer growth, it will be important to identify pharmacologic approaches for targeting aberrant Ral function for cancer treatment. Like Ras, Ral proteins are not tractable therapeutic targets, although recent progress in the identification of direct Ras binders suggests that small GTPases may yet be targeted directly. If not, indirect approaches need to be explored. With kinases implicated as downstream effectors or key regulators of Ral GTPases, can these be exploited for anti-Ral drug development? In summary, with more yet to be learned regarding the function of Ral GTPases, it is quite certain that they will have more than 15 minutes of fame.

Chapter 2: Divergent Roles of CAAX Motif-Signaled Posttranslational Modifications in the Regulation and Subcellular Localization of Ral GTPases¹

Background

As mentioned in Chapter 1, the Ral proteins, like Ras, contain a C-terminal CAAX motif that undergoes prenylation followed by cleavage by RCE1 and carboxylmethylation by ICMT (Fig. 1-4). These processing enzymes are being considered as therapeutic targets in the treatment of cancer since their activities are thought to be necessary for proper subcellular localization and function of CAAX-containing proteins. While their usefulness as therapeutic targets is still not fully understood, progress has been made in further elucidating the roles of RCE1 and ICMT, which will aid in determining how to implement inhibitors against these processing enzymes for the treatment of cancer.

RCE1 was initially discovered as a Ras processing protein in *S. cerevisiae*, where RCE1 disruption was shown to cause Ras mislocalization and signaling [110]. RCE1 is an integral membrane protein of the ER [111]. It is the only enzyme with this particular cleavage activity, and was found to cleave most CAAX sequences efficiently with few exceptions [112-114]. Since targeting Ras has long been of interest in the field of cancer therapeutics, many studies focused on the role of RCE1 catalytic activity in Ras-dependent processes. It has been shown that RCE1 can process all Ras isoforms, and that deletion of RCE1 reduced Ras-driven transformation of mouse embryo fibroblasts [115, 116]. Although there have been numerous discoveries about RCE1 biology, progress toward RCE1 inhibitors has been

¹Adapted from previously published work: Gentry LR, Nishimura A, Cox AD, Martin TD, Tsygankov D, Motohiro N, Elston TC, and Der CJ. "Divergent roles of CAAX motif-signaled posttranslational modifications in the regulation and subcellular localization of Ral GTPases." *J Biol Chem* 2015 290 (37) 22851-62. All figures except Figure 2-6 D and E represent the work of Leanna R. Gentry.

limited. The first documented RCE1 inhibitors were AOMKs ((acyloxy)methyl ketones), which were shown to block K-Ras4B cleavage [117, 118]. Another study showed compounds identified in an *in vitro* screen for small molecule inhibitors of RCE1 that disrupted Ras subcellular localization [119, 120]. However, these inhibitors lack sufficient potency and selectivity and have not been implemented in cancer cell assays. In the absence of useful pharmacologic inhibitors, the assessment of their roles in cancer growth remains unknown and underexplored. Thus, *Rce1*^{-/-} MEFs remain one of the best options for studying the effects of RCE1 loss.

ICMT was initially discovered in *S. cerevisiae* for its ability to methylate a-factor mating pheromone, and it was quickly shown that ICMT methylates Ras as well [121, 122]. ICMT is also localized at the ER [123]. One study found that ICMT was required for Ras-driven myeloproliferative disease. In contrast, a second study demonstrated its absence resulted in exacerbation of K-Ras-driven pancreatic cancer growth. These opposing observations highlight the fact that our understanding of whether ICMT is a useful therapeutic target in Ras-driven cancer remains to be clarified [124, 125]. In contrast to RCE1, there has been better progress in developing and characterizing ICMT inhibitors in cancer models. Many compounds have been found to inhibit ICMT, including methotrexate and lysine nitrilotriacetic acid [126-129]. One such small molecule, cysmethynil, has been shown to decrease both anchorage-dependent and -independent growth and cause mislocalization and impaired signaling of Ras in colorectal cancer cells [130]. However, this inhibitor has off-target activities that complicate interpretation from studies utilizing this inhibitor to assess ICMT as a therapeutic target. In the absence of useful inhibitors, cell models with genetic loss of ICMT function remain the best option. *Icmt*^{-/-} MEFs have been used widely as the cell system most suited to study the impact of ICMT loss on protein function.

Recently, palmitoylation of the second cysteine of CAAX motifs with the sequence CCAX has been reported. This modification was shown to be dependent on prenylation of the first cysteine and

results in a dual prenyl and palmitoyl form of the CCAX-containing protein that then does not undergo subsequent RCE1- and ICMT-mediated processing steps. Palmitoylation at this site of the brain-specific isoform of the Rho family protein Cdc42 was shown to prevent RhoGDI α binding *in vitro* and promote stable Cdc42 membrane association in cells, as opposed to its fully processed form that was instead found in the cytosol in addition to membrane fractions [76]. Furthermore, Cdc42 that was alternatively palmitoylated displayed greater serum response element (SRE) activity than the canonically processed Cdc42, suggesting the palmitoyl form has a greater ability to stimulate downstream signaling. CCAX-containing RalA, RalB, and PRL-3 were shown to be palmitoylated at this site, and other CCAX-containing proteins that undergo prenylation may also be subject to palmitoylation of the motif, but the consequences of this newly-identified processing pathway on Ral GTPase function has not been studied. Unlike Cdc42, Ral GTPases lack a functional counterpart to RhoGDI α . Therefore, the consequences of palmitoylation on Ral function are not known.

Interestingly, although RCE1 and ICMT expression has been shown to be necessary for certain Ras-driven cancers, depletion of either processing enzyme does not fully mislocalize Ras, and it is likely that other RCE1- and ICMT-dependent proteins downstream of Ras are at least partially responsible for mediating these anti-proliferative effects. It is speculated that there may be as many as 400 mammalian CAAX-terminating proteins [131]. Also, while inhibition of palmitoylation is being considered as a therapeutic route in Ras driven cancers given recent findings that palmitoylated KRas4A, as well as N-Ras and H-Ras, are involved in colorectal and other cancers, the consequence of this modification on other small GTPases is not understood. In Chapter 2, I explore the effect of RCE1 and ICMT loss as well as the consequence of palmitoylation on the function of CCAX-containing Ral proteins.

Introduction

The Ras-like (Ral) small GTPases (RalA and RalB) are members of the Ras branch of the Ras superfamily of small GTPases [132-134]. Like Ras, Ral small GTPases function as molecular on-off

switches and cycle between inactive GDP- and active GTP-bound states. The cycle is catalyzed by Ral-selective guanine nucleotide exchange factors (RalGEFs) and GTPase-activating proteins (RalGAPs), which accelerate slow intrinsic exchange and GTPase activities, respectively [106]. Ral GTPases are activated downstream of Ras oncoproteins. Activated Ras-GTP binds to and activates RalGEFs (e.g., RalGDS), stimulating formation of Ral-GTP. Active Ral-GTP binds preferentially to a spectrum of functionally diverse downstream effectors to regulate a diversity of cellular processes that include actin cytoskeletal organization, endocytosis and exocytosis, and mitochondrial function [57, 70, 135, 136] as well as cell proliferation, survival and autophagy [31, 34]. The best validated effectors of Ral are the Sec5 and Exo84 subunits of the exocyst complex and RalBP1/RLIP76, a GAP for Rho family small GTPases [132-134].

RalA and RalB share significant overall sequence, structural, and biochemical identity [134]. Their amino-terminal G-domains (amino acids 12-176) involved in GTP binding and hydrolysis, RalGEF and RalGAP regulation and effector binding share 88% sequence identity. Remarkably, despite their regulation by shared GEFs and GAPs and interaction with shared effectors *in vitro*, RalA and RalB display divergent biological roles in normal and neoplastic cell growth. RalB was found to be specifically required for survival of tumor but not normal cells [89]. In contrast, RalA was dispensable for survival, but was required for anchorage-independent proliferation. Differences in Ral isoform function have also been observed in various cancer types. Distinct aspects of cell migration were differentially regulated by RalA versus RalB in bladder cancer cells [92]. RalA was essential for tumorigenic growth of pancreatic cancer cells, whereas RalB was required for invasive and metastatic growth [105]. In contrast, RalA promoted whereas RalB antagonized anchorage-independent growth of colorectal carcinoma cells [93].

Like Ras, Ral proteins terminate in carboxyl-terminal CAAX tetrapeptide motifs (where C = cysteine, A = aliphatic amino acid, and X = terminal amino acid; Ral amino acids 203-206) which signal for a series of posttranslational modifications that facilitate intracellular trafficking and translocation to the plasma membrane. CAAX-signaled modifications begin with covalent addition of an isoprenoid lipid to

the cysteine residue of the CAAX motif [131]. The X residue determines protein prenyltransferase specificity [74]. RalA and RalB proteins, both of which terminate in leucine, are modified by addition of a geranylgeranyl lipid, catalyzed by cytosolic geranylgeranyltransferase-I (GGTaseI) [75, 137]. Subsequent cleavage of the –AAX tripeptide by endoplasmic reticulum-associated Ras converting CAAX endopeptidase 1 (RCE1) leaves a lipid-modified terminal cysteine. This cysteine is then methylated by endoplasmic reticulum-associated isoprenylcysteine carboxymethyltransferase (ICMT) [138].

The RalA and RalB functional differences identified to date have been attributed to the carboxyl-terminal sequences immediately adjacent to their CAAX motifs [70, 104]. Termed the hypervariable region (HVR; amino acids 177-202), this sequence exhibits the greatest divergence between RalA and RalB (44% identity) and confers secondary membrane-targeting specificity. In particular, the RalA and RalB carboxyl termini possess phosphorylation sites recognized by distinct protein kinases, where phosphorylation causes translocation from the plasma membrane to endomembranes and a change in effector interaction [71, 72, 80-82]. However, posttranslational modifications at other sites may also play an important role in Ral isoform functions.

A recent study of the brain-specific isoform of the Rho family small GTPase Cdc42 concluded that proteins terminating in a CA₁A₂X motif in which the A₁ residue is a cysteine (CA₁A₂X = CCAX) can undergo an alternative processing pathway [76]. While a majority of Cdc42 undergoes the conventional post-prenyl modification pathway involving RCE1 and ICMT, a subset (5-20%) does not undergo RCE1-catalyzed removal of the AAX residues, and instead the A₁ cysteine is covalently modified by palmitoylation. The resulting dually lipid-modified, nonmethylated pool of Cdc42 is no longer regulated by RhoGDI α , leading to enhanced plasma membrane association. The CAAX motif of RalA is CCIL, and that of RalB is CCLL [3], suggesting that, like Cdc42, Ral proteins may also undergo this alternative processing. However, unlike Cdc42, the membrane association of RalA and RalB is not regulated by RhoGDI α . While both RalA and RalB were verified to undergo palmitoylation of the A₁ cysteine of their

CA₁A₂X motifs [76], the impact of dual lipid modification of the CAAX motif on Ral subcellular localization was not determined.

Additionally, the contributions of the CAAX-signaled RCE1 and ICMT modifications to RalA and RalB subcellular localization and membrane association have not been addressed. We therefore determined the consequences of RCE1 or ICMT deficiency on these properties of each Ral isoform. We found that both RalA and RalB required RCE1 for efficient plasma membrane targeting, whereas only RalB required ICMT for this association. Interestingly, while RalA could still concentrate at the plasma membrane in the absence of ICMT, it depended on ICMT for localization to recycling endosomes. We also found that RCE1 deficiency increased steady-state activity of both RalA and RalB, whereas ICMT deficiency increased steady-state levels of RalB but not RalA. Finally, we determined that CA₁AX palmitoylation was essential for plasma membrane association of RalB, but not of RalA. Pharmacologic inhibitors of RCE1 and ICMT are being considered as anti-Ras therapeutic strategies [139]. Since Ral GTPases are key drivers of Ras-dependent cancer growth, it will be critical to understand the complex and differential consequences of RCE1 and ICMT inhibition for Ral function.

Experimental Procedures

Plasmids and Cell Lines – cDNAs encoding green fluorescent protein (GFP)-tagged human RalA was described previously [71] and RalB was generated for this study as described for RalA. Site-directed mutagenesis was done to generate cysteine-to-serine missense mutants of the A₁ cysteine residue of the Ral CCI/LL CA₁A₂X motif, which were then subcloned into pEGFP-C3. The pEGFP-RalA/B chimeric construct was provided by Dr. Christopher Counter (Duke University). The RalB/A chimeric cDNA was subcloned into pEGFP between the BamH1 and Xba1 sites. The pCDH-mCh lentivirus expression vector encoding mCherry-tagged RalA was previously described [72]. All constructs were sequence-verified, and cloning details are available upon request. Mouse embryo fibroblasts (MEFs) deficient in RCE1

(*Rce1^{-/-}*) or ICMT (*Icmt^{-/-}*) were provided by Dr. Steven Young (UCLA) and cultured as described previously [112, 140].

Transfection, Transferrin Labeling, and Microscopy – Cells were plated, transfected, and imaged as described previously [141]. Lipofectamine Plus was used for DNA transfections. Three h after transfection, cells were washed and cultured in phenol-red free Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum. Cells were labeled with transferrin following the manufacturer's protocol (Life Technologies). Cells were imaged in 35-mm culture dishes containing a 15 mm coverslip on the bottom made of number 1.5 glass (MatTek). Cells were examined with an inverted laser-scanning confocal microscope (Zeiss 710 LSM) using an oil immersion 63X objective with a 1.4 numerical aperture. Images of GFP expression were captured by scanning with a 488 nm line of a solid-state laser using a BP 490-555 emission filter. Images of mCh and red fluorescent transferrin probes were captured by scanning with a 555 nm solid-state laser with the variable secondary dichroic set to 580 nm. Images were processed with ImageJ software, and thresholded Manders coefficients using were calculated using the colocalization threshold plugin from the Wright Cell Imaging Facility.

GST Pulldown Assays – Endogenous activated RalA- and RalB-GTP were assayed as we described previously, by pulldown analyses using GST-Sec5-RBD (glutathione S-transferase fusion of the Ral-GTP binding domain of Sec5) [104]. After SDS-PAGE and immunoblotting with the RalA- (BD Biosciences) or RalB- (Millipore) specific antibodies, the amount of active Ral-GTP was normalized to total Ral protein levels in total cellular lysates. Western blot analyses with anti- β -actin (Sigma Aldrich) or anti-GAPDH (Sigma Aldrich) were done to verify equivalent loading of total cellular protein.

Detection of Protein Palmitoylation - Palmitoylated proteins were purified using acyl-resin assisted capture (acyl-RAC). Cells were lysed with RIPA buffer (20 mM Hepes-NaOH pH 7.4, 100 mM NaCl, 3 mM MgCl₂, 1% Triton X-100, 0.5% deoxycholate, and 0.1% SDS) supplemented with protease inhibitor. Cleared lysates were incubated in RIPA buffer containing 0.1% methyl methanethiosulfonate (MMTS) at 42 °C for 30 min with rotation. Proteins were precipitated with acetone, and then resuspended in binding

buffer (100 mM HEPES-NaCl pH 7.4, 1 mM EDTA, and 1% SDS). Samples were mixed with thiopropyl-Sepharose in the presence of 250 mM hydroxylamine to cleave thioester linkages. As a negative control, the same volume of NaCl was added instead of hydroxylamine. After 3 h rotation at room temperature, the resins were washed four times with binding buffer, and samples were eluted in buffer containing 50 mM Tris-HCl pH 6.8, 50 mM DTT, 10% glycerol, and 1% SDS. After immunoblotting with the RalA- and RalB- specific antibodies, the palmitoylation stoichiometry was calculated by a ratio of the precipitate intensity to the input intensity.

Quantification of Fluorescence – The statistics of fluorescence intensity were extracted from images of individual cells as a function of the distance from the cell edge. Cell masks (binary images) were obtained by thresholding with the *MovThresh* module of the *CellGeo* package [142]. Each cell mask was then morphologically eroded using the MATLAB function “imerode” with a disk of radius 1 to 50 pixels as a structural element. At each step of the morphological erosion, the boundary was traced and the intensity of the corresponding boundary pixels was determined from the original image. This allowed the mean, median, and standard deviation to be calculated for each cell as function of the distance 1 to 50 pixels from the cell edge. Finally, the intensity profiles for individual cells were normalized to their peak (max) values, and then averaged over all cells in a given genotype. These four processing steps constitute a single MATLAB code for batch processing.

Results

RalA and RalB show distinct requirements for RCE1- and ICMT-mediated posttranslational modifications for their subcellular localization and plasma membrane association - We first addressed the importance of RCE1- and ICMT-catalyzed modifications in regulating RalA and RalB subcellular localization and membrane association. Since there are currently no potent and selective pharmacologic inhibitors of these enzymes, we utilized mouse embryo fibroblasts (MEFs) deficient in expression of either RCE1 or ICMT [112, 140]. We transiently transfected wild-type (WT), *Rce1*^{-/-} or *Icmt*^{-/-} MEFs with expression constructs encoding RalA or RalB, which were amino-terminally tagged with green

fluorescent protein (GFP) (Fig. 2-1A). To quantitate changes in Ral plasma membrane association, we developed a MATLAB image processing script to analyze fluorescence measurements for localization patterns in individual cells (Figs. 2-1B and 2-1C). In particular, we quantified the GFP intensity as a function of the distance from the plasma membrane to the nucleus. As described previously [70, 72, 136], we found that RalA and RalB each exhibited both plasma membrane and endosome localization in WT MEFs (Fig. 2-1A). However, we found that, in *Rce1*^{-/-} MEFs, both RalA and RalB were mislocalized, with diminished plasma membrane association and increased diffuse cytoplasmic accumulation, although their endosome localization remained intact (Fig. 2-1A). These results were somewhat unexpected, since a previous study suggested that small GTPases modified by the hydrophobic geranylgeranyl lipid attached to Ral proteins would not be highly dependent on ICMT- and RCE1-catalyzed modifications for efficient plasma membrane association [143].

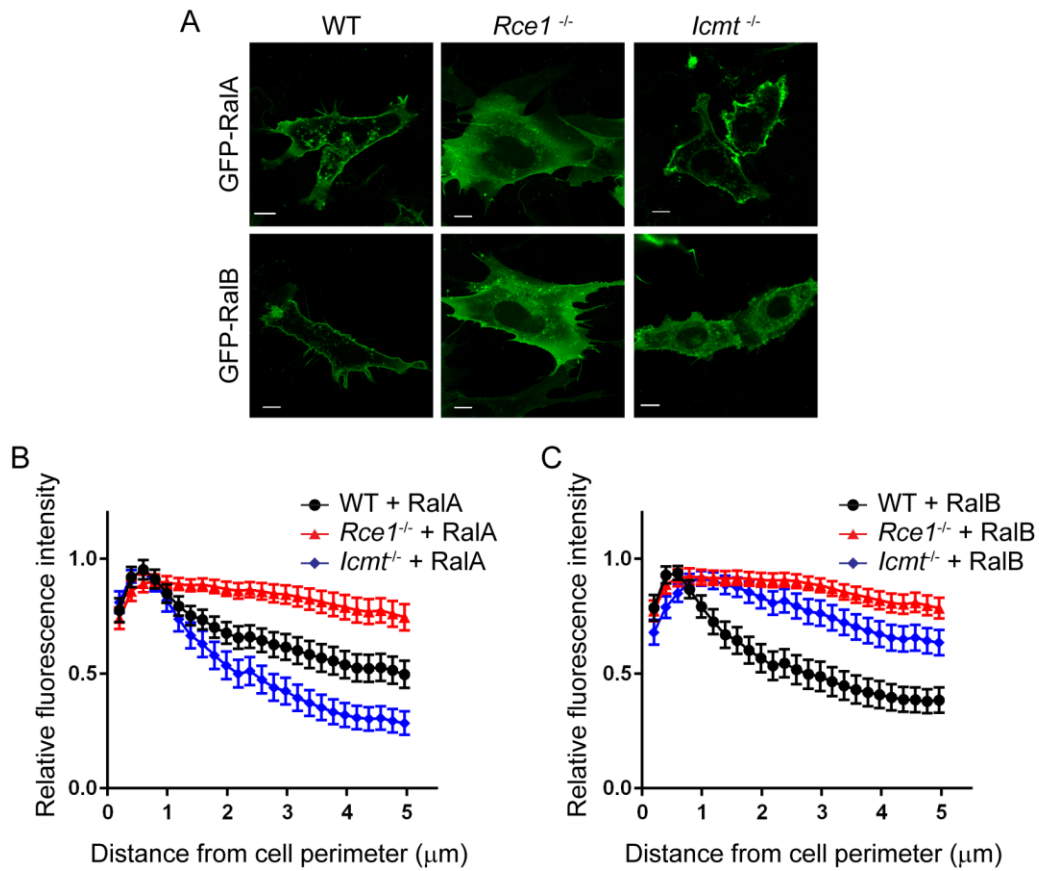


Figure 2-1. Subcellular localization of Ral family proteins is differentially dependent on RCE1 and ICMT processing. Wild-type (WT), *Rce1*^{-/-}, and *Icmt*^{-/-} MEFs were transiently transfected with expression vectors encoding EGFP-tagged RalA or RalB. *A*, live cells were visualized using confocal microscopy. Images shown are representative of three independent experiments, each of which examined >40 cells. Images were analyzed using our MATLAB image processing script for *B*, EGFP-RalA distribution and *C*, EGFP-RalB distribution in WT, *Rce1*^{-/-}, and *Icmt*^{-/-} MEFs. Scale bar, 10 μm.

Since RCE1 deficiency also prevents the subsequent ICMT modification, we next determined the consequences of ICMT deficiency alone. Although the plasma membrane targeting of both Ral isoforms depended similarly on RCE1, surprisingly, we found a differential requirement for ICMT in regulating RalA versus RalB plasma membrane targeting. In *Icmt*^{-/-} MEFs, we observed that RalA remained concentrated at the plasma membrane, similar to its localization in WT MEFs. Thus, the significant disruption of RalA plasma membrane association in *Rce1*^{-/-} cells was caused by the absence of AAX proteolysis. In striking contrast, RalB association with the plasma membrane was reduced significantly in *Icmt*^{-/-} MEFs, instead showing increased diffuse localization in the cytosol. Thus, plasma membrane association of RalB displays a greater requirement for ICMT compared to that of RalA.

We next investigated the basis for the different susceptibility of RalA and RalB to ICMT deficiency. Although their sequence divergence is greatest in the HVR (44% identity), there is also significant sequence divergence in the carboxyl-terminal portion of the G-domain (residues 91-153; 68% identity) (Fig. 2-2A). To distinguish between the importance of these two blocks of sequence divergence in ICMT-dependent Ral localization, we utilized chimeric RalA and RalB proteins that terminate in the reciprocal HVR (Fig. 2-2A). RalA/B is comprised of the RalA N-terminus (residues 1-176), which includes RalA sequences involved in effector and regulatory protein binding, and the carboxyl-terminal HVR of RalB (residues 177-204) involved in membrane targeting. Conversely, RalB/A is comprised of the N-terminus of RalB (residues 1-176) followed by the carboxyl-terminal HVR of RalA (residues 177-204). We transiently expressed GFP-fusion proteins of RalA, RalB, RalA/B or RalB/A in WT or *Icmt*^{-/-} MEFs and observed their localization patterns. As expected, in WT MEFs the RalA/B and RalB/A chimeric proteins retained the predominantly plasma membrane localization patterns of full length RalA and RalB (data not shown). However, in *Icmt*^{-/-} MEFs, whereas RalB/A retained the strong plasma membrane subcellular localization of full length RalA (Figs. 2-2A and 2-2B), the RalA/B chimeric protein exhibited a loss of plasma membrane association similar to that seen with full length RalB. We conclude that the distinct

consequences of ICMT deficiency seen with RalA and RalB are due to sequence divergence in the HVR rather than in the G-domain.

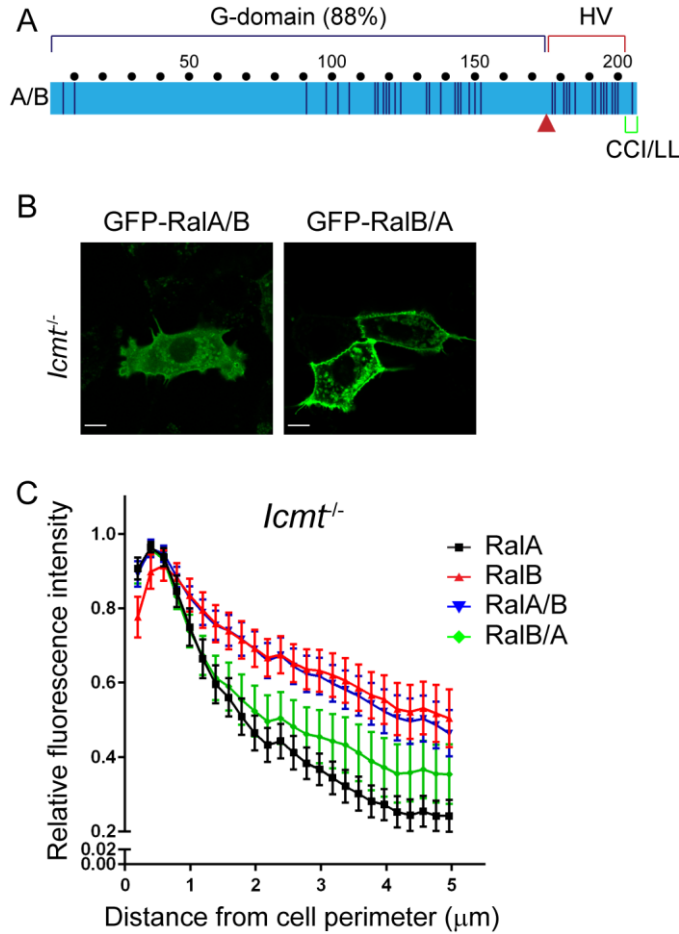


Figure 2-2. Hypervariable domain dictates Ral ICMT dependency. *A*, Chimeric Ral proteins were constructed by a reciprocal switch of the carboxyl-terminal residues 176-206 (red arrow) comprised of the hypervariable domain and the CAAX motif. RalA/B is comprised of the RalA G-domain (residues 1-176) followed by RalB residues 177-206, whereas RalB/A is comprised of the RalB G-domain (residues 1-176) followed by RalA residues 177-206. WT, *Rce1^{-/-}* and *Icm1^{-/-}* MEF cells were transiently transfected with expression constructs encoding EGFP-tagged Ral chimeras RalA/B or RalB/A. *B*, live cells were visualized by confocal microscopy. Images shown are representative of three independent experiments, each of which examined >40 cells. *C*, Images from *B* were analyzed using our MATLAB image processing script. Scale bar, 10 μm .

Icmt regulates RalA localization to recycling endosomes - Although ICMT deficiency did not noticeably impact RalA plasma membrane targeting, we did observe a decreased level of endomembrane-associated RalA in *Icmt*^{-/-} MEFs (Fig. 2-1A). Therefore, we used our MATLAB image processing script to quantify the spatial distribution of GFP-RalA. Our analysis revealed a decreased concentration of RalA closer to the nucleus in *Icmt*^{-/-} MEFs. In contrast, RalB association with internal organelles was not impacted significantly by ICMT deficiency (Fig. 2-1B). Thus, RalA and RalB association with endomembranes exhibited different requirements for ICMT.

Since RalA is known to localize to recycling endosomes in various cell types [70, 136], we speculated that the RalA endomembrane localization that was disrupted in the absence of ICMT included this compartment. To address this possibility, we detected recycling endosomes by live cell microscopy using a fluorescent transferrin probe, and measured the colocalization of GFP-RalA with transferrin-loaded endosomes. Image analysis revealed a significant decrease in colocalization of RalA with transferrin in *Icmt*^{-/-} MEFs compared to WT MEFs, indicating that RalA localization to recycling endosomes was impaired in the absence of ICMT (Figs. 2-3A and 2-3B).

To further validate this finding, we also measured RalA colocalization with a second marker of recycling endosomes, Rab9. For these analyses, we co-transfected expression vectors encoding mCherry-tagged RalA and GFP-Rab9 into WT or *Icmt*^{-/-} MEFs. Colocalization analysis again showed a significant reduction in localization of RalA to Rab9-containing endosomes in *Icmt*^{-/-} MEFs (Figs. 2-3C and 2-3D). These results demonstrate that loss of ICMT significantly impaired RalA localization to recycling endosomes.

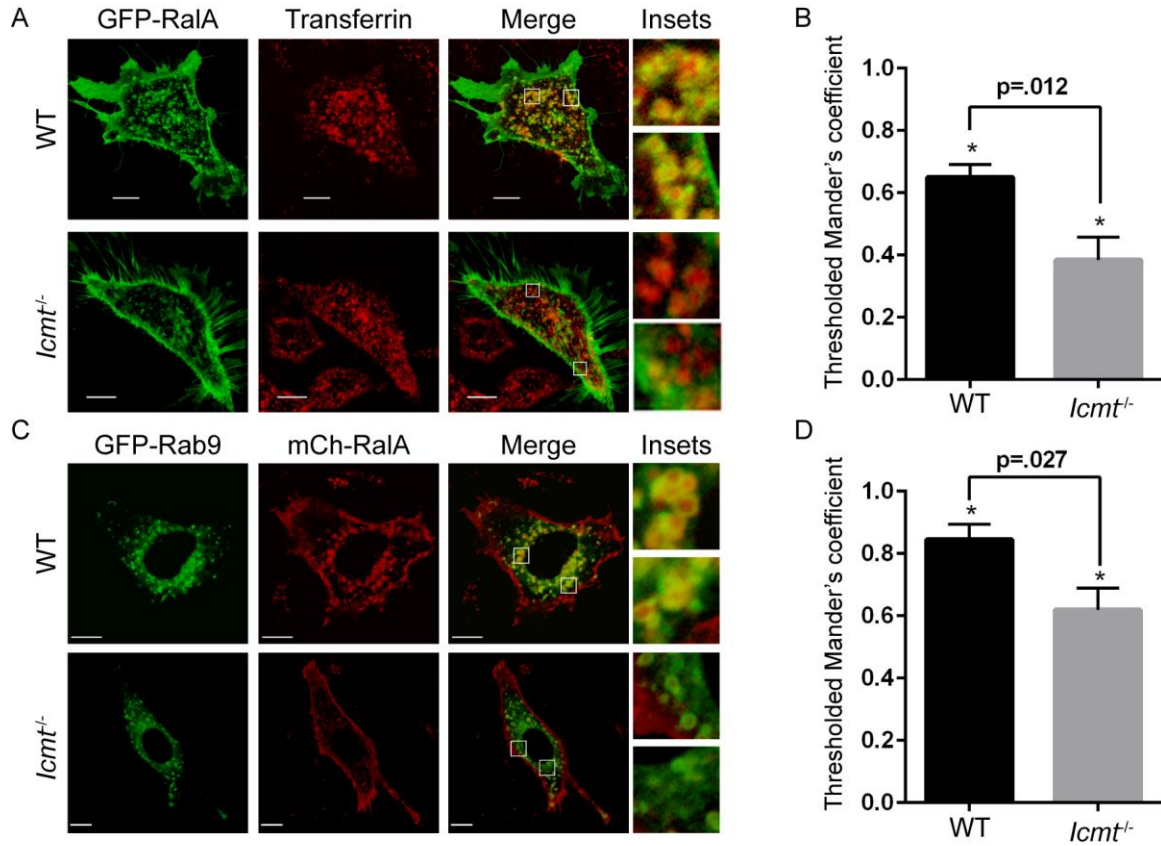


Figure 2-3. RalA localization on recycling endosomes is impaired by loss of ICMT. *A*, WT and *Icm1*^{-/-} MEF cells were transiently transfected with an expression construct encoding EGFP-RalA and incubated with pHrodo red transferrin conjugate for 30 min before visualization by confocal microscopy. The images were overlapped (Merge) to indicate the degree of colocalization on endosomes in WT cells compared with *Icm1*^{-/-}. *B*, Twenty-five images per condition were quantified. Values shown are means of Manders coefficients using thresholds \pm S.E., and an unpaired *t* test was used to determine significance ($p < 0.05$). *C*, WT and *Icm1*^{-/-} MEF cells were transiently transfected with expression constructs encoding EGFP-Rab9 and mCh-RalA. Cells were visualized by confocal microscopy. Images were merged as described for panel *A*, and *D*, were quantified as described in panel *B*. Scale bar, 10 μ m.

RCE1 regulates Ral activity – Since we previously observed that phosphorylation-mediated translocation of RalA or RalB from the plasma membrane to endosomes was associated with an increase in active GTP-bound protein [71, 72], we speculated that the altered subcellular localization of RalA and RalB seen in RCE1- or ICMT-deficient cells might also be accompanied by altered regulation of their GDP-GTP cycle and consequently of their activation state. To address this possibility, we first performed pulldown analyses to determine the level of activated, GTP-bound endogenous RalA and RalB in WT, *Rce1*^{-/-} and *Icmt*^{-/-} MEFs. When normalized to total protein, we found that the steady-state levels of both endogenous RalA-GTP and RalB-GTP were elevated strikingly in *Rce1*^{-/-} MEFs compared to WT MEFs (Fig. 2-4A). In contrast, there was limited increase in RalA-GTP and no increase in RalB-GTP levels in *Icmt*^{-/-} MEFs, although RalB protein levels were markedly higher in these cells compared to WT MEFs.

The elevated activity of the Ral GTPases observed in the absence of RCE1 when compared to WT cells suggests differential regulation of the mislocalized Ral proteins by their regulatory RalGAPs or RalGEFs. To address this possibility, we evaluated the kinetics of RalA-GTP formation in response to external stimuli. Growth factor-stimulated Ral activation is mediated through transient activation of Ras, leading to RalGEF activation and a rapid and transient increase in RalA-GTP [144], with RalGAP activity then terminating the activation. A previous study showed that, in cells depleted of RalGAP, epidermal growth factor (EGF) treatment resulted in an increased level of Ral activation and a decreased rate of termination of activation, leading to sustained Ral activity in stimulated cells [26]. Therefore, we compared the kinetics of endogenous Ral-GTP formation in response to EGF in WT versus *Rce1*^{-/-} MEFs.

We stimulated serum-starved WT and *Rce1*^{-/-} MEFs with EGF and then monitored the GTP-bound levels of RalA and RalB over time by pulldown analyses (Fig. 2-4B). While the activity of both RalA and RalB increased in response to EGF stimulation and returned to near-basal levels at the same rate in both cell types, we did find that EGF stimulation caused a significantly greater initial increase in RalA-GTP and RalB-GTP activity in *Rce1*^{-/-} MEFs compared to WT MEFs (Figs. 2-4C and 2-4D). Thus, the Ral

mislocalization that we observed in *Rce1*^{-/-} MEFs is more likely to alter their regulation by one or more of the 6 RalGEFs than by RalGAPs [134].

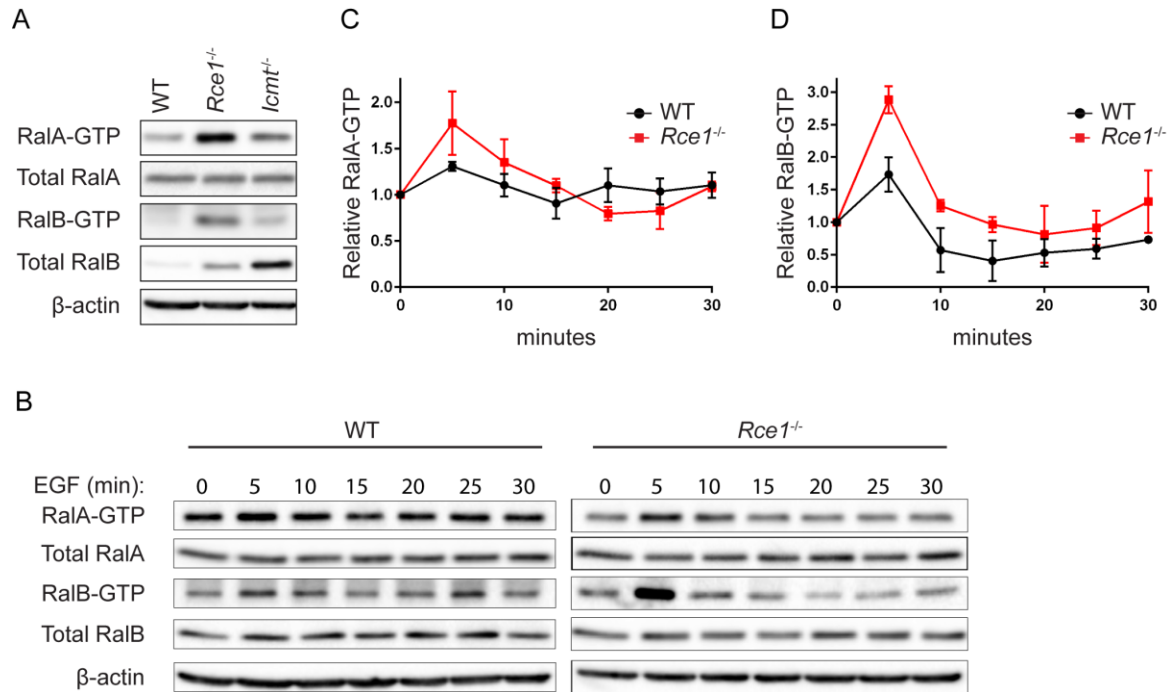


Figure 2-4. RCE1 deficiency increases activated Ral-GTP formed upon EGF stimulation. *A*, Endogenous levels of RalA-GTP and RalB-GTP were compared in WT, *Rce1*^{-/-} and *Icmt*^{-/-} MEFs by a pulldown assay using GST-Sec5 RBD. Western blot analyses were done with anti-RalA or anti-Ral antibody to determine levels of Ral-GTP and total Ral proteins. β-actin was used to verify equivalent total protein loading. *B*, WT and *Rce1*^{-/-} MEFs were serum-starved then stimulated with EGF. Cells were lysed at the indicated time points, followed by western blot analyses as described in panel *A*. *C*, RalA-GTP and *D*, RalB-GTP expression levels were quantified by densitometry of western blot data in panel *B* and normalized to total RalA and RalB, respectively.

Icmt regulates RalB stability – As described above, we observed that levels of endogenous RalB but not RalA proteins were stably elevated in *Icmt*^{-/-} MEFs compared to WT MEFs (Fig. 2-4A). Previous studies demonstrated that RhoA protein was destabilized upon pharmacologic inhibition or genetic loss of ICMT, whereas Ras protein was stabilized [145, 146]. While we did find that the steady-state transcript levels of RalB were slightly greater in *Icmt*^{-/-} MEFs compared to WT MEFs (Fig. 2-5A), this small change likely was an insufficient basis for the large difference in abundance of RalB protein in the two cell types (Fig. 2-5B). We therefore hypothesized that the increases in RalB protein in *Icmt*^{-/-} MEFs were due to altered regulation of RalB stability in the absence of ICMT.

To investigate if RalB turnover was affected by loss of ICMT, we treated WT and *Icmt*^{-/-} MEFs with cycloheximide to block protein synthesis and then measured changes in protein levels as the preexisting RalB protein was degraded over time. We found that RalB exhibited a shorter half-life in WT MEFs compared to *Icmt*^{-/-} MEFs, with only 20% RalB protein remaining in WT MEFs after 18 h of cycloheximide treatment compared to ~60% in *Icmt*^{-/-} MEFs (Fig. 2-5C). This difference indicates that ICMT regulates RalB protein stability and promotes its degradation.

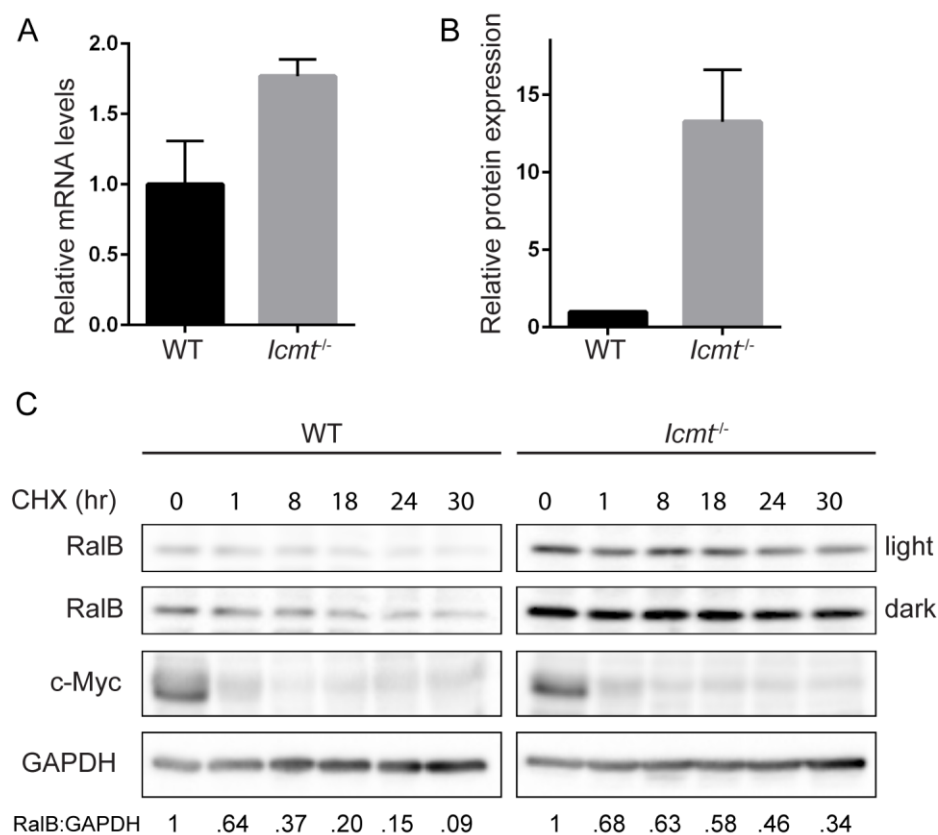


Figure 2-5. ICMT deficiency increases RalB protein stability. *A*, *RalB* transcript levels were determined by TaqMan qPCR analysis and normalized to *GABPB1* in a triplicate set of assays \pm SE. Data displayed as relative to WT MEFs. *B*, Endogenous RalB protein levels were quantified by densitometry of western blot and normalized to total protein. Data displayed as relative to WT MEFs. Experiment performed in triplicate. *C*, WT and *Icmt*^{-/-} MEFs were treated with 25 μ M cycloheximide and lysed at the indicated time points. Lysates were probed by western blot for total RalB, c-Myc, and for GAPDH to verify equivalent total protein. Protein levels from blots in panel were quantified by densitometry and normalized to 0 h.

Dual lipid modification of the Ral CAAX motif affects RalB subcellular localization – In addition to the conventional modification pathway involving RCE1 and ICMT, RalA and RalB can also undergo an alternative pathway, whereby the newly GGTaseI-modified protein does not undergo subsequent RCE1-mediated proteolytic removal of the A₁A₂X residues but instead undergoes palmitoylation of the cysteine residue at the A₁ position [76]. While the mechanism that regulates which pathway will be taken has not been elucidated, it was reported that the level of palmitoylation of RalA was increased in RCE1-deficient MEFs. Thus, impairment of one pathway can then favor a shift to the other pathway. We hypothesized that the altered subcellular localization of RalA and RalB that we observed in *Rce1*^{-/-} MEFs (Fig. 2-1) might reflect not only loss of the RCE1- and ICMT-catalyzed modifications but additionally reflect alterations in dual lipidation by geranylgeranylation and palmitoylation of the CAAX motif.

To determine a role for this alternative dual lipid modification pathway in regulating RalA and RalB localization, we first generated the same palmitoylation-deficient Ral mutants utilized by Nishimura and Linder in defining the pathway [76]. To do so, we introduced a C to S amino acid substitution at the A₁ position of each Ral isoform (C204S), designated RalA-CSIL and RalB-CSLL. This substitution ablates palmitoylation but not geranylgeranylation of the CAAX motif [76]. Although we did not observe a significant alteration in RalA-CSIL subcellular localization compared to WT RalA, we did find reduced association of RalB-CSLL with the plasma membrane, along with increased cytosolic accumulation (Fig. 2-1A, 2-6A). To address the role of geranylgeranylation in Ral localization, we generated nonprenylatable RalB-SCLL and RalA-SCIL C203S mutants that are deficient in both the geranylgeranyl and palmitate modifications. As expected [75], we found that these mutants were completely disrupted in both plasma membrane and endomembrane localization, and showed a diffuse cytoplasmic distribution including nuclear accumulation (Fig. 2-6B). We used our MATLAB image processing script to quantify the spatial distribution of the nonpalmitoylated and nonprenylated mutant Ral proteins (Fig. 2-6C). We found that the alternative dual lipid modification site of the RalB but not the RalA CAAX motif affects subcellular localization to the plasma membrane. In support of this conclusion, analysis of palmitoylation of

endogenous RalA and RalB revealed that the level of RalB palmitoylation is approximately two-fold greater than that of RalA (Fig. 2-6D and 2-6E). An explanation of this result is that disruption of the palmitoylatable cysteine of RalB also disrupted prenylation. To address this, we inhibited geranylgeranylation of the palmitoylation-defective RalB-CSLL mutant with the pharmacological GGTaseI inhibitor GGTI-2017. This treatment resulted in a localization similar to that of the nongeranylgeranylated mutant RalB-SCLL (Fig. 2-6F). We conclude that disruption of the palmitoylatable cysteine does not disrupt geranylgeranylation.

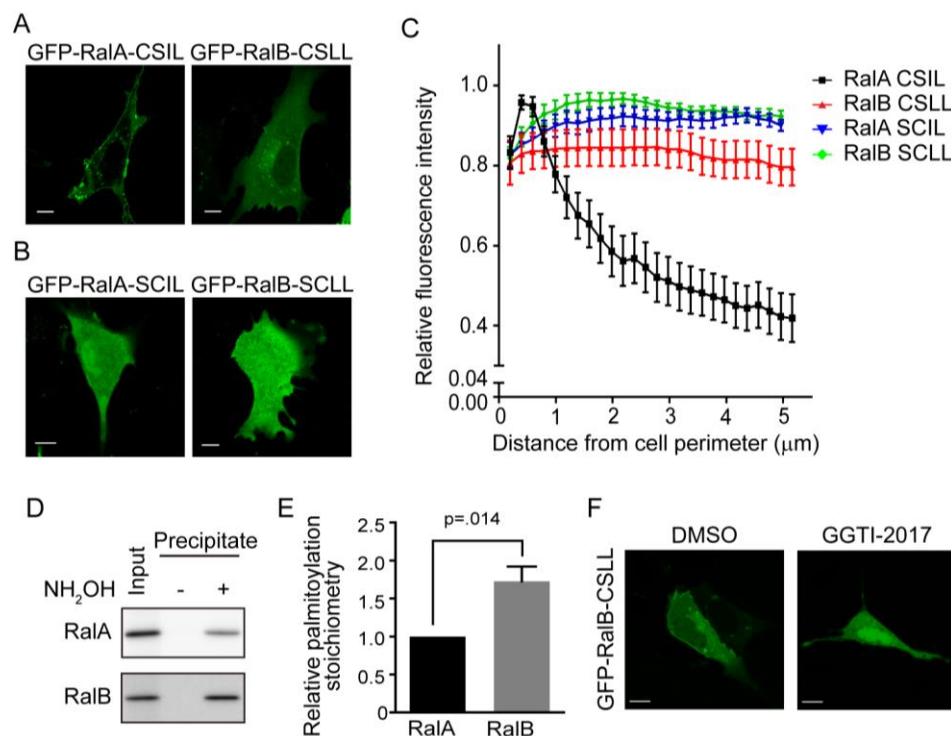


Figure 2-6. CA₁AX palmitoylation dictates RalB subcellular localization. WT MEFs were transiently transfected with an expression construct encoding either nonpalmitoylatable or nonprenylatable CAAX mutants of EGFP-RalA or EGFP-RalB, and then visualized by confocal microscopy. *A*, Nonpalmitoylatable C204S CA₁AX mutants (RalA-CSIL or RalB-CSLL) or *B*, Nonprenylatable C203S SAAX mutant (RalA-SCIL or RalB-SCLL). *C*, Images from *A* and *B* were analyzed using our MATLAB image processing script. *D*, Hydroxylamine (NH₂OH)-sensitive palmitoylated proteins from WT MEF lysates were immobilized on thiopropyl-Sepharose resin using resin assisted capture (acyl-RAC), and palmitoylation of RalA and RalB was detected by immunoblotting. *E*, Palmitoylation stoichiometry was calculated by a ratio of the precipitate intensity to the input intensity. Data represent the mean \pm S.E. (n = 6). *F*, WT MEFs were transiently transfected with the nonprenylatable C203S mutant EGFP-RalB-CSLL as in panel *B*, but then treated with 25 μM GGTI-2017 or DMSO control for 24 h before visualization by confocal microscopy.

Discussion

Despite their significant sequence, structural, and biochemical similarities, RalA and RalB often serve divergent roles in normal and neoplastic cell functions [132-134, 147]. In this study, we determined whether the CAAX-signaled posttranslational modifications of proteolysis, isoprenylcysteine carboxylmethylation, and CAAX motif palmitoylation could contribute to the distinct regulation of RalA and RalB. We found that both RalA and RalB require RCE1 function for association with the plasma membrane but not with endomembranes. In contrast, ICMT deficiency disrupted RalB association with the plasma membrane, whereas it disrupted RalA association with endosomes. Assessing the consequences for Ral regulation, we found that RCE1 deficiency increased the steady-state activation state of both RalA and RalB, whereas ICMT deficiency regulated protein stability of RalB, but not RalA. Finally, we determined that the A₁ cysteine in the CA₁A₂X motif that leads to alternative modification by the fatty acid palmitate was more significant for RalB localization than for RalA. (Ral proteins do not contain the upstream palmitoylatable cysteines present in the HVR of many Ras family small GTPases [3].) We conclude that the differential roles of CAAX-signaled posttranslational modifications contribute significantly to the distinct regulation of RalA and RalB.

Previous reports have reached conflicting conclusions regarding the contribution of CAAX-signaled postprenyl modifications in promoting proper subcellular localization and membrane association of small GTPases. One study showed that both RCE1 and ICMT were critical for localization of farnesylated but not geranylgeranylated Ras and Rho proteins [143], whereas we found that loss of either RCE1 or ICMT did significantly impact the subcellular localization and membrane association of geranylgeranylated Rho GTPases [148]. Although total membrane-bound RalA was shown previously to be unaffected by loss of ICMT, its differential membrane compartmentalization was not examined, and neither the membrane association nor localization of RalB was investigated [143]. In the present study, we have demonstrated that the geranylgeranylated proteins RalA and RalB also require RCE1 and ICMT for proper subcellular localization. Thus, we conclude that the specific prenyl group modifying CAAX-containing proteins is not sufficient by itself to predict dependency on RCE1- and ICMT-catalyzed modifications. Since our

studies utilized RCE1- and ICMT-deficient cells, we recognize that the consequences of their loss on Ral subcellular localization may be indirect and not due specifically to disrupted modification of Ral itself. However, since no known CAAX-terminating proteins have been described that interact with Ral, we feel that our results do likely demonstrate direct roles of these modifications in Ral function.

Although RalA and RalB both localize to the plasma membrane and to endosomes, they exhibited striking differences in the requirement of RCE1 and ICMT to facilitate their association with these two membrane compartments. Loss of RCE1 disrupted association of both RalA and RalB with the plasma membrane but not with endosomal membranes. In contrast, loss of ICMT disrupted RalB but not RalA plasma membrane association, while conversely disrupting endosome localization of RalA but not RalB. Utilizing chimeric Ral proteins, we determined that this differential regulation was due to sequence divergence in the HVR rather than in the G-domain. Both HVRs are largely lysine-rich sequences and contain no other obvious membrane targeting information. Therefore, how each HVR impacts the different consequences of ICMT deficiency is not clear. However, they do diverge in the location and sequence context of the serines that serve as phosphorylation sites for an isoform-specific variety of protein kinases [71, 72, 80-82]. We conclude that inhibition of ICMT is a possible approach to blocking endosomal RalA function, and that this would provide a level of selectivity over inhibition of RCE1. Endosomal RalA function is important for cell processes including exocytosis [135, 149], cytokinesis [136], and cell polarity maintenance [70]. Although it is unclear why prenylation of RalA followed by RCE1 processing alone is sufficient to take RalA to the plasma membrane, we previously found that the RhoB small GTPase also showed the same dependency [148]. Thus, with some prenylated proteins, carboxymethylation is dispensable for plasma membrane association. It is also possible that, since ICMT has additional substrates compared to RCE1, mislocalization of these other proteins may be affecting RalA and RalB differently.

In addition to altered subcellular localization, we also observed consequences for Ral activation in RCE1-deficient cells. We found that loss of RCE1 enhanced the steady-state activity of RalA and RalB.

We speculated that this altered activity is a consequence of their altered subcellular localization and therefore of their interaction with RalGEFs or RalGAPs. Our analyses found that the kinetics of Ral-GTP formation upon EGF stimulation was consistent with increased activation by RalGEFs rather than decreased inactivation by RalGAPs. Since Ral proteins are displaced from the plasma membrane in *Rce1*^{-/-} MEFs, there could be increased exposure of Ral to specific RalGEFs on internal membranes. For example, the RalGEF RGL2 is enriched on recycling endosomes [149]. We conclude that RCE1 deficiency leads to aberrant RalA and RalB activation, and suggest that therapeutic intervention targeting RCE1 in Ras-dependent cancers could have pitfalls in unintentional Ral effector pathway activation.

Another consequence of the loss of CAAX-signaled modifications was the increased steady-state level of RalB protein but not RNA in *Icmt*^{-/-} MEFs. Previous studies reported that ICMT-deficient, mutant K-Ras-transformed MEFs exhibited increased Ras protein stabilization but conversely a decreased half-life of RhoA protein but not RNA. However, no mechanistic basis for these observations was described [145]. While ubiquitination of Ral has been implicated in Ral-dependent lipid raft trafficking [84], a degradation mechanism for Ral has not been described. Perhaps RalB interaction with an E3 ubiquitin ligase controlling Ral stability is affected by mislocalization associated with loss of ICMT. Alternatively, the absence of the methylation modification, independent of altered localization, may alter RalB interaction with an E3 ligase. Consistent with this possibility, there is evidence that prenylcysteine methylation of CAAX-containing proteins can also play a role in regulating protein-protein interactions [150-153]. In summary, our observations provide the first evidence that RalB protein stability may be regulated by a posttranslational mechanism.

Pharmacologic inhibition of CAAX-signaled modifications has been pursued extensively as an approach to inhibit Ras for cancer treatment. Initial efforts targeted the first step, catalyzed by farnesyltransferase. Unexpectedly, farnesyltransferase inhibitors (FTIs) were ineffective in blocking the membrane association of K-Ras and N-Ras, due to FTI-induced alternative prenylation by GGTaseI [154, 155]. Since blocking Ras membrane association remains an attractive approach [156], pharmacologic

inhibition of RCE1 and ICMT has been considered. Initial cell-based studies using RCE1- or ICMT-deficient MEFs found that K-Ras-induced growth transformation was impaired [116, 145]. However, subsequent mouse model studies indicate context-dependent consequences. In a mouse model of K-Ras-induced myeloproliferative disease, loss of ICMT ameliorated tumor growth whereas loss of RCE1 accelerated it [124, 157]. More recently, it was found that ICMT deficiency enhanced K-Ras-driven pancreatic cancer development [125]. A logical explanation for these different consequences for Ras-driven oncogenesis is that ablation of RCE1 or ICMT expression alters the function of other CAAX-terminating prenylated proteins, including small GTPases such as Ral and Rac that function downstream of Ras effector signaling, or of Ras family proteins that can act as tumor suppressors [158]. In conclusion, given the large number of other RCE1 and ICMT substrates, the usefulness of pharmacologic inhibitors of RCE1 and ICMT as anti-Ras drugs will likely be highly context-dependent.

Chapter 3: Targeting TBK1 in *KRAS*-mutant Pancreatic Ductal Adenocarcinoma

Background

Genetic ablation of Ral function has been shown to impair the development and growth of *RAS*-mutant cancers [89, 132, 134]. Therefore, Ral inhibition may be a viable strategy to block Ras for cancer treatment [134]. However, directly targeting Ral GTPases presents the same challenges associated with directly inhibiting Ras. Like Ras, Ral also has a high binding affinity to GTP and a relatively smooth protein surface with few to no hydrophobic binding pockets to accommodate a potent and selective small molecule inhibitor[139]. While several approaches have been taken to overcome these obstacles and directly target Ras and Ral [139, 159, 160], whether these can be advanced to clinically useful inhibitors is unclear. Therefore, as with Ras, indirect approaches for blocking Ral function are being considered as points of intervention. In Chapter 2, I examined the potential for targeting CAAX motif-signaled processing enzymes that are anticipated to contribute to regulating Ral subcellular localization and interaction with specific membrane compartments. Altered localization in turn may regulate Ral interaction with specific effectors, leading to altered signaling and biological activity. Here, I examine another possibility, inhibiting Ral effector signaling. While the best-characterized Ral effectors are RalBP1/RLIP72 (a RhoGAP) and the Sec5 and Exo84 subunits of the exocyst complex [134, 161], these are also not considered highly tractable drug targets. Instead, since protein kinase inhibitors represent the

¹This work was performed in collaboration with research scientists at Eli Lilly and Company. All figures represent the work of Leanna R. Gentry except Figures 3-1 – 3-3.

most successful class of targeted therapies for cancer, I focused on inhibiting the TRAF family member-associated NF-kappa-B activator (TANK) binding kinase 1 (TBK1), a kinase known to be a downstream effector of RalB.

TBK1 is an atypical member of the I κ B kinase (IKK) family with a normal role in innate immunity. It is a mediator of signaling through Toll-like receptor (TLR), leading to activation of interferon regulatory factors that control transcription of genes involved in immune response[162]. TBK1 was discovered less than two decades ago in a yeast-two-hybrid screen to identify binding partners of TANK, and was found to function in a TBK1-TANK-TRAF2 (TRAF2: TNF receptor-associated factor 2) complex, where its activity was required to mediate TANK activation of NF- κ B [163]. Since then, several modes of TBK1-dependent NF- κ B activation have been identified. Phosphorylation of IRF3 by TBK1 has been shown to activate TNF α and subsequently NF- κ B[164, 165]. TBK1 activity has also been shown to play a role in phosphorylation and subsequent degradation of I κ B α , thus releasing NF- κ B to allow translocation to the nucleus. Despite these discoveries, the precise mechanism by which TBK1 activity regulates NF- κ B still is not fully understood, and it is becoming apparent that it is highly context-dependent[166].

Aberrant activation of TBK1 has been implicated in numerous cancers, including breast[167, 168], lung [62, 169, 170], pancreatic[171], and skin[172]. Since its identification as a potential requirement for cancer cell growth driven by mutant K-Ras [62], TBK1 has gained traction as a potential target in the treatment of cancer. Studies have elucidated how TBK1 functions downstream of Ras in cancer. The RalB-Sec5 effector complex has been shown to recruit TBK1 and decrease apoptosis induction by stress on the cell[61], and this complex can also initiate IRF3 response to viral infection[85]. Furthermore, TBK1 activation downstream of an integrin α v β 3-K-Ras-RalB signaling pathway has been shown to promote tumor cell anchorage independent growth and resistance to receptor tyrosine kinase (RTK) inhibitors [173].

This growing line of evidence for the involvement of TBK1 activity in promoting cancer has led to the development of pharmacologic inhibitors targeting TBK1 [174, 175]. In this chapter, I study the effect of a novel TBK1 inhibitor, LSN3090729, on cancer cell growth and signaling, and explore combining the inhibitor with other targeted therapies to find a method of achieving greater efficacy in decreasing PDAC cell growth.

Introduction

TANK-binding kinase 1 (TBK1) is a noncanonical I κ B kinase with a pivotal role in regulating cellular host defense mechanisms. In response to inflammatory cytokines and Toll-like receptor (TLR) activation, TBK1 is activated and phosphorylates substrates including interferon regulatory factor 3 (IRF3) to induce transcription of genes that mediate the innate immune response [162, 176]. Interestingly, there is increasing evidence of aberrant activation of TBK1 having a role in cancer cell proliferation and survival, and the kinase has therefore gained traction as a possible therapeutic target for cancer.

TBK1 is overexpressed in pancreatic, colorectal, breast, and lung cancer, and its aberrant activation has been shown to play a role in proliferation and survival of cancer cells [61, 169, 177, 178]. Specifically, TBK1 can be activated by the RalGEF (Ras-like GTPase guanine nucleotide exchange factor) effector pathway downstream of Ras, whereby RalB engages exocyst component Sec5, promoting Sec5 binding to and activation of TBK1 [61]. TBK1 then phosphorylates various effectors, including Sec5, which ultimately leads to BCL-xL (B-cell lymphoma extra-large) and NF- κ B (nuclear factor κ B) activation and subsequent transcription of survival genes [61, 178, 179]. While TBK1 has been shown to be important for cancer growth and maintenance, the exact mechanisms governing cancer cell dependence on TBK1 activity remain unclear. Studies have shown certain cancer cells harboring K-Ras mutations require TBK1 for survival while others demonstrate that TBK1 is dispensable in other Ras-mutant cell lines [62, 171, 180].

Here, we seek to further understand the role of TBK1 activity in promoting cancer, and specifically address its function in pancreatic ductal adenocarcinoma (PDAC) growth. Since PDAC, a highly lethal cancer with limited treatment options, is characterized by an oncogenic K-Ras mutation in greater than 90% of cases, inhibition of TBK1 has been considered as an approach in the treatment of the disease. Inhibitors against the major effector pathways of Ras, including the Raf-MEK-ERK and PI3K-Akt pathways, have thus far proven ineffective in reducing tumor burden, leading to an alternative approach of targeting the RalGEF-Ral pathway, which has been implicated in PDAC tumorigenesis and metastasis[105, 181]. TBK1 is one of two kinases defined downstream of the RalGEF-Ral signaling axis and therefore is considered one of the most tractable targets in this pathway[134].

The emerging evidence of a role for TBK1 in human cancers as well as continued interest in its infection response mechanism has led to development of small molecule inhibitors targeted against TBK1 kinase activity. The first such inhibitor, BX795, was originally discovered as a PDK1 inhibitor, and its amino-pyrimidine component is a base found in newer compounds that have allowed for the probing of the role of TBK1 [171, 174, 175, 182]. BX795 and a less promiscuous inhibitor, AZ909, were shown to inhibit clonogenic colony formation of N-Ras-mutant melanoma cell lines [172]. Studies of recently discovered inhibitors have begun to define TBK1-dependent mechanisms in cancer cells [172, 183, 184]. However, the efficacy seen due to TBK1 inhibition in certain systems is not applicable to all cancers. One study of genetic and pharmacologic inhibition of TBK1 showed that inhibiting TBK1 activity, measured by disruption of IRF3 phosphorylation, is not sufficient for growth inhibition of PDAC cells, suggesting that a combination therapy approach may be necessary for the therapy to be effective [180].

Here, we used a novel TBK1 inhibitor to further explore the effect of inhibiting TBK1 in PDAC cells. We surveyed a large panel of cancer cell lines and found that, despite inhibiting TBK1 enzymatic activity, treatment with LSN3090729 had a minimal and Ras-independent effect on their anchorage-dependent proliferation. Minimal impact on anchorage-independent proliferation was also observed in a

panel of lung cancer cell lines as well as tumor xenografts of skin, lung, and pancreatic cancer. We found only a small subset of PDAC cell lines tested were sensitive to LSN3090279. Previous studies both in PDAC and in the context of TBK1 show that single-agent inhibition is ineffective, suggesting that combination approaches may be necessary. Interestingly, addition of ERK inhibition to TBK1 inhibition produced a synergistic decrease in anchorage-dependent proliferation and also induced apoptosis in resistant lines. The results of our study suggest that combined TBK1 and ERK inhibition may be effective in reducing PDAC growth.

Experimental procedures

Cell lines and plasmids – PDAC cell lines were obtained from ATCC and maintained in either DMEM-H or RPMI-1640 supplemented with 10% fetal calf serum. A validated short hairpin RNA (shRNA) sequence shT15 for human TBK1 was provided by Dr. Paul Kirschmeier (Dana-Farber Cancer Institute) and was cloned as previously described [180].

Inhibitor treatment assays – Sensitivity of PDAC cells to LSN3090279, alone or in combination with SCH772984, was determined by MTT assay. Briefly, LSN3090279 was serially diluted from 10 μ M to 0.019 μ M in a 96 well plate with or without a constant concentration of SCH772984. Cells were seeded at a density of 3×10^3 cells per well and allowed to proliferate at 37°C and 5% CO₂ for 72 hours. Then, cells were treated with 5 mg/mL MTT for 4 hours at 37°C and formazan crystals were then solubilized with DMSO. The absorbance at 550 nm was recorded.

2D clonogenic growth assays were performed by seeding cells in 6 well plates at 500 to 1,000 cells per well. Cells were allowed to adhere for 24 hours and were then treated with varying concentrations of LSN3090279 for 10 days. Colonies were stained with 2 mg/mL crystal violet in 4% paraformaldehyde for 10 min and counted using Image J.

Immunoblotting – Cells were lysed in NP40 buffer supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktails. Protein concentration was determined by a Bradford Protein Assay (Bio-

Rad). Proteins were separated by SDS-PAGE gel electrophoresis, transferred to PVDF (Immobilon), and probed with either anti-TBK1 (Cell Signaling), anti-phospho-TBK1 S172, anti-IRF3 (Cell Signaling D83B9), anti-phospho-IRF3 S396 (Cell Signaling 4D4G), anti-GAPDH, anti-LC3, anti-cleaved caspase-3 (Cell Signaling), anti-RSK (Cell Signaling 32D7), or anti-phospho-p90RSK S380 (Cell Signaling 9D9). Blots were imaged with the BioRad ChemiDoc and analyzed using Image Lab and ImageJ software.

Results

LSN3090279 is a TBK1 inhibitor – We synthesized LSN3090279, a 4-aryl-2-aminopyrimidine derivative, and have characterized the compound as a selective TBK1 kinase inhibitor with an *in vitro* IC₅₀ ranging from 19 nM to 73 nM (data not shown). We tested LSN3090279 for inhibition of a panel of over 100 kinases and found minimal off-target effects (Fig. 3-1A, 3-1C).

We further characterized LSN3090729 by testing its ability to inhibit TBK1 signaling to known phosphorylation targets IRF3 and Akt in cells. First, we stably expressed TLR4 and IRF3 in HEK293 cells to ensure the known upstream and downstream components of the TBK1 signaling pathway were intact in our model system. We then stimulated TBK1 activity by treatment with lipopolysaccharide (LPS) and used an Acumen-based assay to measure the ability of LSN3090729 to block LPS-induced TBK1-mediated phosphorylation of its direct substrate IRF3 (Fig. 3-1B). LSN3090729 blocked phosphorylation of IRF3 with an IC₅₀ of 125 nM. To extend our cellular analysis, we compared genetic depletion of TBK1 to pharmacologic inhibition of its kinase activity and examined downstream TBK1 signaling to Akt. We either stably suppressed TBK1 expression by shRNA or inhibited TBK1 kinase activity with LSN3090729 in Panc1 cells (Fig. 3-1D and 3-1E). We then treated cells with 100 ng/mL EGF and confirmed that phosphorylation of Akt that was dependent on TBK1 expression was also reduced in the presence of LSN3090729 (Fig. 3-1E). Collectively, these results show that LSN3090279 is active in the cell and is inhibiting TBK1 signaling.

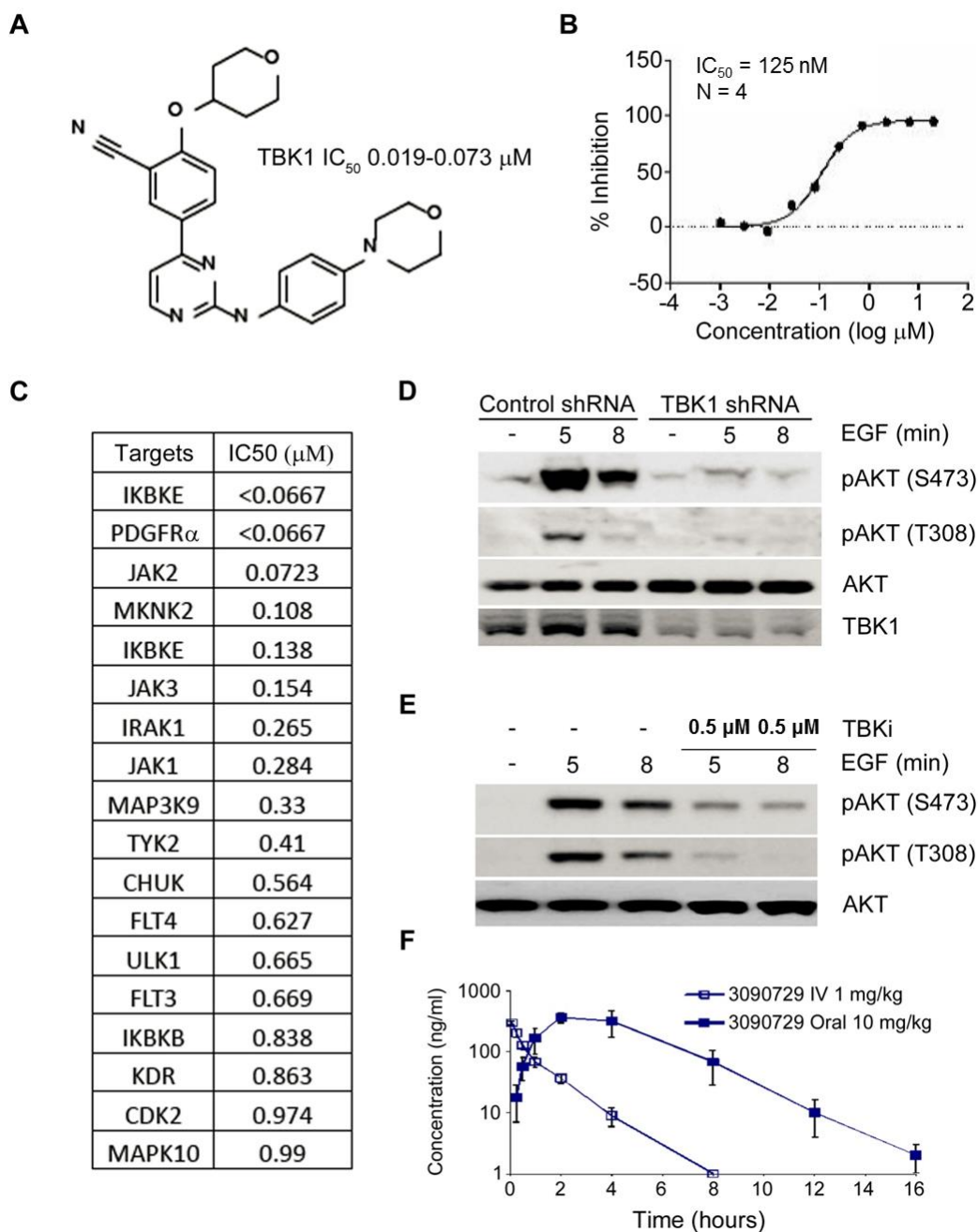


Figure 3-1. LSN3090729 inhibits TBK1. A) LSN3090729 discovered as a TBK1 inhibitor with IC_{50} 0.019-0.073 μ M. B) LSN3090729 has cellular activity. It inhibits LPS-induced phosphorylation of IRF3 in HEK293 cells. C) Off-target effects under 1 μ M. D) TBK1 shRNA and E) LSN3090729 inhibits EGF-mediated AKT phosphorylation in Panc1 cells. F) LSN3090729 pharmacokinetic profile in rats.

LSN3090279 has a minimal effect on cancer cell proliferation *in vitro* and *in vivo* – The effect of TBK1 depletion or inhibition of cell proliferation across different cancer types may be highly context-dependent. TBK1 was shown to be dispensable for the proliferation of a panel of K-Ras-mutant-containing PDAC cells but was required for K-Ras-dependent lung cancer cell proliferation [62, 180]. Furthermore, separate studies have provided conflicting evidence regarding whether TBK1 inhibition is lethal to cells in Ras-dependent cancers. To investigate this further, we sought to determine the impact of TBK1 inhibition by LSN3090729 on cancer cell proliferation, and whether this dependence on TBK1 was dictated by the presence of an activating Ras mutation. To address this, we treated a panel of over 300 cancer cell lines of varying Ras mutation status with LSN3090279 from 0 to 20 μ M and assessed its anti-proliferative activity in 2D culture. We found a large range of GI50 values (where GI50 is the concentration required for 50% growth inhibition), with the majority of cells displaying a GI50 > 10 μ M and with GI50s not dependent on Ras mutation status (Fig. 3-2A). We extended this study to examine the effects of LSN3090729 on anchorage-independent growth in a select panel of cancer cell lines (Fig. 3-2B). Again, we found dependence on TBK1 did not correlate with Ras mutation status. Overall, LSN3090279 was found to have only a minimal effect on cancer cell proliferation *in vitro*.

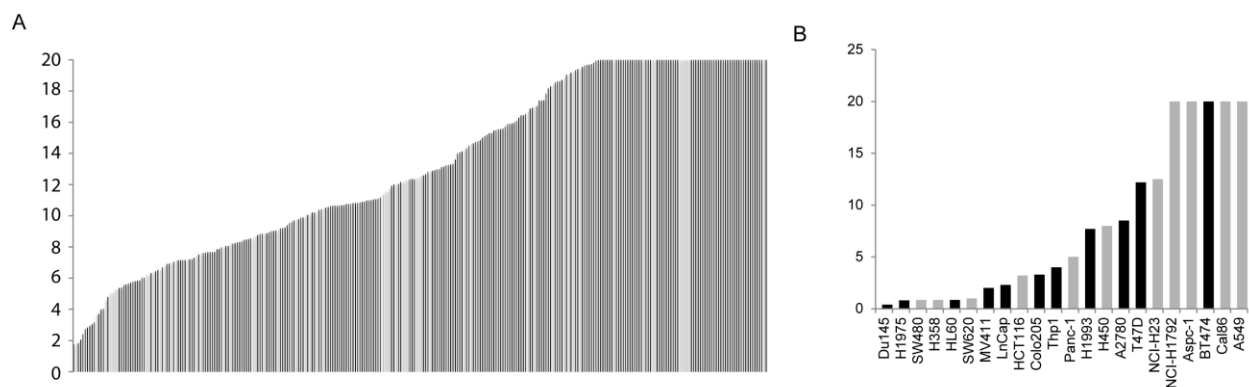


Figure 3-2. LSN3090729 has K-Ras-independent effect on cancer cell growth. A) Panel of cancer cell lines used in 2D Cell-TiterGlo assay (grey: mutant K-Ras; black: wild-type K-Ras) B) Panel of cancer cells in 3D soft agar assay (grey: mutant K-Ras; black: wild-type K-Ras)

PDAC cell lines show varying sensitivity to pharmacologic TBK1 inhibition – Our data suggest that the efficacy seen with TBK1 inhibition is not solely dependent on the presence of a Ras mutation. However, TBK1 is a known mediator of Ras signaling and remains a tractable target due to its kinase activity. Therefore, it remains a potential therapeutic target for Ras-driven cancers. Because of this, we chose to study the effect of LSN3090729 in PDAC, a cancer characterized by an oncogenic K-Ras mutation in greater than 90% of cases. We determined the impact of LSN3090729 on proliferation of PDAC and normal immortalized pancreatic epithelial cells. Anchorage-dependent proliferation was measured after treatment with LSN3090729 for 72 hours (Fig. 3-3A). We found that only two PDAC cell lines, Panc1 and HPAC, were sensitive to TBK1 inhibition (GI50 of 2.59 μ M and 3.41 μ M, respectively) while six PDAC cell lines and the two normal pancreas cell lines were considered insensitive (GI50 > 8 μ M). However, LSN3090729 was found to decrease phosphorylation of IRF3 serine 396 at IC50 values near 2 μ M in both the sensitive and resistant lines (Fig. 3-3C and 3-3D). This demonstrates that TBK1 was not always sufficient to reduce proliferation, consistent with previous studies showing differential effects on the proliferation of PDAC cell lines upon TBK1 inhibition [180]. Cell lines identified as sensitive in 2D culture also showed greater response to inhibitor treatment in clonogenic assays compared to resistant lines (Fig. 3-3B). These sensitivity differences observed in clonogenic assays were not reflected upon depletion of TBK1 by shRNA. However, given that the TBK1 scaffolding function may have different effects on downstream signaling than its enzyme activity does, this result is not surprising and is supported by previous results showing no impact on PDAC cell growth using this TBK1-targeting sequence (Fig. 3-4B).

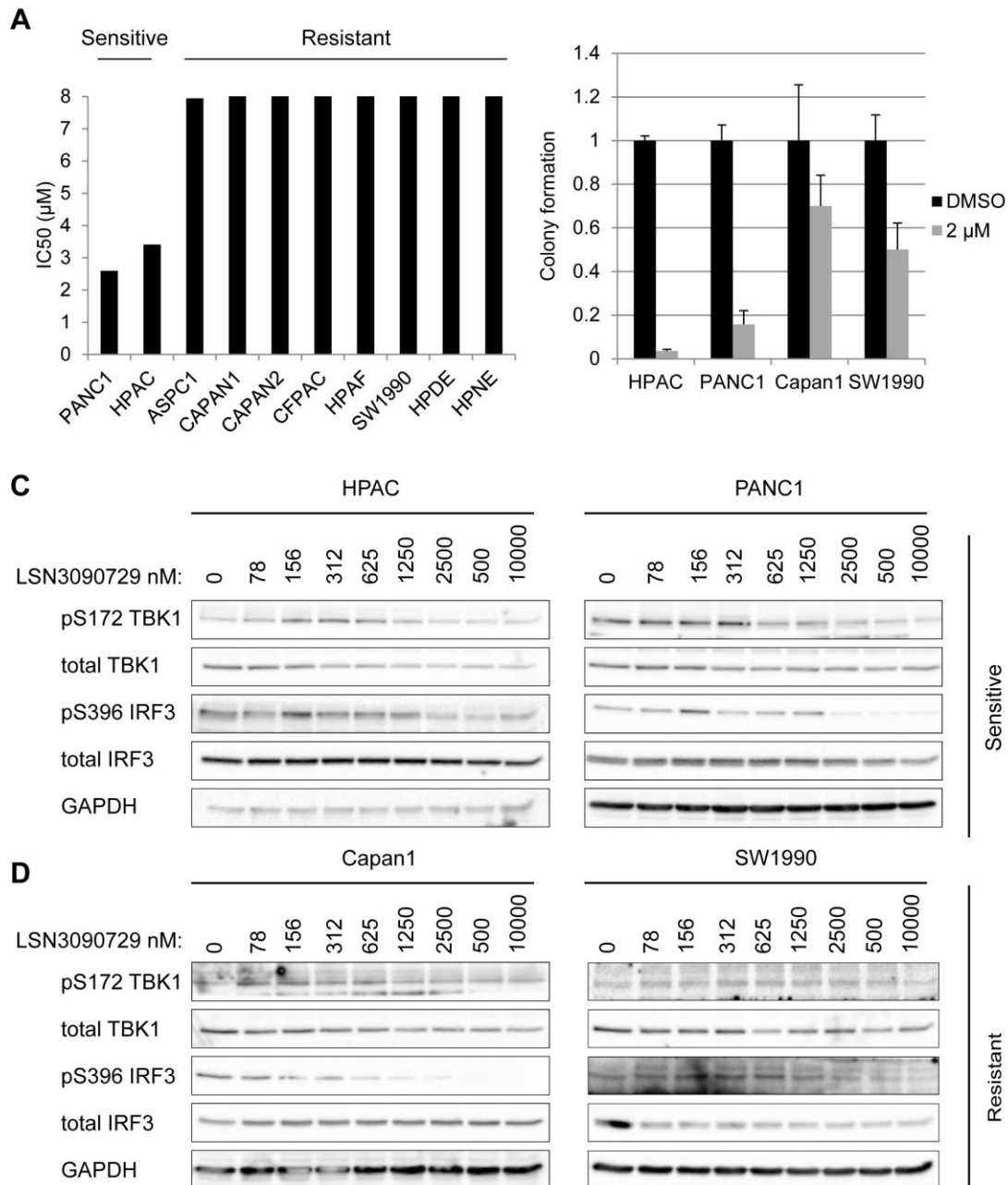


Figure 3-3. PDAC cells are resistant to LSN3090279. A) IC50 of PDAC cells treated with LSN3090279 and analyzed at 72 hours using an MTT assay. B) Cells were seeded for clonogenic assays on day 0, treated with LSN3090279 at indicated concentrations on day 1, and colonies were quantified at day 10. C) Sensitive and D) resistant PDAC cells were probed as indicated at 72 hours post-treatment with LSN3090279.

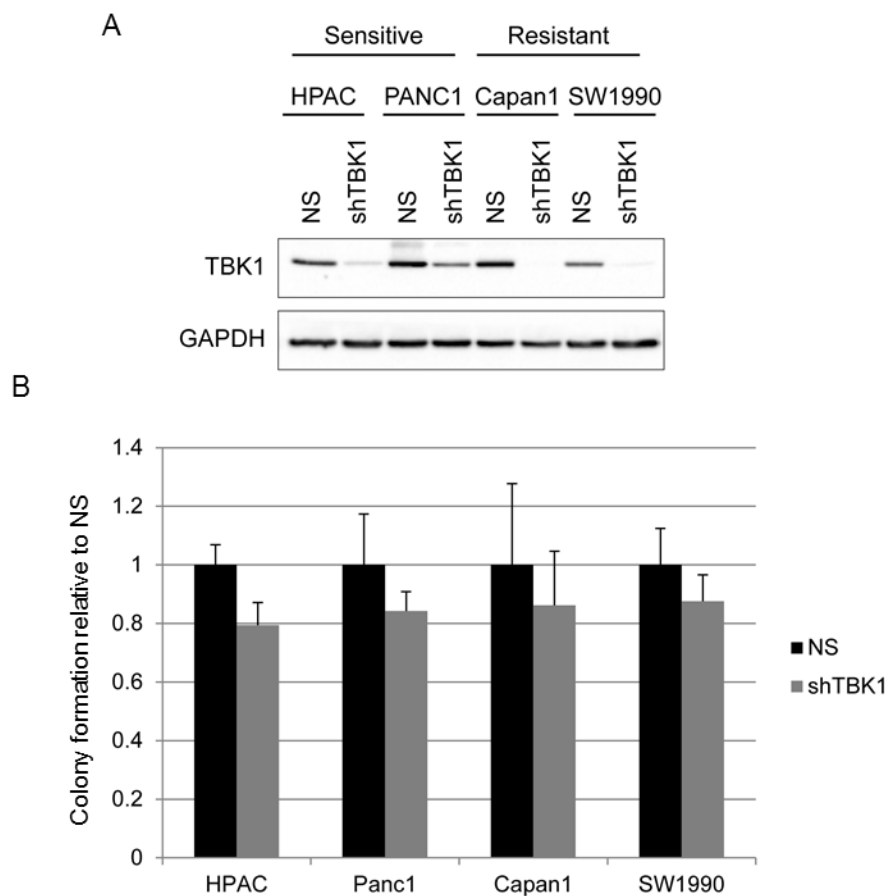


Figure 3-4. TBK1 depletion does not decrease colony formation. A) TBK1 was stably knocked down by shRNA. B) Cells were seeded for clonogenic assays on day 0 and colonies were quantified at day 10.

While TBK1 has an autophosphorylation site at serine 172, this has been found to not be indicative of TBK1 activation, contrary to what is often found in other kinases [174]. Instead, it was previously determined that IRF3 phosphorylation levels are elevated chronically in PDAC cells and that phosphorylation of IRF3 serine 396 is a reliable marker of TBK1 inhibition [180]. We sought to determine if inhibition of phosphorylation of IRF3 or TBK1 by LSN3090729 correlated with sensitivity to growth inhibition using two sensitive and two resistant PDAC cell lines. We found that reduction in phosphorylation of IRF3 tracked closely with increasing doses of LSN3090729, whereas varying effects on TBK1 phosphorylation were observed (Figs. 3-3C and 3-3D), which is also consistent with previous

studies. Furthermore, in our two sensitive cell lines, Panc1 and HPAC, we observed that 50% inhibition of TBK1 as measured by phosphorylation of IRF3 (IC₅₀) correlated with GI₅₀. Conversely, the IC₅₀ of two resistant cell lines, SW1990 and Capan1, was much lower than that of the GI₅₀. These results suggest that, while IRF3 phosphorylation is a reliable marker of TBK1 inhibition by LSN3090729, inhibition of IRF3 phosphorylation does not always correlate with TBK1-dependent proliferation in resistant cells.

Interestingly, upon treatment with LSN3090729, we observed a rapid formation of large vacuoles that remained unresolved after 96 hours (Fig. 3-5A), although these vacuoles did not form after depletion of TBK1 by shRNA. Due to the known role of TBK1 in autophagy and the relation of these vacuoles to autophagolysosome formation in previous studies, we sought to determine if autophagy was misregulated in LSN3090729-treated cells [170, 185-187]. We observed an increase in total LC3BI and LC3BII as well as an increasing ratio of LC3BII to LC3BI, indicating that autophagy was induced and that lysosomal clearance was disrupted (Fig. 3-5B). Further work is required to determine the effect of this LSN3090729-dependent regulation of autophagy on cancer cell proliferation and survival.

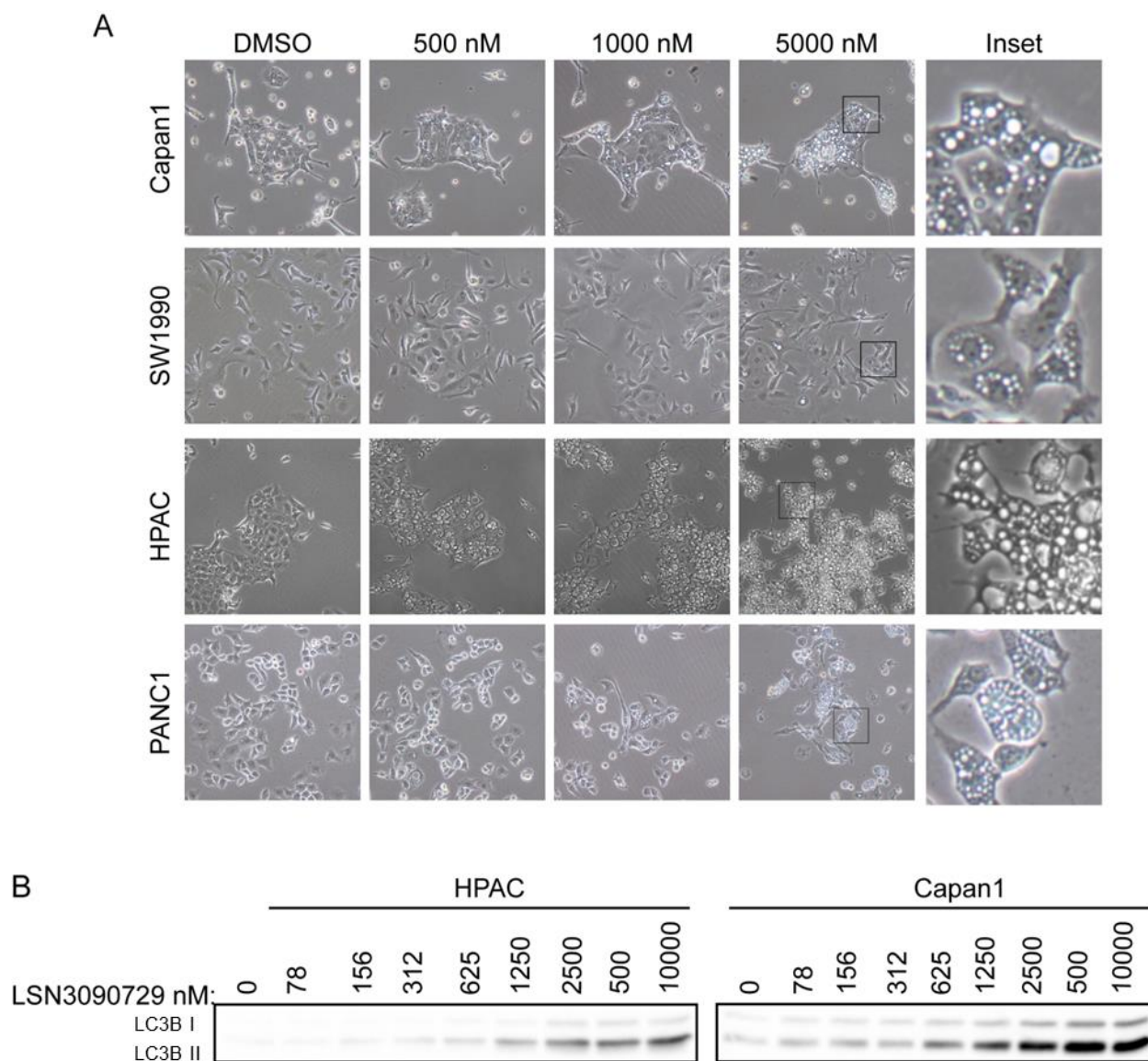


Figure 3-5. LSN3090729 disrupts autophagy in PDAC cells. A) Indicated PDAC cell lines were treated with increasing doses of LSN3090729. Cells were imaged at 24 hours. B) Cells were treated with LSN3090729 for 72 hours and probed for LC3B I and II.

Mounting evidence suggests that differing effects of inhibitor treatment are often observed in cancer cells *in vitro* versus *in vivo* due to tumor microenvironment and heterogeneity [188-190]. Therefore, we examined the effect of LSN3090729 on tumor growth to determine if the sensitivity observed in selected PDAC cell lines was recapitulated in a xenograft mouse model. Pharmacokinetic analysis showed that LSN3090279 has over 70% oral bioavailability and an acceptable half –life of 1.7 hours in rats (Fig. 3-1F). In a pancreatic cancer Panc1 xenograft mouse model, a modest decrease in tumor growth was observed upon treatment with LSN3090729 compared to vehicle control (Fig. 3-6A). We extended this to colorectal cancer HCT116 and lung cancer A549 mouse xenograft models. In the colorectal cancer model, a slight decrease in tumor growth was observed at a higher dosing regimen (Fig. 3-6C). In the lung cancer model, a minimal effect was seen upon treatment with LSN3090729. In the lung cancer model, treatment with LSN3090279 caused only a minimal tumor growth delay, whereas treatment with the PI3K/mTOR inhibitor BEZ-235 dramatically decreased tumor size (Fig. 3-6B).

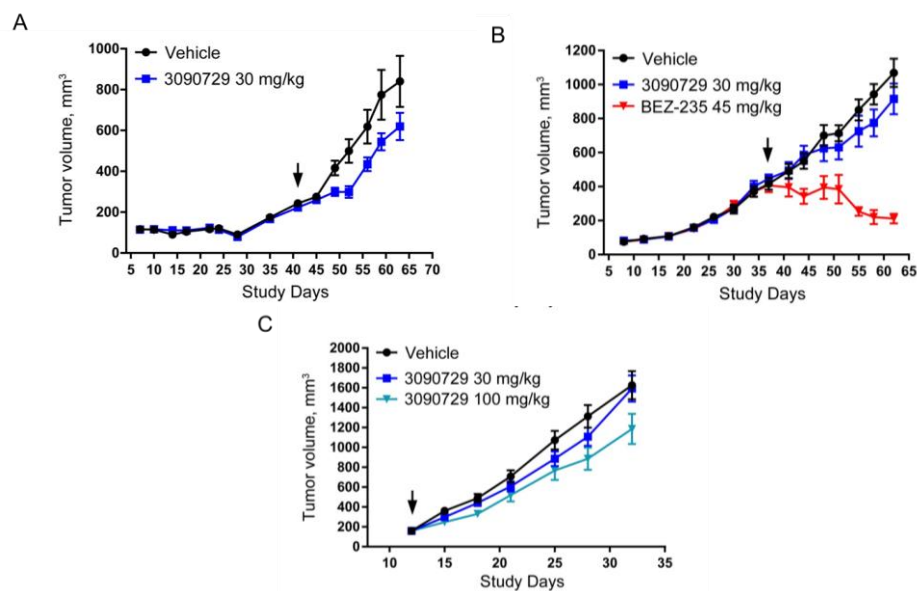


Figure 3-6. LSN3090729 minimally affects K-Ras-mutant tumor xenografts. A) Mice bearing subcutaneous Panc1 tumors in a pancreatic cancer model were dosed with vehicle or a single dose of LSN3090729. Each study group had 10 mice. B) Mice bearing subcutaneous A549 tumors in a lung cancer model were dosed with vehicle, LSN3090729, or PI3K/mTOR inhibitor BEZ-235. C) Mice with HCT116 subcutaneous tumors in a colon cancer xenograft model were dosed with vehicle or one of 2 doses of LSN3090729.

Combined TBK1 and ERK inhibition synergistically decreases PDAC cell proliferation – Small molecule inhibitors against TBK1 in cancer have proven ineffective as single agents, and concurrent inhibition of other cancer-promoting pathways is being explored as a new approach. One study demonstrated that combined TBK1 inhibition with MEK inhibition in N-Ras-mutant melanoma cells induced apoptosis, as shown by increased PARP cleavage and annexin V staining [172]. However, it did not address the impact of the combination treatment on cell proliferation. Previously, we have shown differential growth sensitivity to MEK and ERK inhibition in PDAC cells, with ERK inhibition proving slightly more effective, although the majority of cells remained resistant to both inhibitors (Hayes et al, manuscript in preparation). We sought to determine if simultaneous inhibition of TBK1 and ERK impacted PDAC cell growth.

We have previously determined that the growth of Capan1 and SW1990 cells are resistant to treatment with ERK inhibitor SCH772984 ($GI_{50} > 4 \mu M$) despite inhibition of ERK, as measured by phosphorylation of RSK, at almost 10-fold lower concentrations ($IC_{50} = 450 \text{ nM}$) (Hayes et al, manuscript in preparation). We treated Capan1 and SW1990 cells with ERK inhibitor SCH772984 and TBK1 inhibitor LSN3090729 and measured the effect on anchorage-dependent growth over 72 hours (Fig. 3-7A and 3-7B). Interestingly, we observed that LSN3090729 and SCH772984 acted synergistically in inhibiting proliferation of both cell lines. We observed an increased number of non-adherent cells upon simultaneous treatment with SCH772984 and LSN3090729 simultaneously compared to either inhibitor alone. We also observed an increase in cleaved caspase 3 in adherent cells when combining inhibitors (Fig. 3-7C). Therefore, we concluded that adding SCH772984 to LSN3090279 treatment results in induction of apoptosis in our resistant cell lines.

We compared these results to combined inhibition of MEK using AZD6244. We previously have shown that anchorage-dependent proliferation of PDAC cells is resistant to AZD6244 ($GI_{50} > 8 \mu M$) despite effective disruption of signaling at 100 nM of AZD6244, demonstrated by decreased ERK 1/2

phosphorylation. We found that, while combining AZD6244 with LSN3090279 in treatment of our resistant PDAC lines did cause a further reduction in growth compared to LSN3090729 alone, this did not produce the same synergistic proliferation defect as observed with combined treatment of LSN3090729 and SCH772984 (Fig. 3-7A and 3-7B).

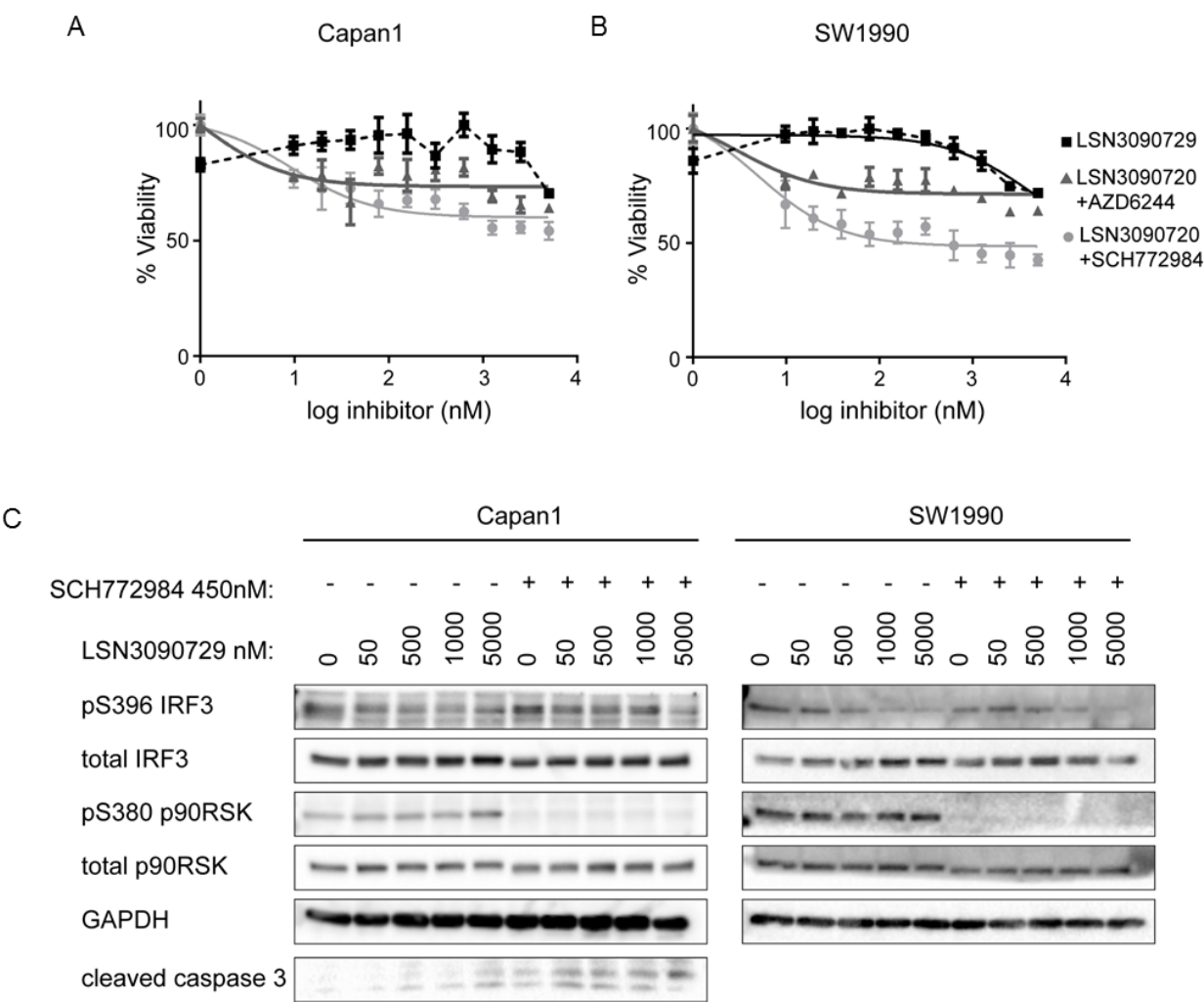


Figure 3-7. Combined TBK1 and ERK inhibition decreases resistant cancer cell growth synergistically. Treatment of A) Capan1 and B) SW1990 cells with increasing doses of LSN3090729 and either 100 nM MEK inhibitor AZD6244 or 450 nM ERK inhibitor SCH772984 gives a synergistic growth defect in 3 day 2D MTT assay, with combination index values in the range of 0.7 – 0.8 (<1 = synergy) over 40 nM – 5 μ M LSN3090729. Signaling in C) Capan1 and SW1990 through pIRF3, pRSK, and caspase 3 cleavage.

Discussion

Mutationally activated K-Ras is a driver in over 90% of PDAC cases [103]. Despite an increased understanding of effector pathways mediating K-Ras-dependent cancer growth, treatment options remain limited [191]. With the exception of the EGFR inhibitor erlotinib, there are no approved targeted therapies for PDAC patients, and chemotherapy remains the standard of care. This is in contrast to other cancers including lung, skin, and breast, for which multiple approved targeted therapies options have contributed to prolonging patient survival [192-194]. While inhibitors of PI3K, Akt, and MEK have exhibited moderate effects on PDAC cell proliferation, the overall efficacy of these inhibitors observed in patients has been minimal, and other possible targets should be considered. Given the increasing evidence for TBK1 activity in promoting cancer, we chose to determine the effect of inhibiting this less-studied Ras effector in PDAC.

Due to the implication of TBK1 overexpression and aberrant activation in promoting numerous cancers, small molecule inhibitors targeting TBK1 have been developed. Inhibition of TBK1 with LSN3090279 had a minimal effect on a large panel of cancer cell lines in anchorage-dependent growth assays, independent of Ras mutation status [180]. This is contradictory to previous reports suggesting that inhibition of TBK1 decreases cancer cell growth selectively in Ras-dependent cells [62]. Furthermore, our findings agree with previous findings that TBK1 inhibition alone is not sufficient to reduce growth in most PDAC cell lines, although a select set of cell lines did show sensitivity to the inhibitor. Future work to define a biomarker that is indicative of TBK1 sensitivity could lead to a more effective implementation of TBK1 inhibitors.

Multiple studies, including ours, have determined that TBK1 inhibition may be a viable approach for targeting Ras-driven cancers when combined with inhibition of another Ras effector pathway. We showed that combining ERK1/2 and TBK1 inhibition produced a synergistic growth defect in PDAC cells resistant to both TBK1 and ERK1/2 inhibition alone, and that this increased sensitivity was accompanied by an induction of apoptosis. These results agree with previous work that has shown induction of

apoptosis upon combined TBK1 and MEK inhibition in N-Ras-mutant melanoma cells and upon combined inhibition of ERK and PI3K in resistant PDAC cells [172](Hayes, manuscript in preparation). Inhibition of TBK1 has been shown to increase phosphorylation of ERK through feedback signaling in lung cancer cells, providing one explanation for the synergy seen with dual inhibition of these kinase pathways [177, 195]. Furthermore, ERK inhibition has been shown result in upregulation of pro-apoptotic signaling through BIM [196]. It is possible that inhibition of TBK1 leads to downregulation of anti-apoptotic proteins through inhibiting NF- κ B signaling, resulting in a second mechanism of permitting this induction of apoptosis and subsequent decrease in cell viability. Another study has shown that combined TBK1 and MAPK inhibition in a mouse model of K-Ras-driven lung cancer caused tumor regression and demonstrated that the disruption of autocrine signaling by a TBK1 inhibitor caused sensitivity in lung cancer cells [195]. Thus, exploring cytokine production by PDAC cells both sensitive and resistant to TBK1 inhibition may shed light on the TBK1-dependent mechanisms dictating PDAC cell proliferation.

Overall, our study demonstrated that TBK1 inhibition alone in PDAC has a minimal effect on cell proliferation, and that combining this with inhibition of a different cancer-promoting pathway downstream of Ras is a viable alternative approach. Future studies combining TBK1 inhibition with blockade of anti-apoptotic pathways may prove efficacious in the treatment of PDAC.

Chapter 4: Concluding Remarks and Future Directions

My work has addressed two approaches for inhibiting Ral small GTPase function and signaling for the treatment of *RAS*-mutant cancer. First, I examined inhibition of posttranslational modifications signaled by the RalCAAX tetrapeptide motif and catalyzed by RCE1, ICMT, and of palmitoylation, as a way to disrupt Ral subcellular localization and association with the plasma membrane. Second, I addressed targeted inhibition of a downstream effector signaling component of the RalGEF-Ral pathway, the TBK1 serine/threonine kinase. Here, I summarize the conclusions from these studies and discuss the next steps to be taken in further determining the effect of Ral inhibition in Ras-driven cancers.

Is Ral responsible for exacerbation of KRas-driven pancreatic cancer in the absence of ICMT?

My work shows that loss of ICMT causes subcellular mislocalization and accumulation of RalB in endomembrane compartments as well as disrupted degradation of RalB. However, we do not know the impact of this on RalB-dependent cancer growth. In previous studies, it was determined that RalB was critical for PDAC invasion and metastasis [105]. Although one study showed that ablation of ICMT decreased K-Ras-induced myeloproliferative disease and thus suggested ICMT targeting was a viable strategy for treating K-Ras-dependent cancers, a recent study in a K-Ras-driven pancreatic cancer mouse model showed that loss of ICMT resulted in accelerated PanIN progression to pancreatic ductal adenocarcinoma[125]. The authors concluded that this exacerbation is due to suppression of Notch. However, I believe that another mechanism may have a role. I hypothesize that misregulation of RalB plays a role in ICMT-dependent suppression of K-Ras-driven pancreatic tumorigenesis. Rather than

inactivating RalB function, the absence of the carboxylmethylation modification may aberrantly activate RalB function or result in increased expression, hence promoting rather than inhibiting cancer growth.

To test this, I would use the previously-described genetically engineered *Icmt flx/+; Pdx1-Cre; LSL-KRasG12D* and *Icmt flx/flx; Pdx1-Cre; LSL-KRasG12D* mouse model. I would culture cells from mouse tumors and compare RalB localization and protein expression in tumor cells with one copy of *Icmt* versus no *Icmt*. Based on my studies, I would expect to see RalB mislocalized away from the plasma membrane and increased in expression in the tumor cells with no *Icmt*. If this is the case, I could then generate an *Icmt flx/flx; RalB flx/flx; Pdx1-Cre; LSL-KRasG12D* mouse model where I could observe K-Ras-dependent tumor formation and progression to metastasis in the absence of both *Icmt* and RalB compared to an *Icmt flx/+; RalB flx/flx; Pdx1-Cre; LSL-KRasG12D* mouse model.

Does loss of RCE1 exacerbate KRas-driven pancreatic cancer?

Surprisingly, I found that absence of RCE1 resulted in mislocalization and hyperactivation of both RalA and RalB compared to levels found in WT MEFs. However, little is known about the impact of RCE1 loss on the activation of other CAAX-containing proteins. While one study has shown *Icmt* loss exacerbates K-Ras-driven pancreatic tumor formation in mice, the consequence of *Rce1* loss in this model has not been studied [125]. Although mutationally activated Ras-induced transformation was decreased in *Rce1*^{-/-} MEFs, loss of ICMT also resulted in decreased transformation by mutationally activated Ras, suggesting this assay is not always indicative of protein's role in tumorigenesis [116, 145]. To test whether RCE1 has an effect on mutant K-Ras-driven PDAC, I could genetically engineer a *Rce1 flx/flx; Pdx1-Cre; LSL-KRasG12D* mouse model with a control model *Rce1 flx/+; Pdx1-Cre; LSL-KrasG12D*. I would examine the rate of PanIN (preneoplastic lesions) progression to PDAC formation to determine if the loss of *Rce1* accelerates PDAC tumor formation, which could be due to an increase in RalA and RalB activation above that caused by K-Ras G12D signaling alone.

Does RCE1 deficiency result in hyperactivation of other small GTPases?

RCE1 deficiency has been shown to impact Ras function, such that transformation by mutationally activated Ras was decreased in *Rce1*^{-/-} MEFs [116]. In studies of CAAX-containing proteins downstream of Ras, RCE1 was found to be required for the subcellular localization of certain Rho family small GTPases and of the Rheb small GTPase, but was dispensable for K-Ras4B- and Rheb-dependent mTORC1 activation [148, 197]. Interestingly, these studies did not address the impact of RCE1 loss on catalytic activity of endogenous small GTPases. Given our finding that loss of RCE1 can cause a significant increase in Ral activity (Fig. 2-4 A), it is worth investigating if there is a similar impact on other non-mutationally activated small GTPases whose functions may contribute to cancer growth. I would compare the consequence of RCE1 loss on GTP loading of Ras, RhoA, Rac1 and Rheb proteins, as all of these have been implicated in promoting cancer. Since RCE1 inhibition is being explored as a point of therapeutic intervention, we must be cautious of the consequences on other CAAX-terminating proteins with known functions as oncoproteins or tumor suppressors such as liver kinase B1 (LKB1) or Di-Ras1 [198, 199]. Furthermore, if this aberrant activation is observed in other small GTPases, a comparison of these proteins sequences, localizations, and functions could reveal why this activation is occurring and if there is a rational approach to safely implementing RCE1 inhibition in cancer treatment.

What is responsible for hyperactivating RalA and RalB in *Rce1*^{-/-} cells?

Although my studies demonstrated that EGF stimulation in the absence of RCE1 results in increased RalA- and RalB-GTP levels above those found in the presence of RCE1, the mechanism by which this occurs remains unknown. Since these activity levels are not sustained, this suggests that aberrant RalGAP regulation is not responsible, but instead that an upstream signaling pathway is likely involved. This leads me to hypothesize that activation by one or more RalGEFs is responsible for mediating this EGF signaling and hyperactivation of the Ral proteins. To address this, I would first express a dominant negative RalA or RalB S28N mutant protein that sequesters endogenous RalGEF

proteins. Stimulation of mutant-containing cells should not show a greater Ral-GTP increase in the absence of RCE1, showing that RalGEFs are indeed necessary for endogenous Ral to be hyperactivated in these cells. I could then stably knock down individual RalGEFs using shRNA in *Rce1*^{-/-} MEFs using a non-targeting shRNA as a control. I would repeat EGF stimulation in these two *Rce1*^{-/-} MEFs and WT MEFs and measure Ral activity to determine if depletion of a specific RalGEF shifts the Ral activation level to that observed in WT MEFs. However, these analyses are complicated by the fact that there are four RalGEFs that act as Ras effectors and two other RalGEFs whose mechanism of regulation is still unclear.

Alternatively, it is possible that misregulation of another small GTPase is resulting in this hyperactivation. RalGEFs have been shown to interact with small GTPases besides Ral, including the Ras family proteins, R-Ras2/TC21, and Rap1A, all of which contain CAAX motifs that are also subject to Rce1 processing [200, 201]. This interaction between alternative GTPases and RalGEFs could be disrupted, indirectly resulting in Ral hyperactivation. A study of TC21 and Rap1A dependence on RCE1 could inform studies of RCE1-dependent Ral regulation.

What region of RalB contributes to stabilization in *Icmt*^{-/-} MEFs?

My studies showed that ICMT absence resulted in increased RalB protein stability and mislocalization that was dependent on the hypervariable C-terminus of RalB. Given this, in order to determine if a specific sequence of RalB is responsible for regulating this ICMT-dependent protein degradation, I would first express chimeric HA-tagged RalA/B and RalB/A in WT and *Icmt*^{-/-} MEFs and monitor degradation by blotting for HA after treatment with the protein synthesis inhibitor cycloheximide. I would expect RalA/B to be stabilized similarly to RalB in the *Icmt*^{-/-} MEFs. Phosphorylation of key serine residues in the C-termini of RalA and RalB by distinct protein kinases is one of the major differences in the Ral protein HVRs and contributes to their diverging roles [71, 72]. If I did observe that RalA/B is stabilized, I would determine if this is linked to phosphorylation of RalB serines 192 and 198,

by immunoprecipitating RalB in WT and *Icmt*^{-/-} MEFs and blotting for phosphorylated serine. If an increase in phosphorylated serine levels was observed, I would express S192A and S198A phosphodeficient mutants to determine if phosphorylation of a specific site is mediating this degradation defect. Examination of RalB protein levels in *Icmt*^{-/-} MEFs after mutation of these sites to phosphomimetic S192D and S198D could provide further insight into whether phosphorylation at these sites is important for efficient RalB turnover.

Is an E3 ligase interaction being disrupted in RalB degradation?

I observed that the steady-state level of RalB protein becomes stabilized in the absence of ICMT. In order to determine if this is due to an inability to be recognized by the relevant E3 ligases, WT and *Icmt*^{-/-} MEFs can be treated with the MG132 proteasome inhibitor and RalB levels monitored for accumulation. If this is seen, then it implicates an E3 ligase-based mechanism of RalB degradation. To determine if ubiquitination of RalB is deficient in *Icmt*^{-/-} MEFs, RalB can be immunoprecipitated and immunoblotted for ubiquitin. I would expect ubiquitinated RalB levels to be greater in the WT MEFs where efficient turnover is occurring.

If this is observed, there could be an E3 ligase interaction disrupted by absence of ICMT. While there are new technologies to find targets of E3 ligases, determining the E3 ligase for a specific substrate is not as straightforward [202-204]. This is due in part to the transient interaction between the E3 ligase and its substrate that limit use of traditional techniques as well as to the little information that is known about the functions of the E3 ligases. To address this question, I would perform an siRNA screen against the 617 putative E3 ligases in WT MEFs expressing a fluorophore-tagged RalB and use a fluorescent readout of relative expression levels. Specifically, I could utilize the Global Protein Stability Profiling (GPS) genetic screening platform [204-206]. Using the GPS system, GFP-RalB and a control DsRed fluorescent protein would be expressed from one mRNA transcript. DsRed would serve as the internal control, and varying GFP-RalB levels could be measured as a GFP – to – DsRed ratio quantified by

fluorescence-activated cell sorting (FACS) [206]. Any E3 ligase identified as modulating RalB stability could be validated by repeating genetic depletion of the specific E3 and monitoring ubiquitination levels and protein expression of RalB by western blot.

Does palmitoylation of the CAAX motif of Ral play a role in Ral-dependent cancer?

Ral proteins have been shown to have roles in tumorigenesis and metastasis *in vivo* as well as anchorage-independent and -dependent growth, motility, and invasion *in vitro* [81, 93, 104, 105]. My studies showing that RalB palmitoylation of the CAAX motif is approximately twice as high as that of RalA and that the palmitoylation-deficient mutant RalB-CSAX is mislocalized lead one to question if palmitoylation of RalB is playing a role in these cancer phenotypes. To address this, I would use PDAC cells, since the RalB-dependent phenotype of cell invasion in this system is well-studied [105]. I would first express CSAX mutants to confirm the same localization patterns as observed in MEFs. I would then stably deplete RalA and RalB in PDAC cell lines known to be dependent on Ral for their cancer properties and rescue with either wild type or a palmitoylation deficient CSAX mutant. I would expect RalB-CSAX to not rescue invasion in PDAC cells due to its mislocalization and higher prevalence of RalB palmitoylation. It would also be interesting to see the impact of the palmitoylation-deficient mutant on RalB activity levels in cancer cells.

Does RalB palmitoylation affect novel effector interaction?

While my studies identified palmitoylation of RalB as regulating its localization, the impact of this modification on RalB effector signaling, enzymatic activity, and contribution to RalB-dependent cancer phenotypes has not been addressed. One question that arises is whether palmitoylation of RalB causes interactions with unidentified Ral effectors or alters interactions with known Ral effectors. Previous studies have utilized constitutively activated RalB to identify interactors, but the effects of posttranslational modifications, such as palmitoylation, on protein-protein interactions have not been examined. To do this, I would express either WT RalB or the palmitoylation-deficient RalB-CSAX

mutant protein in cells. I would co-immunoprecipitate RalB and analyze the isolated product by mass spectrometry. Comparison of proteins in complex with RalB may identify unknown binding partners of RalB, thus giving more insight into the pathways downstream of Ral. Ultimately, this could inform future studies of the best way to target Ral in cancer.

Does synergy found with TBK1 and ERK combined inhibition result from dual inhibition of anti-apoptotic pathways?

My study demonstrated that combined TBK1 and ERK inhibition resulted in an induction of apoptosis. While TBK1 is known to have a pro-survival role, few studies have addressed the mechanism by which this occurs, and it remains unclear how simultaneously inhibiting these two kinases promotes apoptosis. A previous study showed that TNF-induced apoptosis, demonstrated by cleaved caspase 3, was increased in the absence of TBK1, and that this was dependent on both phosphorylation of RelA at serine 536 and RelA-dependent expression of transglutaminase 2 [207]. However, targeting TBK1 activity alone in our system was not sufficient to induce apoptosis. Furthermore, ERK inhibition alone has only a minimal effect on apoptosis. This is likely due to the same mechanism described in a study of MEK inhibition, where suppression of ERK signaling led to increased BIM expression that alone was insufficient to induce apoptosis. When combined with an inhibitor of an anti-apoptotic protein, however, apoptosis was induced [196]. I would hypothesize that combined TBK1-inhibition-dependent suppression of an anti-apoptotic protein and ERK-inhibition-dependent BIM upregulation lead to the synergistic induction of apoptosis. To test this, I could monitor protein expression of BIM and phosphorylation of RelA S536 as well as expression of RelA transcriptional targets after treatment with LSN3090729, SCH772984, or combined treatment. I would expect the increase in BIM from the combination treatment to be greater than the levels of BIM induced from each inhibitor alone.

How does TBK1 inhibition affect autophagy?

My studies demonstrated that treatment with LSN3090279 at low doses caused large vacuole formation that remained unresolved after 96 hours as well as an accumulation of LC3, both indicative of incomplete autophagy. However, knockdown of TBK1 did not cause vacuole formation, and previous studies have shown TBK1 depletion causes a decrease in chloroquine-dependent LC3 accumulation [170]. Therefore, the mechanism by which TBK1 inhibition could deregulate autophagy is not unclear. In this case, it is difficult to determine if the absence of the scaffolding properties of TBK1 upon protein depletion by shRNA may be resulting in a different phenotype than inhibition of TBK1, or if LSN3090279 off-target effects are responsible. To address this, I could utilize alternative TBK1 inhibitors that are chemically distinct with different off-target activities. If treatment of cells with these inhibitors also results in vacuole formation, it is likely that it is a TBK1-dependent phenotype. Alternatively, I could utilize CRISPR to conditionally knock in a kinase-dead K38A mutation into TBK1 and monitor cells for vacuole formation. If vacuoles do not form, I would conclude this is an off-target effect of LSN3090279. However, if they do form, and if LC3 accumulation is observed, I could then determine if upregulation of autophagy was contributing to resistance of cells to TBK1 inhibition. I would first treat with LSN3090279 and then knock down components of the autophagy pathway and measure cell viability to determine if a new dependence on autophagy has occurred in the presence of TBK1 inhibition. Alternatively, I could starve cells resistant to TBK1 inhibition in order to induce pro-survival autophagy. Subsequently, I could treat with LSN3090279 to determine if resistant cells are now sensitive to TBK1 inhibition.

Is sensitivity to TBK1 inhibition dictated by autocrine signaling?

Our study identified a subset of PDAC cell lines that were sensitive to TBK1 inhibition. However, the mechanism controlling this sensitivity remains unknown. One study demonstrated that K-Ras-driven lung tumorigenesis was promoted through TBK1-dependent autocrine signaling; specifically, TBK1 depletion caused a decrease in CCL5 and IL-6 production [195]. To determine if a similar mechanism is

responsible for TBK1 inhibitor sensitivity, I would treat sensitive cells with either LSN3090279 or DMSO. I would then replace media on treated cells with conditioned media from DMSO-treated cells and determine if this rescued cell growth. If cell growth is rescued, I would then assess conditioned medium from DMSO- and inhibitor-treated cells using a cytokine antibody array to determine if any cytokines are depleted or elevated in inhibitor-treated media.

Will TBK1 inhibition sensitize cancer cells to other targeted inhibition?

Combination approaches are gaining traction in the treatment of cancer. However, toxicities associated with simultaneous inhibition of major Ras-dependent pathways like MEK and PI3K have limited this approach, and new combinations are needed to treat patients while minimizing negative side effects. Here, we combined TBK1 and ERK inhibition and found a synergistic proliferation defect. While promising, this was not sufficient to reduce cell viability past 50%. To further determine how TBK1 can be effectively implemented in PDAC patients, I would expand my studies to include other combination approaches. One approach is to use inhibitors targeting lesser-studied pro-survival signaling pathways in PDAC cells. For example, one such target downstream of Ras in cancer is the p21-activated kinase (PAK) family pathway. One member of the PAK family, PAK1, has been shown to be important for BCL-2 expression, and combining this inhibition with TBK1 could decrease expression of two anti-apoptotic proteins, thus inducing apoptosis.

An alternative approach is to use the Drug Sensitivity and Resistance Testing (DSRT) platform to screen for compounds that cooperate or synergize with LSN3090279 [208]. The compounds available for testing by DSRT cover multiple drug classes and include chemotherapeutics, kinase inhibitors, and immunosuppressants, thus allowing for inhibition of targets that may not be considered in the Ras effector pathway approach.

Which proteins confer resistance to TBK1 inhibition?

My studies show that the majority of PDAC cell lines are resistant to TBK1 inhibition in terms of proliferation. However, the mechanism of resistance to LSN3090279 has not been explored. To do this, I would use two approaches. First, I would perform an siRNA screen against targets that have been implicated in the K-Ras signaling network. I would monitor cell viability and determine if the depletion of a specific protein in resistant PDAC cell lines sensitizes cells to LSN3090279 treatment. Second, as a complementary approach to the siRNA screen, I would use the Cancer Toolkit described previously that was used to identify Notch1 activation as a mechanism of acquired resistance to MAPK inhibitors in BRAF V600E-mutant melanomas [209]. Briefly, in cell lines identified as sensitive to LSN3090279, I would express a barcoded library of cDNAs encoding activated mutants of signaling components of 20 known cancer pathways. I would then treat cells with the inhibitor and monitor cell viability in order to determine which activated components conferred resistance to TBK1 inhibition.

Conclusion

My studies have examined targeting the Ras-Ral signaling pathway in cancer both through loss of proteins responsible for Ral association with the plasma membrane and through inhibition of the downstream effector TBK1. I found that posttranslational modification of the CAAX motif of Ral proteins has differential consequences on their activity, stability, and subcellular localization, and this work suggests the impact of these modifications on other CAAX-containing proteins involved in cancer signaling should also be addressed. I also confirmed that pharmacologic inhibition of TBK1 has a minimal impact on cancer cell proliferation, and found that instead combination with another targeted inhibitor may be more effective. In an era when combination therapies for the treatment of cancer are becoming increasingly popular, this finding can inform future studies of how TBK1 inhibitors can be implemented most effectively. In conclusion, these studies serve to advance the field of targeting Ral for the treatment of cancer.

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