Review

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Regulation of Ras signaling and function by plasma membrane microdomains

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Summary **Together H-, N- and KRAS mutations are major contributors to ~30% of all human cancers. Thus, Ras inhibition remains an important anti-cancer strategy. The molecular mechanisms of isotypic Ras oncogenesis are still not completely understood. Monopharmacological therapeutics have not been successful in the clinic. These disappointing outcomes have led to attempts to target elements downstream of Ras, mainly targeting either the Phosphatidylinositol 3-Kinase (PI3K) or Mitogen-Activated Protein Kinase (MAPK) pathways. While several such approaches are moderately effective, recent efforts have focused on preclinical evaluation of combination therapies to improve efficacies. This review will detail current understanding of the contributions of plasma membrane microdomain targeting of Ras to mitogenic and tumorigenic signaling and tumor progression. Moreover, this review will outline novel approaches to target Ras in cancers, including targeting schemes for new drug development, as well as putative re-purposing of drugs in current use to take advantage of blunting Ras signaling by interfering with Ras plasma membrane microdomain targeting and retention.**

Keywords: Ras, lipid rafts, membrane microdomains, tumor progression

1. Introduction

The Ras homologues most prominently associated with cancers, H-, N-, and KRAS, are ubiquitously expressed with overlapping yet non-redundant functions (*1-4*). Ras propagates growth factor signaling, most prominently the MAPK mitogenic pathway (Raf/Mitogen-Activated Protein Kinase/ERK Kinase (MEK)/Extracellular signal-Regulated Kinase (ERK)) and PI3K/molecular target of rapamycin (mTOR) survival pathways (*1,3,4*). Constitutively active (CA) Ras mutations are highly transforming and tumorigenic (*5*). Combined, CA Ras mutations are associated with as high as ~30% of human malignancies (*6*). H- and NRAS mutations account for a substantial proportion, most prominently for HRAS in cervix (9%), salivary gland (15%), and urinary tract (11%), and for NRAS, skin (18%) and hematopoietic cancers (> 10%), as well as many other cancer types; the remainder harbor KRAS mutations (*6*). The molecular mechanisms of isotypic Ras oncogenesis are still not completely understood, and Ras inhibition remains an important anti-cancer strategy (*1,3,4*).

Ras function is modulated by its localization within microdomains at the plasma membrane and putatively at internal membranes. Lipid bilayers are comprised of subdomains enriched in cholesterol and glycosphingolipids, which are referred to as lipid ordered domains, or lipid rafts. The fatty-acid side chains present in lipid ordered membranes tend to be more saturated than those in the surrounding membrane. Due to the presence of cholesterol and fatty acid saturation, a lipid ordered domain exhibits less fluidity than the surrounding plasma membrane. Lipid ordered domains are classically characterized by the resistance to extraction with nonionic detergents. Many proteins involved in cell signaling have been shown to be enriched within lipid ordered microdomains, creating signaling nodes in these domains. By condensing pathway components, lipid

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rafts may promote signal transduction by responding to agonist stimulation causing cluster formation, therefore leading to downstream signal activation. Alternatively, lipid rafts may negatively regulate signal transduction by spatially segregating pathway proteins, leading to reduced downstream signaling (7).

Distinctions in plasma membrane microdomain targeting and regulation between H-/N-/KRAS and related Ras paralogues are proving highly instructive in elucidating the mechanistic basis for Ras signaling in homeostasis and pathophysiology. This in turn provides a basis for design of new anti-Ras drugs and re-appropriation of existing drugs to inhibit Ras, *via* targeting the membrane microdomain localization of Ras. This review will outline the contributions of plasma membrane microdomains and Ras membrane targeting to Ras signaling and functional outcomes, and implications for anti-Ras therapies. The review will focus principally on HRAS, NRAS and KRAS, the isotypes most prominently associated with human cancers, and will include a discussion of the RRAS subfamily, as distinct membrane targeting of RRAS and the other isotypes has revealed important contributions of membrane targeting to Ras signaling, cellular function, and roles in tumor progression.

2. Ras GTPases

2.1. *Ras subfamily*

The Ras superfamily is comprised of over 150 small GTPases, which can bind and hydrolyze guanine triphosphate (GTP). The Ras isotypes most prominently associated with human cancers, H-, N-, and KRAS (that consists of KRAS4A and KRAS4B splice variants), have overlapping yet nonredundant cellular functions, and constitutive activation of each isotype is associated with distinct cancer subtypes. In contrast to the H-/N-/KRAS subfamily, RRAS1 shares similar overall structure but displays different cellular functions. RRAS1 has limited transforming ability in cell lines, and activating RRAS1 mutations are at best weakly associated with human cancers (*8*). In response to growth factor receptor activation, Ras-specific guanine exchange factors (GEFs) are recruited and activated, facilitating GTP binding to Ras by displacing GDP. GTP-loading of Ras enacts a conformational change in the Ras protein, exposing the effector-binding domain to support effector interactions, leading to propagation of downstream signaling pathways promoting cell proliferation, differentiation, and survival. GTP-loading of wildtype (WT) Ras is reversed by the action of GTPase-Activating Proteins (GAPs), which increase the low intrinsic GTPase activity of Ras, causing GTP hydrolysis to guanine diphosphate (GDP), and Ras inactivation. Ras GTPases are generally characterized as "molecular switches" based on the ability to turn signaling on or off through GTP binding and hydrolysis (*9*). Multiple GAPs have been identified as tumor suppressors, including p120-RasGAP (RASA1) and neurofibromin (*10,11*). CA Ras mutations are characterized by 'locking' Ras in the GTP-bound state, either by inhibiting GAP sensitivity, increasing GTP binding affinity or other mechanisms. These Ras mutants are transforming when over-expressed in cells in culture, and drive tumor formation in animal models. Together activating mutations in H-, N-, and KRAS (hereafter referred to collectively as proto-oncogenic (WT) or oncogenic (CA) Ras) are associated with ~30% of all human tumors, and reports have shown Ras mutations in up to 90% of pancreatic cancers (*1,3,4*). Ras signal propagation, and hence cellular and tumorigenic effects, are also tightly regulated by Ras plasma membrane targeting and microdomain association. Although CA Ras is insensitive to activity modulation, spatial regulation by Ras microdomain targeting at the plasma membrane has emerged as a mechanism for modulating Ras signaling.

2.2. *Ras structure*

Ras proteins each comprise an effector-binding domain, guanine nucleotide binding domains, and C-terminal hypervariable region, and include regions of high and low sequence homology across isotypes (Figure 1). Crystal structures of Ras indicate that the exchange of guanine nucleotides GDP-to-GTP results in an allosteric conformational change in adjacent regions, referred to as "switch I" and "switch II" (*12*). The core effector binding domain is located within the switch I region, and is highly conserved among isotypes. Switch I is a loop structure which is exposed upon GTP binding, and is sometimes referred to as the effector loop (*13*). The area which displays the least sequence homology is located in the C-terminal region, named the hypervariable region (HVR). Within the HVR lie residues subject to distinct and isotype-specific post-translational modifications. These post-translational modifications are responsible for the differential membrane targeting among Ras isotypes (*14-16*) (Figure 1).

2.3. *Ras membrane association driven by post-translational lipid modifications*

The C-terminal 23/24 amino acids of Ras constitute the HVR, the sequence of which dictates Ras isotype-specific post-translational modifications (*17*). The extreme C-termini contain a cysteine-aliphatic-aliphatic-X motif (CaaX), in which the X is usually serine, methionine, or glutamine. Following translation in cytosolic ribosomes, the Ras polypeptide is enzymatically isoprenylated with a 15-carbon farnesyl group by farnesyl transferase (Figure 2). The farnesyl transferase binds Ras within the cytosol and attaches the lipid moiety to the cysteine residue of the CaaX motif (*18*). RRAS contains a cysteine-aliphatic-aliphatic-Leucine (CaaL) motif (Table 1). The Leucine in

the CaaL motif drives covalent addition of a 20-carbon geranylgeranyl lipid group to the cysteine residue *via* geranylgeranyl transferase (*19*). Both farnesyl transferase and geranylgeranyl transferase attach the respective lipid group *via* an irreversible thioether bond.

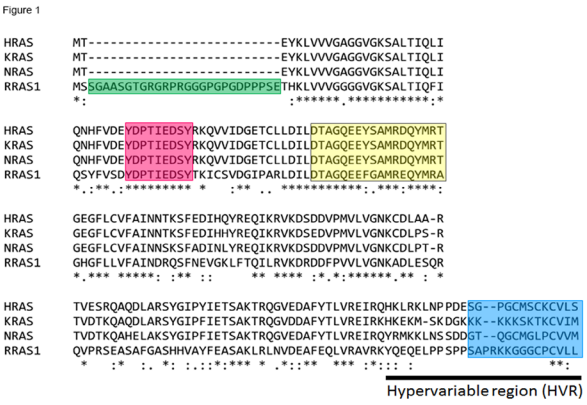

Figure 1. Primary structure alignment of proto-oncogenic RAS subfamily and RRAS1. Green: Unique RRAS N-terminal extension. Pink: Core effector binding domain. Yellow: "switch I" and "switch II" guanine nucleotide-binding region. Cyan: membrane targeting domain. The underlined amino acids indicate the hypervariable region (HVR). Asterisk (*): conserved amino acids.

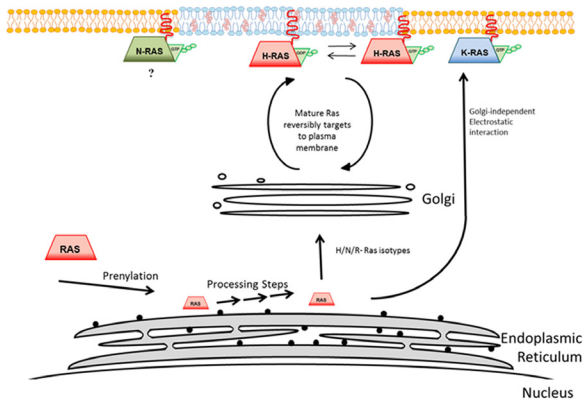

Figure 2. Post-Translational Modifications of Ras and plasma membrane targeting. Ras proteins are translated by cytosolic ribosomes, which are subsequently isoprenylated at the Cysteine of the CaaX consensus sequence. Isoprenylation increases affinity for the endoplasmic reticulum, where the protein undergoes further modification. RceI proteolytically cleaves the –aaX tripeptide, then Icmt carboxy-methylates the newly prenylated Cysteine. Most Ras proteins also require palmitoylation, catalyzed by the DHHC family of isoprenyl transferases, to leave the Golgi and traffic properly to the plasma membrane. Different Ras isoforms laterally segregate at the plasma membrane, to propagate downstream signaling. Upon depalmitoylation, Ras cycles back to the Golgi, where it is capable of being palmitoylated many times during the half-life of the protein.

Once Ras proteins are isoprenylated by either a farnesyl (H-,K-,N-, TC21) or geranylgeranyl (R-, M-) group the protein hydrophobicity is increased, thus causing higher affinity to the endoplasmic reticulum membrane (Figure 2). Thereafter, the CaaX or CaaL sequence targets Ras to the cytosolic surface of the endoplasmic reticulum where Ras and a-factor converting enzyme (Rce1), proteolytically removes the –aaX tripeptide (*20*). Next, the newly C-terminal prenylcysteine is targeted by isoprenylcysteine carboxyl methyltransferase (Icmt), which methyl-esterifies the α carboxyl group (*21*). Covalently linked lipid moieties have been shown to account for specific GEF activation. For example, Ras-GRF1 and Ras-GRF2 can activate HRAS, while Ras-GRF2 is unable to activate RRAS specifically because of the geranylgeranyl attachment (*22,23*).

The isoprenylated Ras proteins weakly bind endomembranes, yet a second motif within the hypervariable region strengthens membrane interaction and supports subsequent Ras trafficking. One common secondary motif is the reversible addition of a 16 carbon palmitate group (*24*). Palmitoyl groups are added to H-, N-, R-, and splice variant K(A)RAS. The more physiologically predominant splice variant K(B)RAS (hereafter referred to as KRAS) contains a polylysine sequence which allows an electrostatic interaction with the acidic headgroups of lipid bilayers (Table 1). KRAS traffics to the plasma membrane through a poorly understood route which is Golgi-independent (*25,26*). N- and K-(A)RAS are monopalmitoylated and thus require a third HVR motif for plasma membrane association, which consists of a stretch of hydrophobic residues (*27*). Proto-oncogenic Ras palmitoylation (H-, N-, K(A)-RAS) is carried out by a heterodimeric complex consisting of Palmitoyltransferase ZDHHC9 (DHHC9) and Golgin subfamily A member 7 (GCP16) (*28*). DHHC9 is one member of a family of DHHC-motif-containing protein S-acyltransferases (PATs) (*28*). Alternatively, the precise function of GCP16 is unclear. GCP16 is required for the heterodimeric complex localization, and plays a role in DHHC9 protein stability (*29*). Another DHHC family member, DHHC19, is responsible for palmitate transfer to RRAS, but not H-, N-, or K-(4A)RAS (*30*). Palmitoylated Ras proteins are mainly targeted to recycling endosomes, which function as a shuttle along the post-Golgi exocytic pathway to the plasma membrane. HRAS is palmitoylated on two

Table 1. Ras Lipid Modifications

Ras isoform	C-Terminal prenylation motif	Isoprenylation	Secondary Lipid Binding Motif	Palmitoylation
HRAS	CaaX	Farnesyl	Palmitoylation	2 sites
NRAS	CaaX	Farnesyl	Palmitoylation, Hydrophobic Residues	1 site
KRAS4A	CaaX	Farnesyl	Palmitoylation, Hydrophobic Residues	1 site
KRAS4B	CaaX	Farnesyl	Electrostatic interaction	None
RRAS1	CaaL	Geranylgeranyl	Palmitoylation	1 site

Cysteine residues, while NRAS is palmitoylated at one site (Table 1). Palmitoylation is required for shuttling of Ras to recycling endosomes, and the lack of proper palmitoylation leads to Ras mis-localization (*16*). In addition to H-, N-, and KRAS4A, RRAS also requires palmitoylation to exit from the Golgi, and to traffic anterograde *via* vesicles to the plasma membrane (*31*).

Palmitate groups are attached *via* a reversible thioester bond. This reversible addition of a lipid moiety allows for spatio-temporal regulation of Ras proteins. Depalmitoylating enzymes, such as acyl protein thioesterase I (AptI) or FKBP12, cleave the thioester bond between the Cysteine residue and the palmitate. Palmitate removal reduces the Ras protein's affinity for the plasma membrane, which triggers recycling back to the Golgi where Ras can be palmitoylated. This cycle can be repeated multiple times during the half-life of the protein (*32-34*).

3. Plasma membrane targeting of Ras

Localization of Ras proteins within the plane of the plasma membrane appears to be more dynamic than originally believed (*35-37*). Biochemical and electron microscopy studies suggest that approximately 50% of the inactive form of HRAS (GDP bound) is localized to lipid ordered domains, while activated HRAS (GTP bound) exits lipid ordered domains and preferentially resides in the lipid disordered membrane(*38,39*). Recently, semi-atomic in silico simulations provided evidence indicating that GTP-bound HRAS is localized to the border between the lipid ordered/lipid disordered domains (*40*). Such evidence provides a handy explanation for GTP-HRAS being observed enriched in both fractions, as gradient sedimentation techniques reveal bulk distributions and a microdomain "split" in GTP-HRAS likely supports its targeting to the microdomain borders. Similarly, inactive (GDP bound) NRAS is found in both lipid ordered and lipid disordered membranes. Atomic force microscopy has shown GTP-bound activated NRAS is also likely shuttled to the lipid ordered/lipid disordered boundary, where it may help reduce line tension at the phase boundary (*41,42*). However, NRAS PM microdomain localization is more controversial, as fluorescence recovery after photobleaching (FRAP) studies indicated that GTP-bound NRAS reside within lipid rafts (*36,43*). KRAS is targeted to the lipid disordered subdomain, regardless of activity state, directly from the endoplasmic reticulum without modification from the Golgi. Despite the electrostatic force generating the association with the plasma membrane, approximately 85% of KRAS is found to localize within the lipid disordered domain. Importantly, KRAS propagates signals from the lipid disordered membrane that is spatially distinct from HRAS (*37,44*). Collectively, the farnesyl lipid moiety appears to preferentially localize Ras proteins to the lipid disordered domain, while the palmitate group prefers the lipid ordered domain. This trend accounts for similar targeting of H- and NRAS to the lipid raft border, and for the nonpalmitoylated KRAS to preferentially propagate signals from the lipid disordered domain. RRAS is geranylgeranylated and palmitoylated (not farnesylated), and in line with the apparent role of Ras lipidation in microdomain targeting, RRAS localizes to lipid ordered microdomains in both active and inactive states, *i.e.*, RRAS does not shuttle to the lipid ordered/disordered domain border upon GTP binding (*40,45*) (Figure 2).

The processes which regulate Ras localization to distinct microdomains at the plasma membrane and lateral movement are incompletely understood. However, recent reports indicate that Galectin proteins play a critical role. Galectin over-expression has been observed in several tumor types, and has been associated with tumor progression (*46*). Galectins are a family of carbohydrate-binding proteins, with high affinity for β-galactosides. GTP-bound HRAS has been demonstrated to selectively bind Galectin-1 (Gal-1), and GTP-bound KRAS selectively binds Galectin-3 (Gal-3) (*47,48*). While Gal-3 does not directly bind to NRAS, increased Gal-3 expression simultaneously increases KRAS signaling while decreasing NRAS activation (*49*). This is due to an interaction of the N-terminus of Gal-3 with Ras exchange factor, RasGRP4, which diminishes NRAS GTP loading (*50*). At the time of this publication, there are no reports of a Galectin protein that selectively interacts with NRAS.

In the case of HRAS, Gal-1 contains a prenyl-binding pocket, which interacts with the farnesyl group in GTP-HRAS, independent of lectin function. This interaction is thought to alter the orientation of the HRAS globular domain with respect to the plasma membrane, and thereby regulate lateral segregation of HRAS and promote MAPK signaling (*47,51*). Indeed, ectopic Gal-1 over-expression or suppression increases or abrogates GTP-bound HRAS nanoclustering, respectively (*52*). Thus, upon GTP-loading, the conformational shift of Ras promotes affinity for Galectin binding, which subsequently allows lateral movement at the plasma membrane, allowing for distinct signaling platforms (*51*).

4. Ras signaling (H, K, N)

Ras can be activated in response to a diverse array of upstream extracellular signals such as growth factors, cytokines, hormones and neurotransmitters which stimulate cell surface receptors that include receptor tyrosine kinases (RTKs), non-receptor tyrosine kinase-associated receptors, and G protein- coupled receptors (GPCRs) (*53,54*) (Figure 3).

Perhaps the most studied pathway of Ras activation involves the mitogen-stimulated RTK epidermal growth factor (EGF) receptor (EGFR) (*54,55*). Upon stimulation, EGFR dimerizes and undergoes autophosphorylation of tyrosine residues in its cytoplasmic domain. These

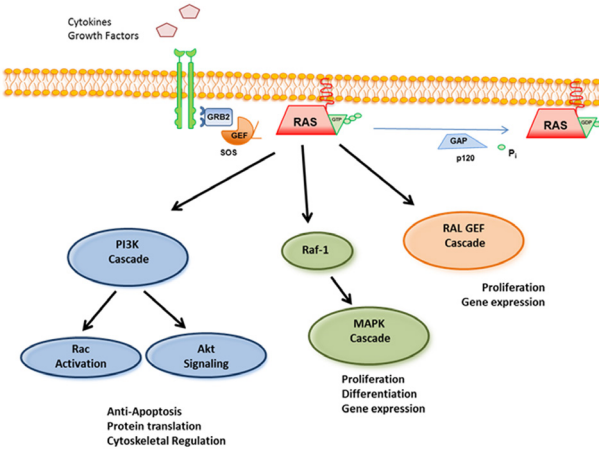

Figure 3. Overview of Ras Signaling Pathways. Ras is activated in response to a variety of extracellular stimuli, which facilitate localization of guanine nucleotide exchange factors (GEFs) to catalyze the replacement of GDP for GTP. Upon GTP-loading (activation) Ras functions like a molecular switch, propagating a variety of downstream signal cascades leading to proliferation, differentiation, altered gene expression, and other biological processes. The intrinsic ability of RAS to hydrolyze GTP to GDP + P*i* is catalyzed by a large family of GTPase activating proteins (GAPs) including p120, which effectively turns off Ras signaling

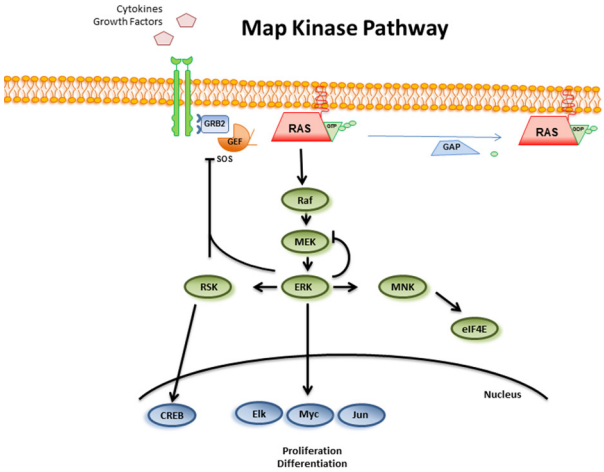

Figure 4. Overview of mitogen activated protein kinase (MAPK) signaling pathway. Upon activation, Ras binds and translocates Raf to the plasma membrane. Activation of Raf subsequently starts a signal cascade which leads to MEK and ERK activation. Activated ERK can translocate to the nucleus and alter transcription, or phosphorylate other cytosolic proteins which push the cell towards proliferation and differentiation. Downstream ERK has been shown to turn off signaling by negatively regulating MEK. In addition, both RSK and ERK can phosphorylate Ras-GEF SOS, which destabilizes its interaction with the RTK and terminates signaling.

phosphorylation events enable binding for adaptor proteins such as Shc and/or Grb2 *via* Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains (*56*). The tri-complex Shc/Grb2/SOS has been implicated as the major pathway for EGF stimulation of Ras (*57*). Upon interaction with the activated RTK, Shc becomes autophosphorylated which creates recognition sites for the SH2 domain of Grb2. Grb2 is stably associated with son of sevenless (SOS), a Ras GEF. Thus, the Shc/Grb2/SOS or Grb2/SOS complex translocation to the plasma membrane mediates an increase in GTP-bound, active Ras (*58,59*). Shc, Grb2 and SOS provide the link between many types of activated cell surface receptors and Ras (*60*). Another receptor which activates Ras proteins is the Tyrosine Kinase Receptor-A (TrkA), which is stimulated by the neurotrophic factor nerve growth factor (NGF) (*61*). Once activated, Ras activates downstream signal cascades (Figure 3).

4.1. *Raf/MAPKinase pathway*

The mitogen-activated protein kinase (MAPK) pathway plays a key role in many physiological responses such as cell proliferation, apoptosis, and differentiation. GTP binding and effector loop exposure in Ras creates a docking site for a high affinity interaction with Raf kinases, which is reversed upon Ras inactivation (*62,63*). The Ras-Raf complex translocates the cytoplasmic Raf to the plasma membrane, which supports activation of Raf through a complex and incompletely understood mechanism (Figure 4) (*64*). Thus, Raf activation by Ras is a function of the ability of Ras to recruit Raf to the plasma membrane where it can be phosphorylated by cognate kinases and not by direct action of Ras itself (Ras lacks a kinase domain and kinase activity); this is supported by constitutive Raf activation by direct fusion of a CaaX sequence to the Raf C-terminus (*65,66*). Once activated, Raf phosphorylates MEK1 and MEK2 proteins at residues Ser218 and Ser222, thus propagating the mitogenic signal (*67*). Raf directly associates and activates MEK through its C-terminal catalytic domain (*68*). Activated MEK 1 and 2 are dual-specificity kinases which phosphorylate tandem residues in the TEY motif in their substrates, the Extracellular Signal Regulated Kinases 1 and 2 (ERK1 and ERK 2, also denoted as p44MAPK (ERK1) and p42MAPK (ERK2)) at Thr202/Tyr204 and Thr185/Tyr187, respectively, allowing for a conformational shift and full enzymatic activation (Figure 4) (*69*). Scaffold proteins, such as protein Kinase suppressor of Ras (KSR), help aid in the MAPK signaling by coordinating the assembly of a Raf-MEK-ERK complex (*70*).

Upon activation, ERK 1/2 can translocate to the nucleus where it phosphorylates and activates multiple transcription factors such as Elk-1, c-Ets-1, and c-Ets-2, c-Myc, c-Jun, and c-Fos (Figure 4) (*71,72*). ERK also has the ability to activate cytoplasmic kinases, such as p90 RSK1, MAP kinase-interacting kinase 1 (MNK1), and 2 (MNK2) (*73-75*). P90RSK can translocate to the nucleus and potentiate transcription *via* the activation of the transcription factor cyclic AMP-responsive element-binding protein (CREB) (*76,77*). MNK proteins may modulate translation by binding and phosphorylating eukaryotic translation initiation factor 4E (eIF4E), which

is a component of the translation initiation machinery (*78*). The various transcription factors regulated by ERK or RSK regulate gene expression to induce cellular phenotypes including cell proliferation, apoptosis, and differentiation (Figure 4).

4.2. *PI3Kinase pathway*

Another well-characterized pathway of Ras signaling involves phosphatidylinositol 3-kinases (PI3Ks), which play important roles in cell growth, adhesion, survival, motility, and transformation (*79,80*). The PI3Ks are heterodimeric lipid kinases with a catalytic and an adaptor/regulatory subunit, encoded by separate genes and subject to alternative splicing to generate multiple subtypes. The PI3K family of enzymes are organized into three main classes (class I, II, and III), and various subgroups have been categorized based on their primary structure, substrate specificity, and regulation (*81*). Class I PI3K is perhaps the best characterized family, and most clearly implicated in human cancer. The catalytic subunits for the class I PI3Ks are p110α, p110β, p110γ, and p110δ (*82,83*). The p110 subunits are divided into two classes; one which binds a p85 regulatory subunit (class IA: p110α, p110β, and p110δ) and the other which does not (class IB-p110γ). The regulatory p85 subunit serves a dual role by protecting p110 from degradation while inhibiting enzymatic activity. However, once RTKs become stimulated, p85 PI3K mediates p110 translocation to the plasma membrane, relocating the heterodimer to propagate downstream signaling (*84,85*).

PI3K activation can occur in multiple ways, which begin with extracellular activation of RTKs. One method of PI3K activation is by the regulatory p85 subunit directly binding to phosphorylated YXXM motifs (consisting of a Tyrosine-X-X-Methionine, where X denotes any residue) within the RTK, which triggers the P110 subunit to be catalytically active. A second form of activation has been characterized by the scaffolding protein Gab1, which binds regulatory p85 PI3K and associates with the EGF receptor both directly and indirectly through adaptor protein Grb2. Gab1 is recruited to the plasma membrane in response to EGFR activation, that is eventually turned off by conversion of Phosphatidylinositol (3,4,5)-triphosphate (PIP_3) to Phosphatidylinositol 4,5-bisphosphate (PIP_2) by Phosphatase and tensin homolog (PTEN), which triggers Gab1 dissociation from the plasma membrane (*86*). Finally, a third method of PI3K activation is through Ras (Figure 5). Adaptor protein Grb2 creates a cascade by binding and activating the Ras GEF SOS, thus activating downstream Ras and p110. Ras binds the catalytic p110 subunit of PI3K in a GTP-dependent manner which can stimulate PI3-kinase activity independently of p85 (*87,88*). Moreover, Grb2 can exist in complex with SOS, Gab1, and Ras which would bring these various PI3K activators in close proximity (*89*). It is unclear which of these pathways predominates in physiological situations; however there is evidence that RTK activation of the p85 subunit and Ras activation of the p110 subunit are complementary, and synergistically activates the pathway (*90*).

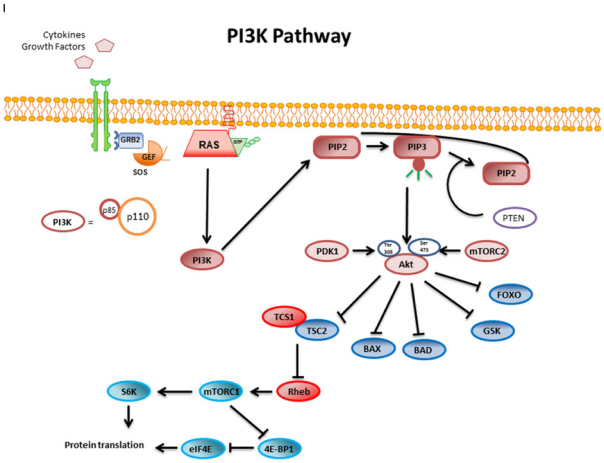

Figure 5. Overview of Ras/phosphatidylinositol-3 kinase (PI3K) signaling pathways. Upon activation, Ras can activate the p110 catalytic subunit of PI3K, which is a lipid kinase that phosphorylates PIP2 to generate PIP3. PIP3 recruits proteins that harbor a PH domain, such as Akt. Akt pushes the cell toward survival by negatively regulating a multitude of pro-apoptotic proteins such as BAX or BAD. Akt also inhibits the Rheb GAP protein TSC2. An increase in active Rheb leads to mTORC1 activation, which regulates multiple proteins that results in increased protein translation. PTEN dephosphorylates PIP3 back to PIP2, and effectively shuts off PI3K signaling.

Upon activation, PI3K generates PIP_3 from PIP_2 by phosphorylation. PIP_3 then acts as a second messenger facilitating downstream signaling, by recruiting proteins that contain a pleckstrin homology (PH) domain to the plasma membrane. PI3K activation and subsequent production of PIP_3 propagate various downstream pathways that regulate a number of cellular functions including those involved in tumor development and progression (Figure 5) (*91*). PTEN dephosphorylates PIP_3 to PIP_2, resulting in release of PH proteins and curtailing PI3K signaling (*92*).

One particularly significant downstream signaling cascade is the recruitment and activation of Akt/Protein Kinase B (PKB) (Figure 5). Akt activation is a multistep process in which residues Thr308 and Ser473 become phosphorylated. Initially, 3-Phosphoinositide-dependent protein kinase-1 (PDK1) and Akt are recruited to the plasma membrane in a PIP_3-dependent manner. Experimentally, PDK1 has been shown to phosphorylate Akt at Thr308, while the kinase for Ser473 has remained subject to controversy. Mammalian target of rapamycin (mTOR) in complex with GβL and Rictor (which is collectively referred to as mTORC2) is the kinase currently thought to be responsible for phosphorylating Ser473 (*93-95*). Akt promotes cell survival *via* negative regulation of numerous pro-apoptotic family members.

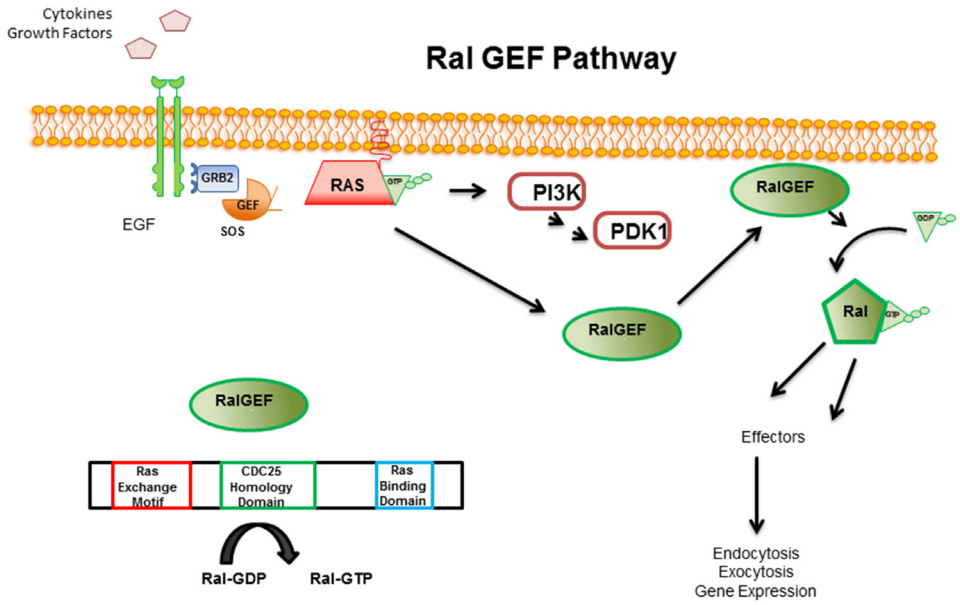

Figure 6. Overview of Ral-guanine exchange factor (Ral GEF) signaling pathway. Upon activation, Ras can bind RalGEFs via a RBD, which triggers translocation of the protein complex to the plasma membrane. At the membrane, the RalGEF encounters Ral, leading towards nucleotide exchange and downstream signaling. *In vitro* the RalGEF protein only requires a CDC25 homology domain for catalytic activity, while *in vivo* studies demonstrate that the upstream REM domain is needed. Activation of Ral alters gene expression, and regulates endocytosis/exocytosis.

For example, Akt can phosphorylate Bcl-2 family members BAD and BAX, rendering them inactive (*96,97*) (Figure 5).

Another important downstream signaling protein of Akt is mTOR in a complex with GβL and Raptor (named mTORC1). This is a multi-step signaling pathway which begins with Akt phosphorylating tuberous sclerosis 2 (TSC2). TSC2 is GAP protein for GTPase Ras homolog enriched in brain (Rheb), which predominately forms a heterodimeric pair with tuberous sclerosis 1 (TSC1). Upon phosphorylation the TSC1/TSC2 complex becomes destabilized, thereafter inhibiting GAP activity for Rheb (*98*). This event relieves a negative regulatory affect, allowing Rheb to activate the mTORC1 complex (*99,100*). The mTORC1 complex phosphorylates S6 Kinase 1 (S6K1) and 4E-BP1, which play a role in the regulation of cell growth and proliferation (*101*). Downstream S6K1 activation ultimately forms a negative feedback loop by inhibiting insulin receptor-substrate function, thus decreasing PI3K activation (*102,103*).

4.3. *RalGEF pathway*

Another well-studied Ras effector is the RalGEF family of proteins, which comprises 4 members; RalGDS, RGL, RGL2/Rlf and RGL3, each of which can interact with members of the Ras family (*104-107*). All the RalGEF family members share a common structure, and display the highest sequence homology in three critical domains: a CDC25 homology domain, Ras Exchange Motif (REM) domain, and a C-terminal RBD (Figure 6) (*108*). RalGEFs act as a guanine exchange factor for Ral, by stimulating the GDP/GTP exchange. The GEF properties come from the CDC25 homology domain. *In vitro* studies show that the CDC25 homology domain is sufficient for RalGDS catalytic activity, while *in vivo* studies show the requirement for the upstream REM domain (*108,109*). The N-terminal region of RalGDS (potentially a portion of the REM domain) has been reported to associate with the catalytic domain and thus blocks GEF activity which may be regulated by phosphorylation by Protein Kinase C (PKC) (*110,111*).

Upon the interaction of activated Ras and RalGEF, the complex is translocated to the plasma membrane where further downstream signaling occurs (Figure 6). Importantly, RalGEFs do not undergo a conformational shift, rather the complex functions by allowing access of the RalGEF with Ral at the membrane (*112,113*). However, the contributions of Ras plasma membrane microdomains to Ras/Ral signaling remain poorly understood. Once Ral is activated in a RalGEF-dependent manner, it subsequently interacts with numerous effectors such as; Sec5, Filamin, RalBP1, and ZONAB, and likely other proteins yet to be identified. Through these interactions, Ral proteins regulate endocytosis, exocytosis, actin organization, and control of gene expression (Figure 6) (*114-116*). Within the literature there is currently debate on the role of RalGEFs in Ras-mediated transformation. Although initial studies in mice had indicated a relatively minor role for RalGEFs in Ras-mediated transformation, following studies indicated a crucial role of RalGEFs in promoting Ras-mediated transformation and tumorigenic growth of human cells. This difference may be in part because of potential variation in mice and human Ras-transformation, which could have some distinctive underlying mechanisms. In

addition, studies which reduce RalGEF function show a diminished effect on cell transformation (*117-119*). RalGDS null mice are viable; however, histological analysis has indicated that proper RalGDS signaling is required for normal apoptosis of papillomas in response to mitogenic signals (*120*) (Figure 6).

Ras-mediated PI3K activation has been shown to increase the GEF activity of RalGDS. EGF-mediated activation of PI3K promotes the association of the N-terminus of the PDK1 with the N-terminus of RalGDS. Currently it is not known whether the N-terminal domains directly interact or if a scaffold protein links them. The PDK1-RalGDS interaction relieves the auto-inhibition of RalGDS, which does not require the catalytic domain of PDK1 (*111*). Collectively, after Ras-dependent movement of RalGDS to the membrane, PDK1 (activated *via* Ras/PI3K/PIP3/PDK1 pathway) is within proximity to associate by some unknown mechanism, which enhances RalGEF catalytic activity.

4.4. *PI3Kinase and MAPK pathway cross-talk*

The PI3K and MAPK pathways interact in numerous ways, which may both negatively and positively regulate downstream cellular responses (*121*). For example, Akt activated by the PI3K pathway has an ability to directly bind and phosphorylate Raf (at Ser259). The phosphorylation of Ser259 negatively regulates the MAPK pathway. The Akt-mediated phosphorylation of Raf allows for the binding partner 14-3-3 to inhibit Raf, and this interaction may shift the cellular response of breast tumor tissue from cell cycle arrest to proliferation through an incompletely understood feedback mechanism (*122*). Alternatively, in cultured mouse fibroblasts, constitutive Raf-MEK1 signaling leads to a negative feedback loop of Ras and PI3K signaling, causing Akt inhibition through Ephrin receptor Eph2A. The Ras-PI3K inhibition from Raf-MEK1 activation is necessary for Ras-induced cell cycle arrest (*123*). The PI3K pathway also activates PAK, which is involved in cytoskeletal dynamics. PAK is regulated by the Rho family of GTPases, and capable of phosphorylating Raf (*124*). This PAK1 dependent phosphorylation of Raf1 may regulate Raf localization to the mitochondria (*125*).

Signals from the MAPK and PI3K pathways have also been shown to converge on common downstream proteins or act synergistically. The mTORC1 complex functions as a point of convergence from multiple signaling networks, and aberrant signaling has been implicated in pathologies including cancer. mTOR is activated downstream of PI3K/Akt; PI3K is a major effector of activated Ras oncogenes. However, mTORC1 is also subject to Akt-independent activation of mTORC1 signaling; *e.g.*, mTOR is activated by mitogenic signaling through activation of the Ras/MEK/ERK pathway. The PI3K/Akt or Ras/MEK/ERK

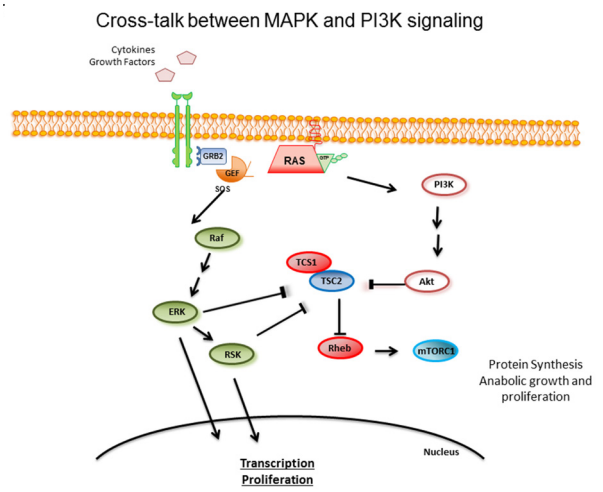

Figure 7. MAPK/PI3K cross-talk in Ras signaling. The Raf/ERK pathway is highlighted in green, and PI3K/mTOR pathways in red and blue.

pathways induce phosphorylation of distinct residues in the mTORC1 negative regulator, TSC2, each resulting in activation of the mTORC1 complex (*126*). Both PI3K/Akt and MAPK/ERK are able to inactivate the proapoptotic protein BAD (*127*). In addition, Raf-MAPK signaling in epithelial cells strongly induces transcription of autocrine expression of EGF-like growth factors, such as HB-EGF, TGFα, and amphiregulin. This autocrine signaling can be a potent inducer of Ras and PI3K, and is implicated in protection of the cells from apoptosis in response to extracellular matrix (ECM) detachment (*128*) (Figure 7).

5. Plasma membrane microdomains and Ras function

Lateral separation at the plasma membrane dictates Ras-isotype specific signaling. Activated HRAS signals from the lipid disordered membrane, which has been shown to be necessary for efficient activation of downstream effector Raf (*38,129,130*). Alternatively, activated RRAS resides within lipid ordered membranes. Initial reports using yeast two-hybrid screening showed RRAS could directly bind Raf-1 (*131*). However, subsequent studies show RRAS does not active the MAPK pathway in cells, suggesting a spatial regulation affecting these differences in HRAS and RRAS signaling (Figure 8) (*132*). Our group recently exploited distinct membrane microdomain targeting of RRAS and HRAS, by swapping the C-terminal targeting domains and stably expressing these Ras variants in non-transformed cells, to explore the contributions of this feature to HRAS signaling, stimulation of proliferation, and tumorigenic potential. Earlier studies had shown that HRAS harboring the RRAS targeting domain (which we refer to as tR), displayed RRAS-like regulation of integrin activation (another Ras function not discussed in detail here), indicating adoption of an RRAS function

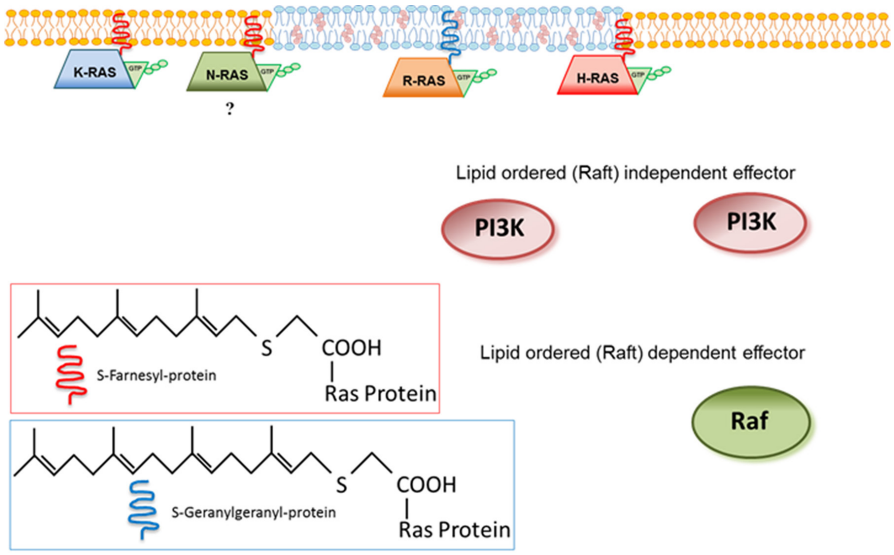

Figure 8. The C-termini modulate Ras signaling by targeting HRAS and RRAS to different membrane microdomains. The lipid ordered membrane (rafts) are distinct microdomains which have increased concentrations of cholesterol and glycosphingolipids. Ras isoforms are laterally segregated at the plasma membrane depending upon the post-translational modifications dictated by the HVR. Shown are the locations in which each isoform signals from upon GTP-loading. The location specific signaling of HRAS has been shown to be necessary for efficient activation of downstream effector Raf, while PI3K activation can occur along the entire membrane.

by HRAS mediated solely by exchange of the plasma membrane targeting domain (*22,45*). We found that the targeting domain (tD) swaps resulted in isotypic switches in Ras mitogenic signaling through the Raf pathway: HRAS harboring the RRAS tD (HRAS-tR) lost the ability to bind and activate Raf, stimulate MEK and ERK activation, and to promote cell proliferation as well as transformation, whereas the converse swap in RRAS, RRAS-tH, showed a gain of all these functions (*39*). However, HRAS-tR retained the ability to activate PI3K, whereas RRAS showed only weak PI3K activation regardless of the associated tD. We proposed a model for regulation of Ras isotypic signaling by lateral segregation in membrane microdomains, outlined in Figure 8. In this model, Ras localized to the lipid ordered/disordered border (*e.g.*, with tH), gains access to Raf interaction, supporting propagation of the MAPK pathway, and mitogenesis, and transforming Ras outcomes in the case of CA Ras (*39*). Thus, targeting to the lipid ordered/disordered membrane border is sufficient for a Ras protein to recruit Raf and propagate the associated signaling and functional effects.

6. Targeting Ras in cancer

There have been a variety of approaches taken to combat Ras oncogenic signaling. The most straightforward approach would be to directly target Ras, though this has proven to be challenging (*133*), in part because Ras binds GTP with an affinity in the picomolar range (*134*). Identification of small molecules which bind with high affinity to the surface of Ras proteins has been difficult due to the globular domain of Ras, which does not have an easily accessible active site or pocket, and Ras was once thought of as 'undruggable' (*135,136*). Few screened inhibitors have shown promising efficacy past pilot *in vitro* experiments (*136-138*). Targeting plasma membrane microdomain localization of H-/N-RAS as well as KRAS holds promise as an alternative approach to interfere with CA Ras signaling and effects.

6.1. *Development of Ras inhibitors and current anti-Ras modalities*

Given the difficulties in targeting Ras directly, some initial efforts focused on intervention of Ras signaling through blocking post-translational modification. Therapeutics designed to interfere with Ras prenylation can be divided into at least two groups. One strategy is development of peptidomimetics that compete with unmodified Ras for farnesyltransferase. Another would be nonpeptidomimetics, such as farnesylpyrophosphate (FPP) analogs, which compete for binding to the farnesyltransferase protein (*139-141*). Collectively, small molecule inhibitors which inactivate the enzymatic function of farnesyltransferase are labeled FTIs (farnesyltransferase inhibitors). Treatment with FTIs (such as SCH 66336) inhibit cell growth in a variety of cancer cell lines when treated *in vitro* and *in vivo* tumor xenografts (*142*). Subsequently, many studies focused on the effect of FTIs on HRAS, showing great efficacy in disrupting membrane association, and blunting colony formation in soft agar (*143,144*). Despite a wealth of data showing blunted cancer growth using various *in vitro* systems, clinical trials using FTIs alone have had disappointingly poor outcomes (*142,145,146*). Further investigation revealed that cells treated with FTIs can

yield alternatively prenylated mutant KRAS or NRAS, by attachment of a geranylgeranyl group (*147,148*). This led to a new approach for developing a class of inhibitors for Geranylgeranyltransferase (GGTIs), though monotherapy or in conjunction with FTIs are not effective due to toxicity issues (*149*). A class of inhibitors targeting both farnesyltransferase (FTase) and geranylgeranyltransferase (GGTase) (such as L-778, 123) were developed and failed to make it through phase I clinical trials. Despite dual inhibition, similar to treatment with FTIs, Ras activity was not inhibited (*150*).

Another strategy for targeting Ras in cancer has been through inhibition of effector pathways directly downstream of Ras. Efforts have largely focused on either the MAPK or PI3K pathways. Sorafenib was the first Raf inhibitor to be approved by the US Food and Drug Administration (FDA) for treatment of carcinomas (*151,152*), though its effects appear largely due to inhibition of tumor angiogenesis (*153*). However, tumors bearing B-Raf activating mutations (such as the most common V600E) do not benefit from treatment with sorafenib, due to the reduced affinity from change in tertiary structure (*154*). This finding facilitated the development for inhibitors targeting CA B-Raf, such as Vemurafenib (PLX4032), which is an ATP-competitive inhibitor of B-Raf V600E (*155*). While successfully inhibiting the mutant Raf, the therapeutic enhances dimer formation and transactivation of the nonmutant Raf dimer, subsequently causing a paradoxical increase in signaling (*156*). Only recently has a new generation of Raf inhibitor has become available, so called 'paradox-breaking' Raf inhibitors. This presents an exciting new avenue, which is expected to make its way to clinical trials within the next few years (*157*).

Increased activity in PI3K signaling is commonly seen in cancer, and p110α is critical for Ras-driven tumorigenesis (*121,158*). Interestingly, Ras and p110α activating mutations are seen together in some cancers, such as colorectal cancer (*159-161*). Quite a few therapeutic agents have been developed to target proteins along this axis which are currently under clinical evaluation. The mTORC1 complex is targeted by Rapamycin analogs, everolimus and temsirolimus, which were the first therapeutics of their kind to be FDA approved to treat cancer (*162,163*). Rapamycin potently inhibits mTOR, and is FDA approved for its immunosuppressant properties, and more recently to treat lymphangioleiomyomatosis (LAM) (*164,165*). Other developed inhibitors currently in clinical trials include targeting of either specific class I PI3-kinase isoforms, or so-called 'pan-PI3K' inhibitors (*166-168*). Despite some preclinical promise, using PI3K-pathway drugs as a monotherapy often leads to acquired resistance (*168-170*).

Often single-pathway inhibitors will induce feedback to the opposite pathway (see PI3K and MAPK pathway cross-talk). As such, the lack of *in vivo* efficacy in targeting either the MAPK or PI3K pathway independently has driven the idea of combinatorial therapies. Studies utilizing *in vitro* systems or animal models which use treatments blocking both pathways have shown some preclinical success, which increases enthusiasm for potential clinical success (*170-172*).

6.2. *Targeting Ras and Ras tumor growth with membrane microdomain-targeting drugs*

6.2.1. *HRAS-induced tumor growth regulated by plasma membrane microdomains*

Based on our model, we predicted that cells stably expressing RRAS-tH would drive tumor progression in mice similar to HRAS cells, due to adoption of HRAS-like signaling by RRAS as a result of tH-directed targeting. We also predicted that HRAS-tR cells would show converse effects, with loss of tumorigenicity on a CA HRAS background by tR-mediated lipid raft sequestration. Surprisingly, both HRAS and HRAS-tR cells yielded robust tumor growth in orthotopic models, yielding tumors up to 2500 mm^3, whereas RRAS-tH cells formed small tumors but they did not progress to volumes > ~50-100mm^3. This was an unexpected result, as we anticipated that tumor promotion by HRAS-tR would be attenuated compared with HRAS. Inhibition of MEK resulted in ~70% attenuation of tumor growth by HRAS cells, but had no effect on HRAS-tR tumor growth. However, blockade of PI3K using LY294002 substantially inhibited growth of both HRAS and HRAS-tR tumors (*39*). Thus, MAPK and PI3K signaling contribute to HRAS-driven proliferation, transformation and tumor progression, and PI3K activation is a major pathway driving tumor progression by lipid raft-sequestered HRAS, which is deficient in MAPK signaling.

We investigated whether mTORC1 pathways represented a point of convergence for HRAS signaling, by monitoring phosphorylation of S6 by S6 kinase, a downstream mTORC1 effector. HRAS induced robust S6 phosphorylation (pS6) at Serine 240/244 in low serum, and PI3K inhibition ablated HRAS-induced pS6, as expected. However, MEK inhibition also diminished pS6. Interestingly, HRAS-tR stable cells, in which HRAS-induced phospho-ERK (pERK) is inhibited, also showed attenuated pS6 in low serum, comparable to MEK-inhibited HRAS expressing cells. Thus, inhibition of MEK partially blocks HRAS-induced mTORC1 signaling. In allograft tumor models, treatment with the mTOR inhibitor rapamycin blunted HRAS cell tumor growth; however, HRAS-tR tumors were much more sensitive to rapamycin. IHC analysis of resected tumors indicated a marked reduction in pS6 in rapamycin-treated HRAS tumors, but also in untreated HRAS-tR tumors. Interestingly, pERK was also reduced in HRAS

tumors with rapamycin (*173*). Together, our recent results indicate that genetically enforced lipid ordered domain sequestration inhibits HRAS-induced mTORC1 signaling, and sensitizes HRAS tumors to rapamycin treatment.

6.2.2. *Gal-1 and combinatorial inhibition*

As described above, we found that dual pathway blockade by a combination of genetically enforced microdomain sequestration (inhibiting Raf pathway signaling) and PI3K (downstream mTOR) inhibition, yields additive effects in blocking progression of HRAS-driven tumor growth. To investigate a more translational approach taking advantage of Ras plasma membrane microdomain targeting, we considered whether inhibition of Gal-1 would alter HRAS PM membrane microdomain localization, and thereby disrupt CA HRAS in a manner similar to genetically-enforced HRAS sequestration (*i.e.*, HRAS-tR). We utilized OTX008, a small molecule allosteric inhibitor of Gal-1, which binds on the opposite side from the β-galactoside-binding site, and has been demonstrated as an anticancer agent (*46,174,175*). We found that OTX008 treatment shifts HRAS to the lipid ordered domain and yields a marked inhibition of tumor growth. However, we found that OTX008 combined with rapamycin provides an additive effect resulting in nearly undetectable tumor growth. These profound results indicate that combined mTOR/Gal-1 inhibition yields stasis of HRAS-driven tumor growth. Thus, inhibition of Gal-1 results in a blockade of GTP-HRAS shuttling from the lipid ordered domain and inhibition of MAPK signaling, and sensitizes tumors driven by CA HRAS to mTOR inhibition (Figure 9A) (*173*). Inhibition of Gal-1 using OTX008 is currently under clinical evaluation, and our findings support the development of Gal-1 targeting schemes to limit progression of mutant HRAS cancers. Moreover, preclinical and clinical trials support using mTOR inhibitors to combat Ras-propagated cancer (*46*); hence, a dual pathway inhibition through Gal-1/mTOR targeting may present increased efficacy over current anti-Ras modalities.

6.2.3. *Gal-3 as a putative approach to combat KRAS cancers*

A majority of Ras-driven cancer cases in the United States (*e.g.*, in lung, pancreas, and colorectal cancers) are caused by mutant KRAS *(6)*. As highlighted above, direct targeting of KRAS has proven clinically intractable due to a lack of drug-binding pockets, and drugs against the membrane anchors have not been successful (*176*). However, alternative strategies to modulate KRAS anchorage to the plasma membrane may prove viable for combating KRAS-driven cancer progression. MEK inhibition alone seems to have

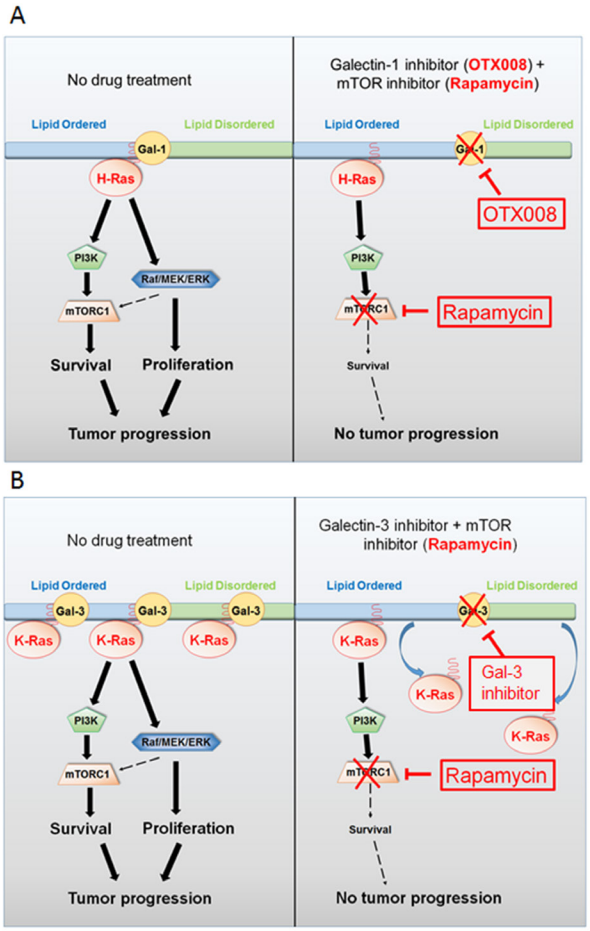

Figure 9. Ras signaling and tumorigenesis in response to inhibition of Galectins and mTOR. (A) Schematic of predicted effects of dual targeting in HRAS cancers. Galectin-1 inhibition using OTX008 results in HRAS mistargeting to the PM and disruption of MAPK mitogenic signaling. Rapamycin treatment potently reduces mTOR survival signaling; analogue mTOR inhibitors should have similar effects. Combinatorial use of these inhibitors results in an additive effect over either monotherapy. **(B)** Predicted effects of dual inhibition of Galectin-3, and mTOR (rapamycin or other mTOR inhibitors), in KRAS mutant tumors. Gal-3 inhibition reduces KRAS membrane anchorage, greatly reducing Raf activation which is lipid ordered domain-restricted, but allowing PI3K activation which is distributed across membrane microdomains. Combined Gal-3 and mTOR inhibition blocks both pathways and inhibits tumor progression.

variable outcomes in KRAS tumors (*177*). Although KRAS does not segregate in PM microdomains like H- and NRAS, targeting KRAS membrane anchorage may be an effective anti-tumor approach in KRAS mutant cancers. Whereas HRAS is localized to the PM with Gal-1, KRAS PM anchorage is supported by the Galectin-3 (Gal-3) scaffold (*178,179*). Gal-3 over-expression leads to chronic KRAS activation, potentiation of Ras signaling, tumor cell activation including increased proliferation and migration, and tumor progression (*180-182*). Gal-3-mediated KRAS activation is associated with ERK, but not PI3K, signaling (*48*). There is substantial evidence for Gal-3 inhibition as a potential tumor blocker (*183-190*).

Thus, a parallel strategy to inhibit KRAS cancers to the Gal-1/mTOR strategy for HRAS, using Gal-3/mTOR inhibition, may provide a new direction (Figure 9B). Our group is currently investigating dual inhibition of Gal-3 and mTOR in blocking progression of KRAS-driven cancers.

7. Conclusions and future prospects

Taken together, how can furthering our understanding of the roles of plasma membrane microdomain Ras targeting help in developing effective therapies to treat patients with Ras-driven cancer? Currently, Ras-positive cancers are treated with a cocktail of therapeutics, generally targeting EGFR or VEGF, or downstream pathway components as described above. However effective the treatment may initially be, it is common to develop an acquired resistance or secondary mutations which requires alternative strategies. Preclinical data largely support positive results using a cocktail of PI3K and MAPK inhibitors, and a large number of inhibitors are currently in clinical trials. We propose a novel rationale for an alternative strategy in HRAS-driven cancers using clinically viable and commercially available therapeutics to disrupt Ras signaling by interfering with localization-dependent effector binding. This approach warrants further analysis as to whether it would be advantageous to individuals who have developed resistance to the common targeted therapies. Future studies are needed to provide further mechanistic insight into the molecular, cellular, and physiological outcomes of combinatorial mTOR/Galectin-1 inhibition.

HRAS mutations are considerably rarer in cancer than mutations in NRAS or KRAS. However, KRAS-positive anti-cancer approaches may still benefit from this basic dual treatment paradigm. Whereas HRAS is localized to the PM with Gal-1, KRAS anchorage is supported by the Gal-3 scaffold. This suggests that inhibition of Gal-3 and mTOR may mimic HRAS results in the context of KRAS mutant cancers. Scaffold proteins that specifically bind NRAS, and PM localization remain controversial, so implications in targeted therapies will require further exploration. However, it is likely that an analogous scaffolding protein which localizes NRAS to the PM exists. This suggests that both N- and KRAS-driven tumorigenesis may benefit through a modified form of the proposed targeted therapy. Overall, new approaches to combat Ras-driven cancers are still needed, and disrupting plasma membrane microdomain-based Ras function holds promise as an effective strategy.

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