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DEVELOPMENT AND CHARACTERIZATION OF NOVEL RAS INHIBITORS

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A

THESIS

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Houston, Texas

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Development and Characterization of Novel Ras Inhibitors

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Ras genes are mutated in 15% of human cancers. Ras GTPases operate as molecular switches regulating cellular processes including proliferation, differentiation, and apoptosis. The three main isoforms of Ras – H-Ras, K-Ras, and N-Ras – inhabit distinct nanodomains of the plasma membrane and intracellular compartments including the Golgi. However, the role of single endogenous Ras isoforms on these compartments remains unclear as most studies have utilized ectopically expressed and mutant forms of Ras proteins. In an effort to develop novel tools that will allow us to abrogate individual endogenous Ras isoforms, we targeted the catalytic domain of p120RasGAP to the plasma membrane with the hypervariable region (HVR) of H-Ras (GAP-CTH) or K-Ras (GAP-CTK) and to the Golgi using the HVR of H-Ras with insertion of a point mutation (GAP-CTH181S). We performed GST-RBD pull-downs on cells expressing each GAP construct and stimulated with epidermal growth factor (EGF). We found that GAP-CTH and GAP-CTK specifically inhibited H-Ras or K-Ras, respectively. However, we did not detect any effect of GAP-CTH181S on Ras activation. Additionally, we used confocal microscopy to verify the ability of GAP constructs to abrogate Ras activation in distinct sub-cellular compartments. We found that GAP-CTH inhibits H-Ras activation on the plasma membrane, while GAP-CTK inhibits K-Ras activation on the plasma membrane. On the contrary, GAP-CTH181S inhibited H-Ras activation on the Golgi. We also analyzed the effects of these GAP constructs on the activation of ERK and Akt in response to EGF stimulation. We found that EGF

stimulation of the MAPK pathway was inhibited by GAP-CTK but none of the other GAP constructs, while Akt activation was not inhibited by any GAP construct. Finally, we assayed cellular proliferation and differentiation. We found that GAP-CTK and GAP-CTH were equipotent inhibitors of cellular growth, whereas GAP-CTH181S was less potent. We also found that GAP-CTK and GAP-CTH inhibited differentiation with similar potency, while GAP-CTH181S was more potent. This approach may be adapted to investigate any Ras-dependent signaling pathway. Therefore, it has the potential to become a powerful tool for studying Ras isoform-specific signaling outputs.

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Chapter 1. Literature Review

1.1 Introduction

Ras genes were originally identified as the oncogenes in the Harvey and Kirsten sarcoma retroviruses responsible for inducing tumors in rats. These retroviral oncogenes were called *ras*, for rat sarcoma, and were later identified in mammalian cells as mutated versions of normal cellular *ras* genes. Three *ras* proto-oncogenes exist in the human genome: *H-ras*, *K-ras*, and *N-ras* (Malumbres and Barbacid, 2003; Prior et al., 2012). The latest gene, *N-ras*, was identified in neuroblastoma cells; however, has not been found in any retrovirus.

Constitutively active *ras* mutations are present in 15% of human cancers. Point mutations in codons 12, 13, or 61 make *ras* oncogenic. There are other mutations that may convert *ras* into oncogenes such as codon 146 in *K-ras*. These mutations lock Ras in the active state by rendering Ras resistant to the effects of GTPase activating proteins (Barbacid, 1987; Lau and Haigis, 2009).

Each *ras* gene encodes a specific Ras isoform except *K-ras*, which bears two gene products (K-Ras 4A and K-Ras 4B) generated through alternative splicing (Capon et al., 1983; McGrath et al., 1983). All Ras isoforms are expressed ubiquitously in cells except for K-Ras 4A which is restricted temporally and spatially to the lung, liver, and kidney (Pells et al., 1997). Ras isoforms are highly homologous with nearly identical sequences in the effector, exchange factor, and guanine nucleotide binding domains (amino acids 1-165), which together comprise the G domain. The C—terminal hypervariable region (amino acids 166-188/189) is the only region of Ras proteins that shows significant sequence divergence between the isoforms (Hancock and Parton, 2005).

Ras proteins are 21 kilodalton peripheral membrane GTPases that bind guanosine triphosphate (GTP) or guanosine diphosphate (GDP) to control signal transduction cascades. Ras signaling regulates a variety of cellular functions

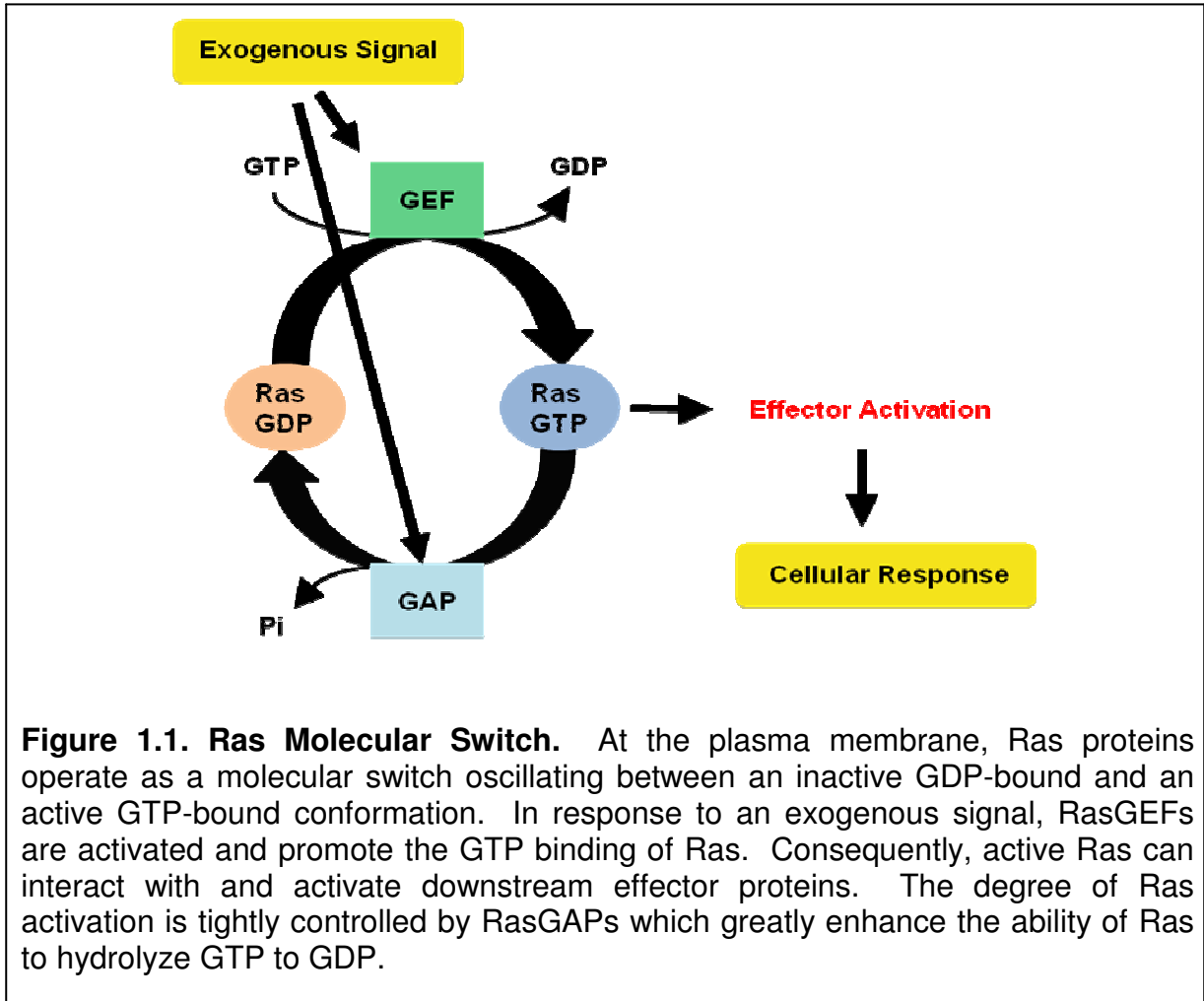


Figure 1.1. Ras Molecular Switch. At the plasma membrane, Ras proteins operate as a molecular switch oscillating between an inactive GDP-bound and an active GTP-bound conformation. In response to an exogenous signal, RasGEFs are activated and promote the GTP binding of Ras. Consequently, active Ras can interact with and activate downstream effector proteins. The degree of Ras activation is tightly controlled by RasGAPs which greatly enhance the ability of Ras to hydrolyze GTP to GDP.

including proliferation, differentiation, and survival. Two sets of proteins, guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) modulate Ras activity. GEFs stimulate the exchange of GDP for GTP. Ras in the GTP-bound state is active (Bos et al., 2007; Satoh and Kaziro, 1992); GTP-loading triggers a conformational change in the Switch 1 (amino acids 30-38) and Switch 2 (amino acids 60-76) regions in the G domain endowing Ras proteins with a high affinity for downstream effectors (Wittinghofer et al., 1993). In contrast, GAPs act

as negative regulators to greatly enhance the intrinsic GTPase activity of Ras to hydrolyze GTP to GDP rendering Ras inactive (Bos et al., 2007) .

Ras is the founding member of a family containing a multitude of small GTPases that regulate signal transduction pathways. The Ras superfamily is comprised of 5 subfamilies: Arf, Rab, Ran, Rho, and Ras (Wennerberg et al., 2005). The Arf subfamily of proteins is involved in vesicular budding (Nie et al., 2003), while the Rab subfamily is involved in vesicle targeting and fusion (Zerial and McBride, 2001). The Rho subfamily is responsible for the control of cytoskeletal organization and cell motility (Etienne-Manneville and Hall, 2002). Finally, Ran is involved in nucleocytoplasmic transport and cell cycle progression (Pemberton and Paschal, 2005; Weis, 2003).

The Ras subfamily is subdivided into groups, which share 40-50% sequence homology with the classical Ras proteins. The Ras protein subfamily includes the following: Ras (H-Ras, N-Ras and K-Ras), R-ras proteins (R-ras, TC21, and M-Ras), Rap proteins (Rap1A, Rap1B, Rap2A, and Rap2B), Rheb, and RalA and RalB. The R-Ras subfamily of proteins regulates mitogenesis and the cytoskeleton and is mutated in some human tumors (Ehrhardt et al., 2002). Rap proteins are involved in the control of cell adhesion and spreading mediated by integrin (Bos, 2005; McLeod et al., 2004). RalA and RalB proteins have various functions including regulation of cell morphology changes, cell motility, and the activity of transcription factors (Feig, 2003). Rheb proteins are associated with the control of cell growth and the cell cycle (Aspuria and Tamanoi, 2004).

1.2 Ras Processing and Trafficking

1.2.1 *Post-translational Modification of the Hypervariable Region*

Ras proteins are synthesized as hydrophilic molecules in the cytosol and are posttranslationally modified by the attachment of a hydrophobic anchor for membrane association (Mor and Philips, 2006). The C-terminal hypervariable region contains two signal sequences that are responsible for targeting Ras proteins to the cytoplasmic face of the plasma membrane. The first of these sequences is the CAAX motif (C for cysteine, A for aliphatic amino acids, and X for any amino acid) located at the extreme C-terminus of the HVR (Hancock et al., 1989). The CAAX motif undergoes a series of posttranslational modifications. The first modification is prenylation of the cysteine by the cytosolic enzyme, protein farnesyltransferase, which is covalently attached (Hancock et al., 1990; Reiss et al., 1990; Schaber et al., 1990). Further modification of the CAAX motif occurs on the cytoplasmic face of the endoplasmic reticulum (Choy et al., 1999). The AAX residue is cleaved by a protease called Ras converting enzyme 1 (Rce1) (Boyartchuk et al., 1997; Gutierrez et al., 1989). Finally, the now C-terminal farnesylated cysteine is methylated on the α -carboxyl group by isoprenylcysteinecarboxyl methyltransferase (Icmt) (Dai et al., 1998). The CAAX motif of Ras proteins increases the hydrophobicity of the Ras C-terminus, but is insufficient for stable membrane binding. A second membrane signal is required for targeting Ras to the plasma membrane (Hancock et al., 1990). In H-Ras, K-Ras 4A and N-Ras, this signal is palmitoylation of one or two cysteines upstream of the CAAX motif by Ras palmitoyltransferase (RPT) (Lobo et al., 2002). RPT is located on the ER and Golgi apparatus in mammalian cells (Apolloni et al., 2000; Chiu et al., 2002). While H-ras has two sites for palmitoylation at C181 and C184, K-Ras

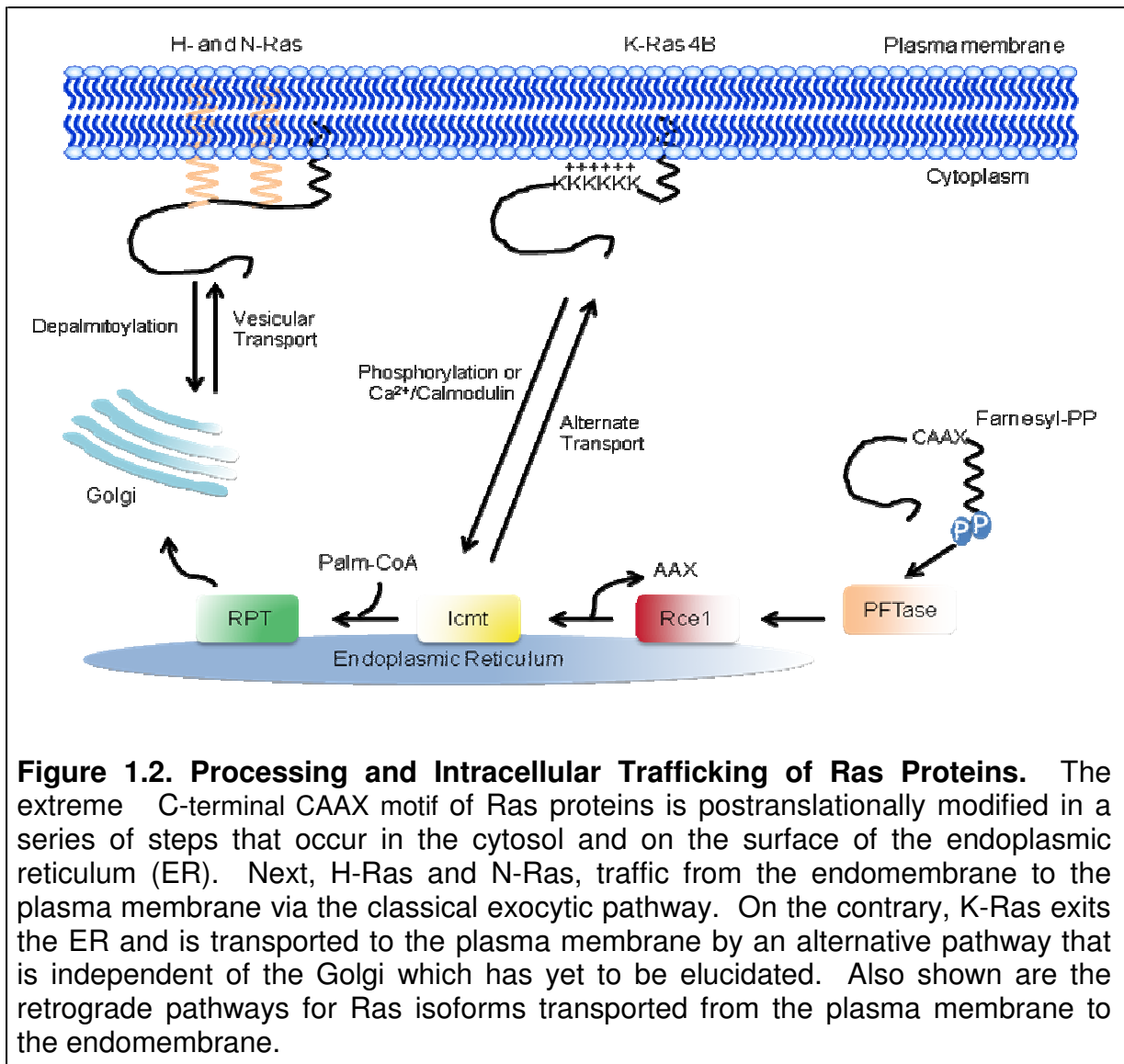
4A and N-Ras have one site at C180 and Ras and N-Ras. The half-life of palmitate on H-Ras is 2.4h while the half-life of palmitate on N-Ras is only 20 min. (Baker et al., 2003; Lu and Hofmann, 1995). In contrast to H- and N-Ras, K-Ras 4B has a polybasic region of six lysine residues responsible for plasma membrane association (Hancock et al., 1990).

1.2.2 From the Endoplasmic Reticulum to the Plasma Membrane

Posttranslational modification of the C-terminal CAAX motif of nascent Ras proteins is completed on the membrane of the endoplasmic reticulum. Further modification of H-Ras and N-Ras by RPT occurs upstream of the CAAX motif on the ER and the Golgi apparatus where H-Ras and N-Ras traffic to the plasma membrane via the secretory pathway, K-Ras is not palmitoylated and traffics to the plasma membrane by a route that is unclear; (Hancock, 2003; Swarthout et al., 2005) however, mechanisms for K-Ras transport have been suggested. One such mechanism is that the positively charged polybasic region of K-Ras contributes to diffusion of this isoform down an electrostatic gradient to the negatively charged lipid head groups on the plasma membrane (Okeley and Gelb, 2004; Roy et al., 2000). The final hypothesis is that K-Ras transport is dependent upon microtubules (Chen et al., 2000; Thissen et al., 1997).

1.2.3 From the Plasma Membrane to the Endomembrane

Recent studies have shown that Ras is not permanently anchored to the plasma membrane, but undergoes retrograde trafficking. H-Ras and N-Ras engage in a continuous cycle of palmitoylation/depalmitoylation that maintains specific localization of these proteins to the plasma membrane and Golgi apparatus.



Palmitoylated Ras proteins on the plasma membrane are depalmitoylated by an unidentified thioesterase causing them to be released from the cytoplasmic face of the plasma membrane where they diffuse through the cytosol to the Golgi membrane. Non-palmitoylated H-Ras and N-Ras encounter RPT on the ER and Golgi and are repalmitoylated (Goodwin et al., 2005; Rocks et al., 2005). After palmitoylation has occurred, Ras proteins traffic to the plasma membrane via vesicular transport (Apolloni et al., 2000). In contrast, K-Ras does not undergo further lipid modification through palmitoylation, but also engages in retrograde

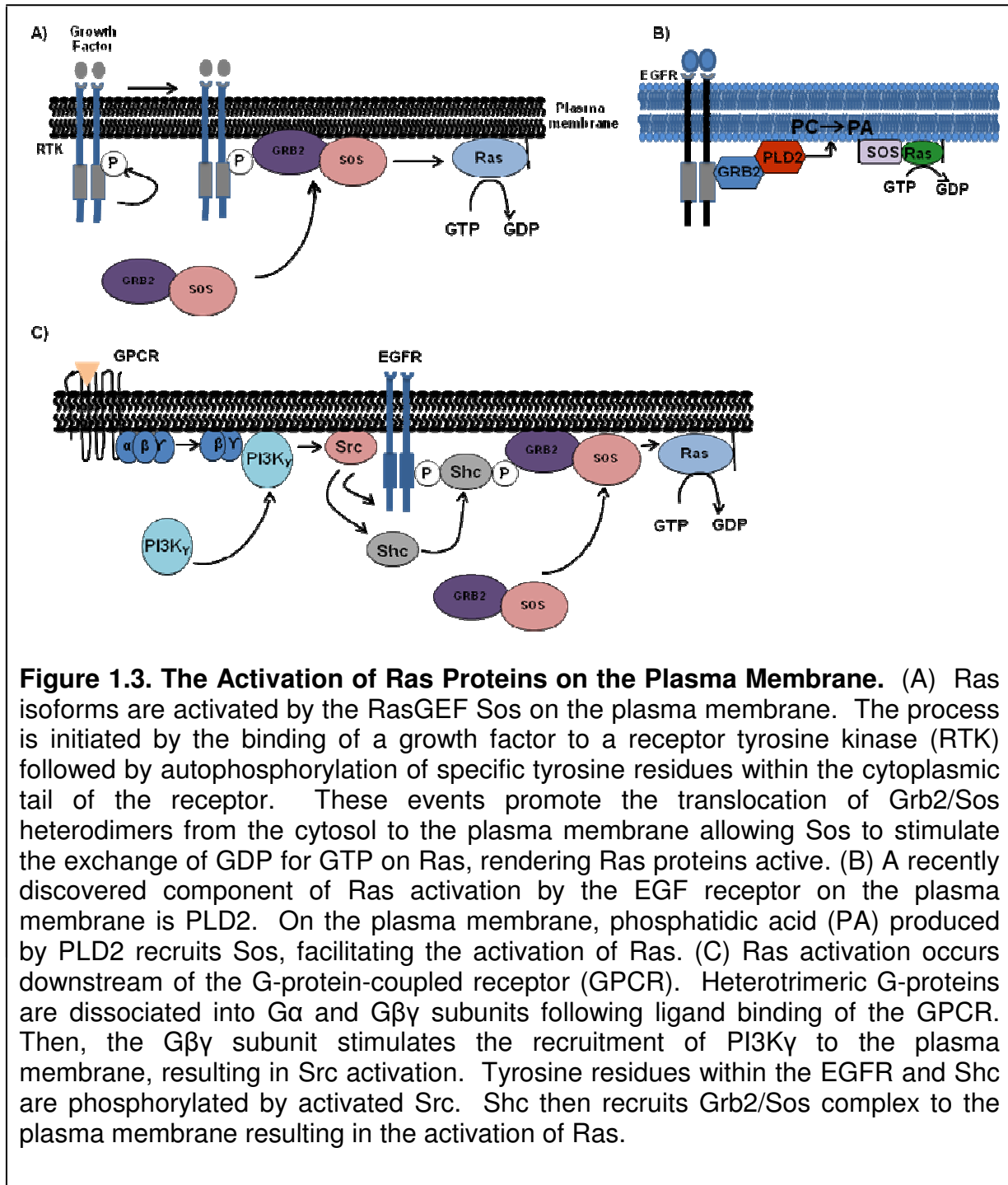
trafficking. In hippocampal neurons, activation of the glutamate receptor induces Ca^{2+} influx and the subsequent binding of calmodulin to the polybasic-prenyl motif of K-Ras. This results in the dissociation of K-Ras from the plasma membrane and the redistribution of K-Ras to the Golgi apparatus and early/recycling endosomes (Fivaz and Meyer, 2005). Like calmodulin, other protein chaperones including smgGDS, phosphodiesterase 6 delta subunit (PDE δ) and prenylated Rab protein acceptor protein 1 (PRA1) may be responsible for the transport of K-Ras away from the plasma membrane. These protein chaperones stimulate the dissociation of K-Ras and other small GTPases from the plasma membrane (Bhagatji et al., 2010; Kawamura et al., 1993). Recent studies have emerged elucidating this role for PDE δ in the intracellular trafficking and signaling of Ras isoforms. The current model for PDE δ -mediated Ras trafficking demonstrates the extraction of K-Ras and H-Ras from the (Bhagatji et al., 2010; Kawamura et al., 1993) endomembrane and plasma membrane, respectively. PDE δ sequesters the farnesyl chain of Ras in a hydrophobic pocket resulting in the solubilization Ras in the cytosol. Non-prenylated small GTPases called Ar12/3 bind to PDE δ stimulating the release of Ras onto the plasma membrane (K-Ras) and Golgi (H-Ras) (Chandra et al., 2012; Ismail et al., 2011; Philips, 2012). An alternative mechanism of K-Ras retrograde trafficking involves phosphorylation of K-Ras on serine 181 by protein kinase C neutralizing the negative charge of the polybasic domain (Bivona et al., 2006). Furthermore, K-Ras is in dynamic equilibrium with a pool in the cytosol (Mor and Philips, 2006).

1.3 Ras Guanine Nucleotide Exchange Factors

1.3.1 Son of Sevenless (Sos)

Son of sevenless is a well-characterized cytosolic Ras guanine nucleotide exchange factor that is responsible for coupling intracellular signals from receptor tyrosine kinases to Ras proteins (Aronheim et al., 1994). Sos1 and Sos2 are two isoforms that are ubiquitously expressed in mammalian cells (Chardin et al., 1993). Although these proteins are highly homologous, Sos2 has a higher binding affinity for Grb2 than Sos1 suggesting that these GEFs may differentially contribute to receptor-mediated Ras activation (Bowtell et al., 1992; Yang et al., 1995). The Cdc25 and Ras exchanger motif (REM) domains of Sos are required for Sos-mediated Ras specific nucleotide exchange. The REM domain connects with a surface of the Cdc25 domain. Ras interacts with Sos entirely through binding of the Cdc25 domain for nucleotide exchange. The product of this exchange reaction, GTP-loaded Ras, interacts with a distal allosteric site on Sos that is between the Cdc25 and REM domains creating a bridge between these domains and enhancing Ras exchange activity. This indicates that Sos is subject to positive feedback regulation by Ras-GTP (Margarit et al., 2003; Sondermann et al., 2004). The generally accepted model for how Sos activates Ras on the plasma membrane is as follows: In response to extracellular stimuli, tyrosine kinase receptors are activated and undergo autophosphorylation at tyrosine residues. In the cytosol, Sos is bound to Grb2 through the interaction of its carboxyl terminus proline-rich sequences and Src homology 3 domain of Grb2 (Buday and Downward, 1993; Egan et al., 1993; Li et al., 1993). The Grb2-Sos complex translocates to the plasma membrane where the Src homology 3 domain of Grb2 associates with the phosphorylated residues of the receptor. Sos is brought into close proximity to Ras

on the plasma membrane and GDP/GTP exchange is catalyzed (Lowenstein et al., 1992; Rozakis-Adcock et al., 1993). This model has undergone review based on a recent study showing that a lipid-protein interaction with phosphatidic acid (PA) is, in fact,



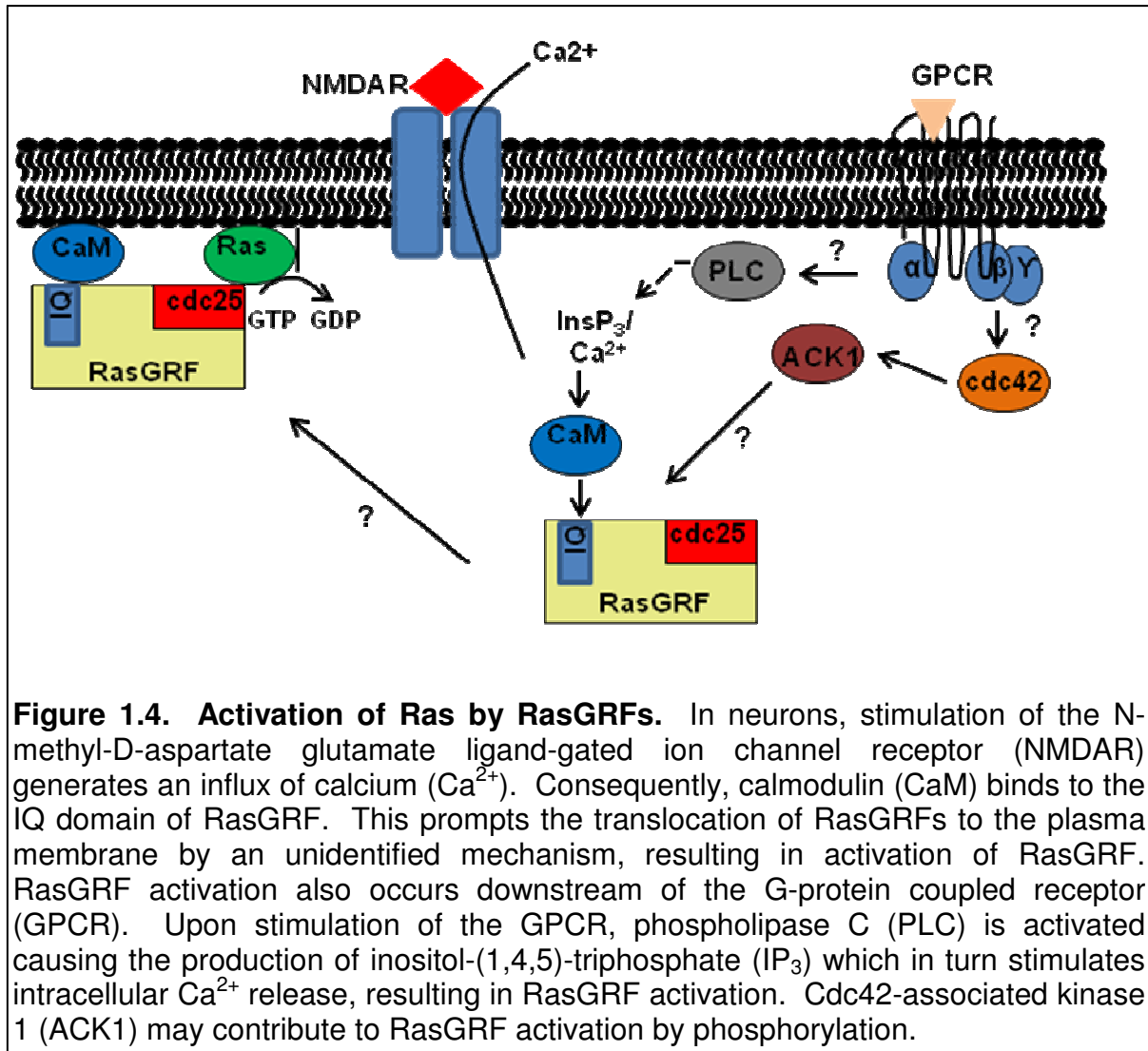
accountable for recruitment of Sos to the plasma membrane upon EGFR stimulation. PA is generated by phospholipase D2 (PLD2) via the hydrolysis of phosphatidylcholine (PC) (Zhao et al., 2007).

1.3.2 Ras Guanine-Nucleotide Release Factors (RasGRFs)

The RasGRF family of guanine-nucleotide exchange factors is comprised of two isoforms, RasGRF1 and RasGRF2. The expression of these proteins is restricted to specific tissue locations. Both isoforms are predominantly expressed in the brain. Traces of RasGRFs expression have also been detected in the heart, lung, and pancreas. The regulation of RasGRFs in signal transduction occurs in a different manner than Sos proteins. RasGRF1 and RasGRF2 are activated in response to elevated levels of cytosolic calcium. A signaling pathway involving activation of the N-methyl-d-aspartate (NMDA) receptor in neuronal cells by ionomycin is known to trigger calcium-dependent activation of RasGRFs. This activation is mediated by binding of calmodulin to the IQ motif of RasGRFs, which stimulates the translocation of RasGRFs to the plasma membrane (Farnsworth et al., 1995). Once at the plasma membrane RasGRFs stimulate Ras guanine nucleotide exchange. Although ionomycin preferentially activates RasGRF1 at the plasma membrane, it also induces activation of RasGRF1 at the endoplasmic reticulum at lower levels. RasGRF1 is also activated in response to ligand stimulation of the G-protein coupled receptor (GPCR). Full activation of RasGRF1 requires serine and tyrosine phosphorylation events and the release of calcium from intracellular stores in response to inositol-(1,4,5)-triphosphate (IP₃) generated by GPCR-activated phospholipase C (Tian et al., 2004). Non-receptor tyrosine kinases such as the Cdc42-associated kinase 1 (ACK1) and Src (sarcoma) have also been shown to phosphorylate RasGRF1 (Kiyono et al., 2000).

1.3.3 Ras Guanyl-Releasing Proteins (RasGRPs)

Another family of Ras guanine nucleotide exchange factors is the Ras guanyl-releasing proteins, RasGRP1, RasGRP2, RasGRP3, and RasGRP4



(Mitin et al., 2005). Like RasGRFs, RasGRP proteins have a restricted pattern of expression. RasGRP1 and 3 are predominantly expressed in the adult brain and lymphoid tissues with some expression present in the kidney (Ebinu et al., 1998; Tognon et al., 1998; Yamashita et al., 2000). RasGRP2 is expressed in a wide range of tissues including the brain, heart, lungs, pancreas, and kidney (Clyde-

Smith et al., 2000). RasGRP4 is expressed in peripheral blood mononuclear leukocytes in adults, while fetal expression is distributed in the heart, lung, kidney, liver, and thymus (Yang et al., 2002). In addition to the catalytic domains REM and CDC25 that all Ras guanine nucleotide exchange factors possess, RasGRPs contain calcium binding EF hands and diacylglycerol (DAG) binding C1 domains. The deletion of these domains results in the inability of RasGRP1 to activate Ras and transform fibroblasts. The membrane recruitment and activation of RasGRPs1,

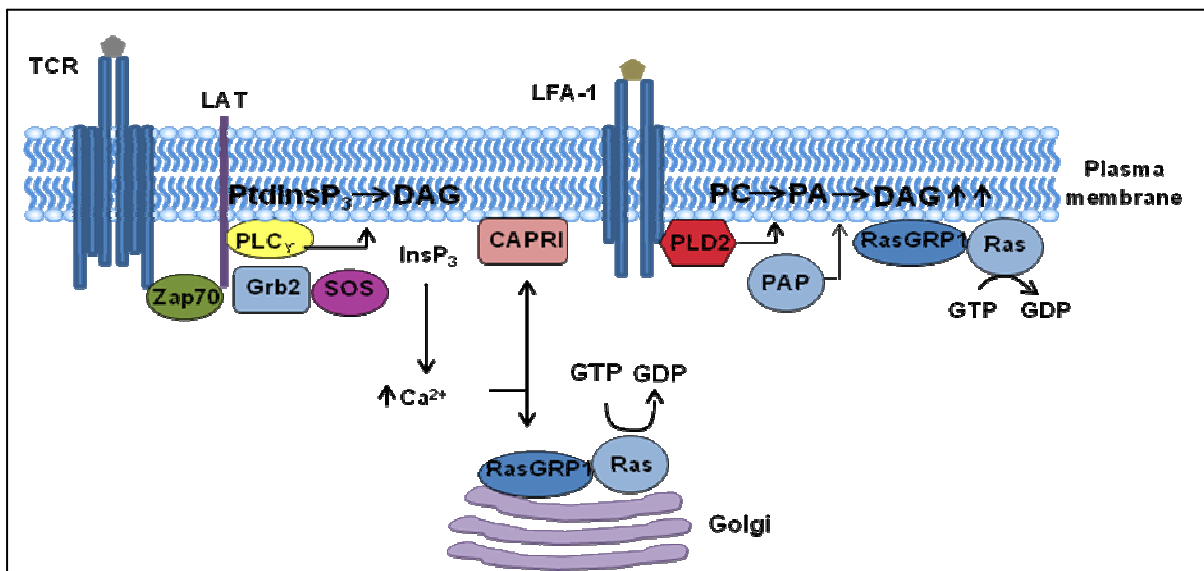


Figure 1.5. Activation of Ras by RasGRPs. Upon activation of the TCR, the scaffold protein LAT is phosphorylated by Zap70 which provides binding sites for the recruitment of PLC and the GRB2/Sos complex. PLC then hydrolyzes PtdInsP2 which in turn generates DAG and InsP3. Calcium is released from intracellular stores by InsP3 recruiting the RasGAP CAPRI to the plasma membrane; therefore Ras activation by Sos is prevented. At the same time, calcium along with DAG drive RasGRP1 to the Golgi, where H- and N-Ras are activated. Co-activation of LFA-1 with the TCR results in increased levels of DAG through PLD2 and PAP. These DAG levels are sufficient to recruit RasGRP1 to the plasma membrane where Ras is activated.

3, and 4 is directly dependent on DAG binding to their C1 domains (Ebinu et al., 1998). In contrast, RasGRP2 is constitutively associated with the membrane by N-

terminal myristoyl and palmitate modifications, but its ability to stimulate nucleotide-exchange on Ras is also enhanced by DAG. DAG production is coupled to phospholipase C activity, which generates the second messengers, DAG and IP₃ (Clyde-Smith et al., 2000). Furthermore, RasGRP1 and RasGRP3 are the only RasGEFs that are localized in the Golgi apparatus (Caloca et al., 2003).

1.4 Ras GTPase-Activating Proteins (RasGAPs)

1.4.1 p120 RasGAP

p120 RasGAP was the first GTPase activating protein (GAP) to be discovered (Trahey and McCormick, 1987). Inactivating mutations in the RASA1 gene, which codes for p120GAP, are found in patients with a capillary malformation-arteriovenous malformation syndrome (Eerola et al., 2003). p120 RasGAP helped to elucidate the reason behind missense mutations at Ras residues G12 or Q61 that result in constitutively active Ras. These Ras mutant proteins are insensitive to GAP activity and are trapped in the active GTP bound conformation. p120 RasGAP interacts with Ras at the effector site (Der et al., 1986; Scheffzek et al., 1997; Seeburg et al., 1984). The mechanism of p120 RasGAP-assisted GTP hydrolysis involves the stabilization of glutamine at position 61 of Ras by GAP. An arginine provided by GAP is positioned into the phosphate-binding site and stabilizes the transition state by neutralizing the negative charge. Mutation of glutamine 61 abolishes GAP-induced hydrolysis (Ahmadian et al., 1997; Coleman et al., 1994).

p120 RasGAP is a widely expressed cytoplasmic protein that is comprised of several domains including a GAP catalytic domain and an N-terminal region containing two SH2 motifs, one SH3 motif, a pleckstrin homology domain, and the calcium dependent lipid binding motif, C2. The two SH2 motifs are responsible for interactions with activated tyrosine kinase receptors and other tyrosine

phosphorylated proteins (Cleghon et al., 1998; Kaplan et al., 1990). p120 RasGAP is also a caspase substrate. When cleaved by caspases this protein negatively or positively regulates apoptosis in a manner that is dependent on caspase activation (Yang and Widmann, 2001).

1.4.2 Neurofibromin

Neurofibromin is a large cytoplasmic protein encoded by NF1, a tumor suppressor gene that is lost in the autosomal dominant disease neurofibromatosis type 1 (Dasgupta and Gutmann, 2003). Clinically, this disease is characterized by learning disabilities, bone deformities, nervous tumors, and gliomas. Neurofibromin is expressed mostly in Schwann cells, neurons, astrocytes, leukocytes, and oligodendrocytes (Dasgupta and Gutmann, 2003; Jouhilahti et al., 2011).

The known functional domains of neurofibromin are limited to the Sec14/PH module and the RasGAP catalytic domain. The Sec14/PH module lies at the C-terminus of the catalytic domain and consists of a Sec 14 homology segment and a neighboring PH-like domain. The Sec 14 homology segment binds to glycerophospholipids and mediates the exchange of phosphatidylinositol for phosphatidylcholine between membrane compartments, a common process associated with lipid metabolism and membrane trafficking. Association of the PH-like domain with the Sec-14 homology segment through an interface allows for regulation of phospholipid binding. A specific region of neurofibromin shares sequence homology with the RasGAP catalytic domain of p120 RasGAP referred to as the GAP related domain (GRD) (Trovo-Marqui and Tajara, 2006). This domain has a higher affinity for H-Ras and N-Ras than p120 RasGAP. The RasGAP domain activity of neurofibromin is inhibited by three lipids: arachidonate, phosphatidate, and phosphatidylinositol-4,5-bisphosphate (Bollag and McCormick, 1991).

1.4.3 GAP1

GAP1 is another family of RasGAPs that includes two subgroups GAP1^m and GAP1^{IP4BP}, and Calcium promoted Ras inactivator (CAPRI) and Ras-GTPase-activating-like protein (RASAL) (Lockyer et al., 1997; Yarwood et al., 2006). Both GAP1^m and GAP1^{IP4BP} are ubiquitously expressed and are activated by binding of phosphoinositides to the pleckstrin homology domain found in both proteins (Lockyer et al., 1999). GAP1^{IP4BP} constitutively associates with the plasma membrane (Lockyer et al., 1997) while GAP1^m translocates from the cytosol to the plasma membrane in response to EGF stimulation (Lockyer et al., 1999). CAPRI and RASAL are activated by calcium influx, but differ in their response to calcium. CAPRI translocates from the cytosol to the plasma membrane when intracellular calcium levels rise (Lockyer et al., 2001; Walker et al., 2004) whereas RASAL cycles between the cytosol and the plasma membrane in response to the frequency of calcium oscillations (Liu et al., 2005; Walker et al., 2004).

1.4.4 Other GAPs

SynGAP is a constitutively membrane associated RasGAP that is mainly present in the brain (Kim et al., 1998). There is little information known about the regulation of this RasGAP; however, its activity is upregulated by serine phosphorylation by calmodulin-dependent protein kinase II (CaMKII) (Oh et al., 2004). Similarly, very little is known about the regulation of the activity of DAP2IP, an abundantly expressed RasGAP in mammalian cells (Wang et al., 2002).

1.5 Ras Effectors

1.5.1 Raf

The Raf kinase family was the first Ras effector identified and is the most thoroughly studied (Moelling et al., 1984; Rapp et al., 1983). The mammalian genome contains 3 Raf genes that encode for three isoforms of Raf, C-Raf, A-Raf, and B-Raf, which are serine/threonine kinases. Alternative spliced forms of A-Raf and B-Raf have also been identified. Oncogenic *B-RAF* mutations have been identified in approximately 70% of human melanomas, making the involvement of B-Raf in oncogenesis far greater than either C-Raf or A-Raf (Dhomen and Marais, 2007).

There are two regions of Raf proteins that associate with GTP-bound Ras: an N-terminal Ras binding domain (RBD) and an adjacent cysteine rich domain (CRD). These motifs bind to specific domains of Ras. The RBD interacts with the switch 1 region of Ras and the CRD binds to the switch II region. In its inactive state, C-Raf is present in the cytoplasm bound to the scaffold protein 14-3-3. The activation of C-Raf is highly complex and involves membrane recruitment, phosphorylation/dephosphorylation events, and protein-protein interactions. Binding of GTP-loaded Ras to Raf via the RBD and CRD results in the recruitment of Raf to the plasma membrane (Bondeva et al., 2002; Stokoe et al., 1994; Thorson et al., 1998). The recruitment event is necessary but not sufficient for Raf activation. Additionally, dephosphorylation of Ser43, Ser233, and Ser259 and phosphorylation of Ser621, Ser338, Tyr341, Thr491 and Ser494 are required. Dephosphorylation of Ser43 removes the steric hindrance on the binding of the N-terminus of Raf to Ras. Dephosphorylation of Ser259 displaces 14-3-3 from this binding site, while phosphorylation of Ser621 allows 14-3-3 to bind to the C-terminus of Raf.

Phosphorylation of Ser338 and Tyr341 imparts a negative charge to the N-region of C-Raf. The activation of A-Raf follows a similar pattern to that of C-Raf, while the activation of B-Raf is solely dependent upon Ras alone (Chong et al., 2001; Dhomen and Marais, 2007; Dumaz et al., 2002).

Active Raf phosphorylates two downstream effectors, MEK1 and MEK2 on two serine residues in the activation segment. Subsequently, MEK1 and MEK2 activate ERK1 and ERK2 through phosphorylating threonine and tyrosine residues in their activation segments. ERK phosphorylates a multitude of cytosolic and nuclear substrates, including transcription factors, cytoskeletal proteins, and kinases and phosphatases. This signaling cascade of Ras/Raf/MEK/ERK is known as the MAP kinase pathway. This pathway leads to the regulation of cellular differentiation, proliferation, and survival and is commonly mutated in human cancers. There are several scaffolding proteins that modulate the activation of the MEK-ERK cascade, including the Kinase suppressor of Ras-1 (KSR1), Connector enhancer of KSR (CNK), Paxillin and MEK partner-1 (MP1) (Kolch, 2005; Shaul and Seger, 2007; Yoon and Seger, 2006).

Another interesting aspect of Raf signaling is the formation of C-Raf/B-Raf heterodimers. This is dependent on GTP-Ras and 14-3-3. These heterodimers have increased kinase activity compared to B-Raf and C-Raf monomers/homodimers due to dramatically elevated C-Raf kinase activity stimulated by B-Raf. In contrast, C-Raf has no effect on B-Raf kinase activity (Garnett et al., 2005).

1.5.2 Phosphatidylinositol 3-Kinase (PI-3K)

A second Ras effector essential for Ras transforming activity is PI3-K. PI3-kinases are heterodimers with a p85 regulatory subunit and a p110 catalytic

subunit. The p85 subunit inhibits p110 catalytic activity of PI3-K. The p110 subunit contains domains for membrane anchoring, interactions with p85 and Ras, and kinase activity. PI3-kinases can be activated through two independent mechanisms: binding of Ras-GTP to the p110 subunit or binding of p85 to phosphotyrosine residues on activated receptor tyrosine kinases, either directly or indirectly. The p85-RTK interaction recruits the inactive p85-p110 complex to the plasma membrane and relieves the inhibitory effects of p85 on p110 (Krasilnikov, 2000). Activated p110 catalyses the phosphorylation of the 3' hydroxyl group of phosphatidylinositol derivatives such as phosphatidylinositol-(4,5)-bisphosphate (PIP₂) to generate phosphatidylinositol-(3,4,5)-triphosphate (PIP₃). PIP₃ activates its main effector, AKT, and other downstream effector pathways. PIP₃ signaling is terminated by the action of phosphatases including PTEN, SHIP1, and SHIP2 (Vivanco and Sawyers, 2002).

The activation of AKT is dependent upon plasma membrane recruitment and phosphorylation at Thr308 and Ser473. The membrane recruitment of AKT is mediated by the direct association of its N-terminal pleckstrin homology (PH) domain with the membrane-associated PIP₃ (Rodriguez-Viciano et al., 1994). Phosphorylation of Thr308 is catalyzed by the PIP₃-dependent protein kinase (PDK1), while Ser473 is phosphorylated by PDK2 (Alessi et al., 1997).

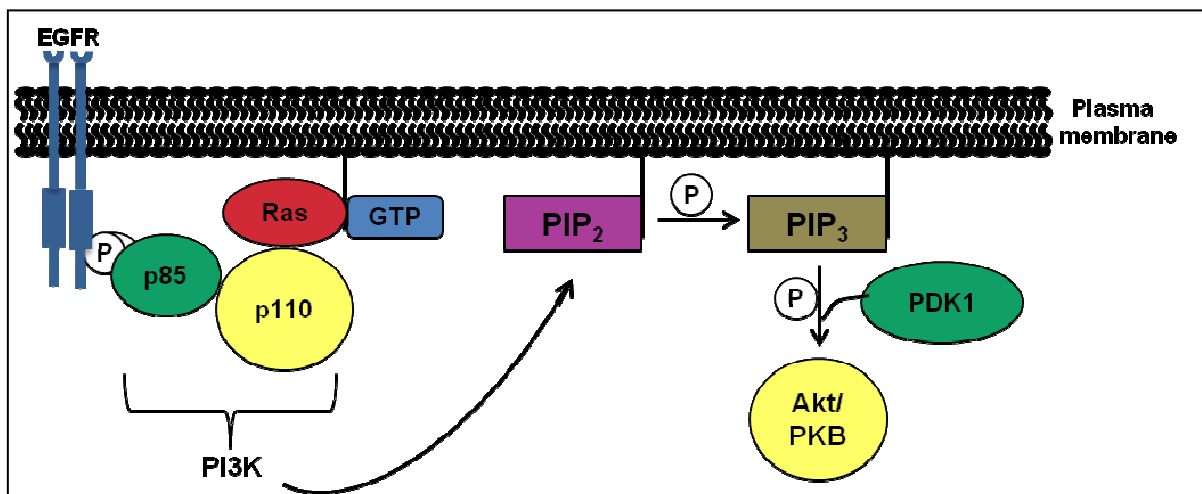


Figure 1.6. Activation of Phosphatidylinositol 3-kinase (PI3-K) by Ras. Activation of the EGF receptor results in binding of the regulatory subunit of PI3-K, p85, to phosphorylated tyrosine residues of the receptor. Consequently, the catalytic subunit of PI3-K, p110, is recruited to the plasma membrane. PI3-K activity is enhanced further by the interaction between p110 and RasGTP. Phosphorylation of PIP₂ by PI3-K produces PIP₃ which recruits and activates PDK1. PDK1 phosphorylates and activates protein kinase B (PKB) also known as Akt.

AKT is a serine/threonine kinase which phosphorylates numerous substrates including BAD, mTOR, and MDM2 to mediate its effects in cell survival and proliferation. PI3-kinases also signal through non-AKT pathways, including Cdc42 and Rac1 which regulate cell motility, and SGK which regulates survival (Vivanco and Sawyers, 2002).

1.6 Non-Redundant Functions of Ras Isoforms

It is well established that Ras isoforms are highly homologous; however, several lines of evidence demonstrate that the functions of each Ras isoform are non-redundant. Nonredundance was first suggested by analyses of the mutation rates of *ras* genes in cancers. Ras mutations occur frequently in certain cancers, but the mutation rates of different isoforms vary significantly across different tumors. For example, mutations in the *K-ras* gene are associated with about 90% of pancreatic cancers, 50% of colorectal cancers, and 35% of non-small cell lung

cancers. In contrast, *H-ras* mutations occur in 10% of transitional cell bladder cancers, while *N-ras* mutations occur in 30% of acute leukemia (Downward, 2003; Roberts and Der, 2007). Interestingly, N-Ras mutations cause Noonan syndrome, a common autosomal dominant congenital disorder characterized as a type of dwarfism (Cirstea et al., 2010). Among all *ras* mutations in cancers, *K-ras* accounts for more than 85%, 12% in *N-ras*, and 2% in *H-ras* (Lau and Haigis, 2009).

Table 1.1 Frequency of Ras Mutations in Different Tumors

Tumor Type	Frequency (%)
Pancreas	90 (K)
Lung adenocarcinoma (non-small-cell)	35 (K)
Colorectal	45 (K)
Thyroid (Follicular)	55 (H, K, N)
Thyroid (Undifferentiated papillary)	60 (H, K, N)
Seminoma	45 (K, N)
Melanoma	15 (N)
Bladder	10 (H)
Liver	30 (N)
Kidney	10 (H)
Myelodysplastic syndrome	40 (N, K)
Acute myelogenous leukemia (Downward, 2003)	30 (N)

Studies in knockout mice provide further evidence for functional divergence between Ras isoforms revealing that only the K-Ras 4B isoform is essential for mouse development. *K-ras*^{-/-} mice die at two weeks of gestation with defects in myocardial and neural development (Johnson et al., 1997; Koera et al., 1997; Umanoff et al., 1995) while *H-ras*^{-/-} and *H-ras*^{-/-}/*N-ras*^{-/-} mice display normal growth, fertility, and viability (Esteban et al., 2001; Ise et al., 2000). Additionally, *K-ras* 4a^{-/-} mice show normal growth and fertility with no abnormal histological pathology up to 20-23 months of age (Plowman et al., 2006; Plowman et al., 2003).

Moreover, all Ras isoforms share a common set of effectors, but engage them with different efficiencies. In transformed fibroblasts, N-Ras binds C-Raf more

effectively than H-Ras. The oncogenic properties of N-Ras are mediated by C-Raf in melanomas. Mutant K-Ras recruits and activates C-Raf with a higher efficiency and potency than N-Ras or H-Ras (Voice et al., 1999; Yan et al., 1998). K-Ras also activates the Rac pathway more efficiently than H-Ras (Walsh and Bar-Sagi, 2001), while H-Ras is a more potent activator of PI3K than K-Ras (Voice et al., 1999; Yan et al., 1998).

1.7 Compartmentalization of Ras Signaling

1.7.1 Signaling on Plasma Membrane Nanodomains

An intriguing area in Ras biology is compartmentalized signaling, in particular, at the plasma membrane. Sophisticated high resolution imaging including electron microscopy (EM), fluorescence resonance energy transfer (FRET), and single particle trafficking (SPT) methods have led to a definitive picture of Ras organization on the plasma membrane (Plowman and Hancock, 2005). The plasma membrane represents a complex mosaic of microdomains that results from the biophysical properties of the constituent lipids, cholesterol, and the actin cytoskeleton and the lateral assembly of sphingolipids and cholesterol into liquid ordered domains; therefore, imposing a non-random distribution on proteins across different nanoscale domains. The plasma membrane is the major Ras signaling platform and is the site where Ras proteins function in distinct nanodomains called nanoclusters. (Hancock, 2006; Kusumi et al., 2005b; Mayor and Rao, 2004). These nanoclusters are transient dynamic structures that are assembled by a complex interplay between components of Ras proteins and the plasma membrane. Vital components of the Ras protein include the farnesyl group and adjacent sequences of the hypervariable region. Vital components of the plasma membrane include the actin cytoskeleton, scaffold proteins, and phospholipid/ cholesterol

composition (Harding and Hancock, 2008b). Ras nanoclusters are comprised of ~40% of Ras proteins that are arranged into clusters of 6-8 proteins on the plasma membrane. The remaining Ras proteins are randomly arrayed as monomers on the plasma membrane (Hancock and Parton, 2005; Plowman et al., 2005; Prior et al., 2003). Ras nanoclusters have a radius of ~9nm and a lifetime of 0.1-0.4s (Hancock and Parton, 2005; Kusumi et al., 2005a; Kusumi et al., 2005b; Murakoshi et al., 2004; Plowman et al., 2005). The function of Ras nanoclusters in Ras signal transduction is to recruit Ras effectors such as Raf, MEK, and ERK. Inhibition of Ras nanocluster assembly abrogates Ras signal output even if Ras is active and localized to the plasma membrane (Plowman et al., 2008; Plowman et al., 2005; Tian et al., 2007). Additionally, in MAPK signaling, Ras nanoclusters function as a digital switch converting graded ligand inputs into fixed outputs of ERK. The generation of Ras nanoclusters that are in direct proportion to ligand input allows cells to build an analogue-digital-analogue circuit relay. This signal is transmitted in high fidelity across the plasma membrane (Harding et al., 2005; Inder and Hancock, 2008; Inder et al., 2008).

Ras isoforms are localized to spatially distinct, non-overlapping nanoclusters on the plasma membrane. GTP-bound H-Ras is mainly localized to cholesterol independent nanoclusters that are stabilized by galectin-1, a cytosolic scaffold protein. In its inactive, GDP- bound conformation, H-Ras is present in cholesterol dependent nanoclusters that require an intact actin cytoskeleton (Belanis et al., 2008; Plowman et al., 2005; Prior et al., 2001; Prior et al., 2003). The GTP-dependent lateral segregation of N-Ras is opposite to that of H-Ras. When GTP-loaded, N-Ras is present in cholesterol dependent domains, but shifts to cholesterol independent domains when GDP-loaded (Roy et al., 2005). On the contrary, GDP-

and GTP-bound K-Ras is localized to cholesterol-independent nanoclusters that are partially dependent upon the actin cytoskeleton. GTP-loaded K-Ras requires the galectin-3 scaffold and GDP-loaded K-Ras does not (Elad-Sfadia et al., 2004; Plowman et al., 2005; Prior et al., 2003).

1.7.2 Signaling on Endosomes

Endosomes were the first intracellular compartment implicated in Ras signaling. Subcellular fractionation studies provided initial insights into endosomal Ras signaling with evidence of Shc, Grb2, Sos, and C-Raf localization to endosomes following EGF stimulation (Di Guglielmo et al., 1994; Pol et al., 1998). Additionally, protein tyrosine kinase receptors (PTKRs), such as the EGF receptor (EGFR), were demonstrated to remain active upon internalization on endosomes (Burke et al., 2001). Moreover, Ras isoforms are localized to endosomes in response to growth factor stimulation or ubiquitination (Jura et al., 2006; Pol et al., 1998). H-Ras and N-Ras signaling require endocytosis, while K-Ras signaling does not (Omerovic et al., 2007; Roy et al., 2002). Recent work revealed that palmitoylated Ras proteins (H-Ras and N-Ras) are directed to recycling endosomes (REs). The current belief is that REs also serve as a way-station for Ras proteins as they traffic to the plasma membrane (Misaki et al., 2010).

1.7.3 Signaling on the Golgi

Live cell imaging using GFP-RBD, a probe that selectively binds Ras-GTP, showed that the Golgi apparatus and plasma membrane are both sites for Ras activation. Activation of Ras on the plasma membrane occurs rapidly and transiently (onset within 1 minute, 10 minute duration) after stimulation with the epidermal growth factor (EGF), whereas activation of Ras on the Golgi is sustained and delayed (onset within 10 minutes, 60 minute duration). The Ras isoforms

involved are believed to be H-Ras and N-Ras, since these isoforms shuttle between the plasma membrane and the Golgi. K-Ras 4B is not present on the Golgi and is not thought to engage in this process. Interestingly, activation of the Golgi pool of Ras was found to occur independently of the endocytic pathway when it was unaffected by dominant negative epsin or a temperature block, factors that block endocytosis and vesicular transport respectively (Bivona and Philips, 2003; Chen et al., 1998; Chiu et al., 2002).

Drawbacks of GFP-RBD include its inability to detect endogenous activated Ras unless a GEF is overexpressed and its dependence on the overexpression of wild-type Ras isoforms (Quatela and Philips, 2006). A similar probe was later created which involved the use of C-terminally tagged RBD constructs with GFP (Walker and Lockyer, 2004) and the use of both the RBD and CRD domains of C-Raf tagged with GFP. Expression of the latter (RBD-CRD) generates a stronger interaction with Ras on the membrane than RBD alone. In striking contrast, active Ras was only observed on the plasma membrane (Bondeva et al., 2002).

Other methods for investigating Ras signaling on the Golgi apparatus have involved the use of Fluorescence Resonance Energy Transfer (FRET)-based Ras activity probes. The first attempt was use of the novel Raichu-Ras FRET probe that assayed active Ras by reporting the sum of GEF and GAP activities. The probe localized exclusively to the plasma membrane. An explanation for this is that the Raichu-Ras probe included the HVR of K-Ras for membrane attachment (Mochizuki et al., 2001; Walker and Lockyer, 2004). Subsequent studies to detect FRET between YFP-RBD and active CFP-Ras also failed to report active Ras on the Golgi (Jiang and Sorkin, 2002; Ohba et al., 2003). Additionally, the use of full-length C-Raf as a FRET reporter for active Ras has given mixed success to demonstrate the

Golgi as a site for Ras signaling. This may be due to the complex regulation of its membrane recruitment (Bondeva et al., 2002; Hibino et al., 2003; Jiang and Sorkin, 2002).

Despite the inconsistency in Golgi studies, there are several lines of evidence that demonstrate Golgi signaling from Ras is important. Research in T cells show that low-grade stimulation of the T cell receptor is specific to N-Ras and only occurs on the Golgi (Perez de Castro et al., 2004). Additionally, in thymic selection, negative selection is related to ERK dynamics on the plasma membrane, while positive selection correlates with Golgi-mediated ERK activation (Daniels et al., 2006). Furthermore, activation of ERK on the Golgi generates a graded response, and plasma membrane-mediated ERK activation produces a digital or switch-like response (Inder and Hancock, 2008).

The mechanism behind Golgi-associated activated Ras was revealed in Jurkat T cells stimulated by the activation of the T-cell receptor. Phospholipase C γ (PLC γ) is responsible for activating Ras on the Golgi. PLC γ is an enzyme that produces diacylglycerol (DAG) and inositol triphosphate (IP $_3$). IP $_3$ releases calcium into the cytosol which leads to the accumulation and recruitment of the Ras GEF, RasGRP1, on the Golgi. RasGRP1 is controlled by both DAG and calcium and has affinity for the Golgi membrane. The requirement of RasGRP1 was revealed when a dominant interfering mutant of this GEF inhibited Ras activation of the Golgi and silencing of RasGRP1 with short interfering RNA abrogated the activation of Ras on this compartment. Furthermore, calcium promoted inactivator (CAPRI), a negative regulator of Ras, was shown to inhibit Ras activation at the plasma membrane in Jurkat T cells (Bivona et al., 2003).

1.7.4 Signaling on the Endoplasmic Reticulum (ER)

Whether or not Ras can signal from the ER remains controversial. The strongest evidence of signaling on the ER comes from studies in yeast. In *Saccharomyces cerevisiae*, an ER-restricted Ras effector was discovered known as ER-associated Ras inhibitor (ERi1). The function of this protein is to preferentially bind to the effector loop of GTP-loaded Ras to inhibit Ras localized to the ER (Sobering et al., 2003). Eri1 is a component of GPI-GlcNAc transferase, an enzyme involved in the rate-limiting step in the biosynthesis of GPI-anchors. Interestingly, GTP-bound Ras2 binds to and inhibits the activity of this enzyme in the absence of Eri1 (Sobering et al., 2004). Further evidence for ER signaling comes from studies in the fission yeast, *Schizosaccharomyces pombe* (*S. pombe*). Since *S. pombe* has only one Ras protein, Ras1, that regulates two different pathways, it provides a simple system that can be used to study compartmentalized Ras signaling. Ras1 controls mating through a Byr2-mitogen-activated protein pathway while maintaining elongated cell morphology through the Scd1-Cdc42 pathway. In these studies, Ras1 mutants were constitutively targeted to the ER/Golgi or the plasma membrane. In *ras1* null cells, the ER/Golgi restricted Ras1 mutant supported morphology, but not mating. On the contrary, the plasma membrane restricted Ras1 mutant supported mating, but not morphology. These observations support the idea that Ras may signal from the ER (Chang and Philips, 2006).

In mammalian cells, the expression of palmitoylated deficient N-Ras or H-Ras results in cytosolic and endoplasmic reticulum (ER) localized Ras (Choy et al., 1999). Coexpression of the Ras-GTP fluorescent reporter, GFP-RBD, with unpalmitoylated H-Ras confirmed that Ras is activated on the ER in response to epidermal growth factor stimulation. Furthermore, restricting constitutively active H-

Ras to the ER with the transmembrane motif of the avian bronchitis virus M1 protein to measure signal output reveals poor activation of Erk and Akt pathways (Chiu et al., 2002). The Ras GEF, RasGRF, is responsible for activation of H-Ras on the endoplasmic reticulum (Arozarena et al., 2004).

1.7.5 Signaling on the Mitochondria and Nucleus

Another potential signaling platform for Ras is on the mitochondria. The three main Ras isoforms are present in mitochondrial fractions of an IL-2-dependent murine T cell line. Further analysis revealed that Bcl-2 targets K-Ras to the mitochondria in an IL-2 dependent manner, while mitochondrial Ras does not require IL-2 (Wang et al., 1996). A recent study has reported that the association of K-Ras with Bcl-2 is regulated by protein kinase C in Jurkat T cells contributing to apoptosis (Bivona et al., 2006). The postulation that Ras may signal from the mitochondria is intriguing; however, the extensive literature on Ras localization by microscopy does not report localization on the mitochondria. Moreover, a novel splice variant of H-Ras, p19, was characterized and is present in the nucleus and the cytoplasm. Another study revealed that nuclear p19 activates p73 with help from the MDM2 protein (Mor and Philips, 2006).

1.8 Perspective

In this study, we will investigate the role of single, endogenous Ras isoforms (H-Ras and K-Ras) on plasma membrane nanodomains and the Golgi. To accomplish our aim, we sought to develop tools whereby the signaling of individual endogenous Ras isoforms can be abolished. The bulk of the aforementioned studies have relied on the overexpression of Ras to comprehend Ras function on the plasma membrane and the Golgi and no isoform-specific anti-Ras tools have been successfully developed; thus the significance of Ras isoforms on these

compartments remains unresolved. The establishment of such tools will potentially increase the understanding of Ras biology.

Chapter 2. Materials and Methods

2.1 Chemicals

All chemicals were purchased from Sigma Aldrich unless otherwise stated.

2.2 DNA Constructs

All GFP-GAP-Ras constructs (GFP-GAP, GFP-GAP-CTH, GFP-GAP-CTK, and GFP-GAP-CTH 181S) were cloned as follows. For GFP-GAP subcloning, pEXVkidGAP (Huang et al., 1993) was digested using Sac1 and EcoR1 restriction enzymes (New England Biolabs). The 1000bp kidGAP fragment was inserted into Sac1/EcoR1 digested pEGFP-C1. The Sac1 site was removed by T4 DNA polymerase (New England Biolabs). For GFP-GAP-CTH, GFP-GAP-CTK, and GFP-GAP-CTH 181S cloning, the HVRs of H- and K-Ras were cloned onto the C-terminus of pEGFP-C1-GAP by PCR amplification. This was performed through the PCR amplification of the CTH, CTK, and CTH 181S sequences from pEGFP-C1-CTH, pEGFP-C1-CTK, and pEGFP-C1-CTH 181S with a reverse GFP sequencing primer (5'CTACAATGGGTATGGC 3') and the following forward primers: 5' CCGAATTCAGCACAAAGCTGCGGAAGCTGAACC 3' for pEGFP-C1-GAP-CTH and pEGFP-C1-GAP-CTH 181S and 5' GGGAATTCAGCATAAAGAA AAGATGAGCAAAGATGG 3' for pEGFP-C1-GAP-CTK. Furthermore, the PCR products were digested with EcoR1/BamH1 and were inserted into EcoR1/BamH1 digested pEGFP-C1-GAP.

2.3 Cell Growth and Maintenance

The following cells were used: BHK, Cos-1, and PC12. All cells were grown and maintained in 37°C with 5% CO₂ injection using Dulbecco's Modified Eagle Medium (DMEM) (Hyclone) with additives including 1% sodium pyruvate and glutamax (Gibco). For PC12 cells, the medium was also supplemented with 10%

horse serum and 5% fetal bovine serum (FBS). BHK and Cos-1 cells were cultured in medium containing 10% bovine calf serum (BCSS) and 10% FBS, respectively.

2.4 Cell Transfection and Stimulation

For Ras-GTP pulldown assays, BHK cells were transiently transfected by the lipofection method with pEGFP, GFP-GAP, GFP-GAP-CTH, GFP-GAP-CTK, or GFP-GAP-CTH181S using Lipofectamine 2000 reagent (Invitrogen) in 15 cm dishes. For GFP, GFP-GAP, GFP-GAP-CTH, and GFP-GAP-CTK transfections, 36µg DNA and 40µL Lipofectamine 2000 reagent was used. To achieve optimal transfection efficiency all dishes were gently shaken for at least 10 times. Cells were returned to growth medium 3hrs after lipofection. For GFP-GAP-CTK transfections, twice as much DNA (72µg) was used and cells were incubated in transfection mixture for at least 8hrs prior to returning to growth medium. Approximately 16hrs after transfection, cells were washed twice with Phosphate Buffered Serum (PBS) (Hyclone) and cells were incubated in serum free medium (DMEM) for 1hr. Subsequently, the serum-free medium containing 50 ng/ml EGF was added to cells for the following time points: 2, 5, 10, 20, 40, and 60min. Immediately following EGF stimulation for each time period, EGF medium was removed and cells were washed twice with ice cold PBS. PBS was then replaced by 1mL Lysis Buffer B (50mM Tris pH 7.5, 75mM NaCl, 25mM NaF, 5mM MgCl₂, 5mM EGTA, 0.33 µg/mL aprotinin, 100µM Na₃VO₄, 3.33 µg/mL leupeptin, 1mM DTT, and 1% NP40). Cells were incubated in Lysis Buffer B for at least 10min before removal from dishes using a cell scraper. Cell lysates were transferred into 1.5 mL eppendorf tubes and vortexed. The insoluble material was removed by centrifugation at 13,000 rpm for 5min and the remaining supernatant stored on ice for further analysis.

For Ras signaling experiments, BHK cells were transiently transfected with Lipofectamine 2000 reagent in 6 cm dishes. For GFP, GFP-GAP-CTH, and GFP-GAP-CTH181S transfections, 8µg DNA and 20µL Lipofectamine 2000 reagent was used. Cells were returned to growth medium 4hrs after lipofection. For GFP-GAP – CTK transfections, 16µg DNA was used and cells were incubated in transfection mixture for at least 8hrs prior to returning to growth medium. Approximately 16hrs after transfection, cells were washed twice with Phosphate Buffered Serum (PBS) (Hyclone), and cells were incubated in serum free medium (DMEM) for 1hr. Subsequently, the serum-free medium containing 50 ng/ml EGF was added to cells for the following time points: 5, 10, 20, 40, and 60min. Immediately following EGF stimulation for each time period, EGF medium was removed and cells were washed twice with ice cold PBS. PBS was then replaced by 150µL Lysis Buffer B. Cells were incubated in Lysis Buffer B for at least 10min before removal from dishes using a cell scraper and cell lysates were prepared as described above.

For confocal microscopy studies, BHK cells were treated the same as Ras signaling experiments; however cells were transfected on coverslips in 6 well plates and fixed instead of harvested. See Confocal Microscopy for fixation conditions. PC12 cells were transiently transfected on coverslips by the lipofection method with pEGFP, GFP-GAP-CTH, GFP-GAP-CTK, or GFP-GAP-CTH181S GFP using Lipofectamine 2000 (Invitrogen) in 6 well plates. PC12 cells were transfected using the same conditions as the Ras signaling experiments. Three hours after lipofection, the cells were returned to growth medium. Approximately 24hrs after transfection, cells were washed twice with PBS and fresh growth medium containing 100 ng/ml NGF was added. Cells were placed in a CO₂ incubator at 37°C for an additional 72hrs to assess neurite outgrowth under a confocal microscope.

2.5 Bradford Assay

Following cell harvesting with Lysis Buffer B, the protein concentration for each sample was determined. Samples were centrifuged at 13,000 rpm for 5 min. The pellet was discarded and the supernatant was sonicated for homogenization. BSA protein standards ranging from 200-1000 ng/ μ L were used. Samples were diluted as needed and were analyzed in triplicates by pipetting 5 μ L of sample into a 96 well plate. The absorbance was measured at 595 nm using a spectrophotometer. The protein concentration of the unknown sample was determined from a graph of the standard curve of ng of BSA verses the absorbance.

2.6 Western Blot Analysis

Proteins were separated on 12% SDS-PAGE. Electrophoretic transfer onto polyvinylidene fluoride (PVDF) membrane was performed at 25 volts for 30min. using a semidry transfer apparatus (Model: Trans-Blot SD Cell, Bio-Ras). After the transfer, blots were stained with Commassie Blue to evaluate transfer efficiency. Membranes were incubated for 1hr at room temperature in blocking buffer (5% dry non-fat milk in 1X TBS (20mM Tris, 137mM NaCl, pH 7.6 with HCL) and 0.1% Tween-20 for non-phospho-blots; 3% BSA in 1X TBS and 0.1% Tween-20 for phosphor-blots. Membranes were subsequently probed with primary antibodies diluted in the appropriate blocking buffer for 2hrs at room temperature. The membranes were washed 3 times for 5min with a washing buffer (1X TBS, 0.1% Tween-20), and then incubated in the appropriate secondary antibody for 1hr at room temperature. All antibody incubations were performed on a shaker. Following antibody incubations, the membranes were treated with chemiluminescence detection reagents (SuperSignal West Dura Stable Peroxide/ Luminal-Enhancer

Solutions or SuperSignal West Stable Peroxide/Luminal-Enhancer Solutions, Thermo Scientific and analyzed by quantifying each lane using the FluorChem Q.

2.7 Antibodies used for Western Blotting

The following antibodies were from Cell Signaling: phosphor-Akt (Ser473), rabbit monoclonal antibody; Akt (pan), mouse monoclonal antibody; p44/42 MAPK (ERK 1/2), rabbit monoclonal antibody; HA-tag, and mouse monoclonal antibody. ERK2 and H-Ras rabbit polyclonal antibodies were from Santa Cruz Biotechnology. Anti-GFP and Anti-K-Ras mouse monoclonal antibodies were from Sigma. Anti-Ras (mouse monoclonal antibody) was from BD Transduction Laboratories. Goat anti-mouse and anti-rabbit antibodies were from Invitrogen.

2.8 Confocal Microscopy

BHK and Cos-1 cells were transfected on coverslips. PC12 cells were transfected on coverslips that were previously coated with poly-L-lysine. Cells were washed 3 times with PBS and fixed with 4% paraformaldehyde/PBS solution for 30 min at room temperature followed by washing steps with PBS and water. Coverslips were mounted on slides in Mowiol for confocal microscopy. Slides were allowed to dry for at least 1hr at 37°C prior to use of the A1 Nikon Confocal Microscope.

2.9 Golgi Staining

BHK cells were transfected on coverslips as previously described. The day after transfection, cells were exposed to the components of the Organelle Lights™ Intracellular Targeted Green Fluorescent Proteins kit (Contents: Organelle Lights reagent, BacMam enhancer, Dimethylsulfoxide (DMSO), Invitrogen) The Organelle Lights reagent consists of a baculovirus which is responsible for directing the expression of autofluorescent proteins (in this case the Golgi resident enzyme, N-

acetylgalactosominytransferase 2) to a specific subcellular location. The growth medium was replaced with diluted Organelle Lights reagent (Component A) as explained in the manufacturer's instructions. Cells were allowed to incubate for 2hrs with gentle rotation at room temperature. Following incubation, the reagent was replaced with growth medium containing diluted enhancer (Component B and C) and cells were placed in the incubator for 2hrs. Finally, cells were fixed as stated before in preparation for observation of Golgi staining by confocal microscopy.

2.10 GST-RBD (Ras Binding Domain of Raf-1) Pull-down Assay

2.10.1 Expression of GST-RBD in bacteria

For GST-RBD expression, 50 ml of Luria broth (L-broth) supplemented with 100 ug/ml of ampicillin was inoculated with plasmid name here GST-RBD K85A glycerol stock and incubated overnight at 37°C with constant agitation. The 50ml culture was then added to 2L of L-broth containing 100 µg/ml ampicillin and incubated at 37°C with agitation for approximately 4hrs or until the culture reached an OD₆₀₀ value of 0.6. After incubation, 0.2 mM IPTG was added to the culture with subsequent incubation for another 4hrs under the same conditions. The bacterial cells were pelleted by centrifugation at 6000 rpm for 15min at 4°C.

2.10.2 Purification and Storage of GST-RBD

For purification of GST-RBD, the bacterial cells above were resuspended in 30ml STE buffer (10 mM Tris (pH 8.0 with HCl), 150 mM NaCl, 1 mM EDTA, 100µg/ml lysozyme, and 1 mM PMSF). The suspension was incubated at 4°C on a rotator. Following incubation, 5 mM DTT and 1.5% N-Laurylsarcosine sodium salt (prepared in STE buffer) was added to the suspension. The cells were shaken vigorously and sonicated on ice for 1min at the maximal power level using the name here. The cells were thoroughly mixed with 3% Triton X-100 (prepared in STE

buffer) and incubated on ice for 30min. After incubation, cells were sonicated as previously described. Insoluble material was pelleted by centrifugation at 13,000 rpm for 20min at 4°C. The supernatant was mixed with 4ml of a 50% suspension of glutathione agarose beads (Sigma Aldrich) overnight on a rotator at 4°C. Following overnight incubation, the beads were washed 6 times for 10min in a mixture of PBS and 0.1% Triton X-100. The beads were resuspended in 4ml of storage buffer (50 mM Hepes (pH 7.4 with HCl), 150 mM NaCl, 5 mM DTT and 10% sterile glycerol) as a 50% suspension. The beads were checked for the bound fusion protein, GST-RBD, by SDS-PAGE gel. Approximately 20µl of GST-RBD beads (50% suspension) and 5µl of 5X sample buffer were loaded onto a 12% gel after being boiled at 95°C for 5min. The gel was stained with Coomassie Blue for approximately 5min and washed 3 times for 3min with destaining solution (10% acetic acid, 50% methanol) to view the GST-RBD fusion protein band. Finally, the beads were stored for future use as aliquots that were snap frozen on dry ice and stored at -80°C.

2.10.3 Ras GTP Pull-down Assay

After each time point of EGF addition, cells were immediately placed on ice followed by 2 washes with ice-cold PBS. The PBS was removed and cells were harvested in 1ml of ice-cold lysis buffer with a cell scraper. Insoluble material was pelleted by centrifugation at 13,000 rpm for 5min. The pelleted material was discarded, and the protein concentration of the supernatant was determined by the Bradford Assay. A total of 1mg of protein was used for the pulldown assay and 20µg of protein was set aside for immunoblotting to determine protein input. For the pulldown assay, the protein was combined with 1ml of binding buffer (50 mM Tris pH 7.5, 10 mM MgCl₂, 0.5 mg/ml BSA, 0.5 mM DTT, 100mM NaCl) and 20µl of GST-RBD beads described previously. The new protein mixture was allowed to

incubate on a rotator overnight at 4°C. The following day, the mixture was spun down at 13000 rpm for 1min to separate the beads from the buffer. The supernatant was removed and replaced with 1ml of binding buffer and placed on the rotator for 5 minutes. This washing procedure was repeated for 2 more times with the final step of separating the beads from the buffer by centrifugation as described previously. The supernatant was removed and discarded. The beads were mixed with 5µl of 5x sample buffer and were boiled at 95°C for 5min in preparation for immunoblotting against Ras proteins.

2.12 Proliferation Assay

BHK cells transiently transfected with GFP-tH or GFP-GAP-Ras constructs were analyzed by FACS (BDFortessa). Prior to FACS analysis, BHK cells were transiently transfected using the same conditions as Ras signaling experiments. Cells were plated in 24 well plates in triplicates. Each day cells were harvested using trypsin/EDTA solution. The number of GFP expressing cells was quantified and recorded for each day.

Chapter 3. Results

3.1 Introduction

It is unclear how individual endogenous Ras isoforms function on plasma membrane nanodomains and the Golgi in cells. Multiple strategies have been utilized in an attempt to reveal the functions of each Ras isoform. K-Ras null cells were used to determine the role of K-Ras. However, these cells do not express both K-Ras4B and K-Ras4A, making the effects of K-Ras4B difficult to isolate. RNA interference had also been used but failed to equally down-regulate all Ras isoforms (Khanzada et al., 2006). Dominant negative mutants of Ras isoforms are also inadequate as they fail to act specifically on one endogenous Ras isoform (Matallanas et al., 2003). Moreover, all the above studies do not acknowledge the localization of Ras proteins to the Golgi making it difficult to differentiate between Ras isoform-specific signaling from the plasma membrane and the Golgi. Tools that will allow us to specifically target endogenous Ras proteins on these compartments are needed to effectively determine the role of endogenous Ras isoform-specific signaling.

Ras signaling from the Golgi is a controversial topic as not all investigations have been able to demonstrate Golgi-localized Ras activity. Nevertheless, at steady state, palmitoylated Ras isoforms such as H-Ras are present on the plasma membrane as well as the Golgi, and furthermore, 2 models for Ras activation on the Golgi have been previously described. In the first model, described in T cells, Ras activation occurs directly on the Golgi where Ras is activated by Src-dependent activation of phospholipase C- γ 1 (PLC- γ 1) resulting in an increase in diacylglycerol (DAG) and the subsequent translocation of the RasGEF RasGRP1 from the cytosol to the Golgi. RasGRP1 can also activate Ras on the plasma membrane in response to co-stimulation by the T cell receptor and the integrin LFA-1. Another

model suggests active Ras-GTP on the Golgi is derived from the plasma membrane due to a rapid and continuous depalmitoylation/re-palmitoylation cycle that occurs after posttranslational modifications of the Ras C-terminus. Either way, recent reports indicate that Ras signaling from the Golgi is important. Studies in yeast reveal that Ras signaling from the endomembrane controls morphology, while Ras signaling from the plasma membrane regulates mating. In mammalian cells, the kinetics of Ras activation on the Golgi are different from the plasma membrane as well as the kinetics of ERK output. In thymocyte selection, endogenous Ras signaling from the Golgi is essential for positive selection, whereas Ras signaling from the plasma membrane is required for negative selection. However, whether or not Golgi activation of endogenous Ras has physiological relevance is still largely unknown.

Here, we design novel tools to target endogenous H-Ras or K-Ras isoforms and abrogate Ras signaling on the compartments mentioned above. A critical component for the localization of Ras to various compartments in the cell is the hypervariable region (HVR) which encodes the membrane anchors and the adjacent sequences required for membrane targeting (Hancock, 2003). Predicated on this vital role of the HVR, we hypothesize that, when fused to GAP, the HVR of Ras isoforms will target GAP to Ras nanoclusters on the plasma membrane and to the Golgi and selectively inhibit endogenous Ras activity. Specifically, we aim to demonstrate that each targeted GAP construct will correctly confine to areas where the cognate Ras isoform is present. This is important, because it alludes to their ability to abrogate specific Ras isoform activation on the plasma membrane and the Golgi. Next, we will investigate the ability of each targeted GAP construct to abrogate the activation of endogenous Ras isoforms and the specificity of these

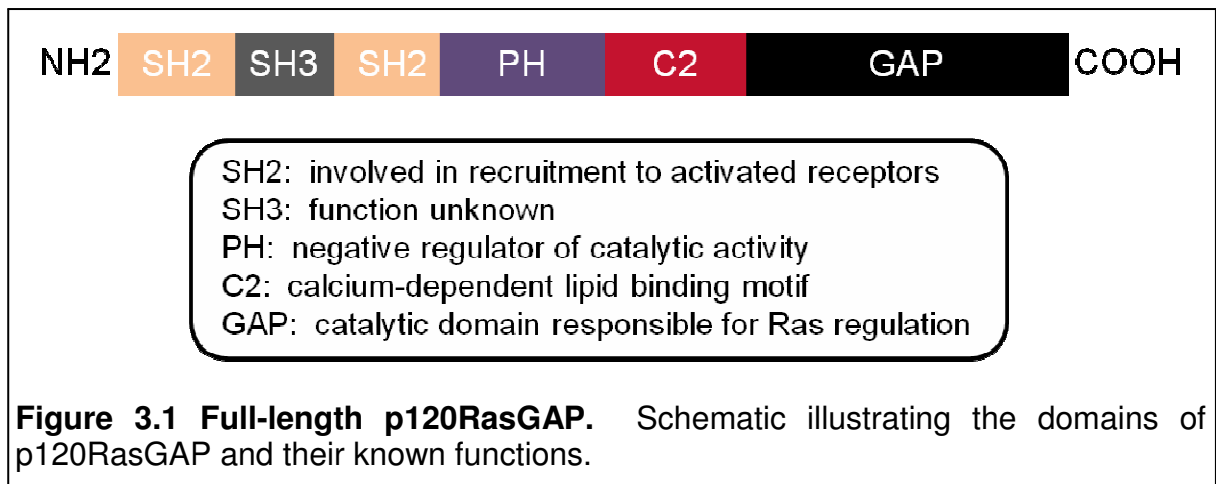
GAP constructs on the corresponding Ras isoform. The inhibition of endogenous Ras isoforms will allow us to investigate the function of Ras isoform-specific signaling on two separate compartments of the cell, an approach which has never been done. This will include an investigation of the ability of Ras isoform-specific inhibitors to abrogate Ras signal transduction. We are particularly interested in two Ras dependent signaling pathways, Raf/MEK/ERK and PI3-K/Akt, because these pathways are the only Ras effector pathways that are also commonly mutated in human cancer (De Luca et al., 2012). Finally, we will examine the physiological relevance of Ras signaling from the plasma membrane and the Golgi. Our findings may provide new insights into Ras function.

3.2 Results

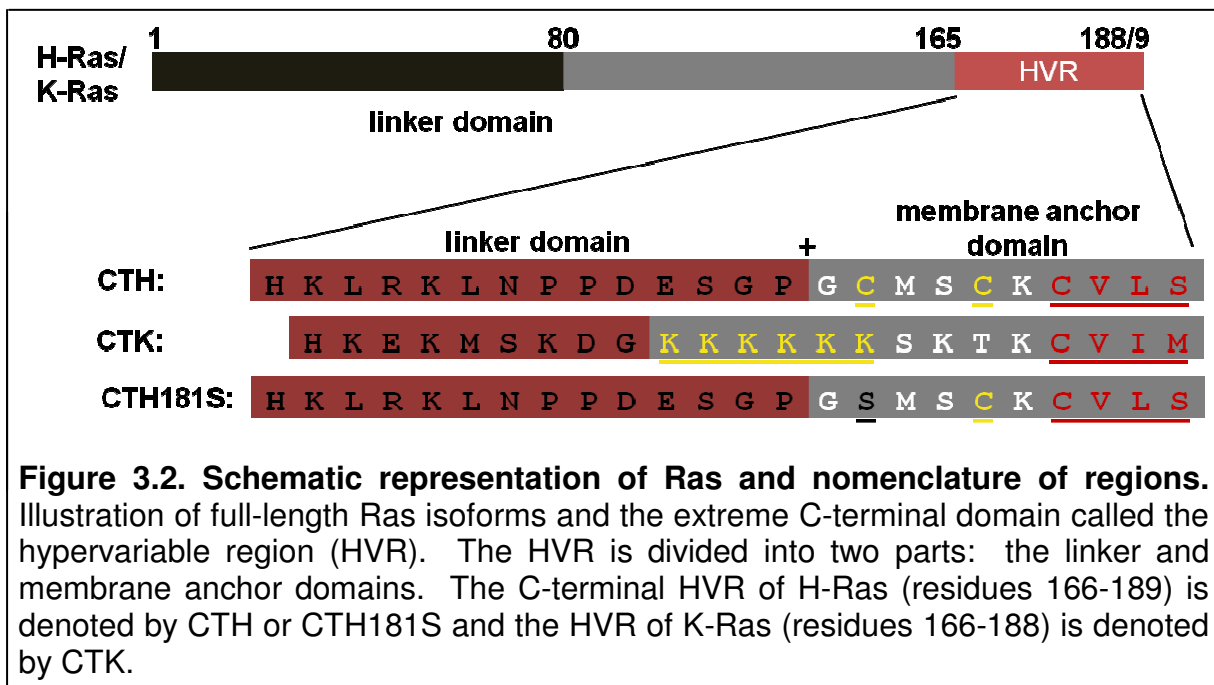
3.2.1 The HVR Targets GFP-RasGAP to the Plasma Membrane and Golgi

In an effort to create proteins that specifically inhibit GTP-loaded H-Ras and K-Ras on plasma membrane nanodomains and on the Golgi, we constructed GAP molecules that are targeted to specific sub-cellular compartments. First, we sub-cloned the catalytic GAP domain (amino acids 701-1044) of p120RasGAP (Huang et al., 1993) onto the C-terminus of GFP in the enhanced green fluorescent protein plasmid, pEGFP. As depicted in Figure 3.1, full-length p120RasGAP has only one region, the catalytic GAP domain, which interacts with GTP-bound Ras and thus is responsible for hydrolyzing GTP to GDP, converting active Ras to inactive Ras (Mitin et al., 2005).

Secondly, we cloned the complete carboxyl HVRs of H-Ras (amino acids

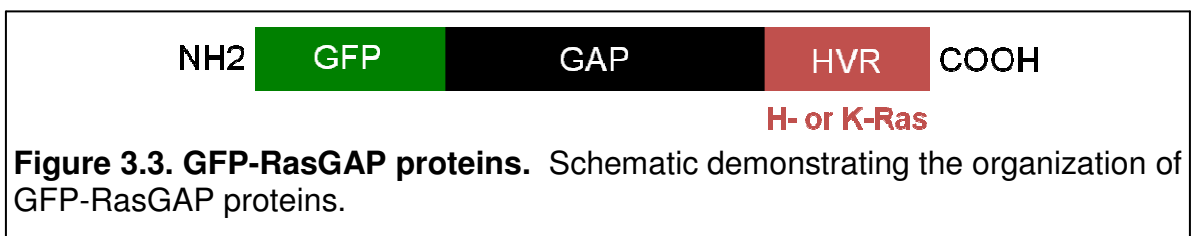


166-189) or K-Ras (amino acids 166-188) by PCR amplification onto the C-terminus of pEGFP-GAP. The HVR or the C-terminus of H-Ras or K-Ras (CTH or CTK) is necessary and sufficient for its membrane targeting. Previous work has shown that GFP-CTH and GFP-CTK undergo the same trafficking pathway as full length H-Ras and K-Ras, respectively (Apolloni et al., 2000).



Additionally, these HVRs target exogenous proteins to specific plasma membrane nanodomains that co-localize with the corresponding GTP-loaded Ras isoform (Inder et al., 2008). Finally, we created a third construct by introducing a point

mutation in the HVR of H-Ras at position C181, changing the cysteine to serine as previously described (Inder et al., 2008). The 181S mutation disables forward trafficking to the plasma membrane causing the protein to remain in the Golgi (Roy et al., 2005). Illustrated in Figure 3.2 are the C-terminal HVRs of H-Ras or K-Ras mentioned above. The organization of these GFP-RasGAP proteins is displayed in Figure 3.3. We predict that GFP-GAP-CTK will localize exclusively to the plasma membrane, while GFP-GAP-CTH will localize predominantly to the plasma membrane and partially to the Golgi. GFP-GAP-CTH181S will localize almost exclusively to the Golgi.



To validate the localization of RasGAP proteins, each protein was transiently expressed into Baby Hamster Kidney (BHK) cells and the localization visualized in fixed cells by fluorescence microscopy. Figure 3.4 shows that the controls, GFP and GFP-GAP, did not localize to any membrane, but instead decorated the cytosol and nucleoplasm. For detection of the Golgi, cells expressing GFP-GAP-CTH and GFP-GAP-CTH181S were stained with a RFP-Golgi marker (N-acetylgalactosaminyltransferase-2) prior to fixation and imaging. As expected, GFP-GAP-CTK exclusively localized to the plasma membrane like K-Ras. GFP-GAP-CTH predominantly localized to the plasma membrane, but with some degree of intracellular staining. GFP-GAP-CTH181S co-localized with the RFP-Golgi

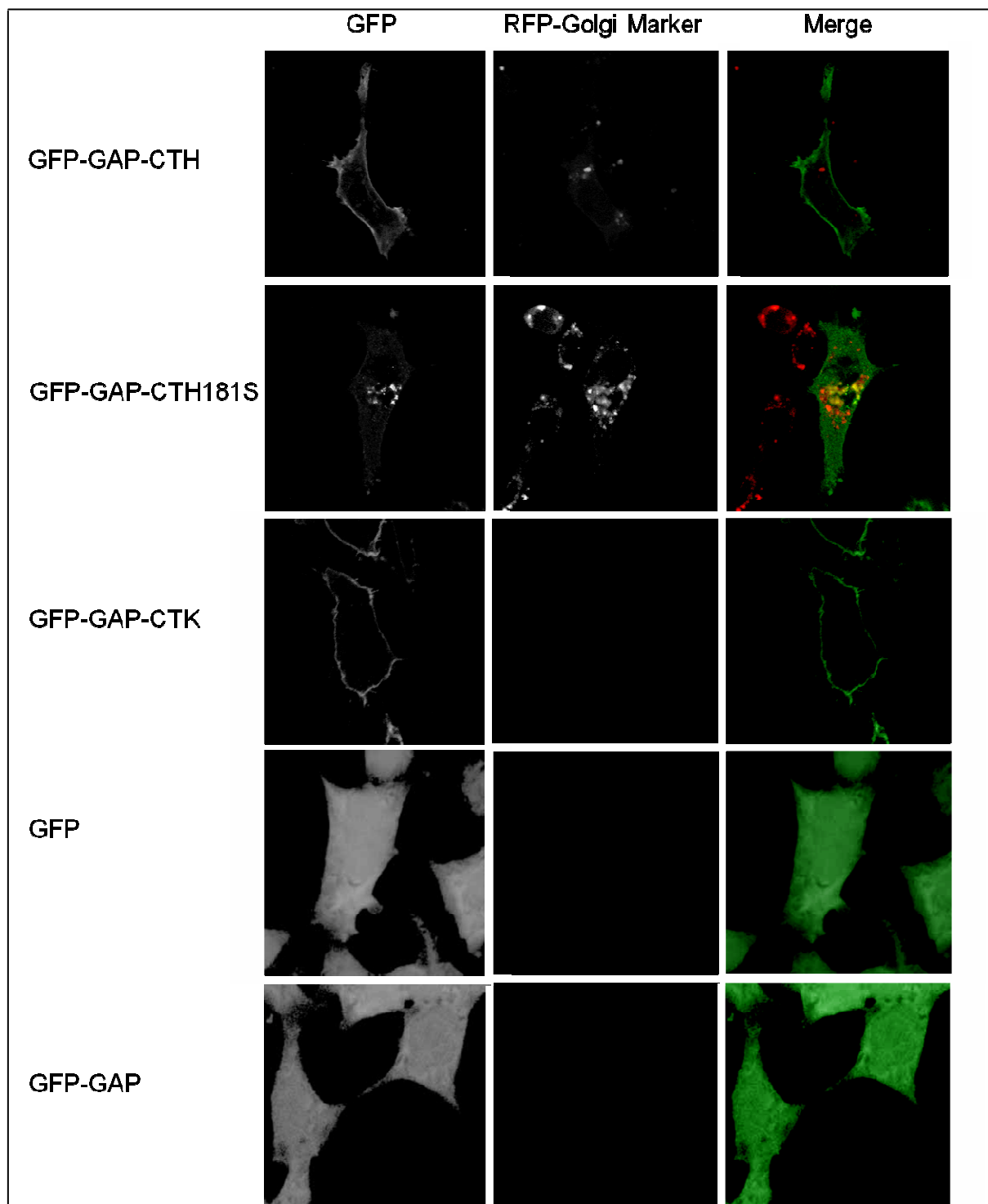
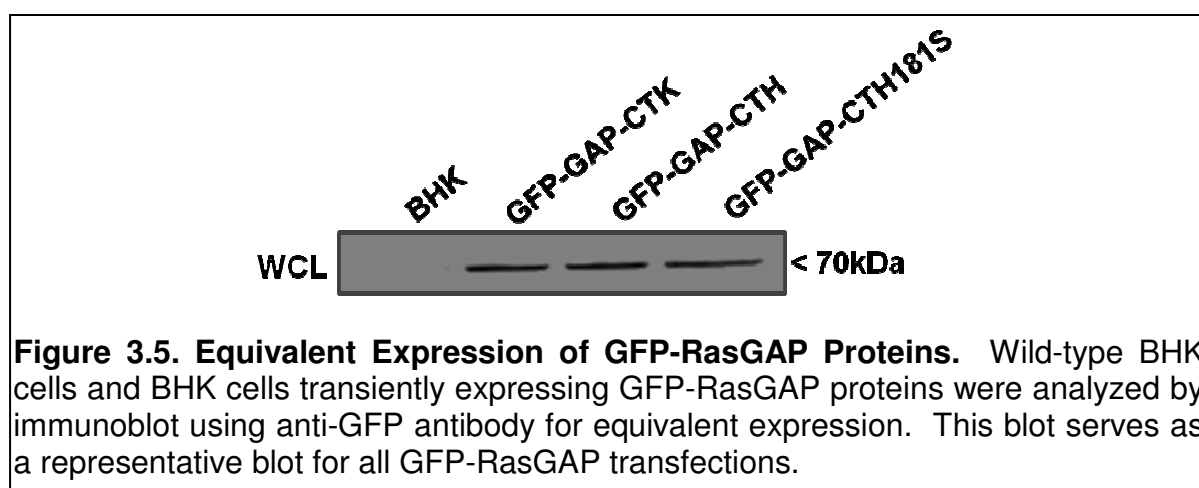


Figure 3.4. The HVR targets GFP-RasGAP to the Plasma Membrane and Golgi. BHK cells transiently expressing GFP-RasGAP proteins were stained with a RFP-Golgi marker followed by fixation on coverslips. The sub-cellular localization of each GFP-RasGAP protein was visualized by fluorescence microscopy.

marker, indicating the presence of a Golgi pool, although a very small amount of the GFP-RasGAP protein was also present on the plasma membrane. Taken together, these observations revealed that the membrane distributions of GFP-RasGAP proteins were directed by the HVR of H-Ras or K-Ras and may co-localize with full-length Ras proteins. Therefore, the cellular localization of each GFP-RasGAP protein was as predicted, demonstrating their potential to specifically abrogate individual endogenous GTP-loaded Ras isoforms.



3.2.2 GFP-RasGAP Proteins Specifically Inhibit Endogenous Ras Isoforms

We next examined the ability of GFP-RasGAP proteins to specifically abrogate activation of individual endogenous Ras isoforms. Therefore, we prepared whole cell lysates (WCLs) from BHK cells and BHK cells transiently expressing GFP-RasGAP proteins. These were then immunoblotted with anti-GFP (Figure 3.5) to confirm equivalent expression of GFP-RasGAP proteins. To observe Ras activation in response to a specific growth factor, quiescent BHK cells transfected with GFP-RasGAP constructs were stimulated with a saturating dose of EGF (50 ng/mL) at various times ranging from 2 to 60min. Following stimulation, endogenous GTP-bound Ras proteins were isolated by GST-RBD pull-down and

the levels of GTP-bound Ras were detected by immunoblotting with a pan-Ras, H-Ras, or K-Ras antibody. As demonstrated in Figure 3.6, the kinetics of Ras activation in control cells (Empty Vector) show maximal levels of GTP-loaded Ras proteins (H-Ras, K-Ras, and N-Ras) occurring rapidly at 2 min. followed by a return to basal levels around 60min. Cytosolic GAP or GFP-GAP did not have any effect on total GTP-loaded Ras levels. This was expected since GFP-GAP did not occupy any membrane structure in BHK cells as visualized by fluorescence microscopy; thus, displaying the importance of the HVR for targeting exogenous proteins to the membrane. However, cells that expressed GFP-GAP-CTK (Figure 3.7) or GFP-GAP-CTH (Figure 3.8) specifically inhibited endogenous GTP-loaded K-Ras or H-Ras, respectively. Significant inhibition was observed at the early times assayed (2-10min). Neither construct had an inhibitory effect on the Ras isoform that was not

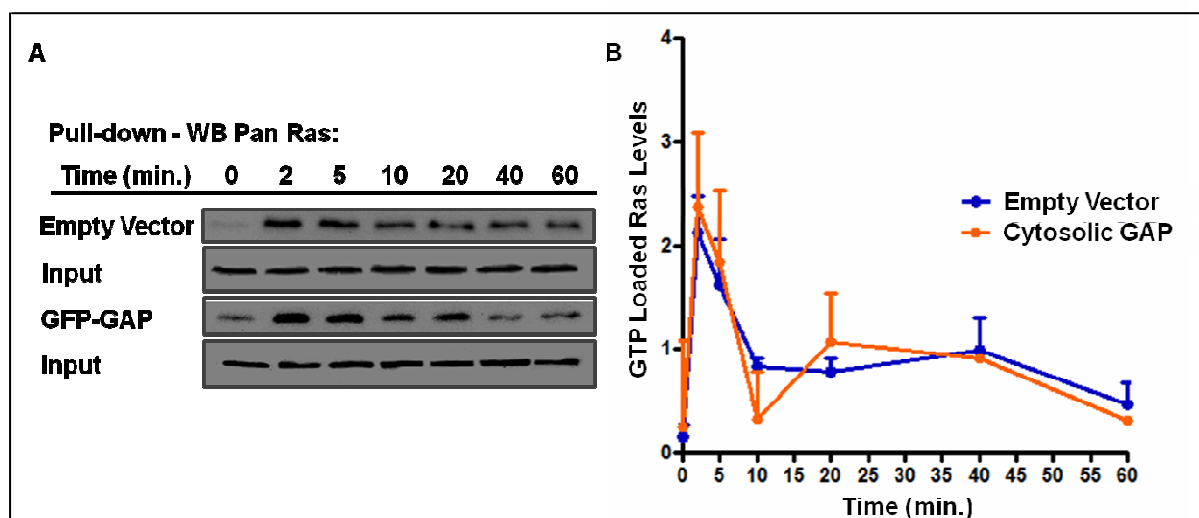
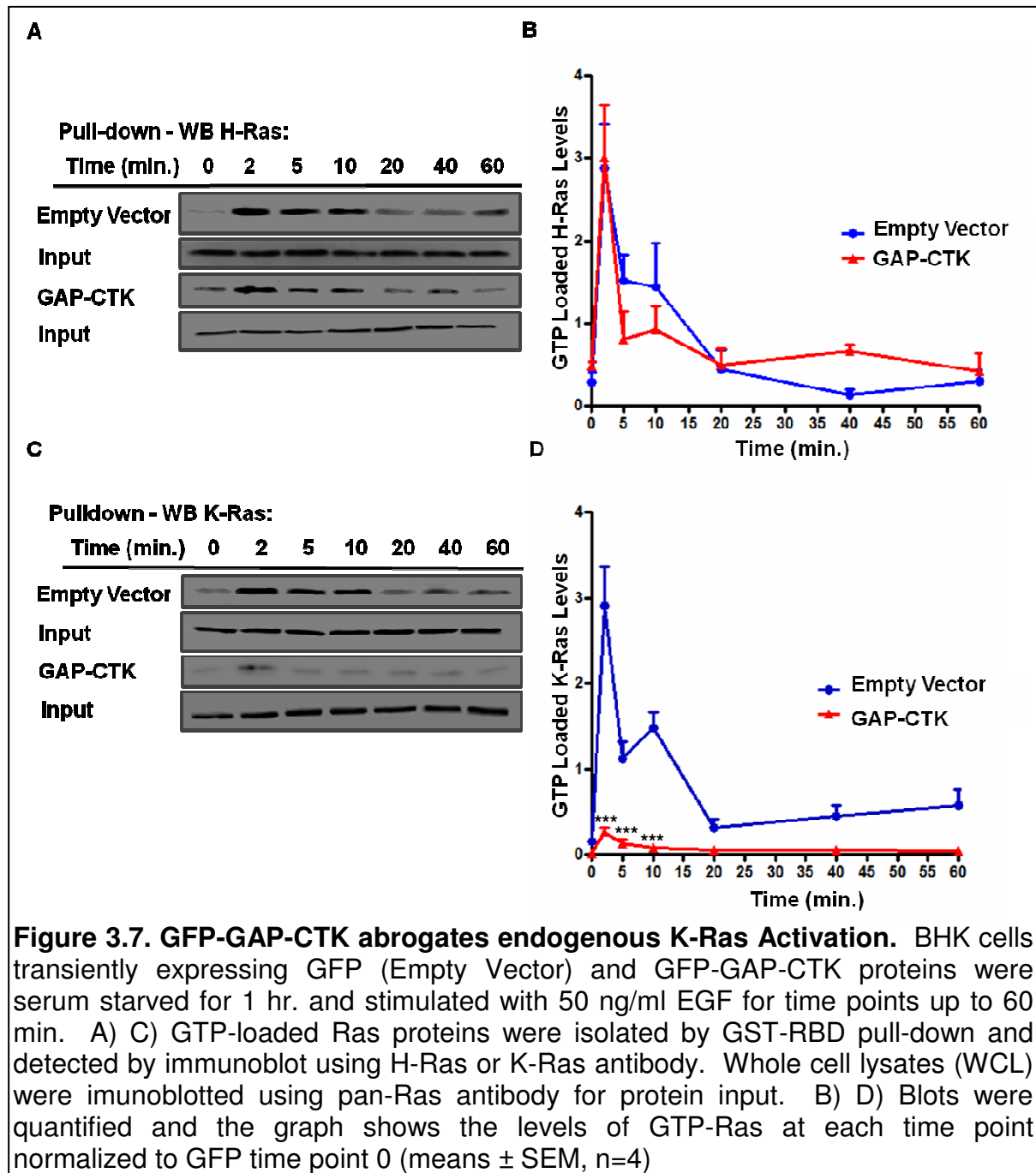
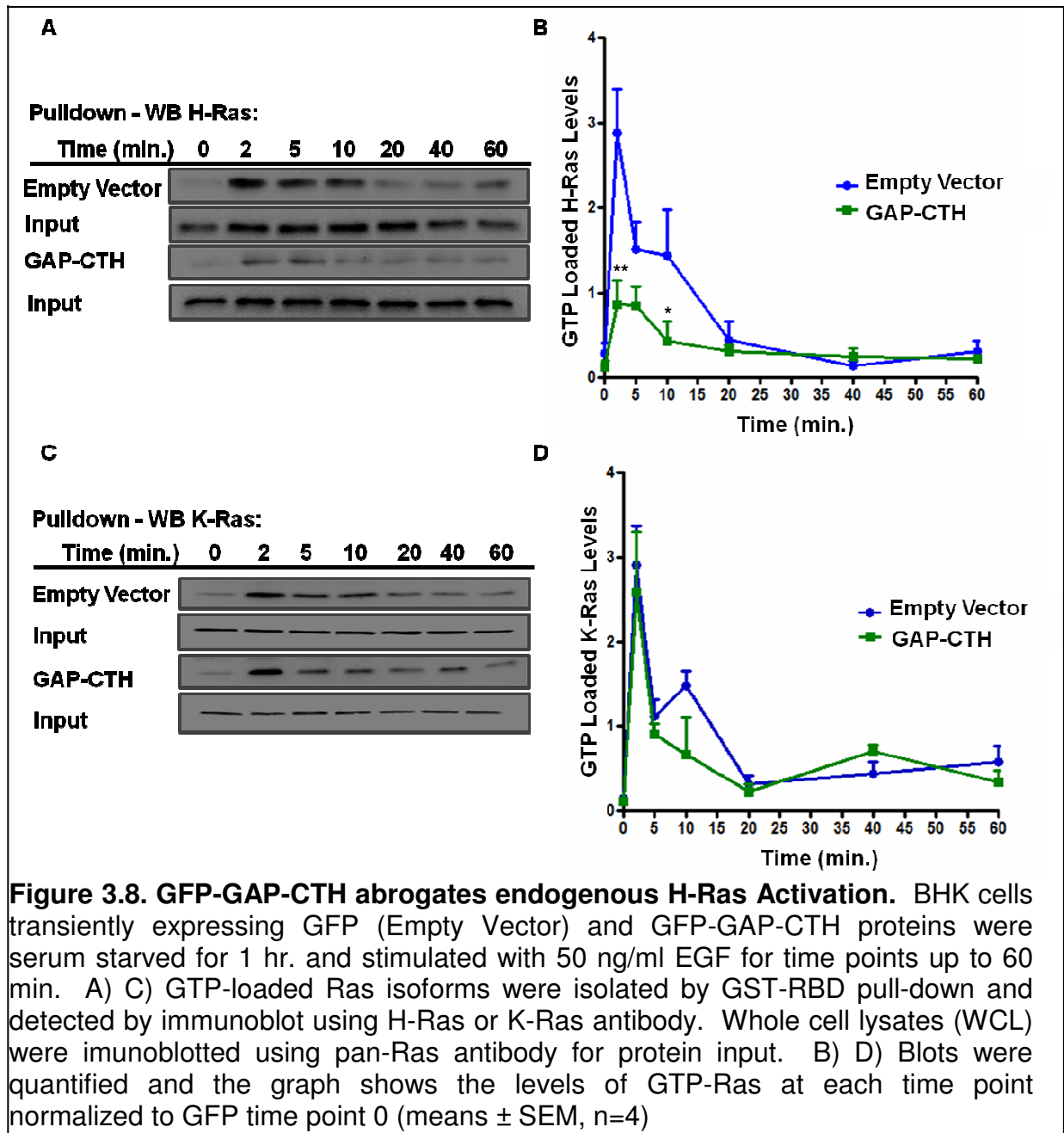
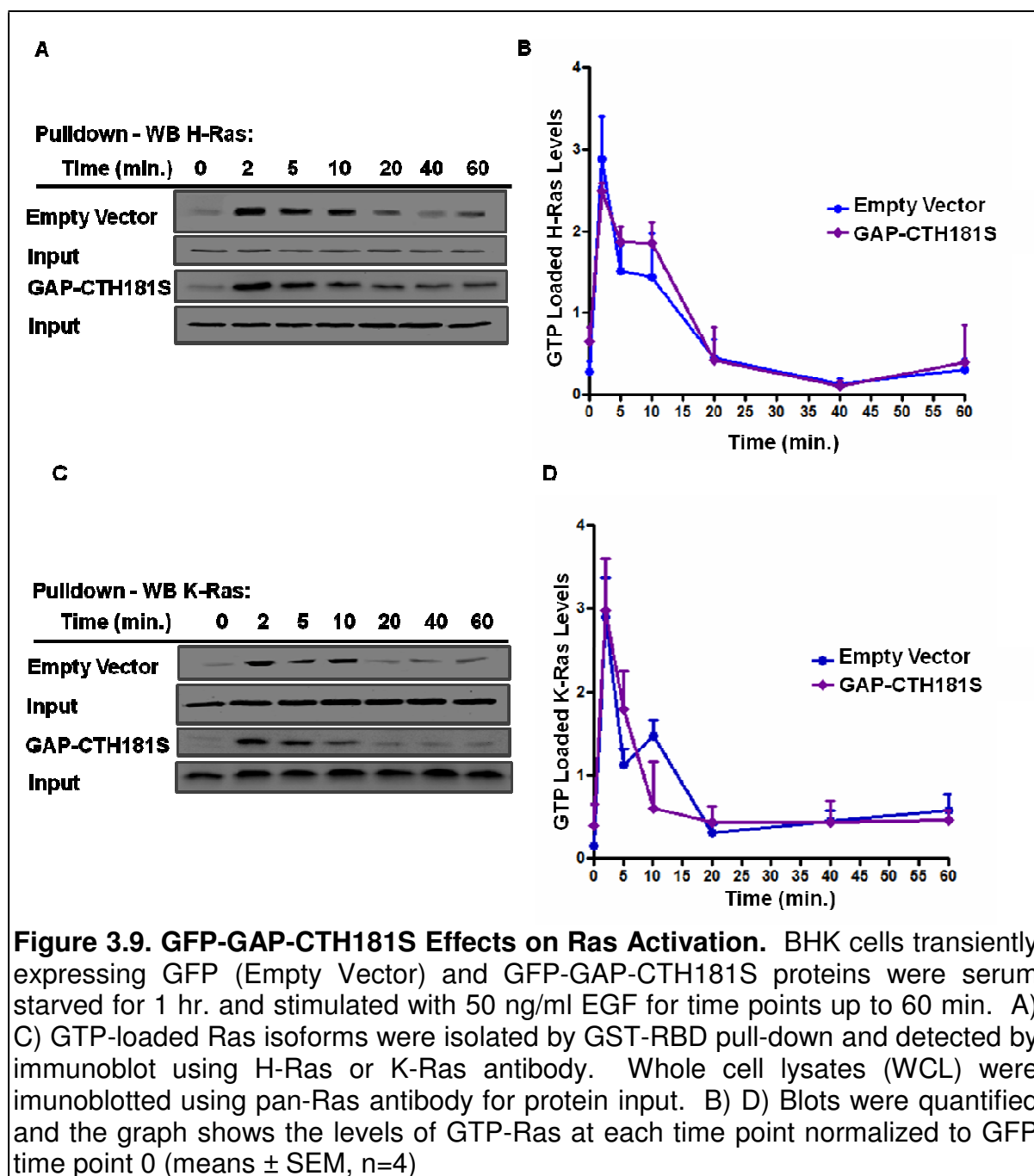


Figure 3.6. Cytosolic GAP Does Not Inhibit Ras Activation. BHK cells transiently expressing GFP (Empty Vector) and GFP-Cytosolic GAP proteins were serum starved for 1 hr. and stimulated with 50ng/ml EGF for time points up to 60 min. A) GTP-loaded Ras proteins were isolated by GST-RBD pull-down and detected by immunoblot using pan-Ras antibody. Whole cell lysates (WCL) were immunoblotted using pan-Ras antibody for protein input. B) Blots were quantified and the graph show the levels of GTP-Ras at each time point normalized to GFP time point 0 (means \pm SEM, n=3)



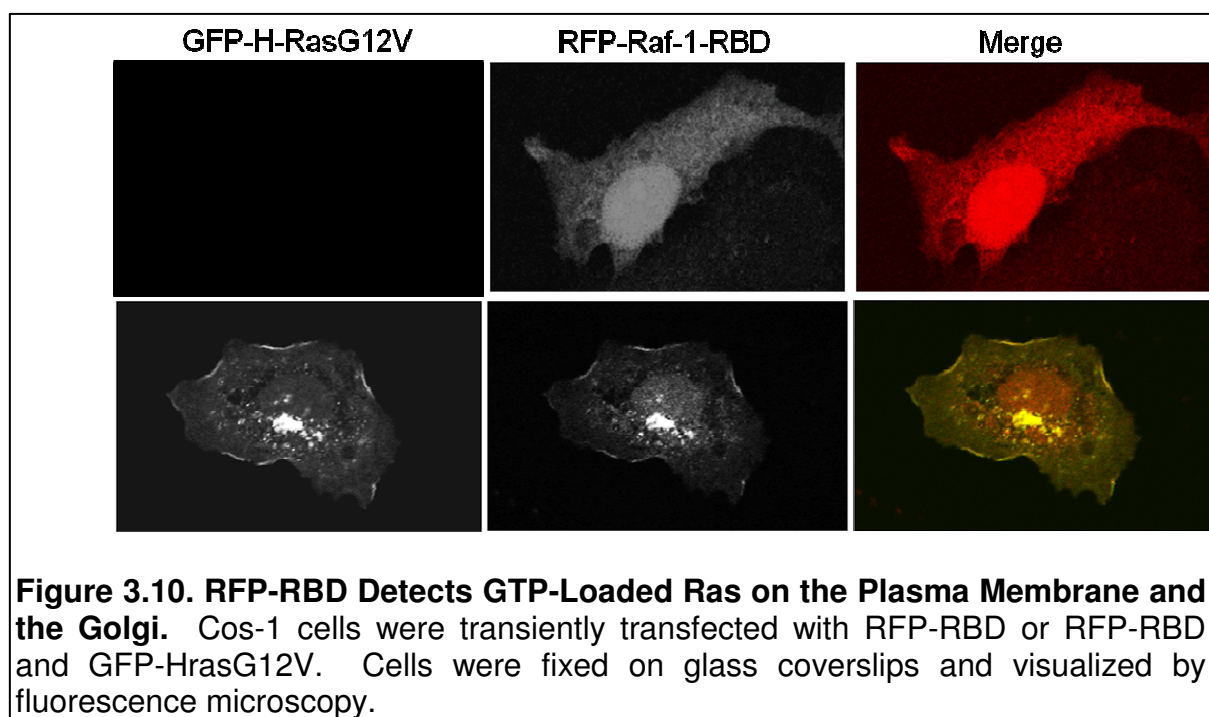




associated with its targeted domain. Therefore, GFP-GAP-CTK had no effect on H-Ras activation, and GFP-GAP-CTH had no effect on K-Ras activation. Unexpectedly, we did not detect any effect of the Golgi localized GFP-RasGAP protein, GFP-GAP-CTH181S, on Ras activation. As depicted in Figure 3.9, there are relatively low levels of GTP-Ras at the late time points (20-60 min) when Golgi Ras activation is maximal. Our method may, therefore, not be sufficiently sensitive to detect changes in this low level of Ras activation. However, there was no abrogation at the earlier times (2-10min) by GFP-GAP-CTH181S where Ras is maximally activated. Since Ras activation on the plasma membrane has been previously shown to occur between 2-10min following EGF stimulation, we can conclude that very little GFP-GAP-CTH181S has access to GTP-Ras generated on the plasma membrane.

3.2.3 GFP-RasGAP Proteins Specifically Inhibit Ras Isoforms on the PM and Golgi

In an effort to resolve the sensitivity issue of the GST-RBD pull-down assay and verify the ability of the targeted GAP proteins to specifically abrogate activation of Ras isoforms in distinct sub-cellular compartments we used confocal microscopy. The Ras binding domain (RBD) C-Raf (amino acids 51-131) has been previously used in imaging studies to report where and when Ras is active in cells (Walker and Lockyer, 2004). We therefore generated an RFP-RBD fusion protein by cloning RFP onto the N- terminus of the RBD. We then tested the ability of this fluorescent probe (RFP-RBD) to detect Ras activation on the plasma membrane and Golgi in fixed cells. As shown in Figure 3.10, expression of RFP-RBD alone in Cos-1 cells resulted in a homogenous localization to the cytosol and nucleoplasm with no evidence of membrane decoration. However, in the presence of a constitutively active form of



H-Ras, H-RasG12V, the RFP-RBD probe was recruited to both the plasma membrane and Golgi, co-localizing exclusively with active H-Ras. Next, RFP-RBD was transiently co-expressed with GFP-RasGAP proteins in the presence or absence of wild-type H-Ras or K-Ras. Cells were deprived of serum prior to stimulation with EGF for 2min in order to observe maximal EGF-induced Ras activation on the plasma membrane. The extent of RFP-RBD localization to the plasma membrane was quantified using Image J software and the fraction of RFP-RBD recruitment to the plasma membrane was calculated (Figure 3.11). As shown in Figure 3.12, in response to EGF stimulation, most of RFP-RBD is localized to the cytosol. When RFP-RBD was co-expressed with H-Ras or K-Ras, a dramatic redistribution of RFP-RBD from the cytosol to the plasma membrane was observed upon EGF stimulation. In cells expressing wild-type K-Ras, the presence of GFP-GAP-CTK significantly reduced recruitment of RFP-RBD to the plasma membrane.

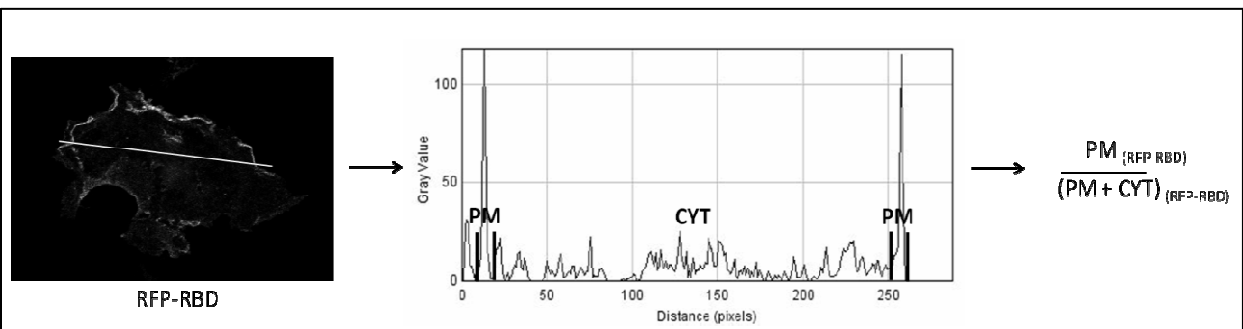


Figure 3.11. Quantification of RFP-RBD Localization to the Plasma Membrane.

In GFP-RasGAP expressing cells, the extent of RFP-RBD localization to the plasma membrane (PM) was determined via quantification by Image J software. A line scan was drawn manually across individual healthy cells for quantification of RFP-RBD localization. The output generated is a plot of the intensity values of RFP-RBD. From the plot, areas representing RFP-RBD intensity on the PM or the cytosol (CYT) were identified and gated as shown. RFP-RBD intensity values for the PM were integrated and the average determined. RFP-RBD intensity values for the CYT were also averaged. The percentage of PM-recruited RFP-RBD, was calculated by a ratio of the average RFP-RBD intensity values on the PM and the sum of the averages of RFP-RBD intensity values on the PM and the CYT. This was done for each cell.

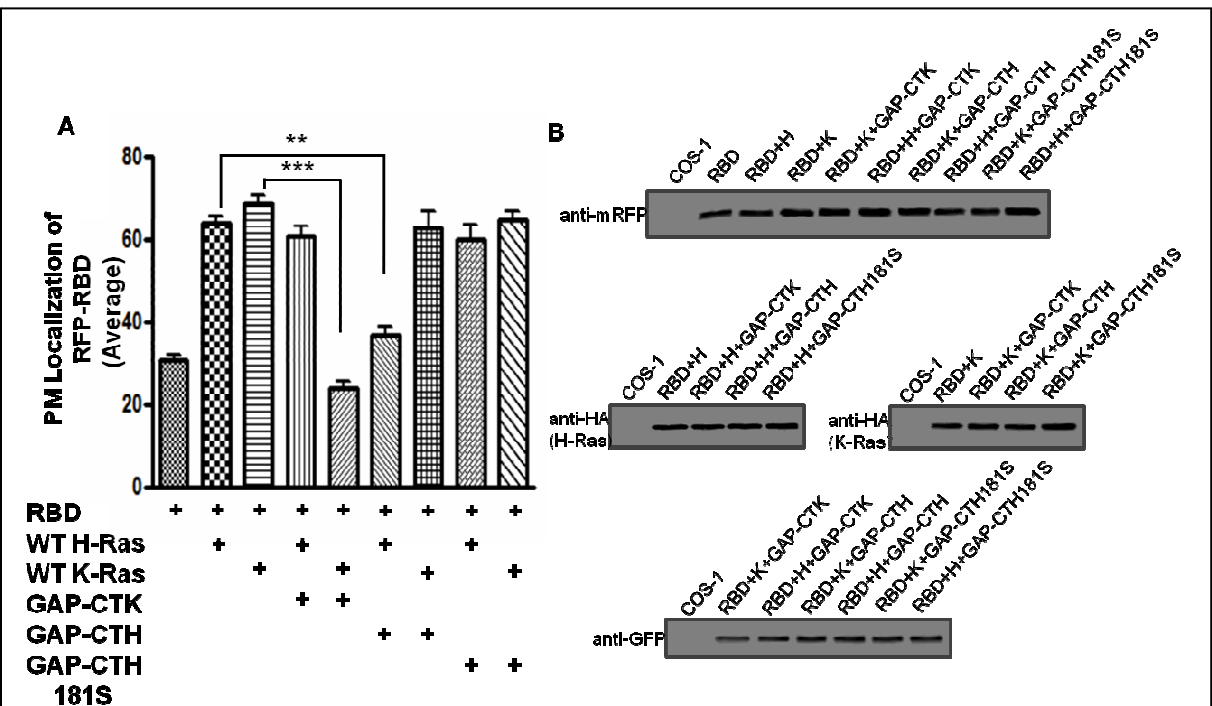


Figure 3.12. GFP-RasGAP Proteins Abrogate Ras Activation on the Plasma Membrane.

Cos-1 cells were transiently transfected with GFP-RasGAP proteins and/or RFP-RBD in the presence or absence of wild-type Ras. Cells were serum starved and stimulated with 50 ng/ml EGF for 2min and subsequently fixed and visualized by fluorescence microscopy. The average RFP-RBD intensity on the plasma membrane was determined by Image J software. A) The graph represents 3 individual experiments with means \pm SEM, n=100. B) Representative blots demonstrating the expression of each construct.

GFP-GAP-CTK did not affect H-Ras mediated recruitment of RFP-RBD to the plasma membrane. Similarly, GFP-GAP-CTH expression abrogated recruitment of RFP-RBD to the plasma membrane in cells expressing H-Ras, but not K-Ras. To observe Ras activation on the Golgi, we co-expressed RFP-RBD with CFP-H-Ras and GFP-RasGAP proteins and imaged cells after 40min of EGF stimulation. Expression of CFP-H-Ras permits visualization of Ras on the Golgi. The extent of RFP-RBD localization to the Golgi was determined only from cells where Golgi staining by CFP-H-Ras was evident using Image J software. Hereafter, we assessed the fraction of RFP-RBD recruitment to the Golgi (Figure 3.13).

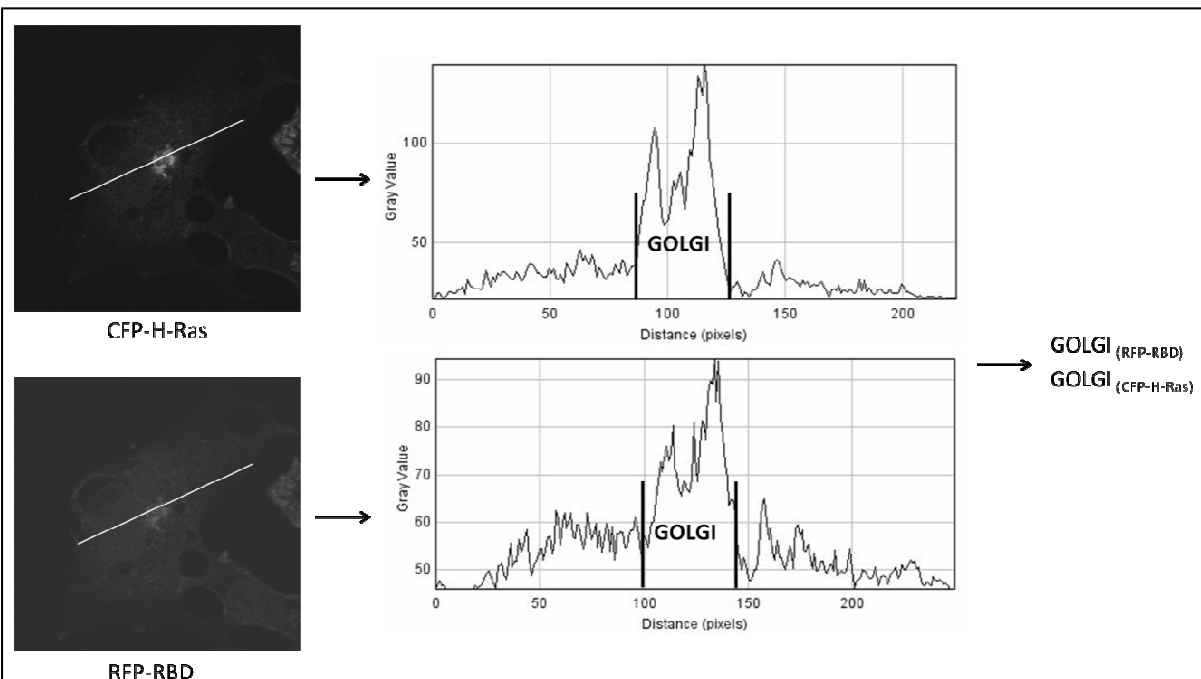
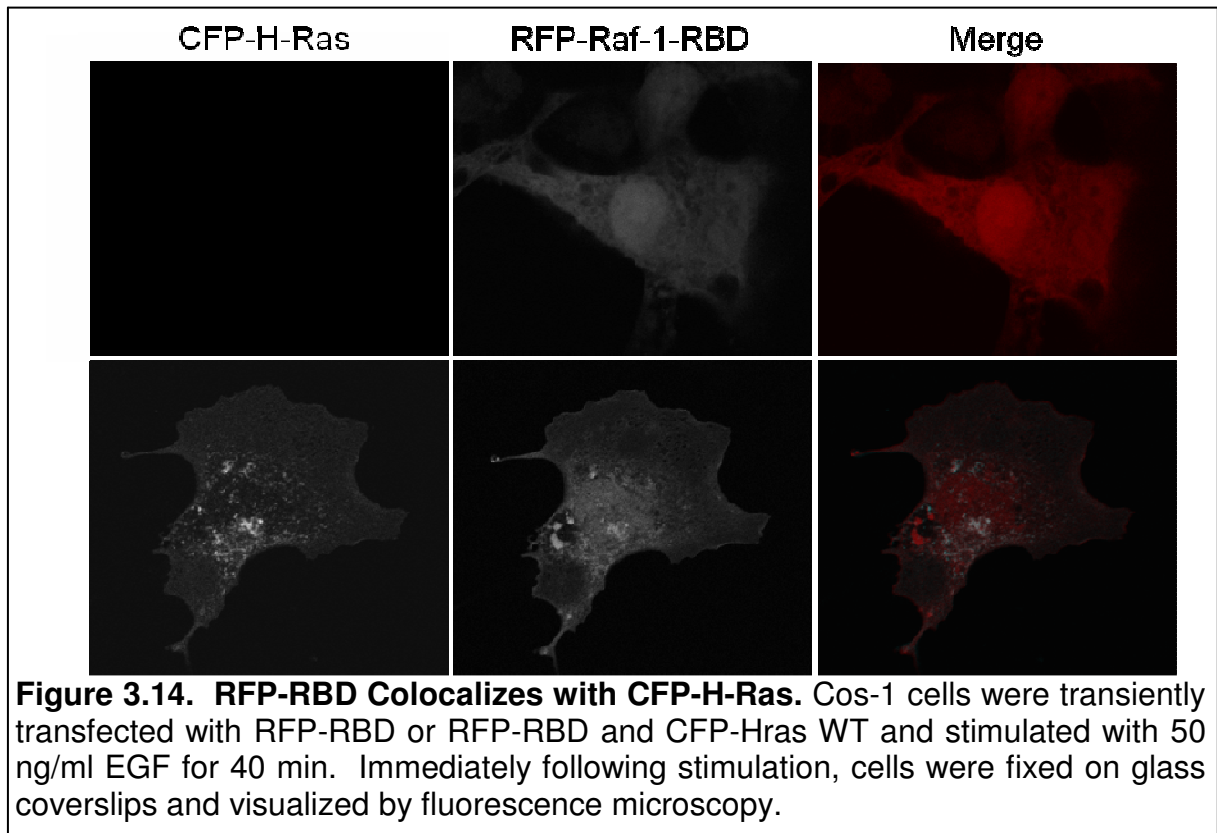


Figure 3.13. Quantification of RFP-RBD Localization to the Golgi. In GFP-RasGAP expressing cells, the extent of RFP-RBD localization to the Golgi was determined via quantification by Image J software. A line scan was drawn manually across individual healthy cells for quantification of RFP-RBD or CFP-H-Ras localization. The output generated is a plot of the intensity values of RFP-RBD or CFP-H-Ras. From the CFP-H-Ras line scan, the Golgi was identified. Then, the areas representing CFP-H-Ras intensity on the Golgi were identified and gated. These marked boundaries were also used to identify and gate RFP-RBD recruitment to the Golgi on the RFP-RBD plot. CFP-H-Ras and RFP-RBD intensity values for the Golgi were averaged. A ratio of Golgi-recruited RFP-RBD and Golgi-recruited CFP-H-Ras was determined. This was done for each cell.

As shown in Figure 3.14, a significant portion of RFP-RBD accumulation on the Golgi is clearly visible after 40 min. of EGF stimulation when wild-type H-Ras is expressed. Figure 3.15 shows that GFP-GAP-CTH did not affect the recruitment of RFP-RBD to the Golgi; however, GFP-GAP-CTH181S expression caused significant reduction of RFP-RBD localization to the Golgi. These data support the



previous findings that all 3 GFP-RasGAP targeted proteins specifically abrogate activation of specific Ras isoforms, which occurs on the plasma membrane and the Golgi.

3.2.4 Effects of GFP-RasGAP Proteins on Ras Signal Transduction

In order to investigate the ability of GFP-RasGAP proteins to abrogate Ras signal transduction, we assayed the effects of GFP-RasGAP on the MAPK and PI-3K/Akt signaling pathways. GFP-RasGAP proteins were transiently expressed in BHK cells that were serum starved and stimulated with EGF for various times

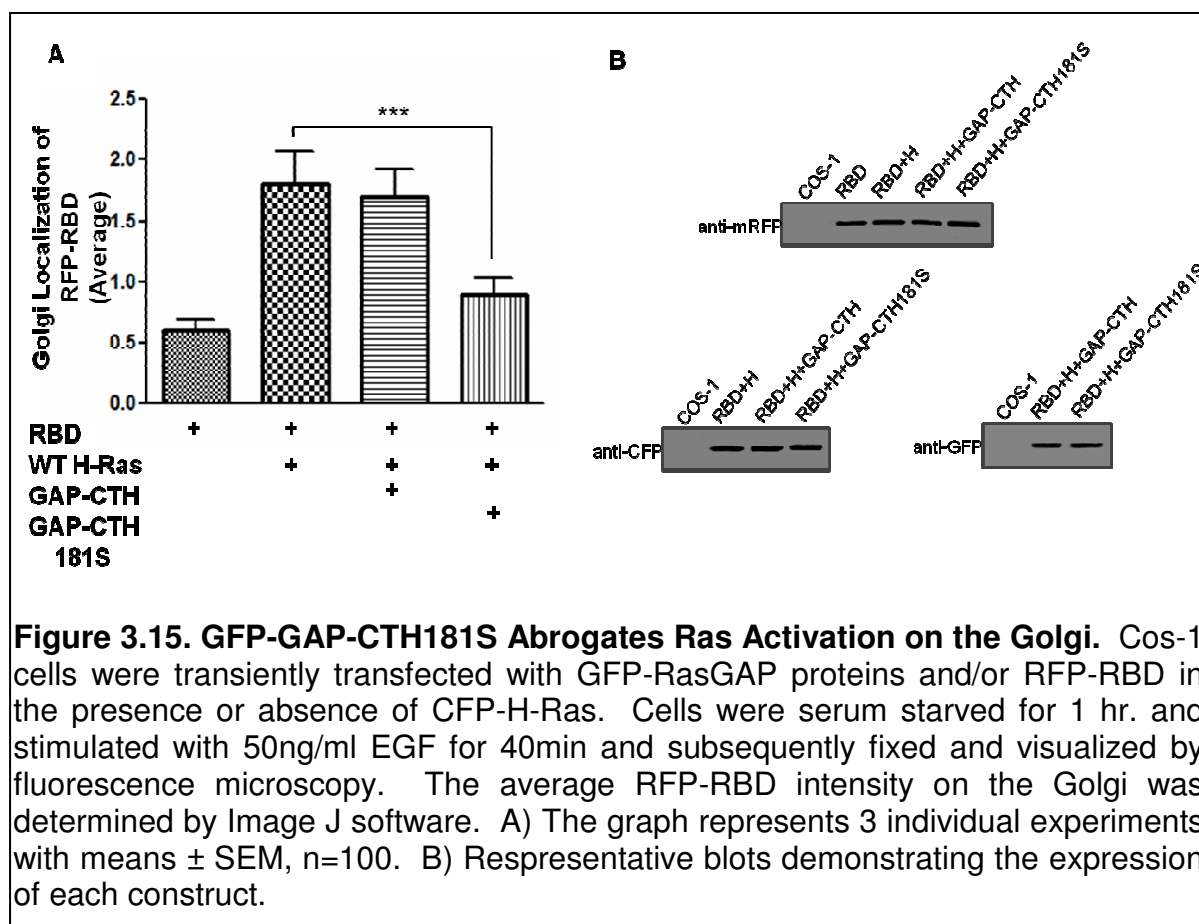
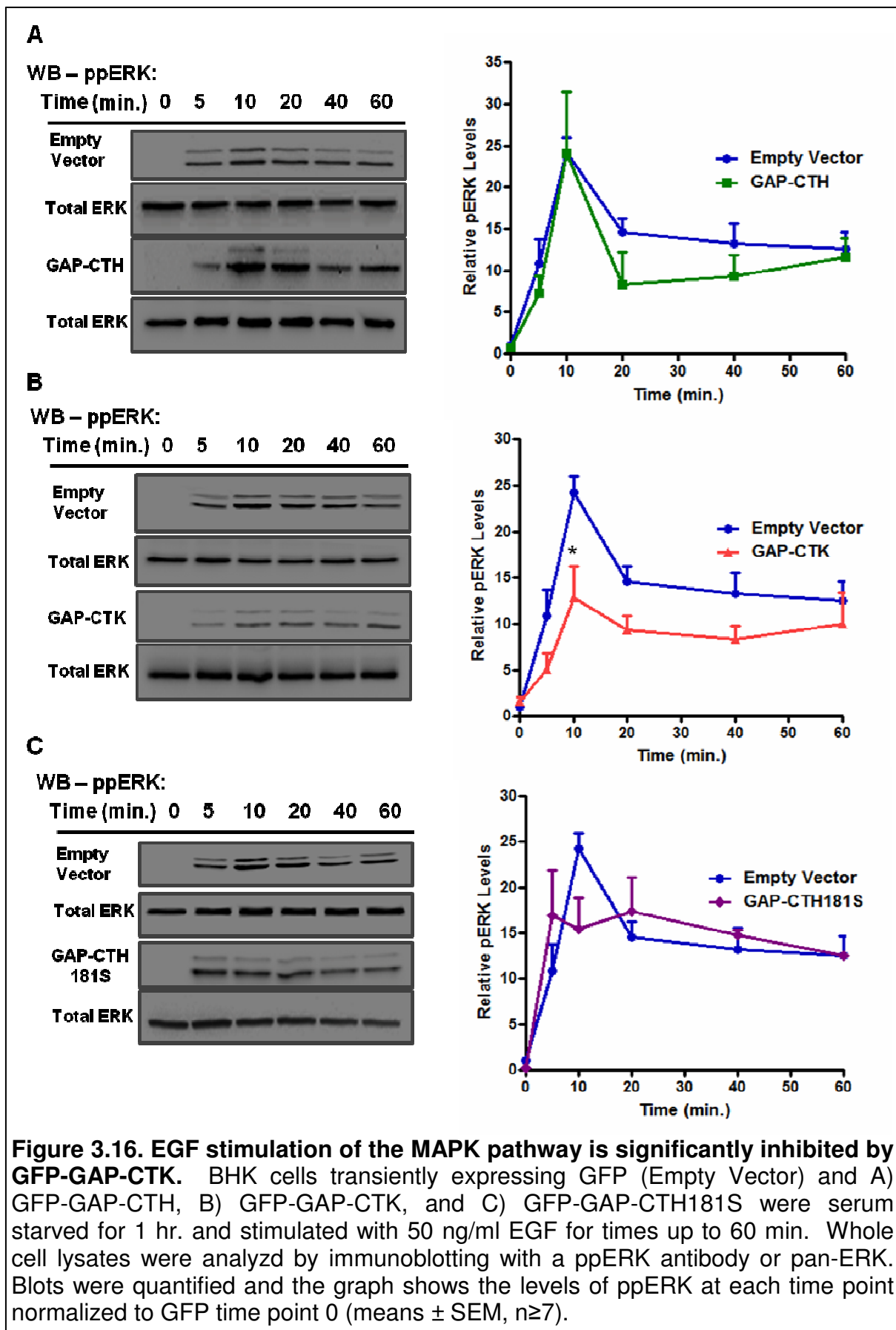


Figure 3.15. GFP-GAP-CTH181S Abrogates Ras Activation on the Golgi. Cos-1 cells were transiently transfected with GFP-RasGAP proteins and/or RFP-RBD in the presence or absence of CFP-H-Ras. Cells were serum starved for 1 hr. and stimulated with 50ng/ml EGF for 40min and subsequently fixed and visualized by fluorescence microscopy. The average RFP-RBD intensity on the Golgi was determined by Image J software. A) The graph represents 3 individual experiments with means \pm SEM, n=100. B) Representative blots demonstrating the expression of each construct.

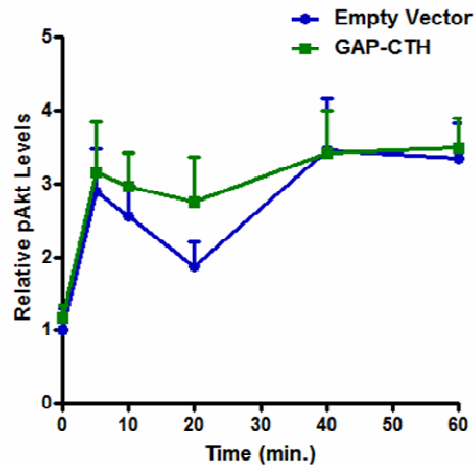
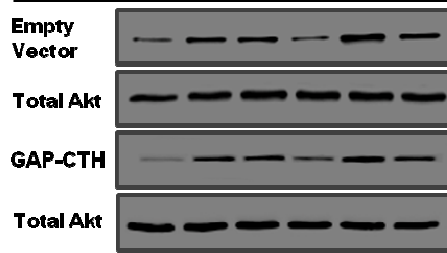
immunoblotting whole cell lysates using antiserum against ppERK and pAkt. As seen in Figure 3.16, maximal ERK activation occurs at 10min of growth factor stimulation and subsequently reduces, but does not return to basal levels on the time cap of this experiment. Figure 3.16 shows that GAP-CTK significantly decreased the level of ppERK when ERK was maximally activated (10min). GFP-GAP-CTH had no significant effect on the level of EGF-stimulated ppERK compared to the control cells (Empty Vector). Similarly, GFP-GAP-CTH181S did not alter the ERK activation profile. EGF-induced activation of Akt differs from ERK activation. Figure 3.17 shows that the activation profile of Akt has two peaks, one at 5min and the other at 40min following growth factor stimulation. Intriguingly, GFP-GAP-CTH181S significantly increased Akt activation at 5min; however GFP-GAP-CTH and GFP-GAP-CTK had no effect on the activation profile of Akt. Since ERK



A

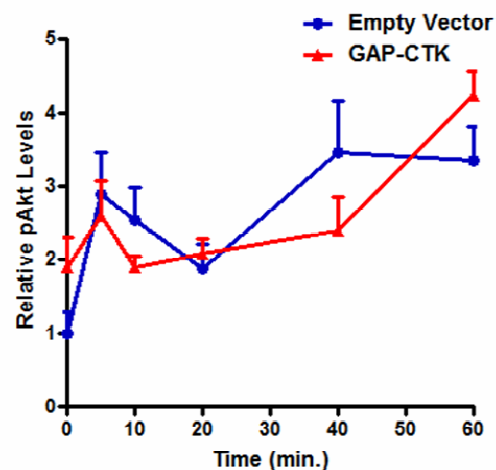
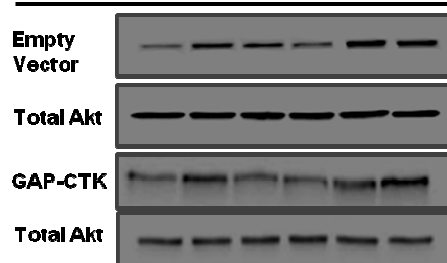
WB – pAkt:

Time(min.) 0 5 10 20 40 60

**B**

WB – pAkt:

Time(min.) 0 5 10 20 40 60

**C**

WB – pAkt:

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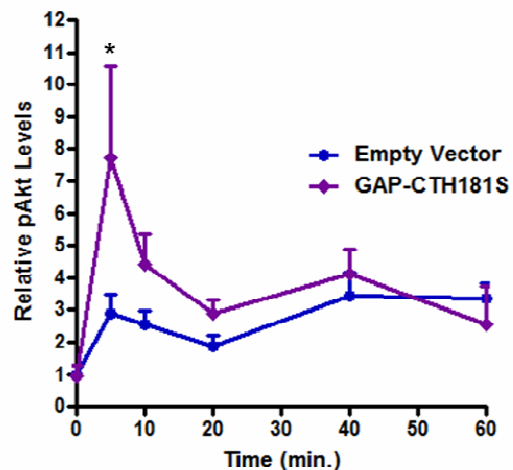
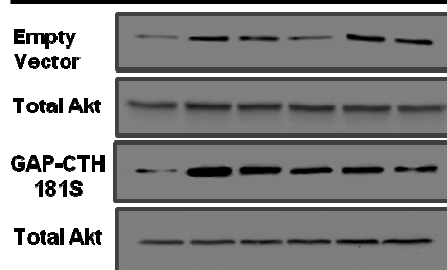


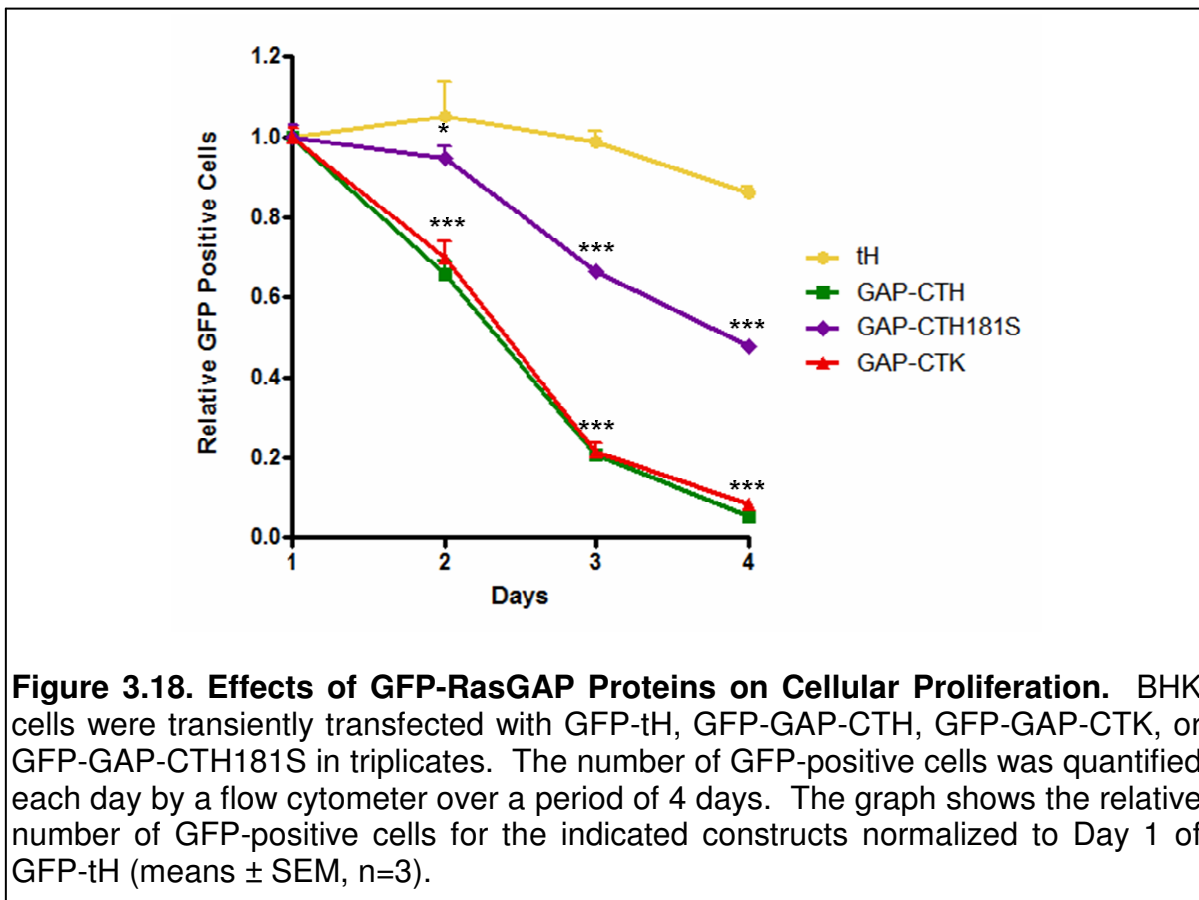
Figure 3.17. GFP-RasGAP Proteins Effect on the Akt Pathway. BHK cells transiently expressing GFP (Empty Vector) and A) GFP-GAP-CTH, B) GFP-GAP-CTK, and C) GFP-GAP-CTH181S were serum starved for 1hr. and stimulated with 50 ng/ml EGF for times up to 60 min. Whole cell lysates were analyzed by immunoblotting with a pAkt473 antibody or pan-Akt. Blots were quantified and the graphs show the levels of pAkt473 at each time point normalized to GFP time point 0 (means \pm SEM, $n \geq 9$).

activation is exclusively inhibited by GFP-GAP-CTK, we can conclude that EGF stimulation of the Raf/MEK/ERK pathway is predominantly mediated by K-Ras, whereas activation of the PI3-K/Akt pathway appears largely Ras independent.

3.2.5 Effects of GFP-RasGAP Proteins on Ras-Dependent Biological

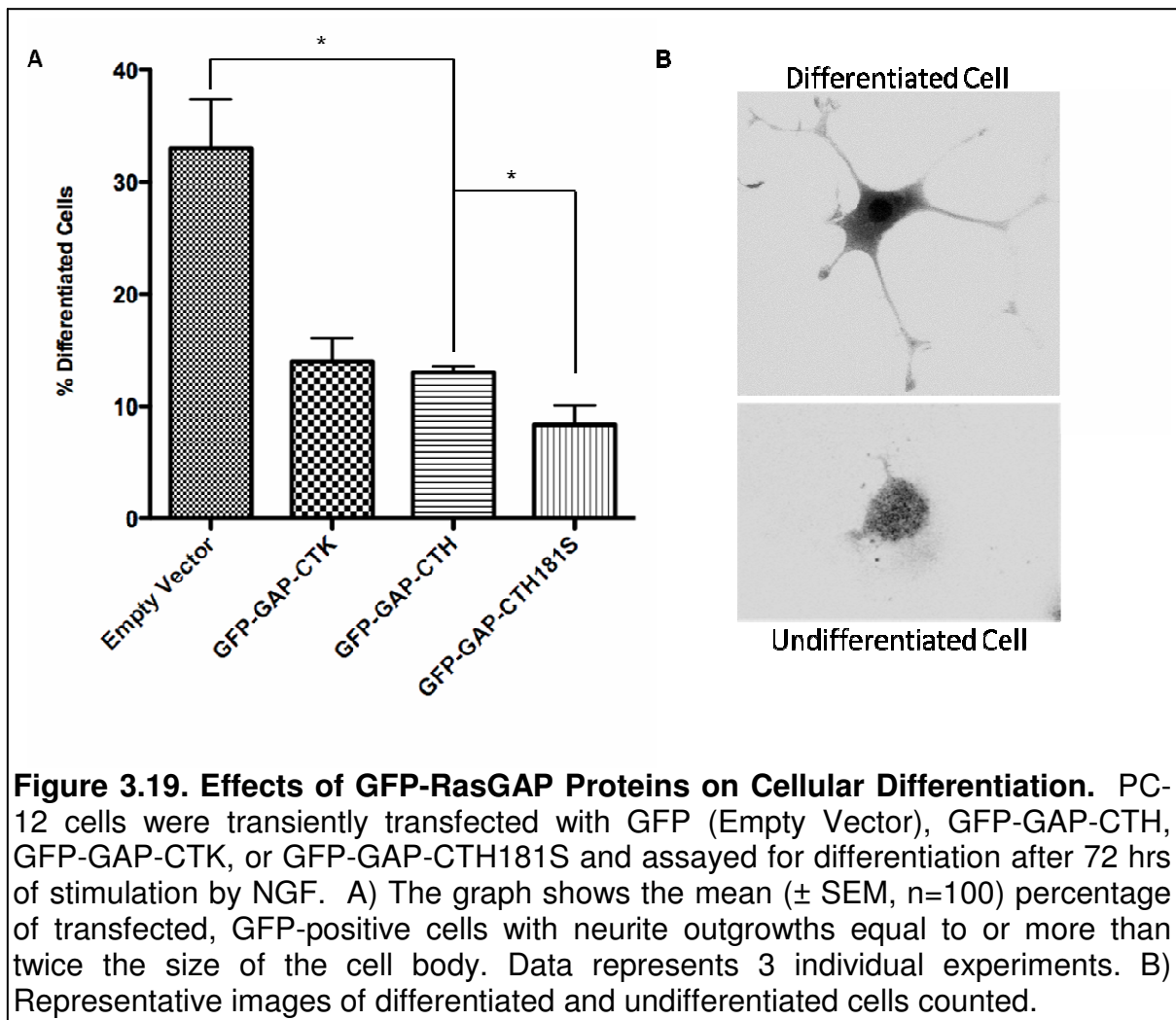
Next, we examined the biological consequences of inhibiting endogenous Ras signaling by assaying cellular proliferation and differentiation. First, we assayed cellular growth and viability via flow cytometry. BHK cells transiently transfected with GFP-RasGAP constructs were grown in culture over a period of 4 days and the number of GFP expressing cells in a total cell population of 10,000 cells was quantified each day using flow cytometry. As a control, we used GFP tagged to the minimal targeting motif of H-Ras, GFP-tH. GFP-tH has been characterized as a plasma membrane marker. In this assay, if the expression of GFP-RasGAP proteins inhibits cellular growth, proliferation, or viability, the number of GFP positive cells will decrease while non-transfected cells continue to grow and eventually take over the cell population. As demonstrated in Figure 3.18, expression of GFP-tH had no significant effect on the fraction of GFP-expressing cells present throughout the 4 day period indicating that expression of GFP-tH does not compromise cellular growth or viability. However, expression of GFP-RasGAP proteins resulted in a significant decrease in the number of GFP positive cells during the time assayed. GFP-GAP-CTK and GFP-GAP-CTH were equipotent inhibitors of cellular growth and viability, whereas GFP-GAP-CTH181S was less potent. Next, we examined the effect of GFP-RasGAP protein expression on cellular differentiation using a PC-12 differentiation assay. Rat adrenal pheochromocytoma (PC-12) cells are widely utilized as a model system for neuronal differentiation since they stop dividing and terminally differentiate in

response to nerve growth factor (NGF) (Santos et al., 2007). Figure 3.19 shows an undifferentiated PC-12 cell and an example of a differentiated PC-12 cell. In this section of the study, we transiently transfected PC-12 cells with or without



GFP-RasGAP proteins and stimulated these cells with 100 ng/mL NGF. Then, we imaged and counted undifferentiated and differentiated transfected cells. Cells with neurite outgrowths equal to or more than twice the size of the cell body were counted as differentiated, while cells with shorter or no neurite outgrowths were considered undifferentiated. Expression of GFP-RasGAP proteins significantly decreased differentiation of PC-12 cells (Figure 3.18). Specifically, GFP-GAP-CTK and GFP-GAP-CTH suppressed differentiation with similar potency, while GFP-GAP-CTH181S suppressed differentiation with greater efficiency than GFP-GAP-CTK and GFP-GAP-CTH. Since differentiation of PC-12 cells is greatly attenuated

by the expression of GAP targeted to the Golgi (GFP-GAP-CTH181S), we can conclude that Ras activation on the Golgi is a major driver of cellular differentiation. On the contrary, GFP-GAP-CTK and GFP-GAP-CTH, but not GFP-GAP-CTH181S, are more potent attenuators of cellular growth of BHK cells; thus Ras activation on the plasma membrane is the major driver of cellular proliferation.



Chapter 4. Discussion

4.1 Introduction

There is clear evidence that Ras proteins can be activated on multiple cellular compartments in addition to the plasma membrane. The significance of Ras signaling from the endomembrane, however, is unclear. To address this, we constructed Ras isoform-specific inhibitors, GFP-RasGAP fusion proteins, targeted to distinct plasma membrane nanodomains and the Golgi. We found that each GFP-RasGAP protein specifically abrogates the targeted endogenous Ras isoform on the plasma membrane and Golgi. We observed striking differences in the ability of GFP-RasGAP proteins to inhibit Ras signal transduction. Furthermore, we found that the expression of GFP-RasGAP proteins significantly and differentially altered the biological output of Ras signaling.

4.2 Role of Endogenous Ras Signaling

By abrogating the activity of an individual, endogenous Ras isoform with the corresponding GFP-RasGAP protein, novel insight into the organization of endogenous Ras proteins on the nanoscale level was discovered. We demonstrated that endogenous GTP-bound H-Ras and K-Ras occupy distinct plasma membrane nanodomains. This supports previous data which used ectopically expressed Ras proteins to show differential nano-localization of each Ras isoform on the plasma membrane (Murakoshi et al., 2004; Plowman et al., 2005; Prior et al., 2003; Roy et al., 2005).

Since one function of Ras nanoclusters in Ras signaling is to recruit C-Raf to the plasma membrane for subsequent activation (Harding and Hancock, 2008a; Harding and Hancock, 2008b; Tian et al., 2007), we were able to investigate the role of endogenous Ras proteins in C-Raf activation in plasma membrane nanodomains. We demonstrated that GFP-RasGAP targeted by the HVR of K-Ras

but not the HVR of H-Ras significantly attenuated the levels of activated ERK in response to stimulation by EGF. We conclude that endogenous K-Ras is the predominant mediator of EGF-induced activation of ERK, whereas H-Ras has no significant role in ERK activation in response to EGF. These results support earlier studies that demonstrated that C-Raf is more potently activated by K-Ras than any other Ras isoform. These earlier studies used ectopic expression of an oncogenic form of the Ras isoform to determine the efficacy of Ras isoform-specific mediated C-Raf activation (Yan et al., 1998). Our new results now show that endogenous K-Ras is also a more potent activator of Raf/MEK/ERK pathway than H-Ras. We also assayed another important Ras signaling pathway, PI3-K/Akt pathway. Previous studies have shown that the ectopically expressed constitutively active form of H-Ras is a potent activator of the PI3-K/Akt pathway (Yan et al., 1998). However, our data here show that neither GFP-GAP-CTH nor GFP-GAP-CTK expression significantly inhibited Akt activation. Unlike ERK activation, which is K-Ras dependent, activation of Akt may be mediated by both K-Ras and H-Ras and/or N-Ras which we did not investigate. Therefore, preventing activation of a single Ras isoform, with either GFP-GAP-CTH or GFP-GAP-CTK, still allows other Ras isoforms to activate PI3-K/Akt. Moreover, PI3-K can also be activated directly by the EGF receptor. Our results could also indicate that PI3-K activation on the plasma membrane, at low doses of EGF, is largely Ras-independent. One approach to test this hypothesis would be to express each GFP-RasGAP protein in EGF receptor null cells, stimulate with growth factor, and assess phosphorylated Akt levels. This will allow us to determine whether or not PI3-K activation is solely dependent upon activation of the EGF receptor.

Perhaps, the most intriguing result yet was the observation of significantly increased levels of phosphorylated Akt levels at 5 min. in EGF-stimulated cells that express GFP-GAP-CTH181S. This data suggests that Akt activation is increased on the plasma membrane when Ras activation on the Golgi is abrogated. An explanation for this result may be possible competition of PI3-K regulatory and catalytic subunits for recruitment to the plasma membrane or Golgi. Activation of PI3-K occurs through at least two independent mechanisms: binding of the catalytic subunit of PI3-K, p110, to GTP-loaded Ras and binding of the regulatory subunit of PI3-K, p85, to an activated receptor tyrosine kinase (RTK) which stimulates p110 activation (Krasilnikov, 2000). By blocking Ras activation on the Golgi, it is possible that the translocation of PI3-K (p110/p85) is enhanced for direct activation by the EGF receptor. In turn higher levels of PI3-K activation on the plasma membrane leads to an increase in the levels of phosphorylated Akt. Another potential explanation could be a reduction in the plasma membrane accumulation of phosphatases in response to abrogation of Ras-GTP on the Golgi. Phosphatases such as PTEN, SHIP1, and SHIP2 can prevent the activation of Akt (Vivanco and Sawyers, 2002); but, how RasGTP on the Golgi might regulate plasma membrane delivery of such phosphatases is unclear.

4.3 Regulation of Endogenous Ras Activation

As previously reported, Ras activation mediated by the EGF receptor occurs rapidly (Murakoshi et al., 2004; Zhao et al., 2007). Immediately following EGF stimulation, we observed Ras GTP-loading that endured until 20min before returning to basal levels with maximum Ras activation by 2 min. Since RasGEFs are responsible for the exchange of RasGDP for RasGTP (Bernards and Settleman, 2007), this data suggests that endogenous RasGEFs are rapidly recruited to the

plasma membrane in response to activation of the EGF receptor. The subsequent return of Ras activation to basal levels is a potential consequence of an up-regulation of endogenous RasGAP activity as well as a down-regulation of endogenous RasGEF activity. In support of these results, earlier studies have demonstrated an up-regulation of RasGAP activity dependent upon activated growth factor receptors. For instance, upon binding of EGF, EGF receptors recruit PLC γ which leads to the generation of DAG and InsP $_3$ through the hydrolysis of PIP $_2$. This stimulates calcium release causing an increase or up-regulation in the recruitment of the RasGAP CAPRI to the plasma membrane. Like CAPRI, the RasGAP GAP1^m is recruited to the plasma membrane due to production of PIP $_3$ from the RasGTP-dependent activation of PI3-K (Lockyer et al., 2001; Lockyer et al., 1999). p120RasGAP also binds to phosphorylated tyrosine sites of activated platelet derived growth factor (PDGF) and EGF receptors (Kaplan et al., 1990; Kazlauskas and Cooper, 1990; Soler et al., 1993). Our results generally support these concepts that p120RasGAP mediated GTP hydrolysis of Ras requires membrane recruitment. Specifically, we show that the expression of cytosolic GFP-GAP does not alter endogenous RasGTP levels on the plasma membrane.

Recent reports have suggested that release of palmitoylated Ras isoforms from the plasma membrane to the Golgi, due to a continuous depalmitoylation/re-palmitoylation cycle, is required for EGF-induced Ras activation at the Golgi. However, our results suggest that retrograde trafficking of Ras from the plasma membrane to the Golgi does not account for the prolonged (EGF stimulation for 40min) accumulation of RFP-RBD on the Golgi. Targeting GAP by the HVR of H-Ras (GFP-GAP-CTH) to the plasma membrane significantly decreased the accumulation of RFP-RBD on the plasma membrane but had no effect on RFP-RBD

localization to the Golgi; yet, we were able to reduce RFP-RBD accumulation on the Golgi by targeting GAP to the Golgi (GFP-GAP-CTH181S). These results suggest that Ras activation on the Golgi is independent of the depalmitoylation/repalmitoylation cycle and may occur directly at the Golgi. These results support a previous study where GFP-RBD was utilized to report Ras activation on the Golgi and the dependence of Ras activation at this compartment on Src. In T cells, a pathway for Ras activation on the Golgi involving the Src-dependent activation of phospholipase C- γ 1 (PLC- γ 1) has been proposed. This pathway includes the translocation of the RasGEF RasGRP1 from the cytosol to the Golgi where it activates Ras.

4.4 Biological Consequences of Endogenous Ras Signaling

Next, we investigated the ability of RasGAP proteins to modify cellular growth. The expression of each RasGAP protein impeded cellular growth; however, with varying efficiency. RasGAP proteins targeted to endogenous H-Ras and K-Ras nanoclusters (GFP-GAP-CTH and GFP-GAP-CTK) on the plasma membrane significantly suppressed cellular growth with similar efficacy. RasGAP targeted to H-Ras on the Golgi (GFP-GAP-CTH181S) suppressed cellular growth to a lesser extent than GFP-GAP-CTH and GFP-GAP-CTK. Therefore, we conclude that activation of endogenous H-Ras and K-Ras on the plasma membrane is more important for growth than Ras activation on the Golgi. Correlating these effects on cellular growth with MAPK activation alone is difficult since only GFP-GAP-CTK significantly reduced ERK activation, yet all 3 GFP-RasGAP constructs inhibited growth to a greater or lesser extent. It seems most probable that other Ras effectors, which we did not assay, are required for cellular proliferation.

Differential regulation of cellular proliferation by distinct pools of Ras activation has been previously described. Palmitoylation-deficient constitutively active H-Ras targeted to bulk membrane or lipid rafts of the plasma membrane similarly enhances cellular proliferation. In contrast, targeting this protein to the Golgi with a KDEL receptor mutant (KDELr N193D) which traps the protein in the Golgi does not enhance growth, but can sustain limited cellular growth. Interestingly, our results support this report providing evidence of differential affects of endogenous Ras activation at the plasma membrane and the Golgi on cellular proliferation.

We also examined whether the expression of RasGAP proteins would alter the cellular fate of PC12 cells. We showed that the expression of GFP-RasGAP proteins significantly reduced the levels of differentiation in PC12 cells. Specifically, GFP-GAP-CTH and GFP-GAP-CTK suppress cellular differentiation with similar efficacy while GFP-GAP-CTH181S expression results in the most profound suppression of PC12 differentiation. In striking contrast to cellular growth, we can conclude that the activation of endogenous Ras on the Golgi is more important for cellular differentiation than H- or K-Ras on the plasma membrane. Our results of endogenous plasma membrane-localized Ras contrast with a previous study where abrogation of endogenous Ras activation on the plasma membrane had no inhibitory affect on cellular differentiation. However, differences in specificity may likely account for this discrepancy. We targeted the GAP catalytic domain of p120RasGAP to specific Ras nanoclusters on the plasma membrane to assess regulation of differentiation by H-Ras or K-Ras, whereas the aforementioned study overexpressed the RasGAP CAPRI and failed to target the protein to specific sites of Ras activation on the plasma membrane. Consequently, all Ras isoforms are

subject to inhibition by CAPRI and the effects on individual Ras isoforms are ignored. Furthermore, oncogenic H-Ras tethered to the Golgi is sufficient to enhance cellular differentiation. Using a different approach, we confirm that endogenous H-ras activation on the Golgi has a key role in cellular differentiation.

PC12 cells are established as a model system for signal specificity of the MAPK pathway. Upon stimulation by NGF, the MAPK pathway (Raf/MEK/ERK) is activated through the receptor tyrosine kinase TrkA giving rise to cellular differentiation. The MAPK pathway is also activated by EGF through the EGF receptor which leads to cellular proliferation. How the MAPK network processes these different inputs into specific biological outputs in PC12 cells has been studied. The underlying cause for these opposite cellular fates (proliferation and differentiation) is the differences in the dynamics of ERK activation. EGF stimulation of the MAPK pathway prompts transient ERK activation where as NGF stimulation produces sustained ERK activation (Marshall, 1995). A proposed model further explains the molecular interpretation of distinct ERK dynamics by immediate early gene products (IEGs). When ERK activation is sustained, IEGs are stabilized by ERK-catalyzed phosphorylation. Consequently, different genes dependent upon sustained ERK activity are expressed (Murphy et al., 2002). Furthermore, it is believed that upstream of ERK activity lies a distinct set of positive and negative regulators which are differentially activated by EGF and TrkA receptors, thereby contributing to opposite cellular fates. Among these regulators are PKC and Raf kinase inhibitory protein (RKIP), respectively (Santos et al., 2007). Interestingly, abrogation of RasGTP on the Golgi significantly decreased differentiation revealing that Ras activity on the Golgi is important for differentiation than RasGTP on the plasma membrane. Since RasGTP on the Golgi has been characterized by

sustained Ras activation (Chiu et al., 2002), it seems likely that sustained Ras activity on the Golgi is responsible for sustained ERK activity and therefore cellular differentiation observed in PC12 cells. As for cellular proliferation, however, precisely which Ras signaling pathways are responsible is not clear from our work.

Finally, our work has focused on H-Ras and K-Ras. It would be interesting to examine the effects of RasGAP on N-Ras activity. To accomplish this, we would simply target the catalytic domain of p120RasGAP to nanodomains that occupy GTP-loaded N-Ras. This can be done by fusing the HVR of N-Ras to the catalytic domain of p120RasGAP. Generally, in plasma membrane localization studies, N-Ras has not been extensively studied, so less is known about the N-Ras nanoclustering. Both H-Ras and N-Ras have been shown to signal from the Golgi. A comparison study between these two Ras isoforms using GFP-RasGAP proteins targeted to the Golgi would be interesting.

Chapter 5. References

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