RAB VESICLE TRAFFICKING IS REQUIRED FOR RAS-MEDIATED PROLIFERATION

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ABSTRACT

The Ras superfamily consists of over 150 members including the well-known: H-Ras, K-Ras and N-Ras. We propose to target Ras related proliferation in cancer cells by inhibiting Rab vesicle formation. H-Ras, K-Ras and N-Ras are carcinogenic; activating mutations in Ras signaling are generally associated with increased proliferation and survival in cancer cells. Ras is mutated in up to 30% of all human cancers and represents an early survival mutation in cancer cells. Vesicle-bound Ras is trafficked to the plasma membrane, which facilitates interaction between Raf, and effector proteins. Previously, farnsesylation inhibitors, (FTIs) and GTP binding agonists of Ras have been tested as potential pharmacological inhibitors of Ras signaling. However, both drug types have proven ineffective *in-vivo*. Therefore, there are currently no effective pharmacological treatments to target Ras signaling. However, unpublished data from our lab has identified co-localization of Ras and Rab proteins, in vesicles. Rab proteins are associated with vesicle budding, and endosome development. Rab activity is driven by GTP binding and geranylgeranylation. Non-geranylgeranylated Rab is cytosolic, and does not facilitate endosome formation or vesicle trafficking. We propose that by targeting the Rab specific geranylgeranylation via Rab-specific geranylgeranyl transferase II, we will inhibit Ras associated proliferation. We will pursue this hypothesis by testing three objectives. First, we will produce and test Rab-geranylgeranyl transferase (RGGT)-shRNA to target Rab specific geranylgeranyl transferase. Second, we will illustrate the effect of RGGT knockdown on a downstream signaling target of Ras, specifically pERK levels. Finally, we will measure the direct effect of RGGT knockdown on Ras mediated cellular proliferation. Our results conclude that RGGT-shRNA is a potential target of Ras mediated tumor proliferation.

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BACKGROUND

The Ras superfamily consists of over 150 members including the well-known: H-Ras, K-Ras and N-Ras. We propose to target Ras related proliferation in cancer cells by inhibiting Rab vesicle formation. H-Ras, K-Ras and N-Ras are carcinogenic; activating mutations in Ras signaling are generally associated with increased proliferation and survival in cancer cells. Ras is mutated in up to 30% of all human cancers and represents an early oncogenic mutation in cancer cells. Ras proteins undergo a series of post-translational modifications to the C- terminal CAAX box, resulting in an increased affinity for lipid membranes. In H-Ras, R-Ras and N-Ras, the final modification is the addition of a palmitoyl group which facilitates interaction with endosomes, and lipid vesicles. Vesicle-bound Ras is trafficked to the plasma membrane, where it enables interaction between Raf, and effector proteins. In current literature it is an area of debate if Ras related survival signaling is driven by Ras localization on the plasma membrane. Previously, farnsesylation inhibitors, (FTIs) and GTP binding agonists of Ras have been tested as potential pharmacological inhibitors of Ras signaling. However, both drug types have proven ineffective in vivo. Therefore, there are currently no effective pharmacological methods to target Ras signaling. However, unpublished data from our lab has identified co-localization of Ras and Rab proteins, in vesicles. Rab proteins are associated with vesicle budding, and endosome development. We have identified that Rabs 4, 5, and 11 co-localize with Ras vesicles in vivo. Rab activity is driven by GTP binding and geranylgeranylation. Non-geranylgeranylated Rab is cytosolic and interacts with the GDP dissociation inhibitor (GDI) within the cytosol. We propose that by

targeting the Rab specific geranylgeranylation via Rab-specific geranylgeranyl transferase II, we will inhibit Ras associated proliferation.

1.1 Ras Isoforms

Ras proteins are mutated in up to 30% of all human cancers (Spiegel, Cromm, Zimmermann, Grossmann, & Waldmann, 2014; Wennerberg, Rossman, & Der, 2005). Activating mutations in H-Ras, N-Ras and K-Ras are all associated with increased survival and proliferation in cancer cells, and an early driving mutation (Spiegel et al., 2014). The theory of oncogenic addiction implies that by targeting these early mutations it will be possible to selectively target a large percentage of the diverse population of cancer cells. However, attempts to target Ras mutations in human cancer have had limited success. Protein inhibitors of GTP binding have proven ineffective because Ras binds GTP at picomolar levels (Baines, Xu, & Der, 2011; Spiegel et al., 2014). However, Ras is selectively active within the cell depending on location. Ras proteins are small GTPases which undergo a series of post-translational modifications altering the C-terminus motif known as a CAAX box (Ahearn, Haigis, Bar-Sagi, & Philips, 2012; Wennerberg et al., 2005). The cleavage and alterations to the CAAX box motif increases the tendency of the Ras protein to interact with membranes (Ahearn et al., 2012). Palmitoylation of oncogenic Ras is the final step, except in K-Ras. In palmitoylated Ras, palmitoylation allows interaction with budding lipid vesicles at the Golgi, and induces trafficking to the plasma membrane (Ahearn et al., 2012; Wright & Philips, 2006). At the plasma membrane Ras protein brings Raf in contact with Raf effectors, present at the plasma membrane. (Stokoe, Macdonald, Cadwallader, Symons, & Hancock, 1994; Udell, Rajakulendran, Sicheri, & Therrien, 2011). Current literature suggests that Ras proteins

clump into the fluid mosaic like structure of the external membrane interacting in formation with high density Rafts, although there is not a consensus in the literature (Barcelo et al., 2013; Janosi, Li, Hancock, & Gorfe, 2012; Kenworthy, 2007; Rotblat et al., 2010). K-Ras also localizes with the plasma membrane, but unlike the other members of the Ras sub-family, K-Ras does not undergoes palmitoylation and localizes with the plasma membrane by electrostatic interaction with the chain of charged amino acids on the C-terminus (Barcelo et al., 2013; Hancock, Paterson, & Marshall, 1990; Spiegel et al., 2014). H-Ras, N-Ras and R-Ras trafficking to the plasma membrane is driven by interaction with budding vesicles from the Golgi. Unpublished data from our lab identifies Rab vesicles co-localize with a significant population of vesicle trafficked Ras.

1.2 Ras Superfamily

The Ras superfamily is made up of over 150 small GTPase proteins, divided into either 5 and 6 sub-families, depending on the methods used (Wennerberg et al., 2005). GTPases contain a GTP switch mechanisms which facilitates the transfer of GDP for GTP to induce activity. Most small GTPases, and all Ras GTPases, are active in the GTP bound state (Bourne, Sanders, & McCormick, 1991). Generally, small GTPases share a common biochemical structure that is highly similar, although they only share up to 30% of their primary structure (Bourne et al., 1991; Colicelli, 2004). External stimuli, such as hormone signals or growth factors, interact with cell surface receptors which promote formation of the GTP-bound Ras superfamily GTPases. The GTPase switch activity is mediated by protein specific GTPase-activating-proteins (GAPs) and Guanine-nucleotide-exchange factors (GEFs) present within the cell (Barr & Lambright, 2010; Bourne et al., 1991). Ras superfamily GTPases contain a high affinity for both GDP and

GTP, and possess a low intrinsic capability for GTP hydrolysis. Therefore, Ras GTPases interaction with GEFs is very important to regulate activity. Ras GEFs act by loosening the association with inactive GDP, and stabilize nucleotide-free Ras, which preferentially binds with GTP (Colicelli, 2004; Wennerberg et al., 2005). The different branches of the Ras superfamily have structurally distinct, but mechanistically similar GAPs and GEFs (Bishop, 2000).

Ras-like proteins in brain (Rab) proteins are the largest branch of the Ras superfamily, with over 61 primary members (many have sub groups). Rab GTPases regulate intracellular vesicle transport and are associated with both early, and late endosome development (Zerial & McBride, 2001). Rab proteins facilitate vesicle formation, budding from donor membrane, and vesicle fusion with the target membrane. Current literature has identified various Rabs associated with different stages of vesicle trafficking; Rab4 is associated with early endosome development and membrane budding and Rab5 is associated with late endosomes and vesicle fusion (Sonnichsen, De Renzis, Nielsen, Rietdorf, & Zerial, 2000). I will go into Rab vesicles in more depth further on.

The Ras superfamily also contains Ras-like nuclear protein (Ran). Ran proteins are related to the Ras proteins primary sequence, but distinguished by specific features. Ran protein is present in the nucleus in high numbers and best associated with nucleo-cytoplasmic transport, and mitotic spindle assembly (Li, Cao, & Zheng, 2003; Wei, Jobling, Chen, Hahn, & Sedivy, 2003). The fifth branch of the Ras super family are the ADP-robosylation factor (Arf) family proteins. Arf proteins are regulated be distinct GAPs and GEFs. Arf1 is the best characterized member, and regulates formation of

vesicle coats, and regulates ARf-coat-protein complex facilitating activity (Memon, 2004; Nie et al., 2003).

The most famous, and founding members of the Ras Subfamily are the oncogenic Ras proteins including H-Ras, N-Ras and K-Ras (Wennerberg et al., 2005). Ras proteins in the GTP bound state are signaling hubs, interacting with a diverse list of downstream effectors. The founder Ras proteins, H-Ras, N-Ras and K-Ras, primarily respond to epidermal growth factor receptor tyrosine kinase and signal through the Raf-MAPK pathway (Colicelli, 2004; Wennerberg et al., 2005). Active Ras proteins bind to the Raf serine/threonine kinase, carrying them to the plasma membrane. Raf phosphorylates and activates the MEK 1/2 protein kinase, which phosphorylates ERK1/2 mitogen-activated protein kinase (MAPK). The active ERK translocate to the nucleus and phosphorylate various transcription factors (Roberts & Der, 2007). The uncontrolled activity of the Raf-MAPK pathway is a common tumor promoter. A few Ras family proteins act as tumor suppressors, rather than tumor promoters (Colicelli, 2004). Overall, the Ras sub-family are known to be powerfully oncogenic proteins, associated with between 20-30% of all human cancers in early development.

1.3 Post-Translational Modifications of Ras

Ras proteins undergo a series of post-translational modifications, which partially define the distinctly different physiological effects of the specific proteins (H-Ras, N-Ras, R-Ras and K-Ras). Ras proteins contain a series of distinct regions, including two switch regions, an SH2 domain and C-terminal hyper-variable region. The switch I region is necessary for GTP binding, and the switch II region regulates binding with the phosphate analog and subsequent phosphorylation of Ras proteins. Ras proteins are largely conserved in their primary and secondary structure, except for the hyper-variable region. The hyper-variable region of Ras oncogenic proteins contains a terminal domain known as the CAAX box proteins which contains a cysteine (C), two aliphatic amino

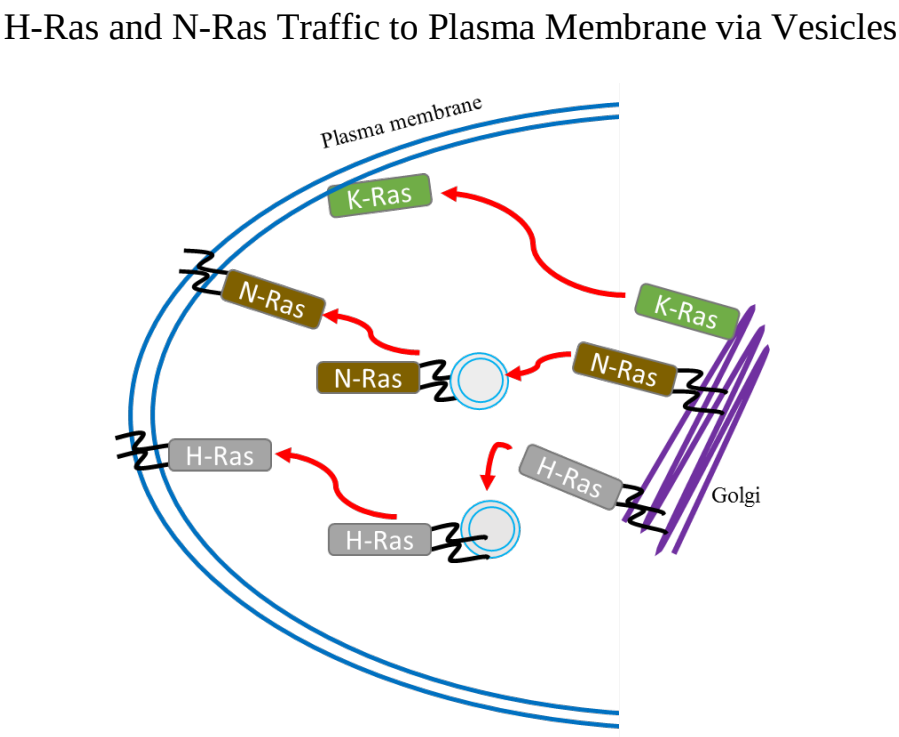

Figure 1. Ras Trafficking to the plasma membrane. N-Ras, H-Ras and R-Ras (not shown) undergoing vesicle-mediated transport from Golgi membranes to the plasma membrane, while K-Ras traffics to the plasma membrane independently of vesicles

acids (A) and X for any amino acid. Ras is the founding member of CAAX proteins, or proteins which contain the CAAX box terminal domain. The CAAX box is a template for a series of enzymes which modify it, creating a hydrophobic domain which allows for membrane targeting. The first step in CAAX box processing is prenylation. Prenylation is the addition of a hydrophobic lipid chain, either a 15 carbon farnesyl isoprenoid or 20 carbon geranylgeranyl isoprenoid (Ahearn et al., 2012). Prenylation forms a substrate for Rce1 which removes the -AAX amino acids (Boyartchuk, Ashby, & Rine, 1997). The prenylated C-terminal cysteine is a target of a highly specific carboxyl methyl transferase, protein-S-isoprenylcysteine O-methyltransferase (ICMT). ICMT methyl esterifies the alpha carboxyl group of the prenyl-cysteine (C. A. Hyrcyna, 1991; Dai et al., 1998).The specificity of the prenylation addition depends on the final amino acid of the CAAX motif. The CAAX processing steps are considered un-regulated with respect to cell signaling, with one exception. One publication in the literature identified a lesser known splice variant of SmgGDS, which selectively associates with non-prenylated members of the Ras superfamily. This publication claims that the SmgGDS splice variant regulates the entry of various Ras superfamily proteins into the prenylation pathway (Berg et al., 2010). Overall, the CAAX processing and transformation of the hydrophilic amino acids to a hydrophobic lipid chain motif is considered necessary for Ras localization (Hancock et al., 1990). A second signal is required for localization to the external plasma membrane. In mammals, an upstream cysteine is palmitoylated by either of the Ras specific palmitoyl transferases, DHHC9/GDP16 (Swarthout et al., 2005). Unlike the other Ras proteins, K-Ras is not palmitoylated, rather the second ‘signal’ is an existing polybasic lysine residue on the C-terminus (Hancock et al., 1990). These post-

translational modifications define localization of Ras (Background 1). The CAAX processing increases the hydrophobicity allowing localization to the ER, then finally the Golgi. Only at the Golgi does Ras interact with its palmitoyl transferase where the final addition of the hydrophobic lipid tail. That final addition allows the palmitoylated Ras proteins, H-Ras, N-Ras and R-Ras, to interact with budding vesicles from the Golgi.

1.4 Vesicles

The formation of the vesicles from the Golgi is referred to as budding. Vesicle budding initiates when coat complexes are recruited to the donor membrane. There are three well studied vesicle coats, clathrin, COPI (Coat protein complex I) and COPII (Barlowe et al., 1994; Malhotra, Serafini, Orci, Shepherd, & Rothman, 1989; Pearse, 1975). The coat corresponds with vesicle activities: clathrin coated vesicles mediate traffic throughout the endocystic system, COPI mediates intra-Golgi traffic and retrograde traffic and COPII mediate anterograde traffic from the ER to the Golgi (Hehnly & Stamnes, 2007). After budding, un-coating occurs, which exposes the tethering and fusion machinery. Un-coating is believed to be induced by interaction with GAPS. Small GTPases such as Arf/Sar activation induces COPI and COPII coat assembly (Barlowe & Schekman, 1993; Boehm, Aguilar, & Bonifacino, 2001; Donaldson, Cassel, Kahn, & Klausner, 1992; Faundez, Horng, & Kelly, 1998; Nakano & Muramatsu, 1989; Ooi, Dell'Angelica, & Bonifacino, 1998; Palmer, Helms, Beckers, Orci, & Rothman, 1993; Stamnes & Rothman, 1993). A cell-free assay of COPI and COPII trafficking demonstrated that fusion could not occur if either Arf or Sar GTP hydrolysis was blocked (Antonny, Madden, Hamamoto, Orci, & Schekman, 2001; Barlowe et al., 1994; Melancon et al., 1987; Tanigawa et al., 1993). Furthermore,

inhibiting GTP hydrolysis of specific small GTPases may inhibit exposure of tethering and fusion machinery. Tethering and fusion machinery are necessary for tethering, docking and fusion with the target membrane (Sutton, Fasshauer, Jahn, & Brunger, 1998; Weber et al., 1998). At the target membrane the tethering factors facilitate interaction between the membranes. During docking soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) initiates fusion by triggering lipid and content mixing (Sutton et al., 1998; Weber et al., 1998). Therefore, SNARES, and vesicle coats increase specificity of vesicle targeting. MTCs and tethers are thought to increase targeting specificity by forming a network between Rabs, SNARES and specific lipids (McNew et al., 2000; Sollner et al., 1993).

1.5 The Cellular Function of Ras

The primary isoforms of Ras are almost identical on a primary protein level. However, the downstream signaling of the various forms of Ras differentiate, dependent on their hyper-variable region and modifications within the cell, as discussed earlier. The varied signaling of Ras protein results in differentiating physiological and cellular effects of Ras signaling.

Typically, Ras signaling is induced by ligands binding to a cell surface activating receptors. The binding of growth factors and other ligands to the Growth Factor Receptors and other binding sites induce tyrosine phosphorylation. The resulting tyrosine binding sites for adaptor proteins mediate the membrane translocation of GEFs, such as sons of sevenless (SOS), which activate the membrane bound Ras (Bowtell, Fu, Simon, & Senior, 1992). Within the cell SOS is in a complex with adapter protein GRB2 (Growth Factor Receptor-bound protein-2). GRB2 binds to active tyrosine binding sites,

localizing the SOS-GRB2 complex (Gay et al., 1999). The membrane bound Ras is inactive until GTP binding is facilitated by GEFs that mediate Ras to dissociate from GDP, allowing Ras to bind with GTP within the cytoplasm (Bowtell et al., 1992) . Ras has a high affinity for GTP, like other members of the Ras superfamily (Baines et al., 2011; Spiegel et al., 2014). In the GTP-bound form, Ras interacts with effector proteins, initiating cascades of down-stream protein interactions. Current literature identifies a variety of downstream signaling pathways activated by Ras, most prominently the Raf/MEK/ERK (Extracellular Signal-Regulated Kinases) pathway. Ras has also been shown to activate the MEKK/SEK/JNK (Jun N-terminal Kinases) pathway, a PI3K (Phophatidylinositol 3-Kinase)/AKT/NF-KappaB (Nuclear Factor-Kappa B) pathway, and a Raf/MERKK1/IKK (I-KappaB Kinase)/I-KappaB/NF-KappaB Pathway. GTP-bound Ras recruits Raf, facilitating Raf interaction with Raf-specific-GEFs at the plasma membrane (Stokoe 1994). Active, GTP-bound Raf initiates signaling by phosphorylating downstream effectors, such as MEK, which in turn phosphorylates ERKs. The Raf/MEK/ERK pathway cumulates with phosphorylated ERK trans-locating from the cytoplasm into the nucleus (Udell et al 2011). The MAPK pathway (Mitogen-Activated Protein Kinases) plays a major role in controlling proliferation and differentiation (Udall 2011, Stokoe 1994).

Various sources in the literature have identified forms of Ras which play specific roles in specific tissues. K-Ras, H-Ras and N-Ras, all signal through the Raf mediated MAPK pathway. K-Ras actually forms an irreversible bond with Raf1, which may regulate downstream signaling effects of K-Ras (Xiangyong Li 2012). For example, K-Ras-4B specifically is essential for embryogenesis, and deficient embryos have a variety

of lethal defects early in gestation (reference Johnson 1997, Koera 1997). However, H-Ras and/or N-Ras deficient mouse mutants are viable (Esteban 2001). Furthermore, a publication in 2005 identified that H-Ras expression when regulated by the K-Ras promoter, may be able to rescue K-Ras deficiency in embryogenesis (Potenza 2005). The different physiological and cellular functions of Ras where, in some cases, first discovered in association to the prevalence of specific Ras proteins in specific cancers.

1.6 Cancer

The Ras oncogenes where first identified in the early 1980s (Defeo, 1981). The Harvey sarcoma virus-associated oncogene was name Ha-Ras (H-Ras in mammals) and the Kirsten sarcoma virus was termed Ki-Ras (K-Ras in mammals). Mutant alleles of both these Ras sequences were soon discovered in various human cancer cell lines (Parada et al 1982, Der et al, 1982, Santos et al 1982). Oncogenic Ras contains point-mutations varying from wild-type, usually on the 12 residue alternating a neutral charged cysteine to a charged amino acid (Tabin 1982, Reddy 1982, Capon 1983). Oncogenic point mutations for Ras can also occur on residues 13 and 61, although less commonly. The most common point mutation on 12 residue leads to an inability for Ras to bind to GAPs and effectively inhibits GTP hydrolysis, leaving Ras in a constitutively active state (Colicelli, et al. 2004). Although Ras proteins are small G-proteins, they hydrolyze GTP at a very low rate without interaction with the Ras specific GAPs. Therefore, mutated active Ras results in increase survival signaling of the MAPK pathway and other cellular functions, outlined above. Ras is considered an early mutation of transformation, however early publications identified that Ras alone cannot initiate cellular transformation However multiple publications in 1983 described H-Ras V12 as a

transformative agent in cell previously immortalized by carcinogens or viral transfection (Neubold 1983, Land 1983, Ruley 1983). Therefore the transformative abilities of Ras are considered limited to cells previously predisposed to transformation. Further research identified that oncogenic Ras initiates senescence by activation of pro-survival signaling pathways (Serrano, 1997, Wei 2003). Ras activating mutations without an earlier 'initiating' mutagenic event undergoes cell-cycle arrest, or senescence, without proliferation. Furthermore, initiators of the RB or p53 pathway, such as SV40 aid a cell to overcome Ras-mediated senescence (Takaoka et al., 2004).

Current literature describe a series of 5 or 6 oncogenic events, (either mutations or viral) to fully transform a cancer cell. One of the most important events is mediation of the p53 pathway, which is aided by oncogenic Ras. The last 30 years have also identified specific forms of cancer associated with the iso-forms of Ras. For example, pancreatic cancer is highly associated with K-Ras, while melanoma and lymphoid malignances are more commonly identified with mutations in N-Ras then H-Ras or K-Ras (Karnoub & Weinberg, 2008; Pylayeva-Gupta, Grabocka, & Bar-Sagi, 2011).

Oncogenic Ras is often considered an early driving mutation in cancer transformation. Oncogene addiction is the theory which proposes that transforming mutations must be maintained within the population of cancer cells to retain tumorgenicity. Therefore, targeting early transformational mutations can be an effective method to target continued proliferation. There have been various attempts at clinically relevant inhibitors of Ras mediated proliferation. The first approach were farnesyltransferase inhibitors (FTIs) designed to block the first step of Ras CAAX modification, and thereby block activity. FTIs were meant to compete with Ras for the

post-translational processing enzymes discussed earlier, and there were very promising *in-vivo* studies in 1995 (Kohl et al., 1995). However, clinical trials of FTIs proved ineffective due to compensation by other prenylation mechanisms, namely geranylgeranylation in the case of N-Ras and K-Ras (James, Goldstein, & Brown, 1995). Further, the apparent effects of FTIs were later attributed to inhibition of other prenylated cellular proteins such as Rheb (Ras homologue enriched in the brain) and RhoB. Alternative strategies to block Ras function were therefore necessary. Other steps of CAAX modifications have been targets of pharmacological inhibitors, such as Rce1 and ICMT1, although initial research suggested disrupting those proteins was not sufficient to completely inhibit Ras signaling (James et al., 1995). Further, GTP competitive inhibitors have been developed but had limited to no effect due to Ras's high affinity for GTP. Furthermore, approaches have been developed to alter Ras functions *in vivo*. For example, Ras inhibition by anti-sense RNAs, and anti-Ras ribozymes (Funato, Ishii, Kambe, Scanlon, & Sasaki, 2000; Y. A. Zhang, Nemunaitis, Scanlon, & Tong, 2000). Or, a large effort has been applied to inhibiting downstream effectors of Ras, including the Raf-MEK-ERK cascade and upstream the GRB2-SOS complex (Gay et al., 1999; Roberts & Der, 2007). Administration of these agents have so far made clinical applications difficult.

1.7 Rabs

Rab proteins are small GTPases, ranging in size from 21 to 25 kD, and contain several highly conserved regions. Ras share regions with many other members of the Ras superfamily, however there are a few conserved regions specialized to the Rab family of proteins (Nikolova et al., 1998; Pereira-Leal & Seabra, 2000). Rab proteins, like Ras,

contain a molecular switch and cycle through inactive GDP-bound state and active GTP-bound state. The molecular switch is facilitated by GEFs and GAPs (Barr & Lambright, 2010). The specificity of Rab mediated signaling is partially regulated by association with GEFs and GAPs. Rab proteins cycle between GTP and GDP bound state, as well as between cytosolic and membrane bound association. Cytosolic Rabs are bound to chaperone guanine nucleotide exchange factors (GDI), or else are largely degraded. Cytosolic Rabs remain untethered to a membrane due to Rab GDI pocket blocking the hydrophobic geranylgeranyl tails. Rab GDI plays the dual role of maintaining the GDP bound Rabs, in their inactive state, and blocking interaction of the hydrophobic geranylgeranyl group from binding to target membranes. Only a small fraction of Rabs are cytosolic. Most Rabs accumulate at their target compartments and membranes. In recent literature Rab GDI dissociation Factors (GDFs) have been hypothesized and may initiate dissociation between Rab-GDP and Rab GDI (Bhuin & Roy, 2014; Sivars, Aivazian, & Pfeffer, 2003). However, it is unclear how, or in what situations GDFs would be active. Currently only one proposed GDF has been identified with limited activity (Sivars et al., 2003).

The membrane domains of the recycling endosome pathway are characterized by three Rabs: Rab4, Rab5, and Rab11. Three populations of endosomes have been observed, one containing only Rab5, the second with Rab4 and Rab5 and the last with Rab4 and Rab11. (Sonnichsen et al., 2000). Rab5 alone is the marker of early endosomes, and mediates traffic from the plasma membrane to the early endosomes (Bhuin & Roy, 2014). Rab4 is associated with early and recycling endosomes, mediating recycling *to* the plasma membrane. Finally, Rab11 associated endosomes are recycling endosomes,

including apical and baso-lateral endocytic recycling endosomes (Sonnichsen et al., 2000). The vesicles containing Rab4-Rab5 together are early endosomes, while the Rab4-Rab11 are recycling endosomes from the plasma membrane. The specificity of various Rabs to target membranes, and endosomes may be mediated by various markers. The Itzen lab in 2013 proposed a Rab5 GEF, RABEX-5, is necessary for specific recruitment to endosomes (Blumer et al., 2013).

As mentioned earlier, Rab association with membranes is mediated by the addition of geranylgeranyl groups. Geranylgeranylation is a post-translational modification, specifically the addition of a 20-carbon lipophilic geranylgeranyl isoprene. Geranylgeranyl groups are added from the geranylgeranyl diphosphate to either one or two hydrophobic C-terminal proteins. Geranylgeranylation of Rabs acts as an anchor to target initial membranes and endosomes (Bhuin & Roy, 2014; Grosshans, Ortiz, & Novick, 2006).

1.8 Geranylgeranyl Transferase

Geranylgeranylation can be catalyzed by one of two geranylgeranyl transferases: geranylgeranyl transferase I (GGTase I), and geranylgeranyl transferase II, AKA Rab geranylgeranyl transferase (RGGT). GGTase I catalyzes the addition of a single geranylgeranyl group to a CaaX motif (A.J., Weimer et al., 2011). RGGT is specific for Rab proteins, and catalyzes the addition of either one or two geranylgeranyl groups to the C-terminal hydrophobic cysteine of Rab proteins (H. Zhang, Seabra, & Deisenhofer, 2000).

RGGT is composed of two subunits alpha and beta, encoded by two genes independently. RGGT catalyzes the addition of geranylgeranyl group from the donor, geranylgeranyl diphosphate. A zinc cation in the beta subunit near the active site, which helps facilitate the SN2 attack on the donor (Bhuin & Roy, 2014; H. Zhang et al., 2000). The alpha subunit contains an immunoglobulin-like (Ig) domain and leucine-rich repeat (LRR) domain, both domains are often involved in protein-protein interactions. The LRR and Ig domains of the alpha subunit may participate in recognition and binding of the Rab escort protein (REP) (H. Zhang et al., 2000). RGGT has only been found to geranylgeranylate Rabs in complex with REP, therefore both subunits are necessary for RGGT activity.

In 2000, the Kingsmore lab identified a point mutation in the alpha subunit of RGGT which alters platelet activity. The G to A mutation was identified in recessive mutant mice known as *gunmetal* mouse, named for the specific grey fur on the affected mice. RGGT activity was found to be reduced up to 4-fold in gunmetal mouse platelets, leading to altered bleeding in the mutants (Detter et al., 2000; Taylor et al., 2011). A mutation in the alpha subunit of RGGT was identified as the cause (Detter et al., 2000). The mice are fully viable, and do not have any other known defects which effect health. However, an FDA approved drug, 3-PEHPC, which targets the alpha subunit of RGGT has proven redundant when gunmetal mice are treated (Coxon et al., 2011; Taylor et al., 2011). 3-PEHPC is a specific RGGT inhibitor, it prevents bone loss and development of osteolytic bone lesions (Lawson, Coulton, Ebetino, Vanderkerken, & Croucher, 2008). The inhibitor 3-PEHPC has been FDA approved for treatment of osteoporosis, with

limited side-effects. Therefore, it has been hypothesized that the 3-PEHPC drug target the alpha subunit of RGGT.

1.9 Hypothesis and Objectives

Targeting lipid modification responsible for Rab binding to target membranes and endosomes will inhibit Ras-vesicle trafficking. Inhibiting the Ras-vesicle trafficking to the plasma membrane will produce a significant, loss of Ras mediated proliferation, and further Ras-induced tumorigenesis.

To test my hypothesis I have set out to prove three objectives

1.9.1 The efficiency and specificity of the RGGT-shRNA to target the Rab specific geranylgeranyltransferase.

1.9.2 The effect of RGGT knockdown on one of the primary downstream signaling targets of Ras mediated proliferation.

1.9.3 The direct effect on RGGT knockdown of specific tumorigenic Ras mediated proliferation.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell Lines, Antibodies, and Reagents

NIH3T3 cells (3T3s) were obtained from the American Tissue Type Collection (ATCC) and used within 10 passages. Secondary antibodies Donkey Anti-Mouse, and Donkey Anti-Rabbit were purchased from Li-Cor Biosciences. The primary Antibodies tERK Anti-Mouse, ppERK Anti-Rabbit and GFP Anti-Mouse were purchased from Santa Cruz technologies. Rab Geranygeranyl transferase (RGGT) primary antibody for the N-terminal region was obtained from Aviva system biology through Fisher Scientific. Donkey anti-Rabbit Cy5-Affini-pure was purchased from Jackson ImmunoResearch Labs Inc. Normal Donkey Serum (NDS) was purchased from Equitech-Bio. DAPI nucleic acid stain was bought from Life Technologies. The BGlII, EcoR1 and HindIII enzymes were acquired from New England Biolabs. Lipofectamine 2000 Transfection Reagent, 4-20% Tris-Glycine Novex ™ protein 15 well gels, and Opti-mem Reduced Serum Media (Optimem) were both purchased from Life Technologies. Odyssey Blocking Buffer was purchased from Li-Cor Biosciences. Dulbecco's modified Eagle's medium (DMEM) high glucose, 2% Bovine Calf Serum(BCS), 1% Penicillin-Streptomycin Solution, 1% nonessential Amino Acids and 0.05% Trypsin were all purchased from ThermoScientific. Protan Ba 85 blotting paper was purchased from Whatman GE Healthcare Life sciences. Soybean Trypson Inhibitor was purchased from Amresco Inc. The pSUPER.retro vector, used to express short hairpin RNA (shRNA), was purchased from Oligoengine. QiaquickGel Extraction Kits used were purchased from Quagen. The Human Plasma Fibronectin used in coverslips was purchased from Millipore.

2.2 Cell Culture

Unless otherwise stated, all cells were grown in 10cm plates in 7-10 ml of DMEM Complete media. Cells were grown at 37 degrees C in 95 % air humidity and 5% CO2. The media used was composed of DMEM, 2% BCS, 1% Penicillin-Streptomycin Solution, and 1% nonessential Amino Acids (DMEM complete).

2.3 Transfection

24 hours before transfection cells were placed in no-antibiotic DMEM Complete media. For the transfection 20 µL of lipofectamine was added to 1.5 ml of Optimem and allowed to incubate for 5 minutes. Concurrently, 12 µg (unless otherwise noted) of DNA was incubated in 1.5 ml of Optimem for 5 minutes. After incubation, lipofectamine mix was added to the DNA Optimem mixture, for a final volume of 3ml. The DNA-lipofectomine was mixed by gentle agitation and incubated at room temperature for 20 minutes to allow lipofectamine lipid plasmids to form. After no more than 30 minutes the 3 ml total mixture was added to the 10 ml cell culture plates. 4-6 hours after the initial transfection No-antibiotic DMEM Media was replaced with DMEM Complete media.

2.4 Protein Gels and Western Blot

4-20% Tris-Glycine Novex ™ protein 15 well gels were used for all Western Blot analysis. The gels were cleaned with filtered, de-ionized water (DI water), and lanes washed out with 1X sodium dodexyl sophate (SDS) prior to loading. All protein samples were boiled in 5X SDS dye with dithiothreitol (DTT). The protein gels were run in 1X SDS buffer at 80 volts for 2-2.5 hours until ladder was 4/5ths of the length of the gel. Samples were transferred onto blotting paper in 2X Transfer Buffer at 4 degrees Celsius

overnight (at least 8 hours). 2X Transfer Buffer was made in 1 liter stock from 200 mL 10X XC-TB, 200 mL Methanol and DI water. After the transfer the sample membrane was incubated in Odyssey Blocking Buffer on a shaker for 1 hour. After blocking, the sample membrane was transferred into primary antibodies at 1:500 dilution in Tris-buffered saline with Tween 20 Buffer (TBST) and .1% bovine serium albumin (BSA) at 4 degrees Celsius overnight. After treatment with primary antibodies the membrane was washed in 10X TBST buffer, 3X for 10 minutes each. The membrane was incubated in secondary antibodies Donkey Anti-Mouse, and Donkey Anti-Rabbit at 1:10,000 concentration in TBST and .1% BSA for 1 hour at room temperature on a shaker. After the secondary incubation the membrane was again washed 3X for 10 minutes each in TBST. The treated membrane can be storied in TBS for up to 6 month at 4 degrees, however for experiments in this protocol imaging was acquired as soon as possible. Imaging of western blot membranes was done on an Odyssey Li-cor Infrared Imaging System.

2.5 Agarose DNA gels

DNA was run on 2% agarose gels freshly made in 1X Tris-Acetate-EDTA (TAE) buffer. Gels were hardened for a minimum of 20 minutes before samples were run in a gel box at 150 volts for 30-40 minutes, or until ladder reached at least ½ the length of the gel. The DNA sample were mixed with 6X SDS dye prior to loading.

2.6 shRNA Primer

The Rab geranylgeranyl transferase (RGGT) short hairpin RNA (shRNA) were designed to target alpha subunit of RGGTase, using previously published examples from the Kingsmore lab (Detter et al., 2000). We designed two primers to fit into the p.SUPER retro vector, containing the shRNA hairpin loop; RGGT alpha shRNA 1 (Top strand: 5'-gatccccCCACTGATGAACAGTTGTTTTCAAGAGAAACAACTGTTCATCAGTGGTTTTTa- 3'. Bottom Strand: 5'-acgttAAAAAACCACTGATGAACAGTTGTTTCTCTTGAAAACAACTGTTCATCAGTGGCggg- 3') and RGGT alpha shRNA 2 (Top Strand: 5'-gatccccGCACCTGGCTCACAAGGATTTCAAGAGAATCCTTGTGAGCCAGGTGCTTTTTa- 3'. Bottom Strand: 5'-acgttAAAAAGCACCTGGCTCACAAGGATTCTCTTGAAATCCTTGTGAGCCAGGTGCggg- 3'). Corresponding primers where annealed in a thermocycler at 95 degrees Celcius. The correctly annealed samples were isolated on a 2% agarose gel. The correctly double stranded primers were extracted from agarose gel using QiaQuickGel Extraction Kit. Annealed primers were ligated into the pSUPER.retro vector. Prior to ligation pSUPER.retro vector was digested with BglII and EcoRI. The correctly digested pSUPER.retro vector was isolated after run and extracted from a 2% agarose gel. RGGT shRNA primers were designed with BglII and EcoRI overhangs in the corresponding directions to allow annealing in frame. Correctly ligated primer-vector resulted in a loss of the BglII cut site and produced a HindIII cut site. The ligation efficiency was confirmed by a 1 hour digestion with HindIII and BglII.

2.7 Serum Starve Protocol

24 hours prior to experiment NIH3T3s were transfected with the desired bacterial plasmid DNA. 24 hours post-transfection all samples were placed in Serum Starve Media. Serum Starve Media was made according to DMEM complete, but with .2% BCS. All the experiments were carried out with two controls transfected with GFP plasmid. The controls were separated into, starved and feedback. Feedback samples were feed with DMEM complete media for ½ hour, 48 hours after being placed into Serum Starve Media. All samples were lysed, or harvested 30 minutes after Feedback. Feedback and starvation control served as controls of protocol efficiency (Figure 2).

2.8 Proliferation Protocol and Cell Counting

All cell counting was carried out with a hemocytometer. Either 10µL of 1ml or 10 µL of 400µL was loaded onto the hemocytometer, according to standard protocol. The four corners of the hemocytometer were counted and averaged with duplicate results. To obtain the final cell count the averages per sample were multiplied by 10,000 (according to standard hemocytometer protocol) and 4/10 (when taken from 400µL sample).

All proliferation experiments in this paper were carried out in a similar manner. NIH3T3 cells were transiently transfected with the desired plasmid DNA as described. Then, 24 hours post-transfection the cells were counted and plated in a 12 well plates at 10,000 cells per ml in 1 ml media. The proliferation media used was a modified version of the DMEM complete, with 1% BCS. The cell count was recorded every 24 hours from initial platting time for a total of 72 hours, and the initial count (0 hours) was taken 6 hours post-platting to standardize plating results.

2.9 Immunofluorescence

Immunofluorescence cover slips were prepped with fibronectin 12-24 hours before use. Prior to incubation in fibronectin the cover slips were cleaned with H20 and 100% ethanol, and all excess ethanol was burned off. Each cover slip was placed independently in a well of a 6 well plate. 1 ml of the fibronectin was incubated on each cover slip until use. Immediately before use all cover slips were washed in phosphate buffered solution (PBS) and replaced in 1mL of media. When imaging, the serum starve experiments .2% BCS media was used in the place of DMEM complete.

The cell samples were trypsinized from 10cm plates by washing 2X with 1X PBS, then incubated for up to five minutes in 2 ml of 1X 0.05% Trypsin at 37 degrees Celsius. The trypsonized cells were spun down and re-suspended in Soybean Trypson Inhibitor. 1:20 of each cell sample was isolated per coverslips. The coverslips were incubated at 37 degrees Celsius for at least one hour. After incubation the wells were washed 2X in TBS, never PBS. The cells were fixed with 1 ml of 3.7% formaldehyde diluted in 1X TBS for 20 minutes at room temperature, in the dark. Once the cells were fixed in formaldehyde, all the samples were kept in the dark as much as possible. Next, the samples were washed 3X for 5 minutes each, in TBS. The samples were then permeabilized in 1 ml of 0.1% TX-100 in 1X TBS for 5 minutes, at room temperature. Post permeabilization, samples were washed in 1X TBS. Then, samples were incubated in 1 ml of .1% BSA in 1X TBS with normal donkey serum (NDS) at a dilution of 1:100 for 30 minutes at 37 degrees C. Finally, the samples were stained with 100 µL/sample of primary antibodies at 1:100 dilution in 1X TBS, at 4 degrees C, overnight.

The next morning, the samples were washed 7X for 5 minutes each in 1X TBS. Then, the samples were incubated in secondary antibodies for 1 hour. For all experiments in this protocol secondary antibody Donkey Anti-Rabbit-Cy5 was used at 1:100 dilution. After incubation the coverslips were washed 3X for 5 minutes in 1X TBS. The coverslips were then stained with 1ml of DAPI 3nM at 1:10,000 dilution in 1X TBS for 10 minutes at room temperature. The coverslips were washed again 3X for 5 minutes in 1X TBS and 1X in 10mM Tris, pH 7.4. The coverslips were mounted on clean slides with a small drop of Prolong Gold (Invitrogen), and were allowed to dry at room temperature for at least 1 hour. The slides were sealed with clear nail polish to preserve the samples. All imaging of coverslips was obtained on Ep1000 fluorescent microscope at 60X magnification. Live cell imaging was obtained with E300 fluorescent microscope at 10X magnification.

2.10 Statistics and formulas

All data analysis was done in Microsoft Excel, and densitometry quantification was completed using imageJ. Anova-one-way test used for proliferation statistical significance analysis using Excel. A P value of .05 or less was considered significant and results of proliferation experiment are averages of cell count from 3 independent experiments.

CHAPTER 3

RESULTS

3.1 Serum Starve Protocol Decreases ppERK/tERK Levels in NIH3T3 Cells

In order to test downstream effectors of Ras-mediated proliferation we set up a serum starve experiment model. ERK phosphorylation, a major step in the MAPK pathway, is mediated by active Ras downstream of Growth Factors (GFs). We designed a 72 hour serum starve experiment protocol to test the efficacy of H-Ras or K-Ras mediated ERK phosphorylation, independent of GFs. NIH3T3 cells were transfected with either active mutant GFP-K-Ras, or an empty GFP vector as a control. The transfected NIH3T3 cells were then plated in a 10 cm plate in DMEM complete media with low levels of serum and allowed to proliferate for 48 hours. After 48 hours in low serum media the feedback control setting was returned to regular DMEM complete media for 30 minutes. After feedback, all plates were lysed in complete lyses buffer and the lysates run on a western blot (WB) to determine the levels of ppERK (Figure 2a). The results were conclusive that 48 hours in a serum starve environment effective decreased the total amount starve experiment effectively decreased the amount of ppERK, to allow specific measurement of K-Ras associated ppERK levels (Figure 2b). Therefore, we determined that the ppERK levels observed in the serum starve K-Ras column are the result of active K-Ras signaling, independent

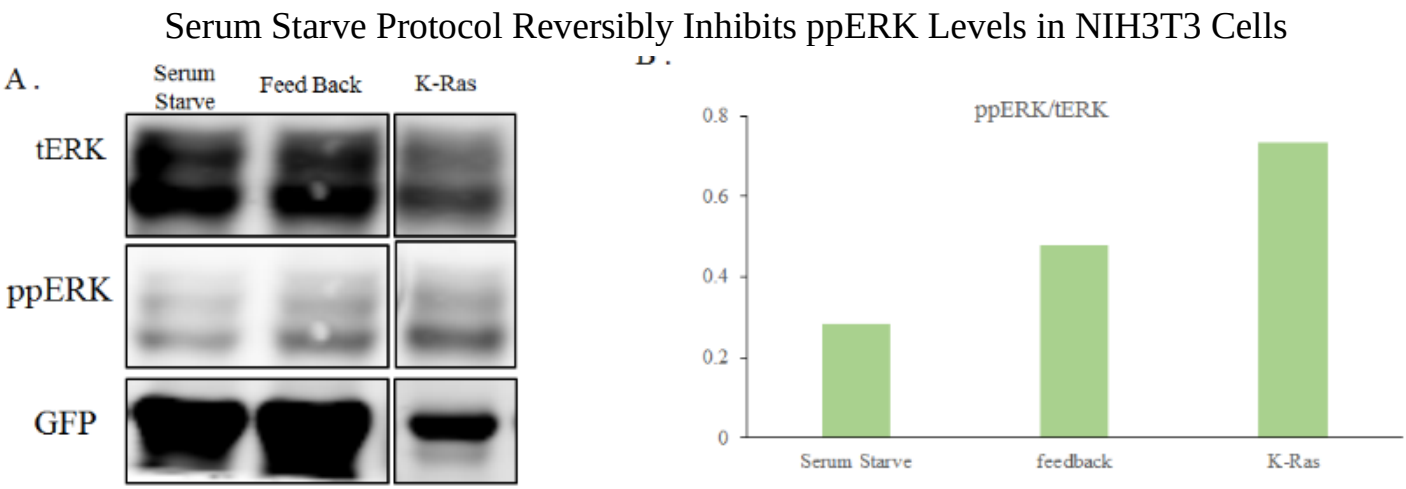

Figure 2. A 48 hour incubation in serum starve media is associated with a reversible decrease in ppERK levels. Mouse fibroblast NIH3T3 cells were transiently transfected with 10µg of either GFP, or active mutant GFP-K-Ras for 24 hours. 24 hours post transfection the cells were placed in serum starve media for a 48 hour incubation. After 48 hours the feedback in DMEM complete media with BCA serum for ½ hour. The lysate of all samples were taken post-feedback (A). Whole cell lysates were treated with protease inhibitors for 30 minutes on ice and proteins were separated by SDS-PAGE, followed by Western Blotting with antibodies to tERK, ppERK and GFP as indicated. Graph at right shows the relative levels of ppERK in cells transfected as indicated, normalized to tERK levels (B).

of GFs or other factors found in serum.

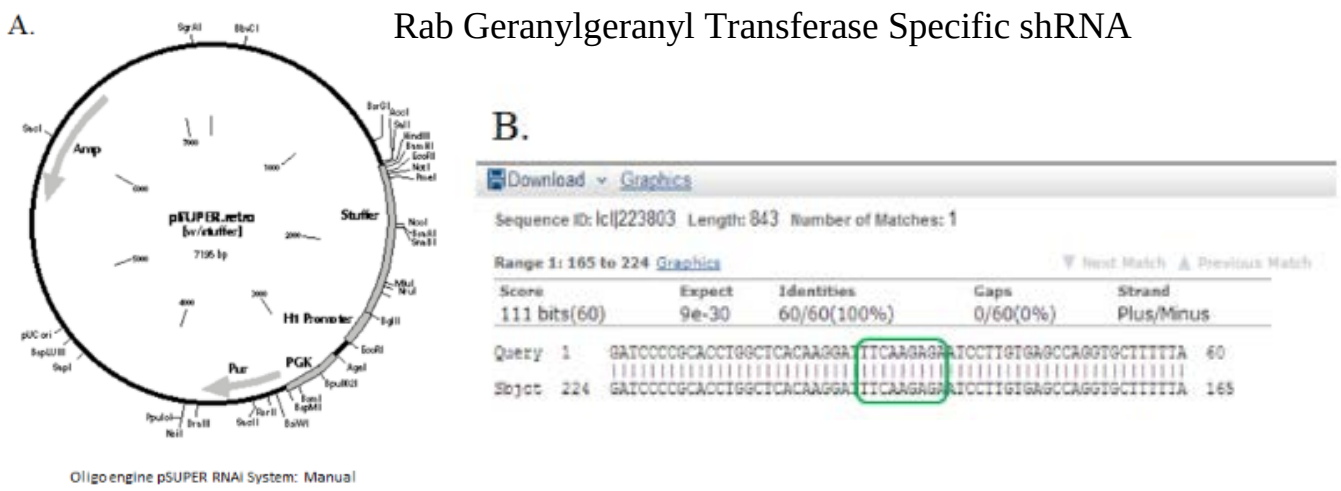

Figure 3. Short hairpin RNA (shRNA) produced targeting Rab-specific geranylgeranyl transferase (RGGT). Two shRNAs were designed to target the alpha subunit of murine RGGTase, using homology to previously published target human sequences from the Kingsmore lab, reporting an 80% knockdown (KD) of RGGTase activity. Primers were annealed in a thermocycler. Annealed primers were ligated into the psuper.retro vector, purchased from OligoEngine (A). Insertion of the shRNA sequence was confirmed by restriction enzyme digestion and by sequencing analysis (B). The resulting transcript is expected to yield a hairpin loop (middle) which will be cleaved by the cell's RNA processing machinery to produce a 19-nt double-stranded targeting shRNA. This is a blast result of our selected sequence of RGGT-shRNA2 against the expected sequence. The complete insert sequence contains reverse complement repeats of the 19-nt shRNA sequence targeting the alpha subunit RGGT, separated by a hairpin loop (green).

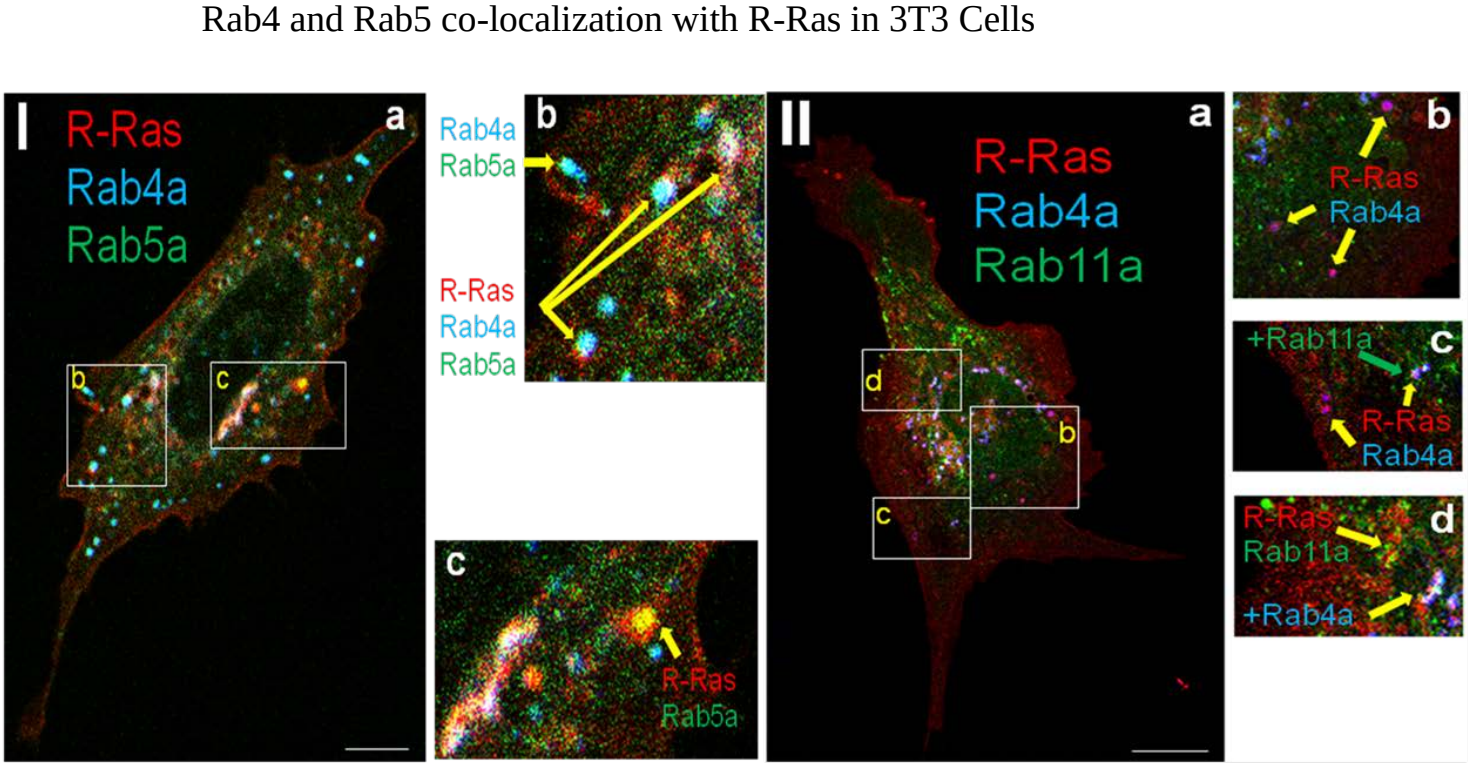

Figure 4. R-Ras traffics through combinations of Rab4, Rab5 and Rab11 vesicles. RFP-R-Ras (red) was co-transfected in NIH3T3 cells with CFP-Rab4a (blue) and either GFP-Rab5a (green, I) or gFP-Rab11a (green, II) and images were acquired in live cells by confocal fluorescence microscopy using sequential scanning. (A) shows the whole cell in each case and (b-d) show images from insets in (a). R-Ras localized in vesicles containing Rab4 only, Rab5 only, and combinations of Rab4, with either Rab5 or Rab11, as determined by comparing fluorescence across channels; in some cases co-localization appears as yellow, purple or white. R-Ras/Rab4 vesicles appeared enriched in peripheral areas. Yellow arrows point to each population of vesicles as indicated.

3.2 RGGT Specific shRNA

In order to consider the effects of targeting Rab mediated vesicle trafficking on Ras-mediated cell proliferation we designed a short hairpin RNA (shRNA) to target Rab specific geranylgeranyl transferase (RGGT). Rabs are a family of small G-proteins involved in early and late endosome development, and vesicle trafficking. Many Rab proteins undergo the post-translational modification geranylgeranylation. Unpublished experimental data from our lab co-localization of Rab and Ras proteins (Figure 4). We propose that Rab vesicles drive Ras targeting to the plasma membrane (PM). A previous publication by the Kingsmore lab produced two independent shRNA to target the alpha subunit of RGGT (Detter et al., 2000). We designed two sets of oligonucleotides to encode shRNAs targeting two distinct sites in the alpha subunit of the murine RGGT, to be ligated into p.super retro vector (Figure 3a). I annealed the corresponding primers with the necessary overhangs and isolated the double stranded samples. (Figure 3b) The double stranded shRNA primers were ligated into the psuper.retro vector. Ligation efficiency was determined with diagnostic enzymatic digestions. Thus, murine shRNA were cloned in a corresponding vector to target RGGT

3.3 RGGT-shRNAs effectively deplete RGGT expression by transient transfection and RGGT-shRNA correspond with phase shift in Rab5 and Rab11

Once correctly ligated samples were isolated we tested the efficiency of RGGT knockdown in NIH3T3 cells. Samples from both completed plasmid were transiently transfected into NIH3T3 cells. I determined that both shRNAs effectively demonstrated knockdown of RGGT expression in NIH3T3 cells. RGGT-shRNA2 had a greater effect, demonstrating up to a 60% knock-down efficiency in transient transfection (Figure 5b). Therefore, for the best results I continued the experiments using shRNA2. I determined knockdown efficacy by comparing total RGGT levels in transfected, versus un-transfected samples. I blotted the total lysate of the transiently transfect 3T3 cells for various Rabs. Unpublished data from our lab identified Rab11, Rab25, Rab 4 and Rab5 co-localize with tumorigenic Ras in vesicles (Figure 4). The WB reveals a slight double band, visible in the Rab 5 blot under control levels (Figure 5a). Transfection with both shRNA2 and shRNA1 correspond with a loss of the secondary band of Rab5. We propose the second band represents geranylgeranylated Rab5. I hypothesis that the geranylgeranylated Rab runs heavier on a WB gel due to the increased charge of the lipid chain. However, the total amount of Rabs 4, Rab5 and Rab11 does not change statistically significantly according to WB (Figure5c). Therefore, any change in the level of downstream effect by RGGT targeting shRNA is specific to the decreased level of Rab geranylgeranylation.

RGGT shRNAs Effectively Deplete RGGT Expression by Transient Transfection

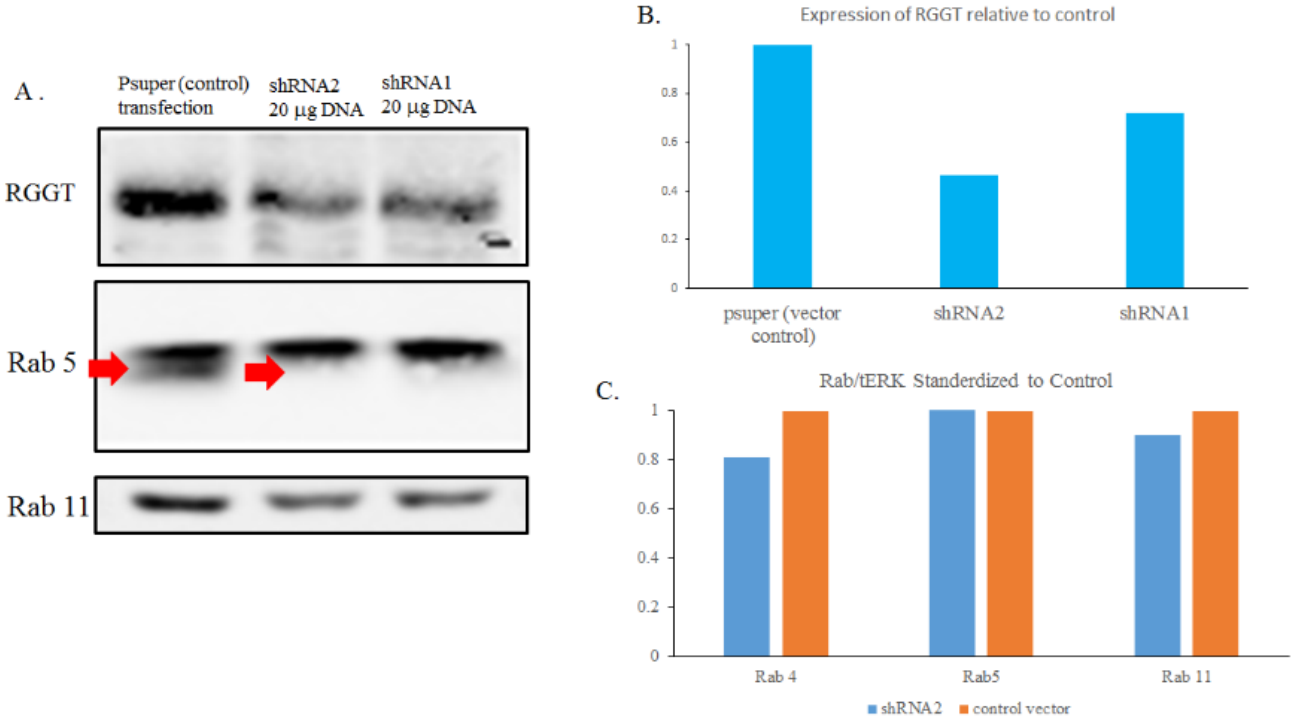

Figure 5. The RGGT specific shRNAs exhibit up to 60% knockdown of RGGT expression and loss of geranylgeranylation of cellular Rab proteins. Mouse fibroblast 3T3 cells were transiently transfected with 20 µg of both RGGT shRNA in the psuper.retro vector for 48 hours. Whole cell lysates were treated with protease inhibitors for 30 minutes on ice and proteins were separated by SDS-Page, followed by Western Blotting with antibodies to RGGT, Rab5 and Rab11 as indicated. (A) Red arrows in the Rab5 membrane indicate geranylgeranylated Rab5, expected to have increase mobility in SDS-PAGE, which is absent in the shRNA-transfected cells. Similarly, Rab11 is mostly geranylgeranylated in control cells, but shows a slight upward mobility shift in the presence of the shRNA,s indicating loss of geranylgeranylation. (B, C) Graph at right shows the relative levels of RGGT in cells transfected as indicated, normalized to a loading control (not shown).

RGGT shRNA Inhibits Rab and Ras Vesicles Anchorage and Ras PM Targeting.

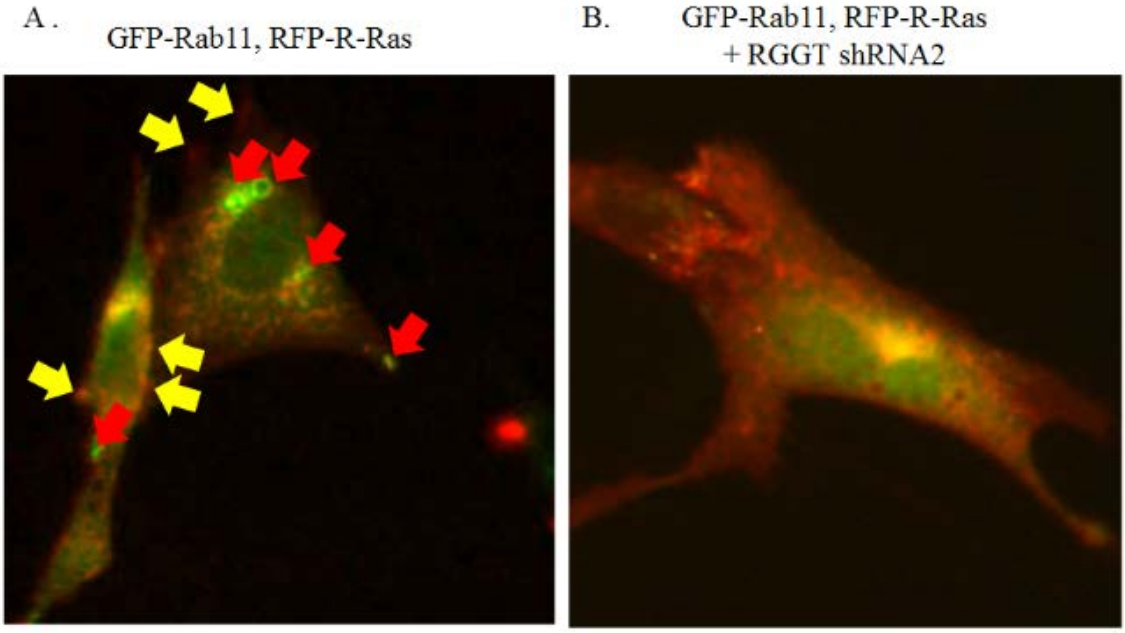

Figure 6. RGGT shRNA correlates with a loss of plasma membrane bound R-Ras and loss of Rab11 and Rab25 vesicles. RFP-R-Ras (Red) was co-transfected with GFP-Rab11 (A) or YFP-Rab25 (B) and RGGT shRNA2 (not visible). Epiflourescence images were acquired in live cells by using a 40x objective. Red arrows point to Rab vesicle formation. Yellow arrows highlight Ras at the plasma membrane.

3.4 RGGT-shRNA inhibits Rab and Ras vesicle anchorage and Ras PM targeting

Next, I tested the ability of the RGGT-shRNA to inhibit formation of Rab vesicles (Figure 6). NIH3T3 cells were transiently co-transfected with RFP-R-Ras (red fluorescent protein) and GFP-Rab11 (green fluorescent protein), in the presence and absence of RGGT-shRNA2. 24 hours post-transfection I acquired 40X epi-fluorescence images. In the control cells Rab vesicles are visible, and co-localization of Rab and Ras vesicles are present in vesicles bound for the plasma membrane. Furthermore, a significant level of Ras is visible at both the Golgi membrane, and PM (Figure 6a). In the majority of cells transfected with 20 μg of RGGT-shRNA Rab vesicles are not visible, and the GFP-Rab is more cytosolic. The figures are representative of transfected cells with both GFP-Rab11 and RGP-R-Ras. The shRNA2 transfected cells display a decrease in Ras at both the internal membrane and PM. The yellow co-localization of RFP-R-Ras and GFP-Rab-11 appears to be limited to the internal membranes of the Golgi and ER (Figure 6b). The RGGT-shRNA2 corresponds with an apparent decrease in overall Rab11 vesicle trafficking, Rab-Ras co-localization and Ras association with the PM.

3.5 RGGT-shRNA has an inhibitory effect on N-Ras mediated ppERK levels

The direct effects of RGGT-shRNA on N-Ras mediated ppERK levels. N-Ras undergoes vesicle mediated trafficking to the PM. We propose that N-Ras trafficking, like that of R-Ras and H-Ras, is mediated by Rabs. We transiently transfected NIH3T3 cells with constitutively active GFP-N-Ras plasmid DNA. The samples were co-transfected with either RGGT-shRNA2, or the control vector. 24 hours post-transfection the samples where incubated in serum starve media, for 48 hours. As per the experimental protocol outlined above. The resulting samples were isolated, and a consistent fraction was added to fibronectin treated coverslips. The sample coverslips were fixed with formaldehyde, and incubated in first ppERK primary antibodies, then washed, and finally Cy5-ppERK secondary antibody. All coverslips were stained with a DAPI DNA stain before imaging. Finally, the sample coverslips were added to clean slides and imaging was obtained at 60X magnification with an epi-florescent microscope. The resulting images show GFP-N-Ras at the PM in the vector controls and ppERK activation consistent with positive transfection of GFP-N-Ras (Figure 7a). The RGGT-shRNA2 transfected samples display a loss of N-Ras at the PM, and a marked decrease in ppERK levels in the positively transfected cells (Figure 7b). The images presented represent what I saw in all the cells. The last cell *appears* to have increased expression (comparable to the control) of pERK levels in the presence of RGGT-shRNA2. I hypothesis that the final cell was positively transfected with the GFP-N-Ras, but was not adequately transfected with the RGGT-shRNA. Considering transfection efficiency, and co-

transfection with two separate DNA plasmids it is possible a few samples were only positively transfected with one plasmid. Therefore the final cell represents a single transfection. We propose that the RGGT-shRNA interrupts N-Ras trafficking to the plasma membrane, and inhibits downstream signaling of N-Ras.

3.6 RGGT-shRNA Has an Inhibitory Effect on H-Ras Mediated ppERK Levels

We repeated the above experiment with GFP-H-Ras instead of GFP-N-Ras. H-Ras, like N-Ras, undergoes vesicle trafficking to reach the PM. NIH3T3 cells were transiently transfected with GFP-H-Ras G12V (the constitutively active mutant), in the presence of either the vector control or RGGT-shRNA. The samples were grown for 24 hours post-transfection, as above, before they were incubated in serum starve media for 48 hours. The samples were plated onto fibronectin-treated coverslips, and stained with Cy5-ppERK, and DAPI. The control cells have a similar phenotype to the N-Ras cells. Positively transfected cells illustrate that H-Ras is present on both the Golgi and plasma membranes and ppERK levels are increased compared to the un-transfected cells in the same field (Figure 8). Levels of ppERK were substantially decreased in H-Ras cells co-transfected with RGGT-shRNA, that is comparable to the un-transfected cells in the serum starve media (Figure 8). The H-Ras mediated ppERK levels are inhibited by the RGGT-shRNA.

3.7 RGGT-shRNA Has no Effect on K-Ras Mediated ppERK Levels

We confirmed our results by repeating the above experiment with an active mutant GFP-K-Ras transfection in NIH3T3 cells. K-Ras signals from the PM, however K-Ras does not undergo vesicle trafficking and interacts with the membrane by electrostatic interaction by the N-terminal chain of charged lysine. Therefore, RGGT-shRNA should have no effect on GFP-K-Ras trafficking and Ras-mediated ppERK levels. GFP-K-Ras transfection serves as a control of the RGGT-shRNAs' proposed vesicle-trafficked-Ras-mediated effects. NIH3T3 cells were co-transfected with GFP-K-Ras in the presence of either the vector control or RGGT-shRNA. All the samples were incubated in serum starve media, as described previously. The NIH3T3 cells positively transfected with GFP-K-Ras displayed increased ppERK levels and uninterrupted localization to the plasma membrane in both the presence and absence of RGGT-shRNA (Figure 9). Therefore, RGGT-shRNAs' ability to interrupt Ras mediated ERK activation is specific to Ras which undergo vesicle trafficking to the PM.

RGGT-shRNA has an Inhibitory effect on N-Ras Mediated ppERK levels and N-Ras Localization

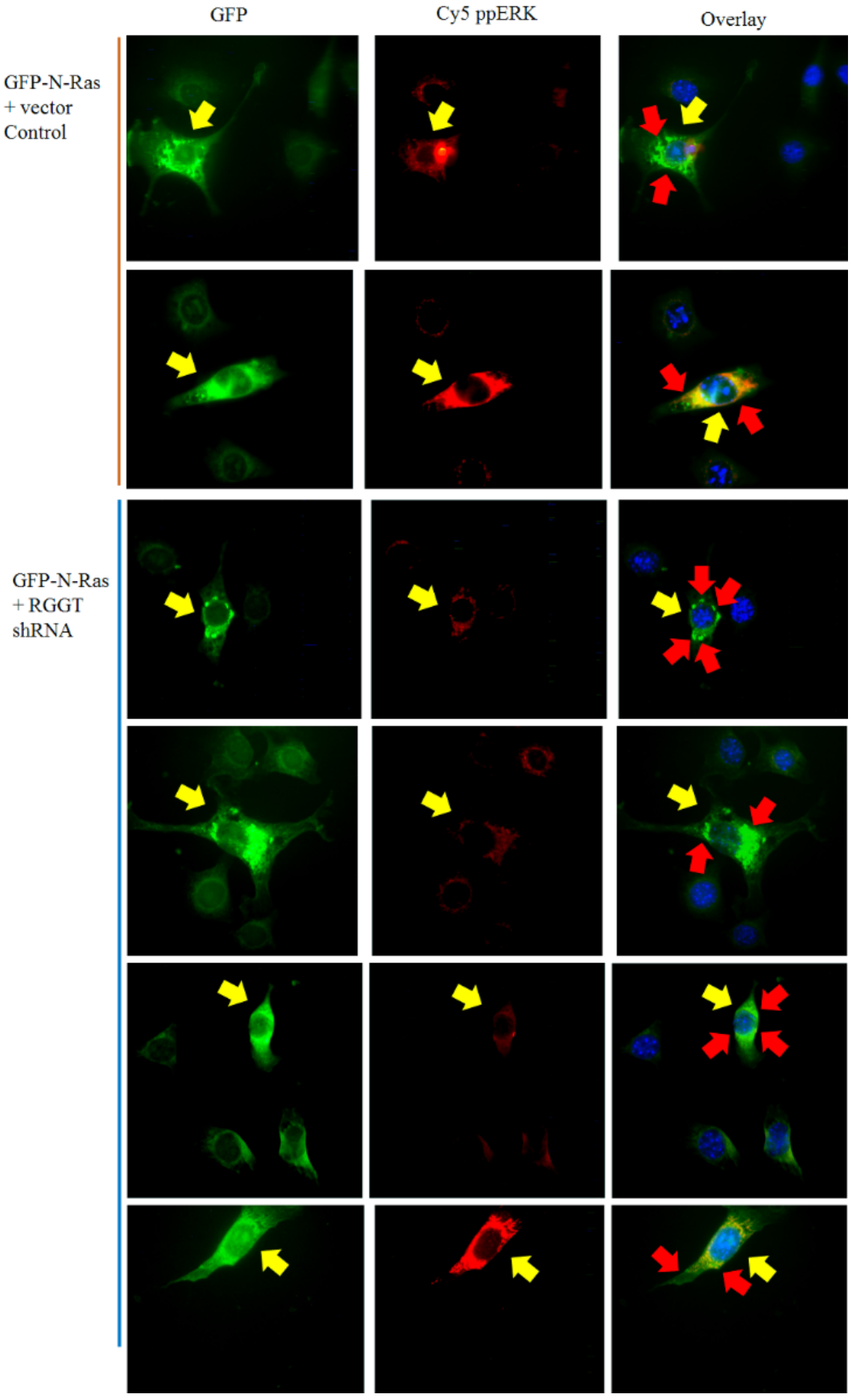

Figure 7. N-Ras associated ppERK levels and N-Ras localization are inhibited by RGGT-shRNA. Samples obtained from NIH3T3 cells under serum starve conditions for 48 hours post transfection.Cy5 (red) was stain with primary AB ppERK. NIH3T3 cells with GFP-N-Ras (green, left), and either RGGT-shRNA2, or psuper.retro vector control (not visible). Epifluorescence images were acquired from fixed cells by using a 60x objective. Red arrows highlight Ras localization. Yellow arrows highlight positively transfected cells

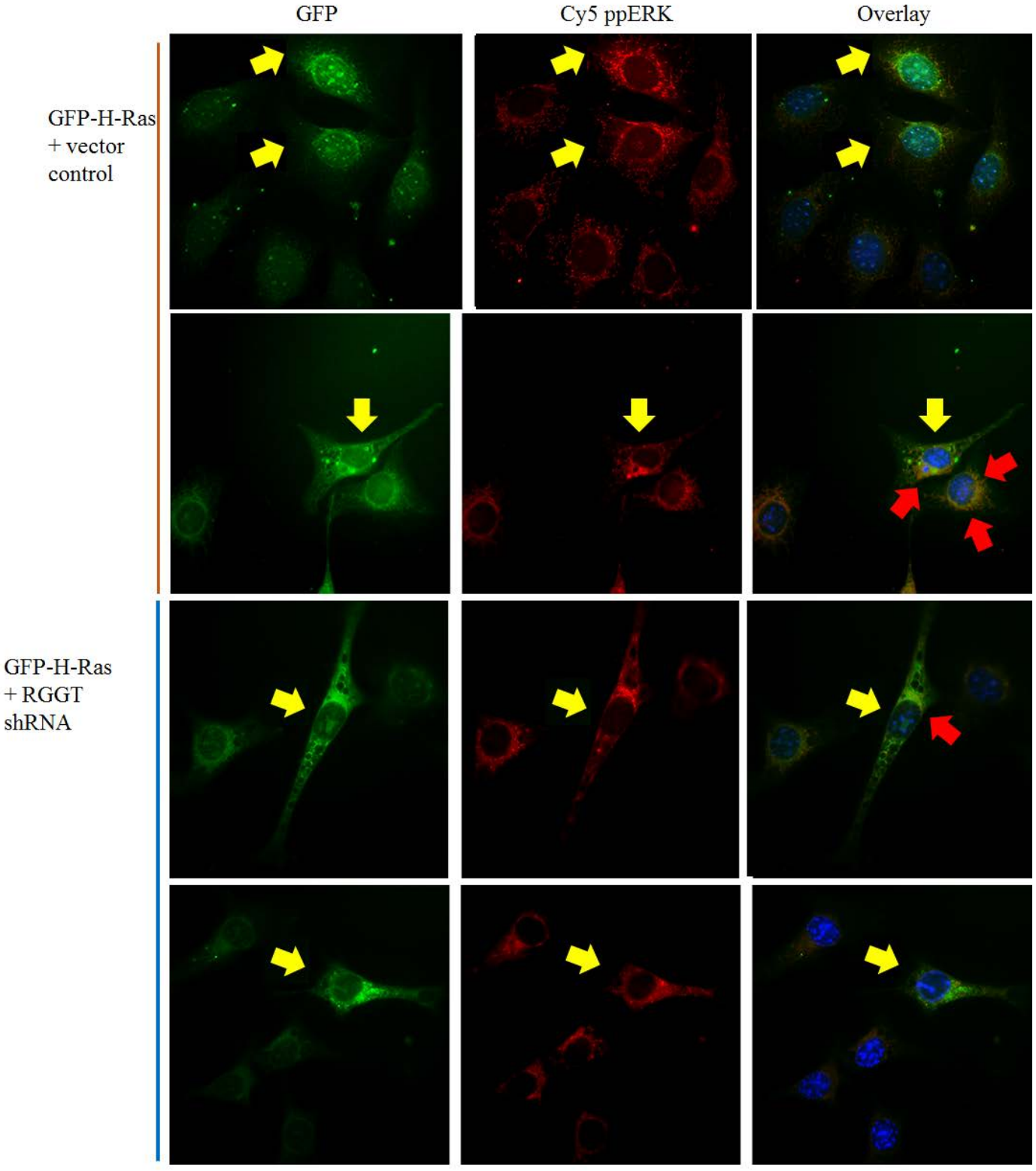

Figure 8. H-Ras associated ppERK leves are inhibited by RGGT shRNA. Samples obtained from NIH3T3 cells under serum starve conditions for 48 hours post transfection.Cy5 (red) was stain with primary AB ppERK. NIH3T3 cells with GFP-H-Ras (green, left), and either RGGT-shRNA2, or psuper.retro vector control (not visible). Epifluorescence images were acquired from fixed cells by using a 60x objective. Red arrows point to Rab vesicle formation. Yellow arros highlight positively transfected cells.

RGGT shRNA Inhibits H-Ras Mediated ppERK Levels

RGGT shRNA has no Effect on K-Ras Mediated ppERK Levels

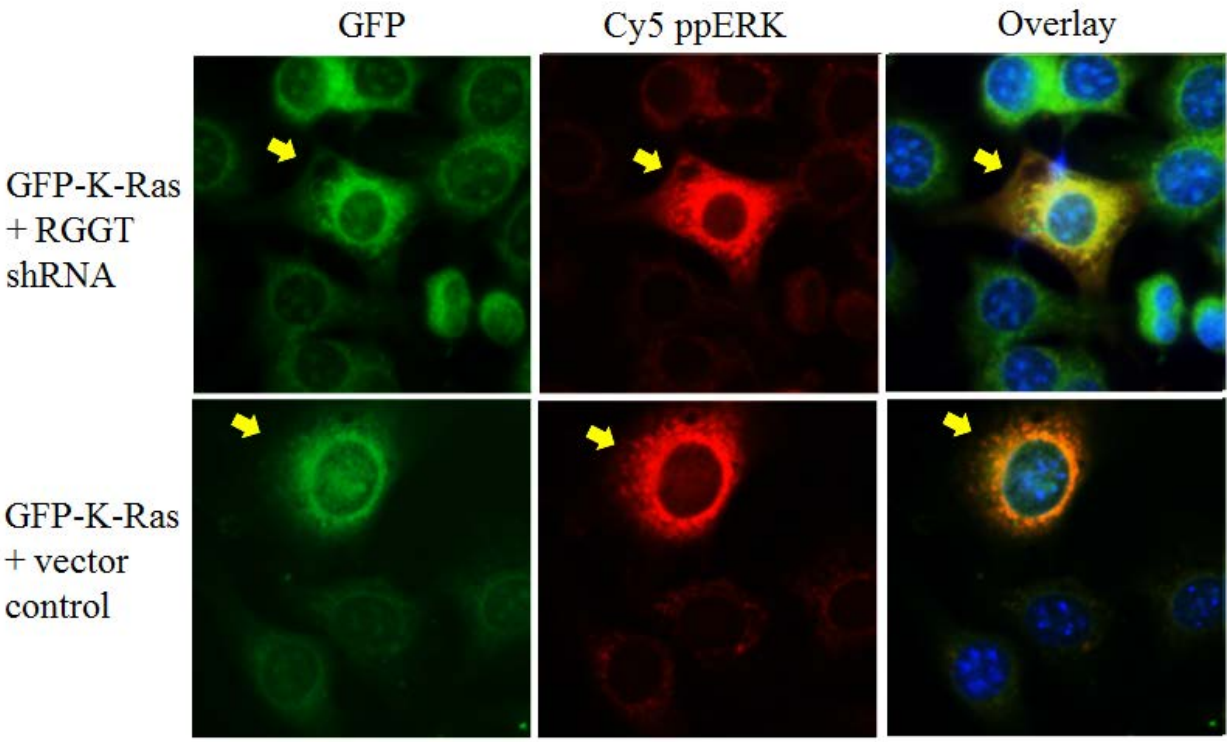

Figure 9. K-Ras associated ppERK levels are unaffected by RGGT shRNA. Samples obtained from NIH3T3 cells under serum starve conditions for 48 hours post transfection.Cy5 (red) was stain with primary AB ppERK. NIH3T3 cells with GFP-K-Ras (green, left), and either RGGT-shRNA2, or psuper.retro vector control (not visible). Epifluorescence images were acquired from fixed cells by using a 60x objective. Yellow arrows highlight positively transfected cells.

Proliferation of NIH3T3 cells Transiently Transfected with H-Ras (G12V) and RGGT shRNA2

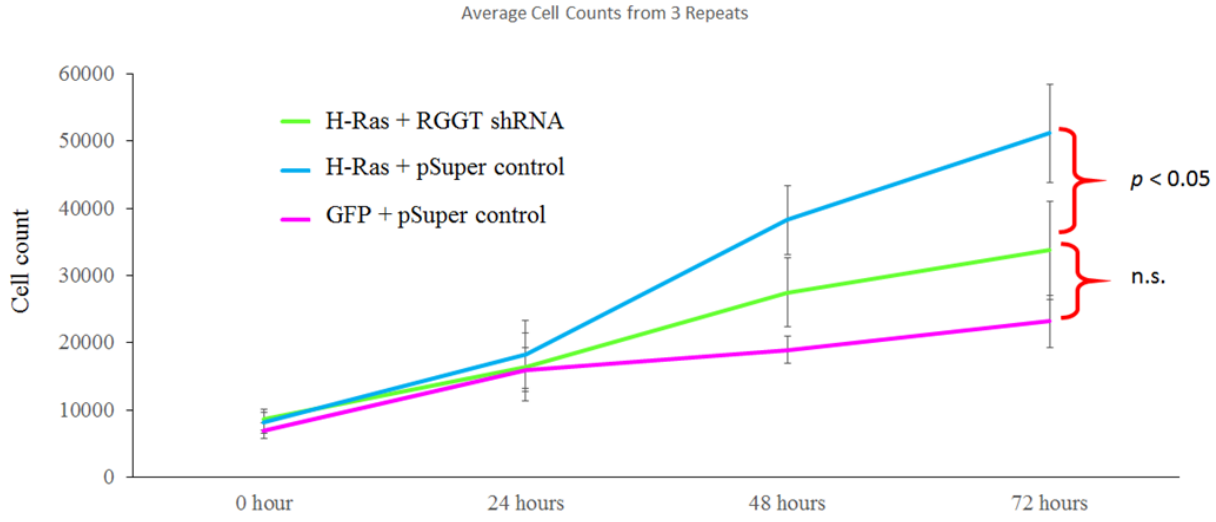

Figure 10. H-Ras induced proliferation is delayed by RGGT-shRNA expression in 3T3 cells. Cells stably expressing GFP or GFP-tagged, constitutively active H-Ras (G12V) were transiently transfected with RGGT-shRNA or psuper.retro control vector. 24 hours after transfection the cells were split into 24-well plates at 10k cells per well. 1 repeat per condition. 0 hour count is taken 6 hours after plating to control for adhesion to the plates, and each subsequent measurement was taken 24, 48 or 72 hours post plating time. n.s. = not significant

3.8 Transient Transfection of RGGT-shRNA Corresponds with Decreased H-Ras Mediated Proliferation

We have demonstrated that RGGT-shRNA effectively inhibits specific Ras-mediated ERK activation. Next, we designed an experiment to test the effects of RGGT-shRNA on H-Ras mediated proliferation. H-Ras active mutants correspond with increased survival and proliferation in tumor cells. We designed a 72 hour proliferation experiment to test the effects of RGGT-shRNA on H-Ras mediated proliferation. NIH3T3 cells were transiently co-transfected with H-Ras G12V (constitutively active mutant) in the presence of either the vector control or RGGT-shRNA. In addition, a control sample was transiently transfected with an empty GFP plasmid and the vector control plasmid. All three samples were plated at 10,000 cells per mL in 1mL of proliferation media (1% BCS in DMEM complete media). The initial (0 hour) cell count was taken 6 hours post platting to standardize results. All subsequent cell counts were taken 24, 48 or 72 hours post platting.

The proliferation experiment supports our initial hypothesis that targeting Rab geranylgeranylation inhibits Ras mediated proliferation. The active mutant GFP-H-Ras transfection induced a rapid proliferation in the low serum conditions. We suspect the H-Ras mediated proliferation is due to H-Ras mediated ppERK. The GFP transfection negative control had a reduced proliferation in the serum starve conditions. While the sample co-transfected with the active H-Ras and the RGGT shRNA presented a decreased proliferation rate. The difference in growth rate between the positive control, and negative control is not statistically significant. The results indicate a statistically significant decrease in cell proliferation in the H-Ras cells co-transfected with RGGT-shRNA from the control active H-Ras cells (Figure 10). The results demonstrate that even an incomplete knockdown of RGGT expression inhibits H-Ras mediated proliferation in NIH3T3 cells. I propose that the inhibitory effect of RGGT depletion on H-Ras-induced proliferation is due to inhibition of Rab-mediated vesicle trafficking.

CHAPTER 4

DISCUSSION AND FUTURE DIRECTIONS

4.1 Discussion

Whether Ras signaling is dependent on localization is an area of debate within literature. Ras proteins at the plasma membrane account for most, if not all, of the downstream effects of Ras activity. Some literature supports that Ras signaling is dependent on localization to the plasma membrane (Mochizuki et al., 2001). However, some literature proposes signaling is observed on endocytic vesicles (Fehrenbacher et al., 2009; Murphy, et al., 2009). The results presented in Background Figure 2 propose that the downstream effects of Ras may be mediated by vesicular trafficking, driven by Rab protein interaction. In order to test the initial findings in the lab, and potential effects of Ras mediated proliferation, I proposed to target Rab vesicle formation by inhibiting Rab geranylgeranylation. Therefore I produced the RGGT-shRNA illustrated in Figure 2. The results of RGGT-shRNA expression on Ras localization, Rab formation, Ras mediated pERK and Ras mediated proliferation are illustrated in our data. Taken together, our results illustrate that RGGT-shRNA inhibits Ras localization to the PM, which has the downstream effect of inhibiting Ras mediated MAPK signaling, and therefore proliferation. More simply, Ras mediated pERK signaling originates from the plasma membrane, not vesicles.

In current literature there are publications which propose Ras signaling is dependent on localization at the plasma membrane, and signaling propagates solely from the plasma membrane. As discussed in depth in the background. However, a few

publications propose that signaling may be propagated from endosomes. In 2001 the Mobley lab presented data which proposed that although, disrupting Golgi and endosome compartments had little direct effect on Ras activation, signaling of MAPK propagated at endosomes (Wu et al., 2001). Later publications by other labs claimed that Golgi or ER bound H-Ras transformed Rat fibroblast cells (Chiu et al., 2002; Bivona et al., 2003). However, neither of these experiments illustrate that Ras mediated signaling originated from the endosomes. Previously, accepted data does illustrate that GTP-bound Ras accumulates at the Golgi. A later publication proposes that the accumulation of GFP-K-Ras, on early endosomes corresponds with increased EGFR signaling (Lu et al., 2009; Fehrenbacher et al., 2009.) An earlier study identified downstream effectors of the MAPK pathway accumulate on endosomes and internal membranes, but are only identified in an activated phosphorylated state at the PM (Galperin & Sorkin, 2008). However, none of these experiments illustrate that the MAPK or direct Ras signaling propagates from the endosomes.

The experiments and results highlighted in this paper have further implications for future research. The initial serum starve experiment (Figure 1) supports the hypothesis that vesicular Ras signals through the ERK/MAPK pathway in the absence of other signaling factors found in serum. As found in previous publications (N. Fehrenbacher, 2009). Secondly, the specificity of the RGGT-shRNA is confirmed by the results in Figure 3; the lack of phase shifted Rabs, and the loss of RGGT expression, coupled with no significant effect on Rab levels. Live cell imaging supports that Ras trafficking in Rab-vesicles, as previously observed in our lab, is inhibited when RGGT-shRNA is expressed. Due to a lack of Rab-vesicle formation. Furthermore, the high-resolution

images in Figure 5 and Figure 6 support that the induced ppERK levels are interrupted by expression of the RGGT-shRNA, which I previously demonstrated interrupts vesicular-Ras signaling. The clear results from K-Ras mediated pERK levels is a negative control to the previous experiment, and further confirms the specificity of the RGGT-shRNA.

The serum starve control experiment in Figure 1 supports our initial hypothesis, and previously published data that Ras activity regulates ERK phosphorylation, independently of other signaling (Colicelli, 2004; Wennerberg et al., 2005;

N. Fehrenbacher, 2009). Our experiment isolates activated Ras in a serum starve environment, and demonstrate that K-Ras can independently induce activity the of the ERK/MAPK pathway. These results are important because, as previously stated, the ERK/MAPK pathway activity has been linked to increased tumorgenisity in tumor cells (Serrano, 1997, Wei 2003). Active Ras induces senescence and promotes survival in tumor cells, however active Ras cannot independently induce proliferation (Takaoka et al., 2004). A secondary mutation from the p53 pathway or a similar pathway is necessary to overcome cell-cycle-arrest. *In-vivo* active mutant activity must signal through a p53 independent pathway. The ERK/MAPK pathway signals independent of the well-studied and expansive p53 pathway. Therefore, our results confirm that Ras-activation of the ERK/MAPK pathway is a potential *in-vivo* target.

Further, when RGGT expression was inhibited Rab proteins were no longer geranylgeranylated. Expression of the RGGT-shRNA corresponds with decreased expression of the RGGT, therefore correctly targeting the RGGT expression. The direct result of RGGT-shRNA expression is in Figure 3. Rab5 proteins exhibit a visible phase shift when run on a western blot. The phase-shift may be due to different charges in Rab

proteins with and without a post-translational modification such as geranylgeranylation (Figure 3a). The phase shift of Rab5 appeared to be lost when RGGT-shRNA was expressed in high levels after transient transfection. However, the shRNA did not alter total Rab levels to a significant amount for either Rab4, Rab5 or Rab11 (Figure 3c). Therefore the further effects I observed where specific to the RGGT levels of the Rabs, and not caused by a change to total Rab levels.

Once the specificity of the RGGT-shRNA was confirmed by experimental results I tested the effect on Ras-Rab co-localization. Initial live cell imaging had promising results. I transiently transfected NIH3T3 cells with active RFP-R-Ras and either RGGT-shRNA or empty control vector (Figure 4). Transfection with RGGT-shRNA corresponds with a loss of Rab-Ras vesicles, and a noticeable decrease in plasma membrane bound Ras. The lack of Rab vesicles supports our hypothesis that RGGT-shRNA effects Rab vesicle formation. Further, the decrease in plasma membrane Ras, and increase in visible cytosolic Ras confirms that RGGT-shRNA alters Ras localization to the P.M. Both corresponding results support the initial hypothesis (Background Figure 2); that Ras trafficking occurs in Rab mediated vesicles.

Further, I have shown that in NIH3T3s both H-Ras and N-Ras mediated ppERK levels are affected by expression of the RGGT-shRNA. Figures 5 and 6 both illustrate that expression of the RGGT-shRNA inhibits the active Ras mediated ppERK levels. Figure 4 supported that RGGT-shRNA inhibits Ras localization. Therefore, the ppERK levels in Figure 4 and Figure 6 further support that localization is necessary for activity in two tested vesicular-trafficked Ras proteins. The results from live cell-imaging coupled with results in fixed imaging, and initial serum results shown in Figure 1, together

demonstrate that a lack of Rab vesicle formation corresponds with a loss of Ras mediated signaling.

The compounding results of the data help to resolve the controversy of Ras localization signaling. My data illustrates that PM localized Ras is required for the MAPK signaling and Ras cellular function (survival). The results illustrated in Figure 3 and Figure 4 taken together illustrate the RGGT-shRNA inhibits Ras localization to the PM. Further, Figure 5, and Figure 6, as discussed in depth above, highlight that expression of RGGT-shRNA corresponds with a loss of MAPK signaling. Therefore, the specific RGGT-shRNA mediated loss of Ras localization to the PM strongly correlates with inhibition of Ras mediated MAPK signaling, as illustrated by a decrease in pERK levels. The final figure of RGGT-shRNA expression inhibiting Ras mediated proliferation (Figure 8) further strengthens my findings.

K-Ras is understood to localize to the plasma membrane in a vesicle independent manner, and therefore will traffic and signal independently of Rab vesicles. Live-cell imaging in NIH3T3 cells transiently expression GFP-K-Ras and RGGT-shRNA act as a positive control to vesicular-Ras proteins (Figure 7). The downstream effects of Rab vesicle trafficking is an area of active research, and not all effectors or pathways have been identified. There may be other tumorigenic or oncogenic proteins trafficked by geranylgeranylated Rabs. Therefore, repeating the ppERK RGGT-shRNA experiment described above with GFP-K-Ras was a control of other downstream effects of Rab vesicle formation as well as a direct control of Ras-mediated activity. The RGGT-shRNA had no effect on ppERK induced by active GFP-K-Ras in the absence of serum and GFs.

Therefore, the GFP-K-Ras experiment confirmed the specificity of altered ppERK levels can be attributed to the vesicular-Ras mediated activity.

The results of the proliferation experiment (Figure 8) strongly support the initial hypothesis: Rab-mediated trafficking of Ras is important for localization, activity and Ras-mediated proliferation. Furthermore, the proliferation experiment results correspond with the initial ppERK results. RGGT-shRNA has a measured, significant effect on proliferation and a noticeable effect on Ras-mediate ppERK. Both results correlate to further support previously published hypothesis that Ras-mediated proliferation signals through the MAPK pathway.

- Vesicular-Ras mediated proliferation is propagated by phosphorylation of ERK and initiation of the ERK/MAPK pathway.
- Vesicular-Ras mediated proliferation is dependent on Ras localization to the plasma membrane.
- Rab-Ras trafficking mediates a large portion of the vesicular-Ras localization to the plasma membrane.
- RGGT-shRNA effects levels of geranylgeranylated Rabs present in 3T3 cells, without inhibiting expression of Rabs.
- Non-vesicular trafficked K-Ras mediated pERK levels are unaffected by interruption of geranylgeranylated Rabs, therefore the effects of RGGT-shRNA on pERK levels are specific to Rab-Ras vesicle trafficking.
- RGGT-shRNA expression inhibits Ras mediated pERK mediated proliferation.

Therefore, I hypothesize that targeting Rab-mediated-trafficking will inhibit Ras-mediated tumor progression and tumorgenisity *in-vivo.*

4.2 Future Directions

Based on the results discussed above I would further hypothesize that RGGT-shRNA would inhibit Ras mediated tumorgenisity *in-vivo*. Therefore, the next step will be to test the effect of RGGT-shRNA expression on both Ras-mediated tumor formation, and Ras-mediated tumor progression *in-vivo*. I hypothesize that expression of RGGT-shRNA in tumor cells with active H-Ras or N-Ras (vesicular Ras) would inhibit tumor formation in a mouse, compared to a control (non-vesicular Ras). I would also hypothesize that active H-Ras or N-Ras positive tumors growth will be inhibited by expression of RGGT-shRNA, compared to control conditions. Further, expression of RGGT-shRNA may lead to a decrease in tumor sizes. In both experiments active K-Ras mutations would serve as the control.

First, we would produce three stably transfected NIH3T3 cell lines expressing GFP-H-Ras, GFP-N-Ras and GFP-K-Ras, respectively. We would then produce cell lines stably transfected with the RGGT-shRNA we made and used in the above experimental protocols, using the selection marker puromyocin. Pyromyocin is a highly selective marker, causing selection at lower concentrations then comparable antibiotics. Therefore we should be able to produce the RGGT-shRNA positive 3T3 cells, even without a selection marker such as GFP. The final result would be NIH3T3 cells stably expression GFP-H-Ras and a second expressing both GFP-H-Ras and RGGT-shRNA etc. Finally, we would need to produce a viral vector expressing the RGGT-shRNA. Cells with stable RGGT-shRNA expression could also be used to repeat the experiments outlined in this

paper, and should confirm a stronger KD effect due to losing the margin of error from incomplete transfection efficiency.

The next experiment would test the effect of RGGT-shRNA inhibition on tumor progression. In the primary experiment we would test the ability of the RGGT shRNA to effect vesicular Ras mediated proliferation *in-vivo*. First, inject a population of the immune-deficient nude mice with either active GFP-H-Ras tumor cells, active GFP-K-Ras tumor cells, or active GFP-N-Ras tumor cells. The GFP-K-Ras cells should be tumorigenic, but as the above experiments suggest, should remain unaffected by the RGGT-shRNA expression. After a selected amount of time, tumors should form on each population. Next, treat half of each condition with the viral vector to induce expression of RGGT-shRNA. In each experimental condition and control measure the change in tumor size and/or tumor number over a period of days, to weeks. I hypothesize that the GFP-K-Ras tumor growth rate would be unaffected by the RGGT-shRNA. The GFP-H-Ras and GFP-N-Ras tumor growth would be inhibited by RGGT-shRNA expression. These experiments will give clear data if targeting Rab vesicle formation or trafficking alters Ras-mediated tumor progression *in-vivo*.

Next, we would design and execute an experiment to test active vesicular Ras mediated tumor formation, and therefore the potential effect on targeting RGGT to limit tumor metastasis. The experimental design would be superficially similar to above. We would test all conditions on immune-deficient mice. Separate populations of mice would be treated with stably transfected active-H-Ras (the more tumorigenic of the vesicular Ras proteins) tumors cells, and stably expressing active-H-Ras + RGGT shRNA tumor cells. The control conditions would be active-K-Ras cells, and co-expression stably

transfected cells with active-K-Ras and RGGT shRNA tumor cells. Separate populations of mice would be injected with either condition and recorded. Tumor formation would be measured and recorded. After a specific time the final tumor formation would be counted, and weighed. We hypothesize that the size and number of tumors in the K-Ras mutant mice would be statistically unaffected by RGGT shRNA expression. However, we would hypothesize that expression of RGGT-shRNA will statistically inhibit the formation of H-Ras active tumors in the immune-deficient mice, although it would be unlikely to inhibit all tumor growth.

As stated earlier, an effective pharmacological target of Ras mediated proliferation has been hard to identify. The traditional, direct means of targeting Ras activity have proven either redundant, or purely ineffective at altering Ras activity (Baines, et al., 2011). However, we have identified a target for Ras mediated proliferation that has an existing potential pharmacological drug. The RGGT alpha subunit, which our RGGT-shRNA targets, has a known FDA approved pharmacological inhibitor, 3-PEHPC (Coxon, et al., 2001; Coxon, et al., 2011; Roelofs, et al., 2006). As I discussed earlier, 3-PEHPC is redundant to a known mouse mutant model, the gunmetal mouse. The gunmetal mouse, named for the grey-silver coat, has a known mutation on the alpha sub-unit of RGGT. Therefore, 3-PEHPC is a phosphono-carboxylate which inhibits the alpha subunit of RGGT, thereby blocking its activity, and increasing the level of cytosolic un-geranylgeranylated Rabs (Detter, et al. 2000; Coxon, et al. 2011). The RGGT-shRNA I produced also targets the alpha subunit of RGGT. Therefore, I further hypothesize that 3-PEHPC should have a similar effect on Ras mediated proliferation as the RGGT-shRNA we made, and tested. The experiments I have outlined in this section would further

demonstrate if the RGGT-shRNA is a valid method to target Ras proliferation *in-vivo*. Therefore, if RGGT-shRNA has the desired effects *in-vivo,* we hope that 3-PEHPC would exhibit the same specificity and effect on Ras mediated proliferation.The use of geranylgeranyl inhibiters, targeting both geranylgeranyl transferase I and II are areas of active research being considered (Wiemer, et al., 2011). Our RGGT-shRNA should have the same effect as the pharmacological inhibitor 3-PEHPC.

Therefore, a statistically significant inhibition of vesicular-Ras mediated tumor growth, or proliferation by RGGT-shRNA in either experimental model proposed above will allow us to consider the FDA approved 3-PEHPC as a target of Ras mediated proliferation. In the long term considerations duel use of the RGGT-shRNA we have produced, and the existing drug could further our understanding of Rab-Ras interactions, as well as lead directly to an effective target in clinical trials.

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APPENDIX

ACRONYMS AND ABBREVIATIONS IN TEXT

Abbreviation	Full name
3T3	NIH3T3
Arf	ADP-ribosylation factor family proteins
BCS	Bovine Calf Serum
BSA	Bovine Serium Albumin
COPI	Coat Protein Complex I
DAM	Donkey-anti-mouse
DAR	Donkey-anti-rabbit
DI Water	Filtered de-ionized water
DMEM	Dulbecco's Modified Eagle's Medium
DTT	Diotheiothreitol
ERK	Extracellular signal-Regulated Kinases
FTI	Farnesylation inhibitor
FTIs	Farnesyltransferase Inhibitors
GAPs	GTPase-activating-proteins
GDI	GDP Dissociation Inihbitor
GEFs	Guanine-nucleotide-exchange factors
GRB2	Factor Receptor-bound protein-2
ICMT1	Isoprenylcysteine Carboxymethyltransferase-1
IKK	I-KappaB Kinase
JNK	Jun N-terminal Kinase

MAP	Mitogen-activated Protein
MAPK	Mitogen-activated protein kinase
NDS	Normal Doneky Serum
NF-KAppaB	Nuclear Factor-Kappa B
Optimem	Opti-mem Reduced Serum Media
PBS	Phosphate Buffered Solution
PI3K	Phosphatidylinositol 3-Kinase
PM	Plasma Membrane
Rab	Ras-associated Brain Protein
Ran	Ras-like Nuclear Proteins
RCE1	Ras-converting Enzyme-1
RGGT	Rab Geranylgeranyl Transferase
Rho	Ras-homologous Proteins
SDS	Sodium Dodexyl Sophate
shRNA	short hairpin RNA
SNARE	Soluble N-ethylmalemide sensitive factor attachment protein receptor
SOS	Son of Sevenless
TAE	Tri-Acetate-EDTA buffer
TBS	Tris-buffered Saline
TBST	Tris-buffered saline with Tween 20 Buffer