Intersectin: A Regulator of Cell Signaling

BY

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THESIS

Submitted as partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Pharmacology
in the Graduate College of the
University of Illinois at Chicago, 2012

Chicago, Illinois

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This thesis is dedicated to my parents, Ann and Ron Wong, without their love and support who knows where I would be.

ACKNOWLEDGEMENTS

I would first like to acknowledge my advisor, Dr. John O'Bryan, without his guidance and training I would not have been able to accomplish my doctorate degree. Secondly, I would like to thank my thesis committee members, Dr. Richard Ye, Dr. Richard Minshall, Dr. Karen Colley, and Dr. Graeme Carnegie. I appreciate all the time, energy, and insightful comments they have contributed to aid me in the completion of my doctoral degree.

I would also like to thank members of the O'Bryan lab past and present. When I started in the lab Sara Dunn and Dr. Yun-Ju Chen were invaluable in my training. I am also especially grateful to Joseph Sennello who was an enjoyable lab mate and friend.

I am also grateful for all the friends I made during my graduate school career. Emily Welch, Brendan Quinn, Jackson Hoffman, and Chris O'Donnell who all made graduate school fun and exciting. Thank you for the summer of 2008, turtle races, La Bamba's, Hawkeye's lunches, and all other fun times. I am especially thankful to my better half, Erica Southgate. She is truly the greatest friend that anyone could ask for. I would also like to give a special thank you Adam Wieschhaus. Thank you for putting up with me and taking care of me. Graduate school is long and challenging, thank you all for also making it fun.

Finally, I would like to thank my family. It is a true gift to have such a strong support system and unconditional love. Thank you is not enough to describe the gratitude I feel for my parents, Ann and Ron Wong. Thank you for your patience, support, love, and guidance. My parents raised three daughters; my older sister Kari who received her doctorate degree from the University of Chicago, and my younger sister Keely who received her nursing degree from California State at Chico. Without them there is no way I would be where I am today.

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LIST OF ABBREVIATIONS

AD	Alzheimer's Disease
aPKC	atypical Protein Kinase C
C2	Ca^{2+} binding
CC	Coiled-coiled
DH	Dbl homology
DMEM	Dulbecco's Modified Eagle Medium
DN	Dominant negative
DS	Down Syndrome
EEA1	Early endosomal antigen I
EGFR	Epidermal growth factor receptor
EH	Eps15 homology
ER	Endoplasmic Reticulum
ESCRT-II	Endosomal sorting complex required for transport-II
FYVE	Fab1, YOTB, Vac1, EEA1
GAP	GTPase activating protein
GDS	Guanine nucleotide dissociation stimulator
GEF	Guanine nucleotide exchange factor
HA	Hemagglutinin
HSA21	Human chromosome 21
HV	Hypervariable
Icmt	Isoprenylcysteine transferase

ITSN	Intersectin
ITSN1-L	Intersectin1-Long
ITSN1-S	Intersectin1-Short
ITSN2-L	Intersectin2-Long
ITSN2-S	Intersectin2-Short
MAPK	Mitogen activated protein kinase
NMJ	Neuromuscular junction
Nwk	Nervous Wreck
PDGFR	Platelet derived growth factor receptor
p-EGFR	Phospho-EGFR
PH	Pleckstrin homology
PHA2	Pseudohypoaldosteronism type 2
PI	Phosphoinositides
PI3K	Phosphoinositide 3' kinase
PI3K-C2α	PI3K Class II alpha
PI3K-C2β	PI3K Class II beta
PI3K-C2γ	PI3K Class II gamma
PIK PI3K	PI3K accessory domain
PKC	Protein Kinase C
PM	Plasma membrane
polyQ	Poly-glutamine
PRD	Proline-rich domain

PTB	Phosphotyrosine binding domain
PtdIns	Phosphatidylinositol
p-Tyr	Phosphotyrosine
PX	Phox homology domain
RA	Ras association
RBD	Ras binding domain
Rce1	Ras-converting enzyme 1
RILP	Rab-interacting lysosomal protein
ROMK1	Renal outer medullar potassium1
RTK	Receptor tyrosine kinase
SH2	Src homology 2
SH3	Src homology 3
WNK	With-no-lysine kinases

SUMMARY

The human body is made up of cells that need to communicate with themselves and each other in order to function. Strict organization of proteins and intracellular compartments is necessary to ensure appropriate activation and propagation of cell signaling. When proteins are mislocalized or improperly activated or inactivated disease occurs. Scaffolding proteins are organizers of cell signaling. Intersectins (ITSN) are a family of multi-domain scaffolding proteins involved in multiple signal transduction pathways. My work on ITSN has revealed two novel roles for ITSN in cell communication.

First, I discovered that ITSN regulates a novel Ras-phosphoinositide-3-kinase Class II beta (PI3K-C2β) pathway. ITSN interacts with the Ras guanine nucleotide exchange factor Sos leading to Ras activation on intracellular vesicles (1-3). ITSN also regulates a cell survival pathway through the activation of PI3K-C2β. The presence of a putative Ras binding domain (RBD) on PI3K-C2β combined with the co-localization of ITSN, Ras and PI3K-C2β at intracellular vesicles suggested that Ras is involved in ITSN activation of PI3KC2β. Indeed, I observed that nucleotide-free Ras directly interacts with PI3K-C2β to inhibit kinase activity. In addition, point mutations in the effector domain of Ras or in the RBD of PI3K-C2β disrupt complex formation indicating that PI3K-C2β is a novel Ras effector that complexes with nucleotide-free Ras. My data demonstrates for the first time that nucleotide-free Ras plays a role in cell signaling and offers a new mechanism for GTPase regulation. Delayed Ras activation at intracellular sites distal to exchange factors adds another level of Ras regulation. It has been a long held assumption that nucleotide-free Ras is rapidly turned over *in vivo*; however guanine nucleotide exchange factors associate with this intermediate, raising the possibility that other proteins may do the same. My data suggests that PI3K-C2β is such a protein. Additionally, my

data is the first example of nucelotide-free Ras mediated signal regulation. There are multiple lines of evidence indicating that oncogenic Ras is not the only form of Ras responsible for downstream signaling. My model for PI3K-C2β mediated Ras signaling is the first to address this issue.

Secondly, I discovered a role for ITSN in compartmentalized signaling. ITSN was initially characterized as an endocytic adaptor protein, but my work has demonstrated that ITSN is localized with effectors on multiple endocytic compartments. Indeed, ITSN has been shown to regulate cell signaling independent of a role in endocytosis [as reviewed in (4)]. ITSN itself does not have any enzymatic activity or an "on/off" state, but is regulated by subcellular localization. The various pools of ITSN allow ITSN to regulate multiple pathways.

The work covered in my dissertation defines a new mechanism for Ras signaling and provides insight into how cells organize proteins in order to propagate signals.

I. ITSN, PI3K-C2β, RAS: THE THREE AMIGOS OF CELL SIGNALING

A.   <u>Introduction</u>

Cells communicate with themselves and each other to grow, multiply, move, and die. There are hundreds of different kinds of proteins that promote cell signaling to determine what function a cell needs to perform and when. Many intracellular signaling pathways overlap and diverge at many points. Spatio-temporal regulation prevents signaling from going awry as well as allowing for a single protein to participate in multiple pathways. Due to the numerous proteins involved in cell communication, strict organization of these proteins is necessary. Scaffold proteins serve as organizers for these signaling cascades. Scaffolding proteins consist of multiple domains that promote protein-protein interactions and serve as a junction point for other signaling molecules to come together and signal more efficiently. Intersectin (ITSN) is a multi-domain scaffolding protein that mediates multiple signal transduction pathways. ITSN has been implicated in endocytosis, kinase activation, transcriptional regulation, receptor trafficking, cell cycle progression, monomeric GTPase regulation, and cell survival.

B.   <u>Intersectin</u>

1.   **ITSN Genes**

There are two ITSN genes (ITSN1and ITSN2) located on human chromosomes 21 (21q22.1-q22.2) and 2 (2pter-p25.1), respectively (5-7). Each of these genes is alternatively spliced into short (S) and long (L) isoforms. ITSN-S isoforms consist of two Eps15 homology (EH) domains, followed by a coiled-coiled (CC) domain, and five Src homology 3 (SH3) domains. The longer isoforms contain an additional Dbl homology (DH) domain, plekstrin

homology (PH) domain, and a C2 domain (Figure 1). In addition, there are multiple minor splice variants that have altered binding to ITSN effectors (8). ITSN1-S has 53% homology with ITSN2-S. ITSN1-L is 60% homologous to ITSN2-L. ITSN1-S is the isoform that is well conserved across many species including: humans, rodents, *Xenopus*, and *Drosophila* (Table I) (9-11). Higher mammals, such as humans and rodents, have two ITSN genes and express short and long isoforms, while lower level vertebrates such as *Drosophila*, have only one ITSN gene and express only a short isoform. While ITSN1-S and ITSN2 are ubiquitously expressed, ITSN-1L is mainly found in the nervous system, with only low levels being detected in other tissues (8, 12). For the remainder of this dissertation, ITSN1-S will be the focus and referred to as ITSN.

2.   ITSN and Disease

Trisomy of human chromosome 21 (HSA21) results in overexpression of ITSN and Down Syndrome (DS), suggesting ITSN plays a role in DS pathogenesis (12, 13). DS is manifested in numerous ways including mental retardation, heart defects, and development of an Alzheimer's Disease (AD) like neuropathology in patients of 40 years of age (14). Indeed, one of the earliest phenotypes observed in DS and AD patients is the presence of abnormally large early endosomal compartments, suggesting a defect in a vesicle trafficking pathway (15-17). ITSN's role in clathrin coated pit formation and neuromuscular junction (NMJ) organization further support ITSN's role in these neurodegenerative diseases. The T65Dn mice, a model for DS, have a partial trisomy of the murine counterpart to HSA21. These mice have increased levels of ITSN and a subset of the phenotypes related to DS, including growth retardation, age-realted degeneration of basal forebrain cholinergic neurons, increased frequency of obesisty, craniofacial
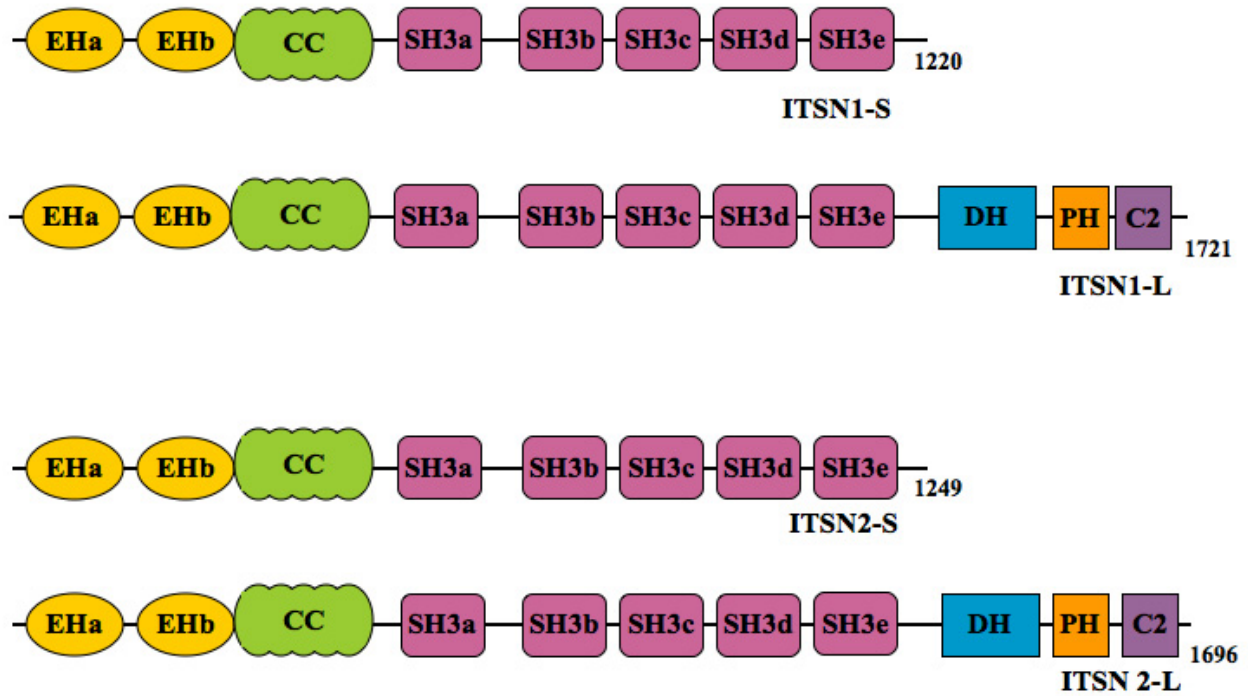
Figure 1. Intersectin isoforms and domains. There are two ITSN genes that each code for a short and long isoform. ITSN1-S and ITSN2-S consist of two Eps15 homology (EH) domains, a coiled-coiled (CC) region, and five Src homology 3 (SH3) domains. ITSN1-L and ITSN2-L contain additional Dbl homology (DH), pleckstrin homology (PH), and C2 domains.

defects, astrocytosis, and impaired spatial learning and memory (18, 19). Mice overexpressing ITSN in the brain have high levels of ITSN in the striatum resulting in reduced motor activity compared to control animals (13).

ITSN has also been linked to other neurodegenerative diseases including Huntington's Disease and Kennedy's Disease. These pathologies are characterized by aggregation of polyglutamine (poly-Q) containing proteins. In Huntington's Disease, the protein huntingtin is mutated resulting in expansion of poly-Q repeats and aggregation of the resulting protein. ITSN overexpression increases aggregation of huntingtin in a JNK-dependent manner (20). In addition, ITSN activates Elk in a JNK-dependent manner, and this activation is blocked by overexpression of mutant huntingtin (20). ITSN also increases aggregation of polyQ-expanded androgen receptor as observed in Kennedy's disease (20). These data suggest that ITSN regulates neurodegeneration through JNK and poly-Q aggregation.

With-no-lysine kinases (WNK) are serine-threonine kinases (21). Mutations in WNK1 and WNK4 lead to pseudohypoaldosteronism type 2 (PHA2) that causes hypertension and hyperkalemia in patients (22, 23). Enhanced endocytosis of the renal outer meduallar potassium 1 (ROMK1) channel leads to inhibition of the channel and defects in potassium secretion, resulting in the hyperkalemia seen in PHA2 patients (24). WNK1 interacts with ITSN (25) to enhance internalization of the ROMK1 channel and therefore inhibition and hyperkalemia. In addition, mutations in WNK found in PHA2 enhance ITSN association, suggesting a link between ITSN and PHA2.

ITSN has also been implicated in neuroblastoma tumorigenesis. Human primary tumors and neuroblastoma cell lines have high ITSN expression. Additionally, ITSN is essential for anchorage-dependent growth of neuroblastomas in cell and mouse models (25). Furthermore,

ITSN-S and ITSN-L overexpression results in oncogenic transformation of NIH-3T3 cells and ITSN plays a role in JNK and Ras signaling, suggesting broad role for ITSN in cancer development (26, 27)

3.   ITSN and Signal Regulation

ITSN's multiple domains allow for involvement with several signal transduction pathways. The EH domains of ITSN bind to Asn-Pro-Phe sequences in target proteins, the CC domain promotes homo- and heterodimerization, and the SH3 domains bind to Proline-rich regions within target proteins. The additional DH-PH domains found on the long isoform function as a specific guanine nucleotide exchange factor (GEF) for Cdc42, a Rho family GTPase (28, 29).

Initially ITSN was characterized as an endocytic adapter protein due to its localization to clathrin-coated pits(30) and its association with members of endocytic machinery including dynamin (9, 11), epsin (9), Eps15 (6), SNAP-25 (11), and synaptojanin (9). Transient silencing of ITSN blocks internalization of the epidermal growth factor receptor (EGFR) and transferrin (31, 32) whereas stable silencing does not have an effect on transferrin internalization or bulk endocytosis (Figure 2) (33). This difference is most likely due to compensation by ITSN2 and other endocytic proteins. In addition, concentration dependent overexpression of ITSN can inhibit endocytosis (6, 34), presumably due to the concentration sensitivity of scaffold proteins. Low levels of scaffolding protein prevent the formation of signaling complexes, while high levels of a scaffold result in non-productive complexes (35). Indeed global ITSN and ITSN-1L specific knockout mice have defects in endocytosis and enlarged endosomes, further demonstrating ITSN's role in endocytosis and trafficking (36). ITSN also plays a role in synaptic
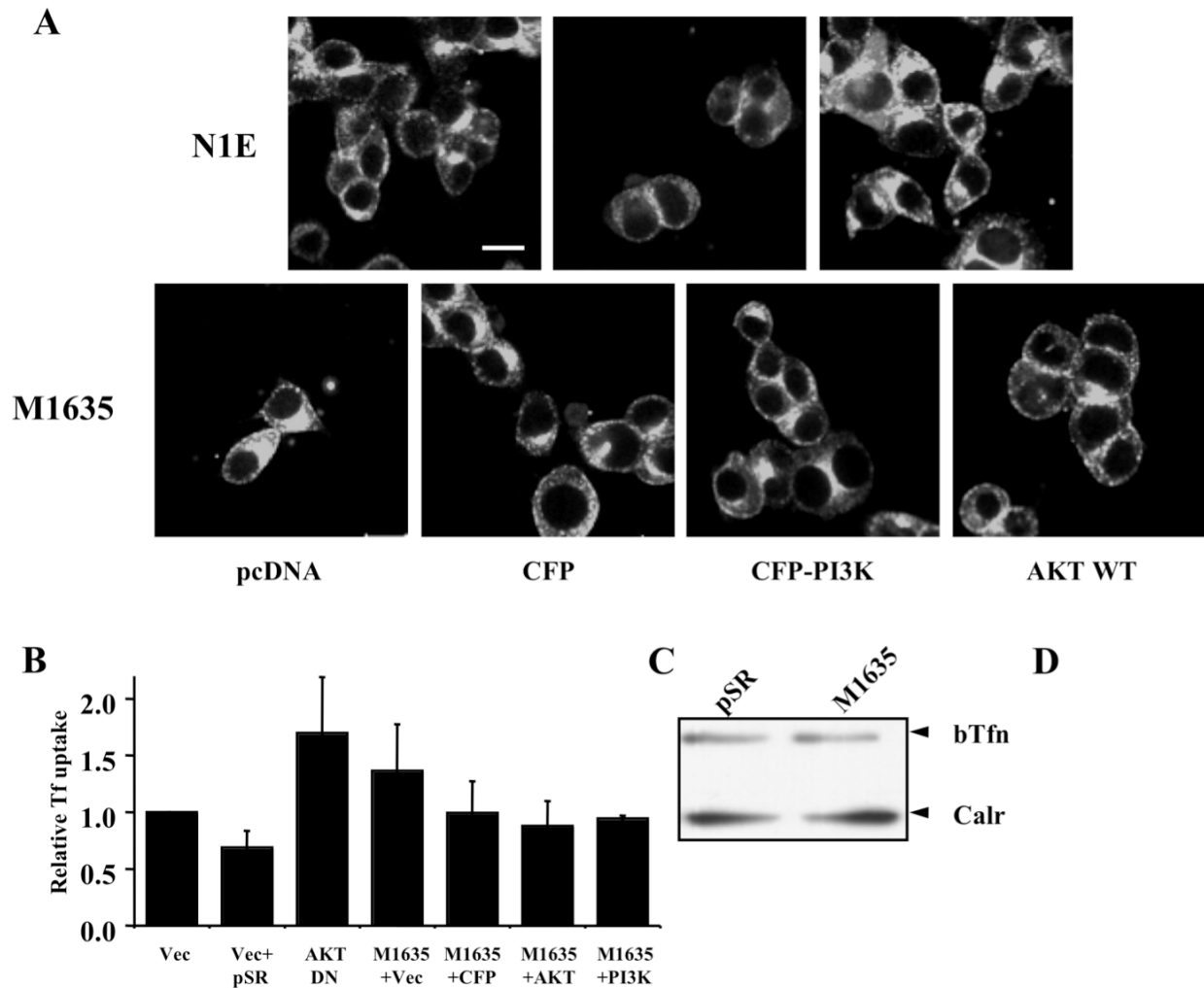
Figure 2. ITSN silencing does not inhibit transferrin receptor or bulk internalization. (A) Images indicate that there is no difference in Alexa 488-transferrin internalization in N1E-115 control (pcDNA, pSR, or AKT-DN) or M1635, ITSN silenced cells that overexpress vector (pcDNA, CFP) or PI3-C2β or AKT. **(B)** Transferrin internalization was quantified for the different cell lines. The graph represents the means ± SEM of the results from two independent experiments. **(C)** N1E-115 cells transfected with the indicated pSR construct were incubated with biotinylated transferring (bTfn). Transferrin internaliztion was quantified by Western blot using an HRP-strepavidin antibody. **(D)** N1E-115 cells stably silenced for ITSN (M1635) or vector control cells (pSR) were incubated with HRP and then assayed for uptake. Copyright © American Society for Microbiology, Copyright © American Society for Microbiology, [Molecular and Cellular Biology, 27, 2007, p.7906-791, doi:10.1128/MCB.01369-07].

vesicle internalization and NMJ organization (37). Loss of the *Drosophila* ITSN homolog, Dap160, lead to the de-stablization of NMJs due to the mis-localization of multiple proteins including, synaptojanin, endophilin, Nervous wreck (Nwk), and dynamin (37-40). Disruption of ITSN at the NMJ increases dynamin recruitment, but inhibits vesicle fission, suggesting that dynamin activation is blocked in the absence of ITSN (41). Dap160 interacts with Eps15 at NMJs (38). Loss-of-function mutations of Dap160 in *Drosophila* are lethal, but less severe mutations result in temperature sensitive paralysis, decreased endocytosis, and irregular boutons and vesicles at the NMJ (37, 39). ITSN null *C. elegans* have a decreased number of synaptic vesicles and reduced synaptic events suggesting that ITSN is involved in synaptic vesicle recycling (42). ITSN silencing in cultured rat hippocampal neurons did not inhibit synaptic vesicle endocytosis or alter bouton morphology, but did decrease transferrin uptake and dentritic spine maturation (32). The difference between these phenotypes is likely due to differences in ITSN expression. Mammals have two ITSN genes, while *Drosophila* and *C. elegans* have only one (Table I). In addition to clathrin mediated endocytosis, ITSN has been linked to caveolae internalization. ITSN depletion inhibits caveolae release from the plasma membrane in endothelial cells(34).

Although ITSN plays a significant role in endocytosis, ITSN also regulates other signal transduction pathways, including mitogen activated protein kinase (MAPK) and GTPase mediated pathways. ITSN itself does not activate ERK1/2, but does cooperate with receptor tyrosine kinases (RTKs) to stimulate Elk-1-mediated transcription through the activation of JNK (1, 26). In endothelial cells, transient ITSN silencing lead to decreased ERK1/2 and MEK phosphorylation, possibly due to a decrease in internalization of caveolae (34, 43). Indeed, in HEK293T cells, transient ITSN silencing decreased EGFR internalization resulting in decreased

TABLE I. ITSN GENES AND ORTHOLOGS

Species	#genes[a]	ITSN1 like	ITSN2 like	GEF domain[b]
Homo sapiens	2	Chr 21	Chr 2	Yes
Pan troglodytes	2	Chr 21	Chr 2A	Yes
Macaca mulatta	2	Chr 19	Chr 2	Yes
Canis lupus familiaris	2	Chr 31	Chr 17	Yes
Felis catus[e]	2	ND	ND	Yes
Bos taurus	2	Chr 1	Chr 11	Yes
Mus musculus	2	Chr 16	Chr 12	Yes
Rattus norvegicus	2	Chr 11	Chr 6	Yes
Sorex araneus	2	ND	ND	Yes
Tupaia belangeri[e]	2	ND	ND	Yes
Oryctolagus cuniculus	2	ND	ND	Yes
Loxodonta africana[e]	2	ND	ND	Yes
Monodelphis domestica	1	Chr 4	-	Yes
Erinaceus europaeus[e]	1	ND	ND	?
Echinops telfairi[e]	2	ND	ND	Yes
Gallus gallus	2	Chr 1	Chr 3	Yes
Myotis lucifugus[e]	1	-	ND	Yes
Xenopus laevis[d]	1	ND	-	No
Xenopus tropicalis	2	ND	ND	No
Ornithorhynchus anatinus	2	ND	Chr 2	Yes
Oryzias latipes	2	Chr 24	Chr 3	Yes
Danio rerio	3	Chr 1	Chr 20 (ITSN2-like, Chr 17)	Yes
Takifugu rubripes	3	ND	ND/ND	Yes
Tetraodon nigroviridis[c]	3	Chr 17	Chr 5 / Chr 14	Yes
Gasterosteus aculeatus	3	Group VI	Group II & Group XVIII	Yes
Ciona savignyi	1	ND	-	No
Ciona intestinalis	2	ND	ND	Yes
Nasonia vitripennis	1	ND	-	No
Acyrthosiphon pisum	1	-	ND	Yes
Anopheles gambiae[d]	1	3R	-	No
Aedes aegypti[d]	1	ND		No
Drosophila pseudoobscura[d]	1	ND	-	No
Drosophila melanogaster[d]	1	2L	-	No
Tribolium castaneum[g]	1	LG3	-	?[f]
Caenorhabditis briggsae	1	ND	-	No
Caenorhabditis elegans	1	Chr 4	-	No

TABLE I. ITSN GENES AND ORTHOLOGS (continued)

Species	#genes[a]	ITSN1 like	ITSN2 like	GEF domain[b]
Caenorhabditis remanei	1	ND		No
Hydra magnipapillata	1	ND	-	?[f]
Crytococcus neoformans	1	ND	-	Yes

[a] Denotes the number of distinct genetic loci encoding ITSN-related proteins in the indicated species. The human genome encodes two highly homologous ITSN genes present on Chromosomes 21 (ITSN1, 21q22.1-q22.2) and 2 (ITSN2, 2pter-p25.1). NOTE: many species also contain one more more ITSN pseudogenes.

[b] Indicates whether the ITSN genes encode a long splice variant encoding a Cdc42 GEF.

[c] This species possesses three ITSN genes present on Chromosomes 5, 14, and 17. The increase in ITSN genes likely arose from duplication of the genome [49].

[d] These orthologs possess 4 SH3 domains in contrast to the 5 SH3 domains found in most ITSN orthologs.

[e] only has 3 SH3 domains, Due to ambiguous sequence, protein appears to have less than 5 SH3 domains

[f] Partial clones.

[g] Only has 2 predicted SH3 domains possibly due to incomplete isolation of the gene.

Information was derived by searching NCBI MapView (http://www.ncbi.nlm.nih.gov/projects/mapview/ for intersectin related sequence, and the SMART database (http://smart.embl-heidelberg.de) for entries possessing EH and SH3 domains. Additional comparisons were done by comparing various ITSN-related proteins using BLAST programs to determine relationships between orthologs.

ERK and Elk-1 activation (31). Interestingly, inhibition of endocytosis blocks MAPK activation without effecting activation of the monomeric GTPase, Ras (2). ITSN forms a complex with the Ras exchange factor Sos and activates Ras on a pool of intracellular vesicles (1-3). Overexpression of ITSN leads to an increase in activated Ras, but no increase in MAPK activation, suggesting this pool of Ras activates a different downstream target. In addition, overexpression of the SH3 domains, but not the full-length protein, inhibits MAPK activation, presumably due to the sequestration of Sos and inhibition of endogenous Ras (2). In addition to Ras, ITSN also regulates Rac1 activation through inhibition of the Rac1 GTPase activating protein, CdGAP (44). ITSN-L is also involved in GTPase regulation. The DH-PH domains of ITSN-L act as a GEF for the Rho family GTPase, Cdc42 (29). The SH3 domains of ITSN-L auto-inhibit this GEF activity, but N-WASP or EphB2 binding relieves this inhibition and increases GEF activity leading to an increase in activated Cdc42 (28, 29, 45, 46).

ITSN has been implicated in receptor down regulation through interaction with the E3 ubiquitin ligase Cbl. ITSN and Cbl form a complex to enhance the ubiquitylation and degradation of the EGFR (31). Recent results suggest that ITSN enhances Cbl activity by sequestering Sprouty2, an inhibitor of Cbl. ITSN also forms a complex with CIN85, a Cbl interacting scaffold protein, suggesting ITSN is involved in receptor down regulation at multiple steps (47). Additionally, a yeast two-hybrid screen of ITSN pulled out multiple regulators of receptor trafficking, including Rabaptin-5, an effector of the early endosomal GTPase Rab5, and KIF16B, a kinesin involved in EGFR recycling further supporting the role of ITSN in receptor regulation (48).

ITSN regulates cell polarity through two different pathways mediated by atypical protein kinase C (aPKC) and Notch. aPKC is important in establishing cell polarity for

asymmetrical cell division. Dap160 directly interacts with and activates aPKC to regulate neuroblast proliferation (49). Numb and Notch are important for asymmetric cell division of neuronal precursor cells and are differentially segregated during cell division to generate two different precursor cells. Dap160 EH and SH3 domains interact with Numb and Dap160 overexpression leads to Notch inhibition suggesting a role for ITSN in cell division (50). Numb is also associated with ITSN-L in dentritic spines of rat hippocampal neurons. The GEF activity of ITSN-L is increased by Numb binding, suggesting a role for this complex in neuronal cell development (51).

ITSN has also been shown to regulate apoptosis in endothelial cells. ITSN knockdown leads to the activation of the pro-apoptotic protein Bax (43) and this cell death can be rescued by the anti-apoptotic factor Bcl-X_L. ITSN also plays a role in cell survival through phosphoinositide 3' kinase (PI3K) dependent activation of AKT. ITSN silencing in mouse neuroblastoma cells (N1E-115) leads to cell death upon differentiation. Interestingly, stable silencing of ITSN in these cells lines did not inhibit transferrin internalization, indicating that this cell survival response is independent of ITSN's role in endocytosis (Figure 2). Additionally, ITSN activates phosphoinositide-3 kinase Class II beta (PI3K-C2β) and PI3K activity is necessary for ITSN activation of AKT indicating that ITSN is mediating a PI3K-AKT cell survival pathway. Indeed, N1E-115 cell death can be rescued by PI3K-C2β or AKT (33). PI3Ks have been shown to be important signaling molecules and are involved in multiple signal transduction pathways.

C.   **Phosphoinositide 3-Kinases**

PI3Ks phosphorylate the 3'-OH position on the inositol ring of phosphoinositides (PIs) and their precursor phosphatidylinositol (PtdIns). PI3Ks have been found in all eukaryotic cell types and have been linked to multiple signal transduction pathways including cell survival, growth, proliferation, motility, and membrane trafficking [reviewed in (52)]. PI3Ks are activated downstream of numerous types of receptors including, RTK, G-protein coupled receptors, B-cell receptors, T-cell receptors, and integrins leading to diverse signaling output. PI3K's also play a role in oncogenesis and the immune response, underlying the importance of these proteins in cell signaling (53-58). There are eight different PI3Ks, which can be divided into three classes based on their domains, substrate specificity, and adaptor proteins (Figure 3). These PI3Ks can generate different 3'-PI's including, PI_3P, $PI_{3,4}P_2$-, and $PI_{3,4,5}P_3$(59-61).

1.   **Three Classes of Phosphoinositide 3-Kinases**

Class I PI3Ks are subdivided into Class IA and IB. Class IA includes three catalytic subunits, p110α, p110β, and p110δ. Each catalytic subunit associates with an adaptor (p50, p55,or p85) subunit that contains two Src homology 2 (SH2) domains. These SH2 domains facilitate binding to phospho-tyrosine (pTyr) sites on RTKs at the plasma membrane (59). Class IB consists of a p110γ catalytic subunit that forms a dimer with the adaptor p101, and is activated by the $\beta\gamma$ subunit of heterotrimeric G-proteins(52). *In vitro*, Class I PI3K's utilize PI, PI_4P, or $PI_{4,5}P_2$ as substrate, but *in vivo* they have a preference for $PI_{4,5}P_2$ to produce $PI_{3,4,5}P_3$ (62-64). There are three isoforms of Class II PI3Ks, C2α, C2β, and C2γ. All three isoforms can utilize PI or PI_4P *in vitro*, but the *in vivo* lipid product of these kinases remains unclear (65, 66).

Class	Catalytic		Adaptor	Regulation	Lipid Products *in vivo*
I A	p110α,β.δ		p85α,β & p55γ	Tyr Kinases, Ras	PI(3,4,5)P3
B	p110γ		p101	Heterotrimeric G-proteins, Ras	
II	PI3KC2α,β,γ			ITSN	?
III	Vps34p analogues		p150	Constitutive	PI(3)P
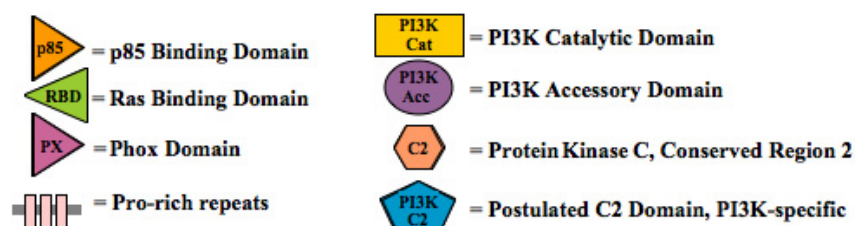
Figure 3. Phosphoinositide-3-Kinases. There are three classes of PI3Ks that are divided by domains, substrate specificity, and adaptor proteins. Class I PI3Ks are sub-divided into Class IA and Class IB and are activated by RTKs and heterotrimeric G-proteins, respectively. Class II PI3Ks do not have an adaptor subunit, but contain a C2 domain. Class III PI3K is homologous to the yeast PI3K.

Class III PI3K, Vps34p, was originally found in yeast (67). Class III PI3Ks are involved in membrane trafficking through the generation of their lipid product PI$_3$P (68).

2.   PI3K Inhibitors

Much of the research on PI3Ks utilize two major inhibitors, wortmannin and LY294002. LY294002 is a reversible competitor for ATP binding(69). Wortmannin covalently inactivates PI3K through an interaction with a lysine in the catalytic domain (70, 71). Although these inhibitors work on all classes of PI3Ks, the *in vitro* sensitivities of the classes are different. Class I PI3Ks are the most sensitive to LY294002 with the IC$_{50}$ of p110α ranging from 0.8μM-1.2μM (72, 73). PI3K-C2β (IC$_{50}$=6.9μM) and especially PI3K-C2α (IC$_{50}$=19μM) are less sensitive to LY294002 (72, 73). PI3K-C2α (IC$_{50}$=420nM) and PI3K-C2γ (IC$_{50}$=32nM) are more resistant to wortmannin compared to Class I PI3Ks (IC$_{50}$=2-5nM) (65, 72). PI3K-C2β has an IC$_{50}$ of 1.6nM for wortmannin, comparable to what is seen with p110α (71). Often the use of these inhibitors blocks all PI3K activity making it difficult to decipher the isoform responsible for a specific biological output. More recently, isoform specific inhibitors have been developed (Table II), but the widespread usage of these inhibitors has not been reported. Additionally, the development of these isoform specific inhibitors have focused on the Class I PI3K's, while inhibitors for Class II and III PI3Ks are lacking.

TABLE II. ISOFORM SPECIFIC PI3K INHIBITORS

Inhibitor	PI3K	IC50	Reference
PI-387	p110α	n.d.	(74)
2a[a]	p110α	0.67μM	(75)
2g[b]	p110α	1.8nM	(75)
12 [c]	p110α	2.8nM	(75)
15e[d]	p110α	2nM	(76)
PIK-75	p110α,p110γ	5.8nM, 76nM	(77)
TGX221	p110β	5nM	(78)
TGX-286	p110β,p110δ	0.12μM,1μM	(77)
PIK108	p110β,p110δ	0.057μM, 0.26μM	(77)
TGX115	p110β,p110δ	0.13μM, 0.63μM	(77, 79)
IC87114	p110δ	0.13mM-0.5μM	(77, 80)
PIK-39	p110δ	0.13μM	(77)
PIK-23	p110δ	0.097μM	(77)
AS252424	p110γ	33nM	(81)
AS604850	p110γ	0.25μM	(82)
AS605240	p110γ	0.008μM	(82)

[a] 3-{1-[(4-Fluorophenyl)sulfonyl]-1*H*-pyrazol-3-yl}-2-methylimidazo[1,2-a] pyridine

[b] 6-Bromo-3-{1-[(2-methyl-5-nitrophenyl)sulfonyl]-1*H*-pyrazol-3-yl}[1,2-*a*]pyridine

[c] 6-Chloro-3-{2-[2-methyl-5-nitrophenyl)sulfonyl]-1,3-thiazol-4-yl}imidaza[1,2-*a*]pyridine hydrochloride

[d] 3-(4-Morpholinothieno[3,2-d]pyrimidin-2-yl)phenol hydrochloride

3.   **Class II PI3K**

Class II PI3Ks have been identified in *Caenorhabditis elegans* (F39B1.1),

Drosophila melanogaster (PI3K_68D/cpk), mouse (p170/cpk-m), and human (83-85). Humans

have three isoforms coded by three distinct genes. PI3K-C2α was initially cloned from a

lymphoma cell line (U937) (73). PI3K-C2β, first called HsC2-PI3K, was cloned from a breast

cancer cell line (MCF-7), and finally PI3K-C2γ was found in human breast tissue, mouse, and rat

(66, 86-88). PI3K-C2α and PI3K-C2β are widely expressed, while PI3K-C2γ is mainly found

in the liver (52, 72, 89). The Class II PI3K's are grouped together due to the presence of a

protein kinase C (PKC)-related Ca^{2+} binding (C2) domain and a Phox homology (PX) domain

located at the carboxyl-terminus (90). Similar to Class I PI3Ks, Class II PI3K's contain a Ras

binding domain (RBD), PI3K-C2 domain, PI3K accessory domain (PIK), and PI3K catalytic

region (Figure 3). Class II PI3Ks differ in that they do not have a p85 binding domain. In

addition, PI3K-C2β orthologs have amino-terminal Proline-rich domains (PRD).

4.   **Class II PI3K-beta**

PIK3C2B is located on human chromosome 1 (1q32) (86). PI3K-C2β is mainly

found on intracellular vesicles with low levels detected in the cytosol and plasma membrane (72,

91). Immunofluorescent staining of PI3K-C2β reveals a punctate, perinuclear distribution (92).

PI3K-C2β has also been shown to translocate to the nucleus where it is cleaved by calpain and

activated (93, 94). The *in vivo* lipid product of PI3K-C2β is controversial with evidence

suggesting that it maybe PI$_3$P or PI$_{3,4}$P$_2$ (33, 92, 93, 95, 96). *In vitro*, PI3K-C2β can generate PI$_3$P

and to a lesser extent, PI$_{3,4}$P$_2$ (72). In addition, in these assays PI3K-C2β may utilize Mg^{2+}, Mn^{2+}

or Ca^{2+} as a cofactor, a characteristic unique to Class II PI3Ks (72, 73, 83).

Unlike Class I PI3Ks, PI3K-C2β does not bind a regulatory subunit increasing the complexity of possible mechanisms for regulation. The C2 domain of PI3K-C2β has been shown to inhibit kinase activity by competing for substrate. Indeed, carboxyl-terminal deletions of PI3K-C2β have increased lipid kinase activity (72). Additionally, clathrin binding to the amino-terminus of PI3K-C2β increases kinase activity, presumably through relief of this inhibition(91). PI3K-C2β may also be regulated by the Ca^{2+}-dependent protease, calpain, presumably through cleavage of the C2 domain. PI3K-C2β is activated and cleaved in response to liver injury and this effect is blocked by calpain inhibitors(93). In addition, calpain inhibition blocks PI3K-C2β activity downstream of platelet activation and hepatocyte growth factor stimulation (97, 98).

PI3K-C2β has also been implicated in insulin signaling. Insulin stimulation leads to the recruitment of PI3K-C2β to p-Tyr complexes and prolonged insulin stimulation increases PI3K-C2β activity (65, 99). Indirectly, PI3K-C2β has also been implicated in the production of a pool of PI_3P important for glucose transport (100, 101).

PI3K-C2β may play a role in cell cycle progression and differentiation. In HL-60 cells, PI3K-C2β activity in the nuclear envelope is increased as cells progress into the G_2/M phase(102). This activity is the result of calpain cleavage. In addition, when HL-60 cells are stimulated with all-trans-retinoic acid they differentiate into neutrophil like cells. Under these conditions PI3K-C2β activity is increased in the nuclei and nuclear envelope, possibly, through tyrosine phosphorylation (94). The role of PI3K-C2β in epidermal differentiation is questionable due to the results observed in mouse models. In cell culture PI3K-C2β can induce epidermal differentiation, but epidermal-specific overexpression of PI3K-C2β in a mouse model does not effect the process (103). In addition, knock-out of PI3K-C2β from the mouse does not result in defects in epidermis (103).

PI3K-C2β is activated downstream of multiple receptor types, including G-protein coupled receptors, T-cell receptors, and RTKs. LPA stimulation increases a PI3K-C2β-dependent pool of PI$_3$P necessary for cell migration (96). Activation of PI3K-C2β downstream of T-cell receptors leads to K$^+$ efflux through the KCa3.1 potassium channel and T-cell activation (104). PI3K-C2β is also activated downstream of the c-Met tyrosine kinase receptor in the brush border plasma membrane of the kidney (97).

PI3K-C2β is activated downstream of the EGFR and the platelet-derived growth factor receptor (PDGFR) (65). The PRD directly interacts with Grb2 to mediate recruitment of PI3K-C2β to the EGFR (91, 105). In addition, both Grb2 and EGFR stimulate the *in vitro* kinase activity of PI3K-C2β (105). Downstream of EGF stimulation PI3K-C2β has been shown to activate AKT (33, 95). Dominant negative (DN) PI3K-C2β blocks AKT activation in response to EGF, but does not have any effect on ERK (95). In addition, a C-terminal deletion mutant increases AKT activation, but the full-length protein does not (95, 106). This is in agreement with the C2 domain blocking kinase activity of PI3K-C2β by competing for substrate (72, 95). Silencing PI3K-C2β also results in a decrease in AKT activation without effecting ERK (95). PI3K-C2β also induces cell survival in ITSN-silenced cells, presumably through AKT activation (33). ITSN overexpression increases the *in vitro* lipid kinase activity of PI3K-C2β as well as activates AKT in a PI3K-dependent manner(33).

In addition to regulating the AKT pathway, PI3K-C2β functions downstream of EGFR to regulate Rac activity. At the EGFR, PI3K-C2β forms a complex with Grb2, Eps8, Abi1, and Sos (106). Abi1 functions as a scaffold for Eps8 and Sos, and together this complex functions as a Rac-GEF (107, 108). There are conflicting reports on the role of PI3K-C2β in Rac activation with evidence for PI3K-C2β increasing as well as decreasing levels of activated Rac

(92, 106). In both cases PI3K-C2β increases cell migration and actin rearrangement. One report suggests the effects of PI3K-C2β is due to Cdc42 activation and loss of $\alpha_{IV}\beta_1$ integrin expression (92). PI3K-C2β has also been shown to be activated downstream of the $\alpha_{IIb}\beta_3$ integrin in platelets (98). Other data indicates that PI3K-C2β increases activated Rac levels leading to JNK activation (106). In addition to Rac, the presence of a RBD on PI3K-C2β suggests PI3K-C2β, like class I PI3K's, may be regulated by the monomeric G-protein Ras.

D. <u>**Ras**</u>

Ras is the founding member of the Ras superfamily of GTPases and is the most frequently mutated oncogene in human cancers (30% prevalence) (109). The high frequency of Ras mutations in human tumors has led to intense interest in the biological activity of Ras over the past 30 years. Ras has been implicated in proliferation, cell survival, differentiation, cytoskeleton organization, and cell migration.

1. <u>**Ras Genes and Structure**</u>

Ras genes were first identified in Harvey and Kristen strains of rat sarcoma viruses (110, 111). Ras genes are well conserved across all eukaryotes, including, human, rats, mice, *Drosophila,* and yeast. There are four mammalian Ras isoforms (H-Ras, K-Ras4A, K-Ras4B and N-Ras) that are coded for by three separate genes (112-115). The K-Ras gene uses alternative fourth exons to code for two isoforms, A and B (116-118). Human H-Ras was first cloned from a bladder carcinoma cell line (113, 119), while human K-Ras was cloned from a lung carcinoma cell line (Lx-1) (114). Finally, N-Ras was identified in SK-N-SH neuroblastoma cells (115). All four isforms are remarkably similar (85% identical) (120), with the 85 amino-

terminal residues of all four isoforms showing 100% identity (Figure 4.). This region encodes the effector domain (25-45aa), the switch I region (30-40aa) and the switch II region (60-76aa) (121). The variation between the isoforms occurs in the carboxyl terminal 25 residues (4% identity). The carboxyl- terminal region of Ras contains a CAAX sequence and the hypervariable (HV) domain, both involved in membrane targeting of Ras (122).

2. Ras GTPase

Ras is regulated by cycling between GDP and GTP-bound states(109). The intrinsic GTPase activity and GDP/GTP exchange activity of Ras is very slow, therefore GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs) mediate nucleotide exchange. GAPs (p120 GAP and neurofibromatosis 1) promote the hydrolysis of GTP back to GDP, while GEFs (Sos, RasGRF, RasGRP) promote the loading of GTP onto Ras. GTP binding to Ras induces conformational changes in the switch I and II regions of Ras promoting effector binding. Mutations at amino acids 12, 13, and 61 in Ras prevent GTP hydrolysis leading to constitutive activation of Ras and Ras-regulated pathways (123). In addition to these activating mutations, dominant negative mutants have been generated. These mutants have substitutions at positions 15,16, or 17 (123). Dominant-negative Ras mutants bind exchange factor and prevent the activation of endogenous Ras (123).
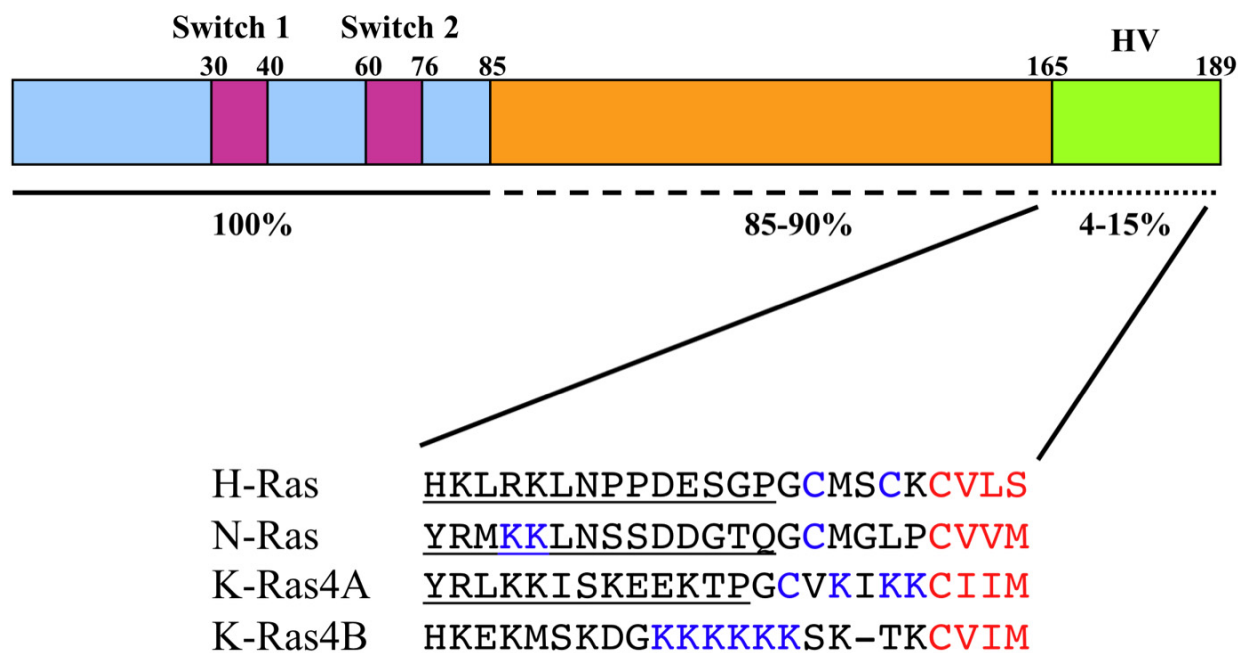
Figure 4. Ras. H-Ras, N-Ras, K-Ras4A, K-Ras4B differ only in the carboxyl-terminal region. Switch 1 and 2 regions (pink), the HV (green), linker regions (underlined), lipid modified CAAX domain (red), and secondary signal for PM localization (blue), [adapted from (124)].

3. **Ras Modification and Trafficking**

Lipid modification of Ras is necessary for full activation of Ras signaling. The carboxyl-terminal CAAX motif of Ras undergoes postranslational modification to target Ras to the plasma membrene (PM) and endomembrane compartments. First, a prenyltransferase adds either a farnesyl or geranylgeranyl group to the cysteine of the carboxyl-terminal CAAX motif, where C is the prenylated cysteine, A is any aliphatic amino acid, and the X amino acid determines the type of modification Ras will undergo (Figure 4). Generally, Ras is farnesylated due to a serine (H-Ras) or a methionine (N-Ras, K-Ras4A/4B) in the X position, but N-Ras, K-Ras4A, and K-Ras4B have been shown to be substrates for geranylgeranyl transferase as well. K-Ras4B especially has a high affinity for the geranylgeranyl transferase, 10-20 fold greater compared to the other Ras isoforms, due to a poly-lysine region amino-terminal to the CAAX motif. Secondly, the -AAX peptide is then cleaved by Ras-converting enzyme 1 (Rce1), a protease located in the endoplasmic recticulum (ER) (125, 126). Finally a methyl group is added to the farnesylated cysteine by a prenylcysteine carboxyl methyltransferase (also called isoprenyl cysteine transferase, Icmt) also located in the ER (127, 128). Prenylation is sufficient to generate Ras on the Golgi and ER, but a second signal in the HV region is necessary for PM targeting (129, 130). H-Ras is dipalmitoylated on Cys181 and Cys184 for targeting to the PM (131). Both K-Ras4A and N-Ras utilize a combination of monopalmitoylation and a short basic region for PM localization, while in K-Ras4B a lysine-rich region is used for targeting to the PM (130, 132).

At the molecular level the biggest difference between the Ras isoforms is the post-translational modification and trafficking of K-Ras4B. H-Ras, N-Ras, and K-Ras are initially associated with the ER (122, 133). From the ER, H-Ras and N-Ras are transported to the Golgi

and then to the PM through an endocytic pathway, while little K-Ras4B is found on the Golgi

(122, 133). H-Ras has also been shown to be trafficked from the Golgi to recycling

endosomes(134). *In vitro* evidence suggests the route of K-Ras4B to the PM is dependent on

microtubules. K-Ras4B directly interacts with microtubules and pharmacologic disruption of

microtubules decreases the PM pool of K-Ras4B (133, 135, 136). Alternatively, pharmacologic

disruption of microtubules has been shown to re-localize K-Ras4B from the PM to endosomes,

but not microtubles (133). Furthermore, the polybasic region of K-Ras4B interacts with the

negatively charged PM raising the possibility that K-Ras4B may traffic to the plasma membrane

by diffusion through the cytosol (137). Once at the PM H-Ras and N-Ras palmitoylation is

reversible, allowing for recycling back to the Golgi (138). This palmitoylation cycle is

independent of activation and generates a fluid pool of H-Ras and N-Ras that traffic between the

Golgi, PM and ER (139, 140). K-Ras4B does not undergo palmitoylation, but is also

dynamically associated with the PM with a half-life of minutes (141). Additionally, Ras is

activated at these various compartments to activate multiple signaling pathways (142).

4.   Ras Signaling

Classically, Ras effectors are defined as proteins that interact with the effector

domain (25-45aa) of Ras in a GTP-dependent manner. Over 20 Ras effectors have been

identified, but there are three canonical Ras effector pathways: Raf, Ral guanine nucleotide

dissociation stimulator (GDS), and PI3K pathways (Figure 5) (124). Raf is a serine/theronine

kinase that directly interacts with Ras-GTP (143). Upon growth factor stimulation, RTKs

dimerize and become autophosphorylated. These p-Tyr residues recruit proteins with SH2
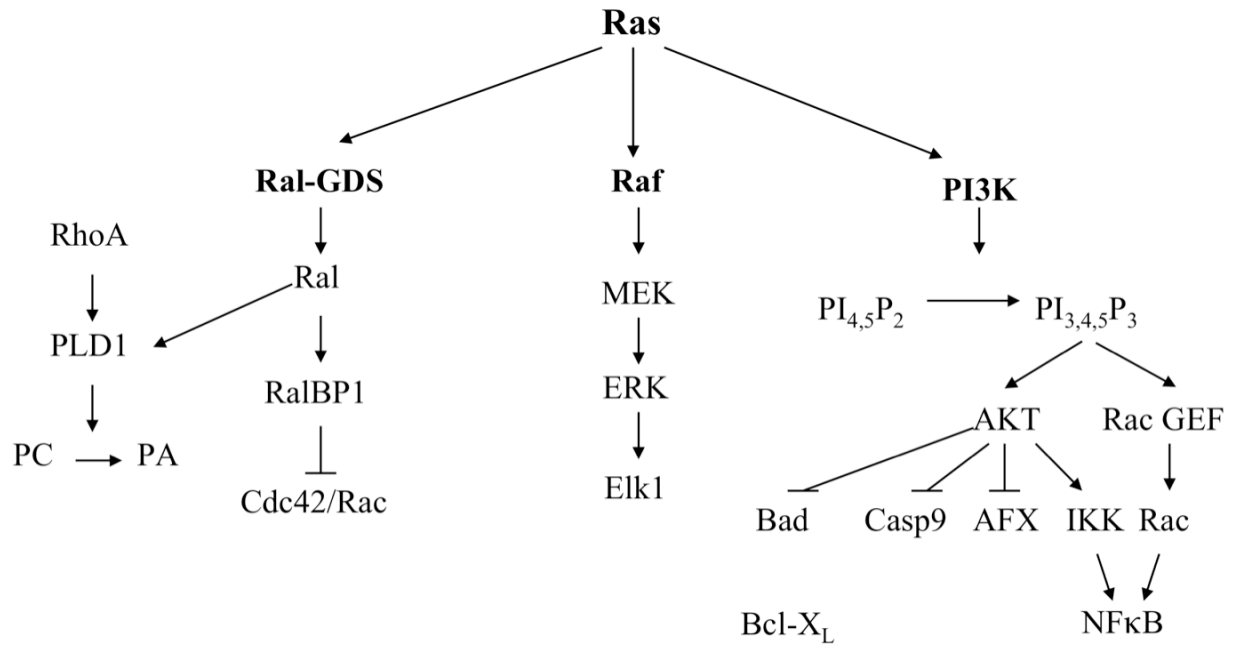
Figure 5. General schematic of Ras regulated signaling pathways. Ral-GDS, Raf, and Class I PI3K are the prototypic Ras effectors, [adapted from (142)].

domains such as the adapter protein Grb2. In the cytosol, Grb2 is constitutively associated with the Ras GEF Sos, and following RTK activation, Grb2-Sos translocates to the PM. Sos stimulates the release of GDP from Ras. Given the relatively high ratio of GTP:GDP, GTP then binds nucleotide-free Ras leading to activation (144, 145). Ras-GTP at the PM recruits effectors, such as Raf. Once at the PM, Raf is activated to phosphorylate MEK1/2 which are dual specificity kinases that phosphorylate ERK1/2. ERK1/2 then translocates to the nucleus to phosphorylate multiple substrates such as the Elk-1 transcription factor.

The second characterized Ras effector is Ral-GDS, a Ral GEF. Ral-GDS specifically interacts with Ras-GTP. Ras binding increases the GEF activity of Ral-GDS leading to an increase in Ral-GTP (146-149). Active Ral regulates cytoskeleton rearrangement as well as vesicle transport.

Finally, Class I PI3Ks have been shown to be Ras effectors. Generally, it is thought that PI3K is recruited to activated Ras. The p110 catalytic subunit of PI3K directly interacts with Ras in a GTP dependent manner (150), and Ras-GTP stimulates the lipid kinase activity of PI3K to produce $PI_{3,4,5}P_3$ (150-152). The localized production of $PI_{3,4,5}P_3$ at the PM leads to the recruitment of $PI_{3,4,5}P_3$ binding proteins such as AKT via its PH domain. Here, AKT becomes phosphorylated and fully active leading to the activation of cell survival pathways (61). Interestingly, the p110α catalytic subunit is an oncogne, and mutant p110α has been found in human tumors (54-56). Additionally, mouse models have indicated that PI3K binding to Ras is necessary for Ras tumorigenesis (57, 58). Evidence also indicates that PI3K is necessary for Ras-mediated tumor development, but not maintenance of these tumors (58, 153).

5. **Compartmentalized Signaling of Ras**

Initially, it was thought that downstream signaling from all Ras isoforms was similar due to the high degree of homology, but evidence suggests that the Ras-mediated signaling events are much more complicated. Indeed, all isoforms are ubiquitously expressed (154-156), but are alternatively mutated in various human tumors(157). In addition, mice null for H-Ras and N-Ras are viable, while the K-Ras knockout is embryonic lethal(158-161). One explanation for this difference is the alernative post-translational modifications the Ras isoforms undergo, resulting in compartmentalized specific signaling. As mentioned previously, H-Ras and N-Ras are localized to the PM, Golgi, ER, and endosomes, while K-Ras is predominantly found on the PM (122, 133). Ras is constantly being trafficked between the PM and Golgi. This trafficking is dependent on palmitoylation, but independent of activation of Ras (139). Both constitutively active Ras12V and dominant negative Ras17N are found on endosomal compartments (139, 162). This compartmental restriction may result in alternative exposure to GEFs, GAPS, or effectors leading to alternative downstream signaling. Indeed upon growth factor stimulation, Ras is quickly and transiently activated at the PM, but also at the Golgi and ER where Ras activation is delayed and sustained (139, 140, 163). FRET experiments utilizing the RBD of Raf as a probe for activated Ras have been used to follow the kinetics of Ras activation at the PM and Golgi. These data show that Ras is activated at the PM with a $t_{1/2}$~3min while Golgi Ras is activated with a $t_{1/2}$~17min (139). There are reports of Ras activation at the Golgi and ER independent of internalization, suggesting that Ras-GTP is not simply being translocated to these compartments from the PM (163). Activated Ras localized at these compartments is able to stimulate downstream siganling. Constitutively active Ras mutants that are localized to either the Golgi or ER differentially activate effectors. Golgi localized Ras more

efficiently activates ERK and AKT, but not JNK, while ER localized Ras better activates JNK, but not ERK or AKT (163). Contradictory to this report, Golgi localized constitutively active Ras has also been shown to inhibit ERK and AKT, but stimulate JNK and Ral-GEF, whereas ER localized constitutively active Ras activates ERK, AKT, and JNK, but not Ral-GEF (164).

Although there are conflicting reports it is clear that the localization of Ras effects activation of downstream signaling. In addition to the Golgi and ER, Ras also signals from endosomes. Blocking endocytosis prevents H-Ras, but not K-Ras activation of Raf indicating that Ras localization to endosomes is necessary for signaling (165). Indeed activated H-Ras is found on early and recycling endosomes (162, 166, 167). There is also evidence that internalization is not necessary for Ras activation, but this report does not distinguish between Ras isoforms (168). K-Ras has also been shown to localize with early endosomes where is it trafficked to late endosomes and into the lysosme (169). Additionally, protein kinase C phosphorylation of K-Ras4B also results in internalization and trafficking of K-ras4B to the mitochondria to induce apoptosis (170). These data demonstrate that Ras is biologically active on multiple membrane compartments. Interestingly, how Ras gets activated on these intracelluar compartments is still not fully understood. There is evidence that in T-cells the Ras GEF, RasGRP1 is localized to the Golgi to activate Ras, but there are low levels of RasGRP1 in other cell types suggesting this mechanism is not universal (140, 171, 172). My studies would suggest that nucleotide-free Ras is trafficked to various intracellular compartments where is it activated in response to ITSN binding and high GTP concentrations (Chapter 2). This mechanism corresponds with the delayed onset of Ras activation on the Golgi as well as the kinetic data that shows the delay coincides with the trafficking timeline (139). Additionally, there is a higher concentration of intracellular GTP compared to GDP, and these levels are not equimolar across

the cell, but rather compartmentalized, further supporting my model for nuclotide-free Ras activation at these sites (144, 145, 173, 174).

6.　　**Nucleotide-free Ras Signaling**

Although the prevailing hypothesis is that Ras-GTP is the only biologically functional version of Ras in the cell, multiple lines of evidence suggest otherwise (175-178). Over-expression of wild-type Ras, which is predominantly GDP-bound due to its intrinsic GTPase activity, exerts a suppressive effect on oncogenic transformation by constitutively activated Ras alleles (175-178). In a chemical carcinogenesis model of lung tumorigenesis, mice hemizygous for K-Ras deletion develop 4-5 times more lung tumors than wild-type mice suggesting that the wild-type allele plays a protective role (178). A number of human tumors exhibit loss of heterozygosity of K-Ras further supporting the notion that wild-type Ras functions as a tumor suppressor (175, 178). Indeed, a similar oncosuppressive effect is seen with dominant-negative Ras, Ras17N, which exerts its inhibitory effect by competing for GEFs due to its low affinity for nucleotides (compared to wild-type Ras) and therefore longer lifetime in the nucleotide-free state (123). Ras17N can inhibit oncogenic Ras transformation as well as inhibit Elk activation, but not Erk (179, 180). Ras17N has been isolated from cells in a GDP-bound form, but *in vitro* data indicates that Ras17N has a 30-fold lower affinity for nucleotide compared to wild type Ras (179, 180). This low affinity results in rapid turnover of nucleotide and a prolonged nucleotide-free state suggesting that nucleotide-free Ras may play a role in intracellular signaling.

In addition to Ras17N, many other Ras mutants have decreased nucleotide affinities. Amino acids 116, 117, and 119 of Ras are conserved in all GTPases and necessary for

GTP binding (181). Mutations at these sites in the background of an activating mutation drastically decreases the affinity of these mutants for GTP, but they retain transforming activity, suggesting that GTP binding may not be necessary for Ras transformation (181, 182). A single mutation at amino acid 119 retains its transforming activity and is suggested to have concentration-dependent dominant negative and oncogenic effects (183, 184). At low concentrations, Ras119N functions as a dominant negative by sequestering exchange factor, while at high concentrations (presumably above exchange factor levels), Ras119N is activated due to its increase in exchange activity and de facto GTP loading (184). Contrary to this hypothesis, Ras119A has a 20-fold lower affinity for nucleotide compared to WT Ras and can be purified from *E. Coli* in a nucleotide-free form, suggesting it is predominantly in a nucleotide-free state and not GTP bound (183). Ras16N has 100-fold lower affinity for nucleotide compared to WT Ras, and has also been purified in a nucleotide-free state, indicating that this intermediate is stable *in vivo* (183). Finally, Ras146V has a 1000-fold higher in nucleotide exchange activity, but is still transforming (185). These data, taken together, suggest that nucleotide-free Ras plays a role in cell signaling and Ras-mediated transformation.

II. A NEW DIMENSION TO RAS FUNCTION: A NOVEL ROLE FOR NUCLEOTIDE-FREE RAS IN CLASS II PHOSPHOINOSITIDE 3-KINASE BETA (PI3K-C2β) REGULATION

A.   Introduction

The intersectin (ITSN) scaffold stimulates Ras activation on endocytic vesicles without activating classic Ras effectors. The identification of Class II phosphoinositide 3-kinase beta, PI3K-C2β, as an ITSN target on vesicles and the presence of a Ras binding domain (RBD) in PI3K-C2β suggests a role for Ras in PI3K-C2β activation. Here, we demonstrate that nucleotide-free Ras negatively regulates PI3K-C2β activity. PI3K-C2β preferentially interacts *in vivo* with dominant-negative (DN) Ras, which possesses a low affinity for nucleotides. PI3K-C2β interaction with DN Ras is disrupted by switch 1 domain mutations in Ras as well as RBD mutations in PI3K-C2β. Using purified proteins, we demonstrate that the PI3K-C2β-RBD binds nucleotide-free Ras and that this interaction is not disrupted by nucleotide addition. Finally, nucleotide-free Ras but not GTP-loaded Ras inhibits PI3K-C2β lipid kinase activity *in vitro*. Our findings indicate that PI3K-C2β interacts with and is regulated by nucleotide-free Ras. These data suggest a novel role of nucleotide-free Ras in cell signaling in which PI3K-C2β stabilizes nucleotide-free Ras and that interaction of Ras and PI3K-C2β mutually inhibit one another.

B.   Materials and Methods

1.   Cell lines, Transfection, and Reagents

COS cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. COS Cells were transfected with Lipofectamine

(Invitrogen, Carlsbad, CA) as recommended by the manufacturer. Antibodies used include anti-hemagglutinin (HA) (Covance, Emeryville, CA), anti-Flag (Sigma, St. Louis, Missouri), anti-GST conjugated to HRP (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Ras (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Rab5 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-AKT (Cell Signaling Technology, Danvers, MA), anti-AKT-473 (Cell Signaling Technology, Danvers, MA).

2.   DNA Constructs and Generation of mutants

The BiFC vectors pFlag-VN-173N and pHA-VC155N were gifts from Dr. Chang-Deng Hu (Purdue University, West Lafayette, IN). CFP-PI3K was cut with XhoI, blunt ended with Klenow, digested with KpnI, and then cloned into the pHA-VC155N vector that had been cut with EcoRI, blunted ended with Klenow, and then digested with KpnI. All Ras constructs used in this manuscript were to the H-Ras isoform and are subsequently referred to only as Ras. pBABE-Ras constructs were a gift from Dr. Lawrence Quilliam (Indiana University, Indianapolis, IN). All Ras mutants were PCR amplified using the following primers, 5'CACCCGGGATCCTCAGGAGAGCACACAC3', 5'TGAGGATCCATGACGGAATATAAG, digested with BamHI and cloned into the BamHI site of pFlag-VN-173N. The RBD of Raf (aa 1-148) (1) was removed from pGEX with EcoRI, treated with Klenow fragment of DNA pol I, and then digested with BamHI. The Raf-RBD was then cloned into the EcoRV and BamHI sites of the pEFG vector. The RBD of PI3K-C2β (aa 364-466) was PCR amplified from full-length PI3K-C2β using the following primers: 5' CGCAGATCTGCTGTCACCCCTAGC 3', and 5'CGCCCCGGGAACCTTCTGCTCC ATCAGC3' digested with BglII and SmaI and then subcloned into YFP digested with

BglII/SmaI. The pEFG-PI3K-C2β-RBD was constructed by sequential digestion of the YFP-RBD construct with SmaI then BglII and cloning the resulting RBD fragment into pEFG SmaI and BamHI sites. The GST-PI3KC2β-RBD construct (aa 368-463) was constructed by PCR amplification using 5' GAGAGAGACATATGAGCCCAGAGCA CCTCGGGGAT 3' and 5' GAGAGAGGATCCCTCCATCAGCTGTA GCCGAAT 3'. The resulting fragment was cloned into pcr4-TOPO (Invitrogen, Carlsbad, CA) and sequenced. The TOPO-PI3K-C2β-RBD was digested with NdeI and BamHI, treated with the Klenow fragment of DNA pol I, then cloned into the SmaI site of pGEX-4T1. The Lys379Ala point mutation in the RBD of PI3K-C2β was generated using 5'-CTCGGGGATGAGGTCAACCTGGCGGTGACT GTG-3', 5'-CCTGTCACACAACACAGTCACCGCGAGGTTGAC3'. The Thr392Asp point mutation in the RBD of PI3K-C2β was generated using, 5'-GACAGGCTTCAAGAGGC ACTCGATTTCACCTGC-3', 5'-GGAGGAACAGTTGCAGGTGAAATCGAGTGCCTC-3', and cloned into CFP-PI3K as XhoI/BmgB1 fragments. CFP-PI3K-C2β-K/A, and CFP-PI3K-C2β-T/D were digested with EcoRI, treated with Klenow fragment of DNA pol I, then digested with KpnI. These fragments were then cloned into pHA-VC155N digested with XhoI, treated with Klenow fragment of DNA pol I, then digested with KpnI.

3. **Protein Purification**

GST and GST-Raf-RBD were purified from DH5α cells. GST-PI3K-C2β-RBD was purified from BL21 (DE3) cells. Bacterial cultures were grown overnight at 37ºC. The following day bacterial cultures were diluted 1:10, grown to an OD_{600} of 0.6, and induced with 0.1mM - 0.2mM IPTG for either 3hrs at 37ºC or overnight at room temperature. Cells were resuspended in MTPBS (16mM Na_2HPO_4, 4mM NaH_2PO_4-H_2O, 150mM NaCl, 50mM EDTA,

1% TritonX-100, pH 7.3) and sonicated. The supernatant was then incubated with Glutathione

Sepharose beads (GE Healthcare) for 1hr at 4ºC. Beads were then washed 3x with MTPBS and

3x with loading buffer (20mM Tris-HCl pH 7.6, 10mM EDTA, 5mM $MgCl_2$, 1mM DTT, 0.1mM

PMSF, 10% glycerol). pQlink Ras 1-166 was a kind gift from Dr. Sharon Campbell (University

of North Carolina, Chapel Hill, NC) and was used to express Ras in BL21 cells. Bacterial

cultures were grown overnight at 37ºC and the following day diluted 1:50, grown to an OD_{600} of

0.6, and induced with 100 μM of IPTG overnight at room temperature. Bacteria were lysed in

NiA buffer (50mM HEPES, 150mM NaCl, 5mM $MgCl_2$, 20mM imidazole, 50uM GDP, 100uM

TCEP) and sonicated. The supernatant was incubated with nickel beads (Clontech, Mountain

View, CA) for 30min at room temperature, then washed with NiA and NiB buffer (50mM

HEPES, 150mM NaCl, 5mM $MgCl_2$, 80mM imidazole, 50uM GDP, 100uM TCEP). His-Ras

was eluted off the nickel beads with NiC buffer (50mM HEPES, 150mM NaCl, 5mM $MgCl_2$,

300mM imidazole, 50uM GDP, 100uM TCEP) and dialyzed ON at 4ºC against Loading Buffer

(20mM Tris-HCl pH 7.6,10mM EDTA, 5mM $MgCl_2$, 1mM DTT, 0.1mM PMSF, 10% glycerol).

The His tag was then cleaved off His-Ras with TEV protease (Promega) ON at 4ºC. The TEV

protease and uncleaved His-Ras was removed from the reaction by incubation with nickel beads.

4. ***In vitro* Pulldowns**

Purified Ras was incubated in Loading Buffer (20mM Tris-HCl pH 7.6, 15mM

EDTA, 5mM $MgCl_2$, 1mM DTT, 0.1mM PMSF, 10% glycerol) with either 1mM GDP or 1mM

GTPγS for 30min at 30ºC. The reaction was stopped with 20mM $MgCl_2$ and kept on ice. 250nM

nucleotide loaded Ras was incubated with 850nM GST-Raf-RBD, GST-PI3K-C2β-RBD, or GST

alone bound to glutathione beads for 1hr at 4ºC. Beads were then washed 3x with Washing

Buffer (20mM Tris-HCl pH 7.6, 10% glycerol, 5mM MgCl$_2$, 1mM DTT, 0.1% TritonX-100, 0.1mM PMSF) and run on an SDS-PAGE gel.

5.   Nucleotide-free Assays

Purified Ras 250nM was incubated with either 850nM GST-Raf-RBD, GST-PI3K-C2β-RBD, or GST bound to Glutathione beads in Loading Buffer (20mM Tris-HCl pH 7.6, 15mM EDTA, 5mM MgCl$_2$, 1mM DTT, 0.1mM PMSF, 10% glycerol) for 30min at 30ºC in the absence of added nucleotide. Beads were then washed 3x with Loading Buffer. For the competition assays, 1mM GDP or 1mM GTPγS was added either directly to the binding reaction or to the third wash of the beads and incubated for 30min at room temperature. Samples were then run on an SDS-PAGE gel.

6.   Biochemical analyses

Cells were treated and analyzed by Western blot as described previously (31). BiFC, confocal imaging and analysis were performed as described (186). Transient reporter assays using the Gal-Elk reporter were performed in HEK293T cells as previously described (26). AKT activation assays were performed as in (33).

7.   *In vitro* Kinase Assays

To measure the effect of Ras on PI3K-C2β activity, pulldowns with Ras were performed as described above, but using full length GST-PI3K-C2β (Invitrogen, Carlsbad, CA). 2x Kinase Buffer (40mM Tris-HCl, 200mM NaCl, 2mM DTT) was added to GST-PI3K-C2β:Ras beads. The reaction was started by addition of ATP (40uM), phosphatidylinositol (0.2

mg/mL), MgCl$_2$ (3.5mM), and [γ-^{32}P]-ATP (10μCi). Samples were incubated for 15min at 37°C. 100μL of 1N HCl was added to stop the reaction, followed by 200μL of CHCl$_3$:MeOH (1:1) to extract the lipid. The organic phase was collected and a second extraction was performed by adding 70μL of MeOH:1N HCl (1:1). The organic phase was collected, the samples dried, re-suspend in 30μL of CHCl$_3$:MeOH (1:1), and spotted onto a TLC (Whatmann, Kent, ME) plate that had been pre-treated with 1% oxalic acid, 1mM EDTA, 40%MeOH, and baked at 110°C for 1hr. Plates were developed in 34mL water, 65mL n-propanol, and 1mL glacial acetic acid. Phospho-images were developed on a STORM 860 phosphoimager (GE Healthcare) and quantified using ImageQuant 5.2 (Molecular dynamics).

C. Results

1. PI3K-C2β and Ras interact and co-localize with ITSN on intracellular vesicles.

Co-localization of PI3K-C2β to ITSN-positive vesicles (33) coupled with ITSN activation of Ras on similar vesicles (1) and the presence of a putative RBD on PI3K-C2β (1) prompted us to examine whether PI3K-C2β was a target of the ITSN-Ras pathway. Although a previous report suggested that PI3K-C2β and Ras do not interact *in vitro* (72), we sought to determine whether PI3K-C2β interacted with Ras in cells. Using bimolecular fluorescence complementation (BiFC) (187, 188), I observed significant interaction between PI3K-C2β and Ras on a perinuclear population of vesicles, reminiscent of the localization of ITSN and Ras as visualized by FRET (Figure 6A) (1). Ras interaction with PI3K-C2β required the RBD as deletion of this region significantly reduced the BiFC signal. Although mutation of the PRD does not significantly reduce Ras-PI3K-C2β interaction, ITSN association with PI3K-C2β is

Figure 6. Ras, PI3K-C2β, and ITSN co-localize on intracellular vesicles. (A) Ras interaction with PI3K-C2β is disrupted by deletion of the RBD (ΔRBD) but not by mutation of the Pro-rich, ITSN1 binding sites (PRD-PA). The graph represents the average fluorescence intensity per cell ±S.E.M. from at least three independent experiments (34-59 cells were counted for each condition, *$p<0.05$, WT vs ΔRBD, PRD-PA vs ΔRBD). Western blot analysis demonstrates equal expression of all constructs (size bars=20μm). **(B)** VN-Ras and VC-PI3K-C2β (green) were co-transfected with CFP-ITSN1 (red) into COS cells. a) The PI3K-C2β-Ras BiFC complex co-localizes with ITSN1, represented by yellow in the overlay panel; b. The VN-Ras and VC-PI3K-C2β YFP signal (green) does not bleed into the CFP channel; c.The CFP-ITSN1 signal does not bleed into the YFP channel (size bars=20μm). Note: the differences in signal strength of the BiFC signal in (A) vs (B) are due to a lower power setting for the laser in (A) so that pixel intensities can be accurately quantified and are not saturated. In (B) a higher laser power was used to illustrate the punctate localization of the Ras-PI3K complex throughout the cell.

Figure 6. Ras, PI3K-C2β, and ITSN co-localize on intracellular vesicles.
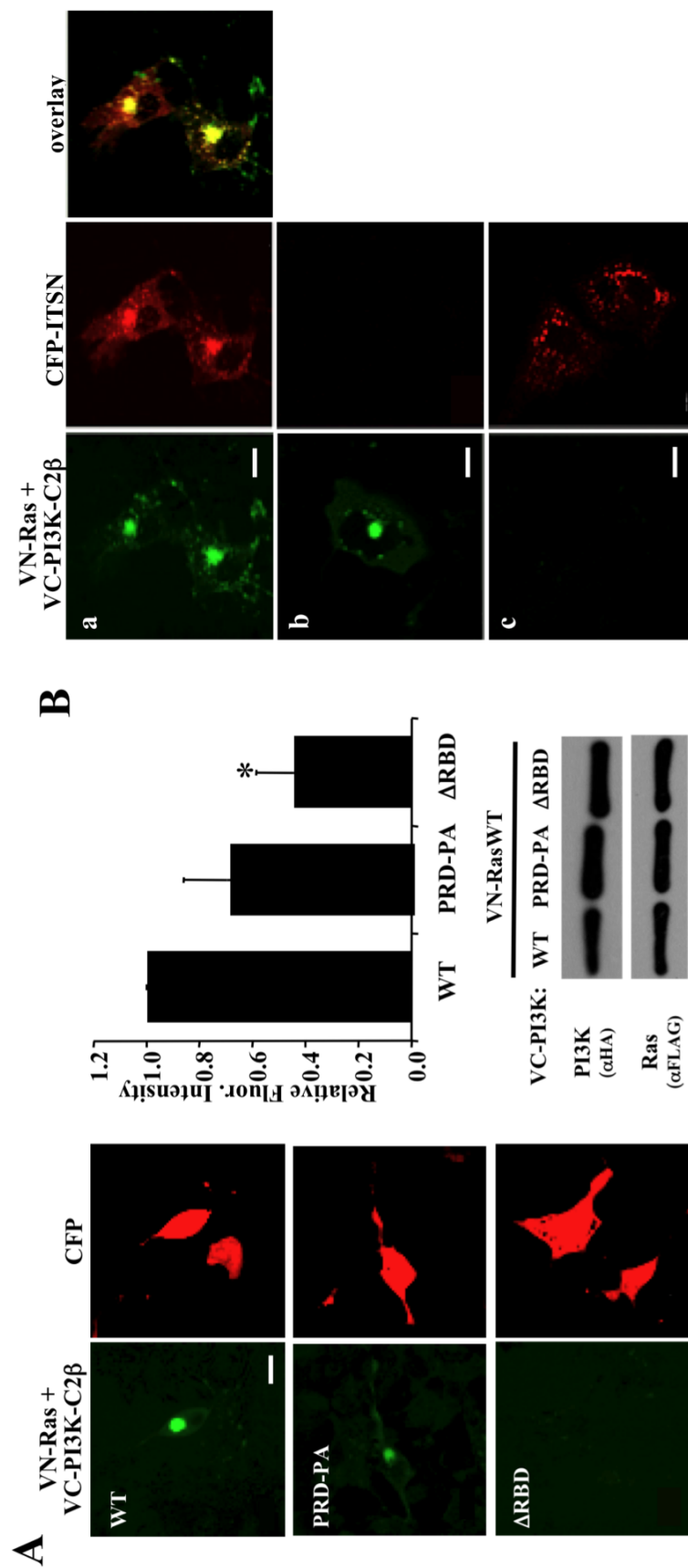
abolished (33). Interestingly, deletion of the RBD also reduces ITSN interaction with PI3K-C2β suggesting that Ras is important for ITSN/PI3K-C2β association. To address whether all three proteins interact in the same complex, I combined BiFC with conventional co-localization. The BiFC complex of Ras and PI3K-C2β co-localized with CFP-ITSN (Figure 6B). These results suggest that all three molecules are indeed in a trimolecular complex.

2.   **Ras is necessary for ITSN activation of AKT.**

PI3K activity is necessary for ITSN activation of AKT and for regulation of an ITSN survival pathway in neuronal cells (33). Given the co-localization of ITSN, Ras, and PI3K-C2β, I tested the importance of Ras in the mechanism of ITSN induced activation of AKT. Expression of the dominant negative Ras mutant, Ras17N, inhibited ITSN activation of AKT (Figure 7) indicating that Ras is necessary for ITSN mediated activation of the PI3K pathway. ITSN also directly interacts with the Ras exchange factor Sos (3). To determine if Sos is mediating ITSN activation of AKT a second Ras mutant, Ras17N/69N, was utilized. Mutation of Asp 69 to Asn (69N) disrupts the interaction of Ras with GEFs such as Sos and RasGRF (189). Interestingly, Ras17/69N only partially disrupted ITSN activation of AKT indicating that ITSN activation of the PI3K pathway and subsequent activation of AKT is not dependent on Sos (Figure 7).

3.   **PI3K-C2β forms a complex with Ras on intracellular vesicles.**

The presence of an RBD on PI3K-C2β and the co-localization of Ras with PI3K-C2β suggest that PI3K-C2β is a *bona fide* Ras effector. Surprisingly, constitutively activated Ras, Ras61L, exhibited little interaction with PI3K-C2β (Figure 8). Similar results were observed
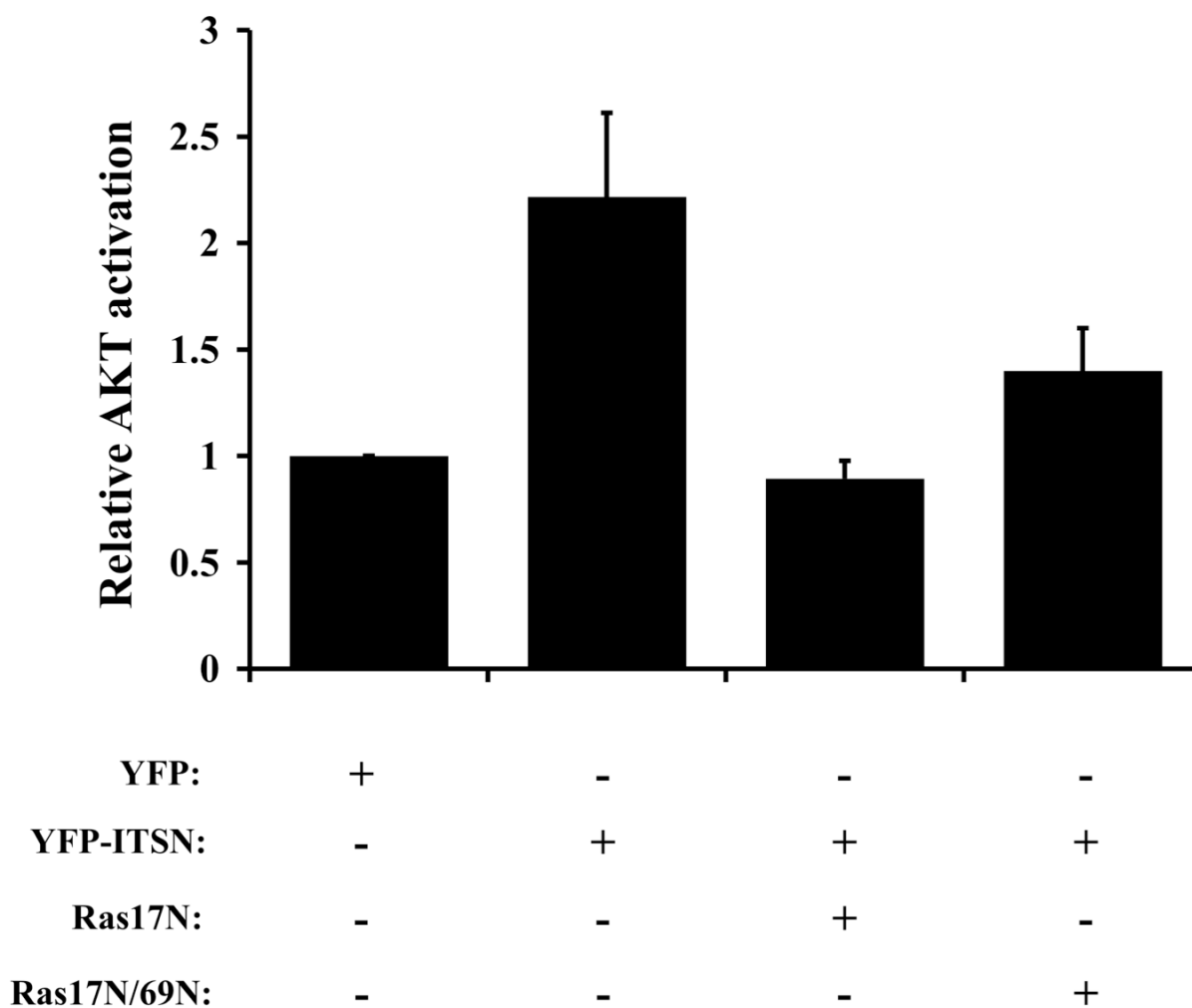
Figure 7. Ras is necessary for ITSN activation of AKT. YFP-ITSN activation of AKT is inhibited by co-expression of Ras17N. Ras17N/69N only partially reduced ITSN activation of AKT. Results represent the average fold activation of AKT ±S.E.M. from at least three independent experiments. (Assay performed by Angela Russo, Ph.D)
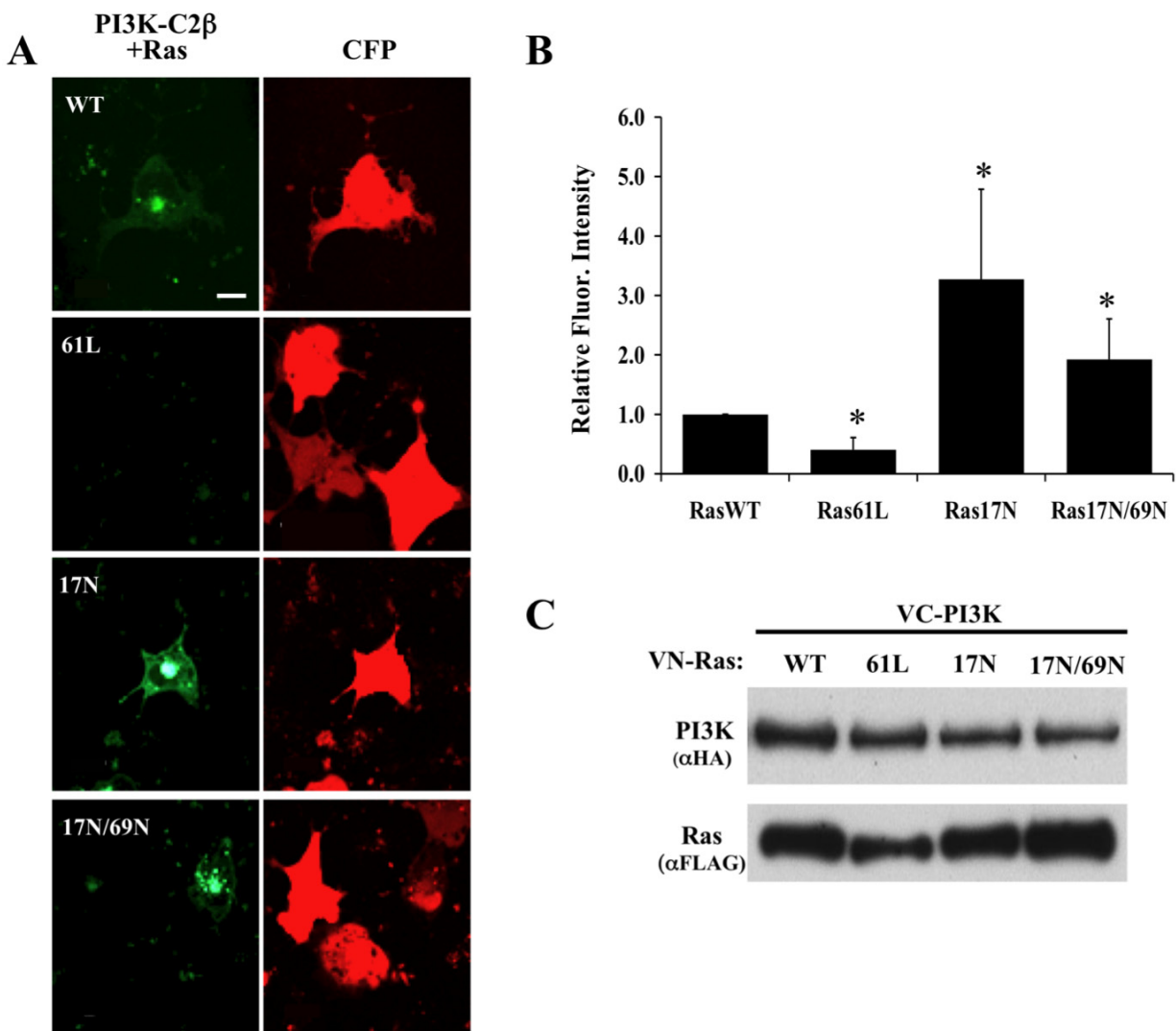
Figure 8. Ras forms a complex with PI3K-C2β. (A) PI3K-C2β preferentially interacts with Ras17N. VC-tagged PI3K-C2β was co-transfected with one of the following VN-tagged Ras constructs: WT, 61L, 17N, or 17N/69N. BiFC signal (green) demonstrates that PI3K-C2β interacted with Ras17N>17N/69N>WT>61L. CFP (red) was used as a transfection control. **(B)** The graph represents the average fluorescence intensity per cell ±S.E.M. from at least three independent experiments (66-76 cells were counted for each condition, *p<0.05). **(C)** Western blot analysis demonstrates equivalent expression of all constructs.

with Ras12V (Figure 9). In contrast, Ras17N interacted most strongly with PI3K-C2β followed by RasWT (Figure 8). Since Ras17N acts as a dominant-negative by sequestering Ras GEFs and preventing nucleotide dissociation from endogenous Ras (123), I examined if exchange factors mediated the interaction of PI3K-C2β and Ras using the Ras17N/69N mutant (189). PI3K-C2β interaction with Ras17N/69N was only moderately reduced when compared to interaction with Ras17N (Figure 8) indicating that exchange factors are not responsible for PI3K-C2β-Ras17N interaction. In contrast, ITSN interaction with Ras17N was dramatically reduced by the 69N mutation consistent with the finding that ITSN activates Ras through direct binding of Ras GEFs (Figure 10) (3). Interestingly, the use of another Ras dominant negative, Ras15A, did not result in a BiFC signal (Figure 11). Ras15A has reduced affinity for nucleotide, but an increased affinity for exchange factor(190-192). The lack of BiFC signal suggests that the high affinity of Ras15A for exchange factor is preventing association with PI3K-C2β. Mutation of the ITSN-binding site in PI3K-C2β did not affect Ras-PI3K-C2β interaction (Figure 6A) further supporting the conclusion that exchange factor binding (through ITSN) is not necessary for Ras-PI3K-C2β interaction. However, deletion of the RBD dramatically reduced Ras-PI3K-C2β interaction (Figure 6A).

4.   The PI3K-C2β RBD directly interacts with nucleotide-free Ras.

While Ras17N is reported to be GDP-bound in cells (180), *in vitro* studies indicate that Ras17N binds nucleotides with a ~30-fold reduced affinity compared to wild-type Ras suggesting that it exists in the nucleotide-free state for extended periods *in vivo*(179). Thus, the interaction between PI3K-C2β and Ras17N raises the possibility that the PI3K-C2β-RBD interacts with either RasGDP or nucleotide-free Ras. To determine the nucleotide dependence for

Figure 9. PI3K-C2β does not interact with oncogenic Ras. (A) VC-tagged PI3K-C2β was co-transfected with one of the following VN-tagged Ras constructs: WT,17N, or 12V. BiFC signal (green) demonstrates that PI3K-C2β interacted with Ras17N>WT>12V. CFP (red) was used as a transfection control. **(B)** The graph represents the average fluorescence intensity per cell ±S.E.M. from at least three independent experiments (60-62 cells were counted for each condition, *p<0.05). **(C)** Western blot analysis demonstrates expression of all constructs (size bar=20μm).
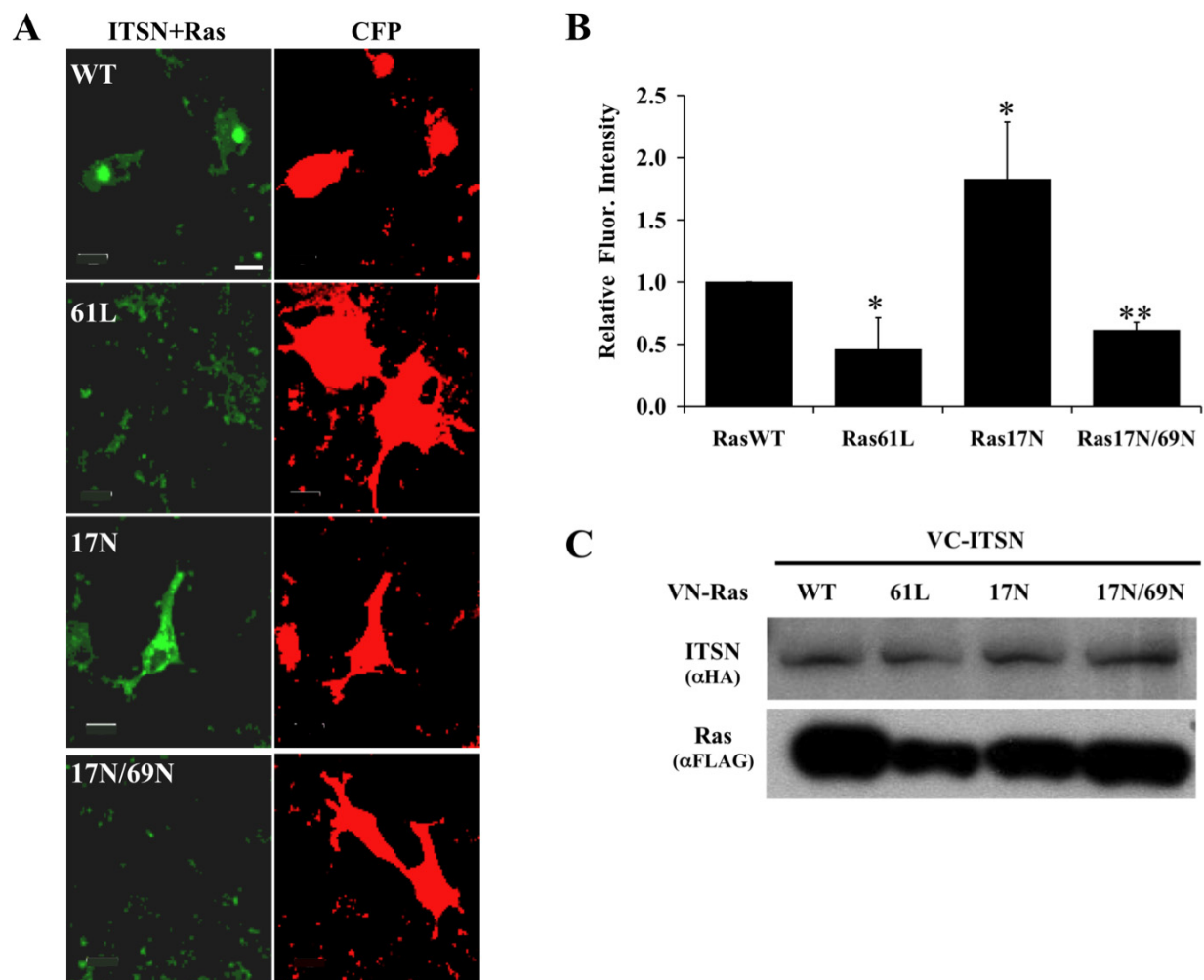
Figure 10. ITSN and Ras form a BiFC complex. (A) VC-tagged ITSN1 was co-transfected with one of the following VN-tagged Ras constructs: WT, 61L, 17N, or 17N/69N. ITSN1 formed a complex (green) with Ras17N>WT>17N69N>61L. CFP (red) was used as a transfection control. **(B)** The graph represents the average fluorescence intensity per cell ±S.E.M. from at least three independent experiments (44-66 cells were counted for each condition, * $p<0.05$, ** $p<0.01$). **(C)** A Western blot was performed to demonstrate equal expression of all constructs (size bar=20μm).
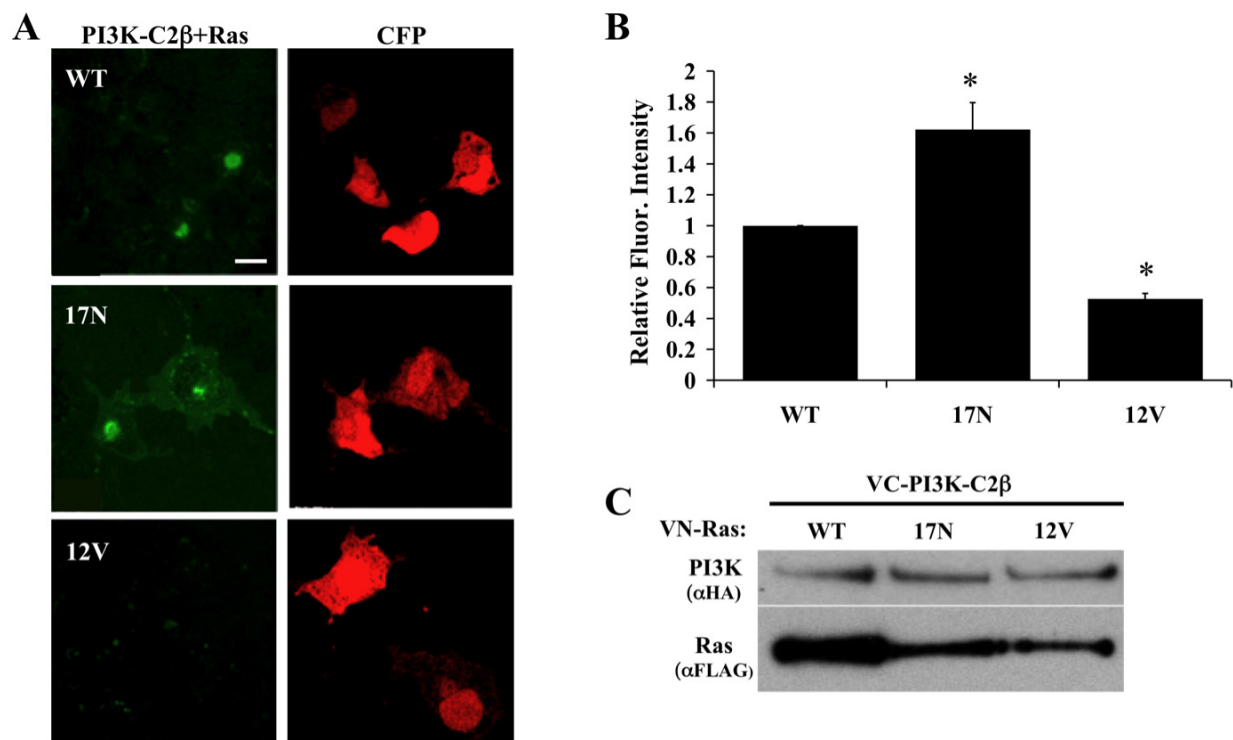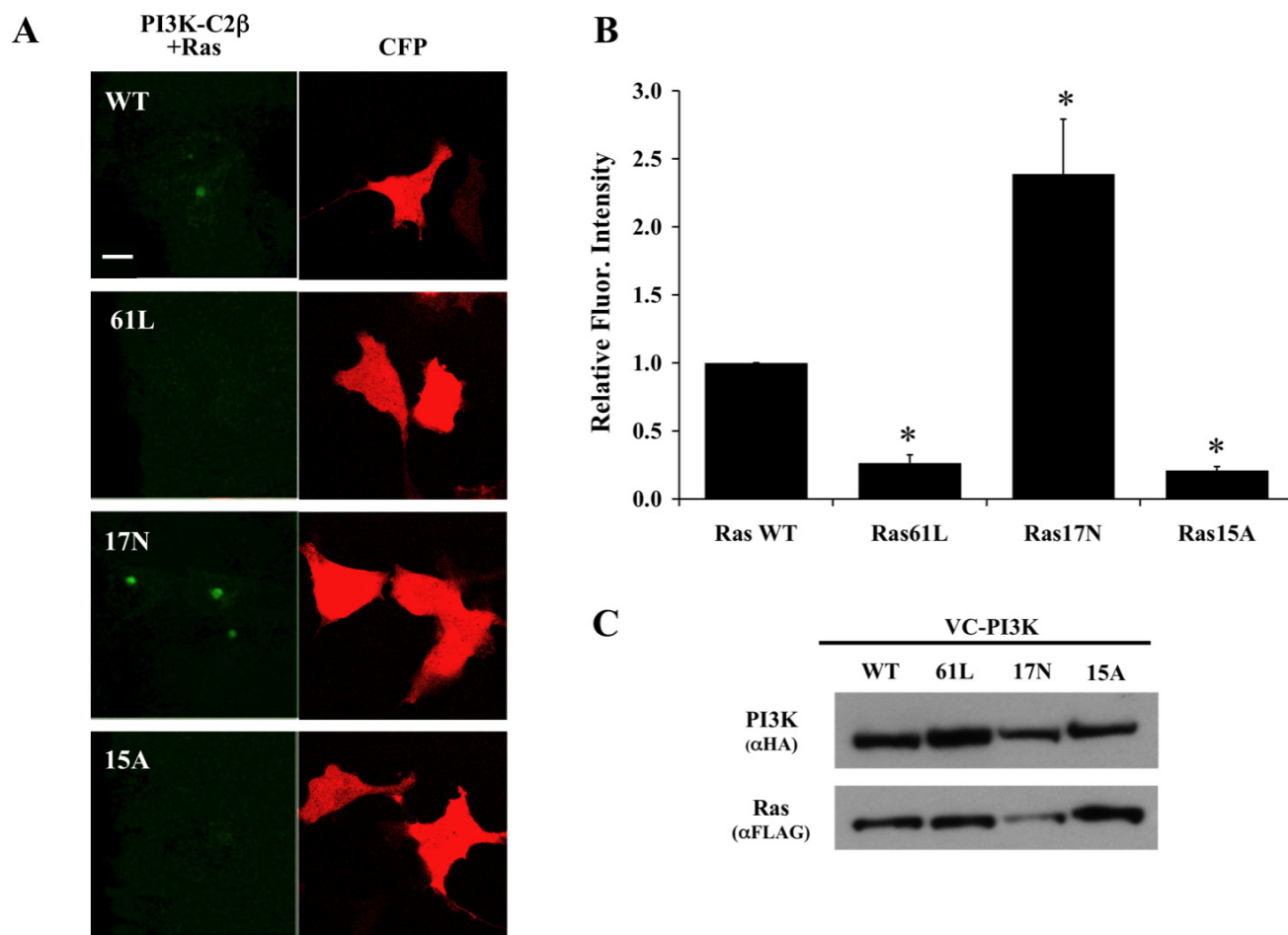
Figure 11. PI3K-C2β does not interact with Ras15A. (A) VC-tagged PI3K-C2β was co-transfected with one of the following VN-tagged Ras constructs: WT, 61L, 17N, or 15A. BiFC signal (green) demonstrates that PI3K-C2 interacted with Ras17N, but has reduced complex formation with the other mutants. CFP (red) was used as a transfection control. The graph represents the average fluorescence intensity per cell ±S.E.M. from at least three independent experiments (49-67 cells were counted for each condition, * p<0.05). **(C)** A Western blot was performed to demonstrate equal expression of all constructs (size bar=20μm).

Ras-PI3K-C2β binding, I examined the interaction of Ras and PI3K-C2β *in vitro* using purified

proteins. Bacterially expressed and purified Ras (aa 1-166) was loaded with nucleotide (GDP or

GTPγS) and tested for interaction with GST, GST-Raf-RBD (aa 1-148), or GST-PI3K-C2β-RBD

(aa 368-463). As expected, GST-Raf-RBD bound preferentially to RasGTPγS(143). In contrast,

neither GST-PI3K-C2β-RBD or GST interacted with nucleotide-bound Ras (Figure12A). To

determine if the PI3K-C2β-RBD interacts with nucleotide-free Ras, I generated nucleotide-free

Ras *in vitro* (see Materials and Methods). GST-PI3K-C2β-RBD directly bound nucleotide-free

Ras, while little binding was observed between GST-Raf-RBD or GST alone (Figure 12B).

Addition of nucleotide (either GDP or GTPγS) to the binding reaction prior to incubation at 30°C

inhibited interaction of Ras and GST-PI3K-C2β-RBD. Addition of GTPγS to the GST-Raf-RBD

binding reaction, however, induced Ras-Raf interaction (Figure 12C). These results indicate that

under these experimental conditions, nucleotide-free Ras is generated and in the presence of

nucleotide, Ras becomes nucleotide loaded. Next, I examined whether nucleotide could compete

off nucleotide-free Ras once bound to the PI3K-C2β-RBD, a function previously observed with

the Ras GEF, CDC25 (191). Addition of 1 mM nucleotide, either GTP or GDP, was not

sufficient to disrupt the complex of nucleotide-free Ras-PI3K-C2β-RBD (Figure 12D). These

results suggest that PI3K-C2β directly interacts with nucleotide-free Ras, conceivably stabilizing

this nucleotide-free intermediate.
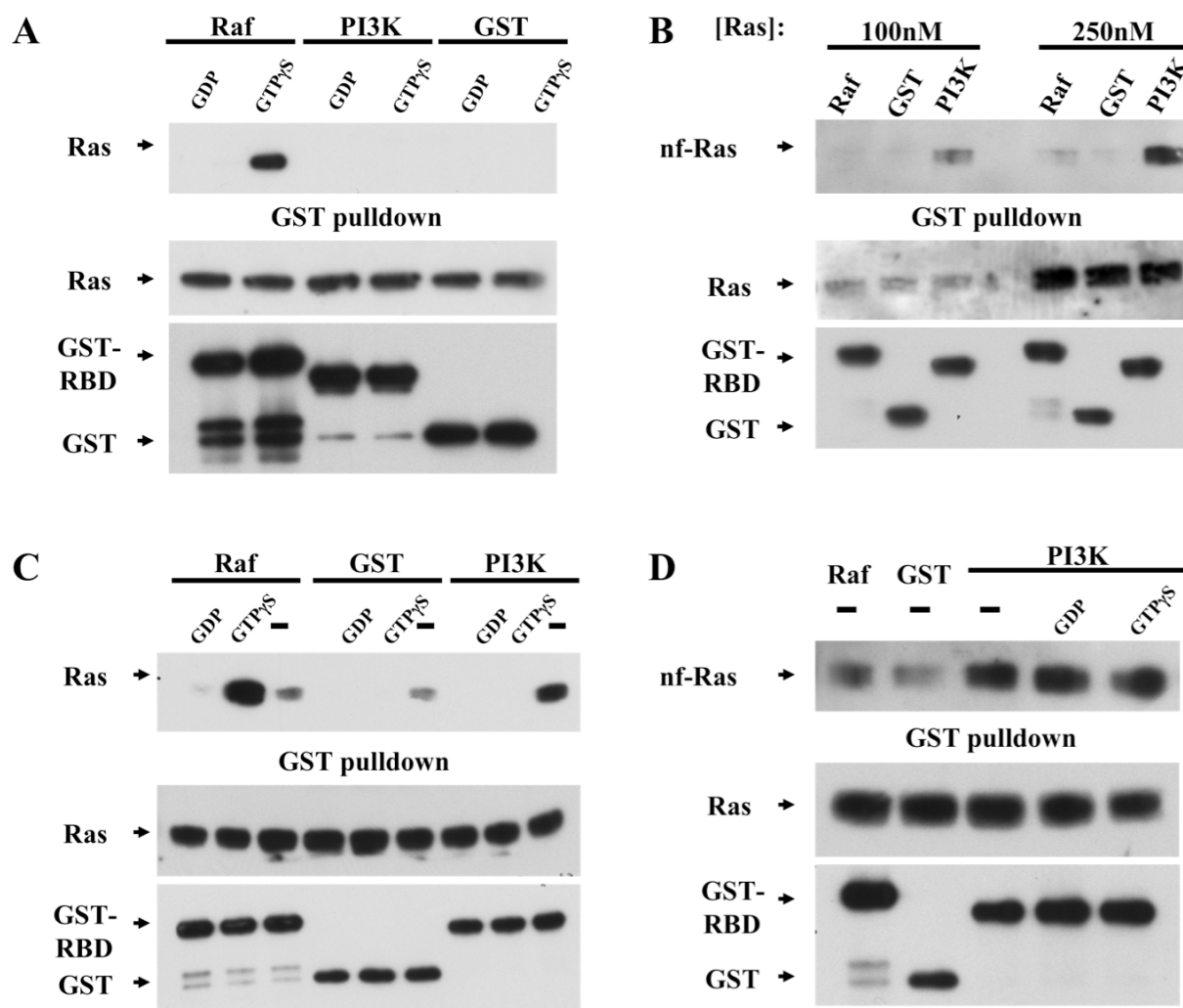
Figure 12. Binding of PI3K-C2β-RBD to Ras. (A) Nucleotide loaded Ras does not directly interact with the RBD of PI3K-C2β. Ras-GDP or Ras-GTPγS were incubated with GST-Raf-RBD, GST-PI3K-C2β-RBD or GST alone as a negative control. Bound proteins were then analyzed by Western blot with a Ras antibody. The RBD of Raf specifically bound Ras-GTPγS. Neither GST-PI3K-C2β-RBD nor GST alone interacted with Ras-GDP or Ras-GTPγS. Top panel, Ras bound to GST proteins. Bottom panels, input amounts of proteins. **(B)** Nucleotide free-Ras was generated *in vitro* as described and then tested for binding to the various GST proteins as in A. GST-PI3K-C2β-RBD directly binds nucleotide free-Ras while little association was seen with the GST-Raf-RBD or GST alone. Panels are same as in A. **(C)** Repeat of **(B)** except nucleotide (1 mM) was present during the binding reaction. Panels are same as in A. **(D)** Addition of nucleotide (1 mM) does not disrupt pre-bound PI3K-C2β-RBD-nucleotide free-Ras. GST-PI3K-C2β-RBD was first bound to nucleotide-free Ras. Following binding, the complex was incubated with 1mM GDP or GTPγS at RT for 30 min and then washed with buffer. Bound proteins were then analyzed as in A.

5. **Nucleotide-free Ras inhibits PI3K-C2β activity.**

Ras17N inhibits PI3K-dependent ITSN activation of AKT (Figure 7), suggesting that nucleotide-free Ras may negatively regulate PI3K-C2β activity. To determine the effect of nucleotide-free Ras on PI3K-C2β activity, *in vitro* PI3K-C2β activity was measured in the presence of nucleotide-free or GTP-loaded Ras (Figure 13). Treatment with LY294002 (10 uM) inhibits *in vitro* PI3K-C2β activity as expected. Interestingly, addition of nucleotide-free Ras, but not RasGTP, dose dependently inhibits PI3K-C2β activity.
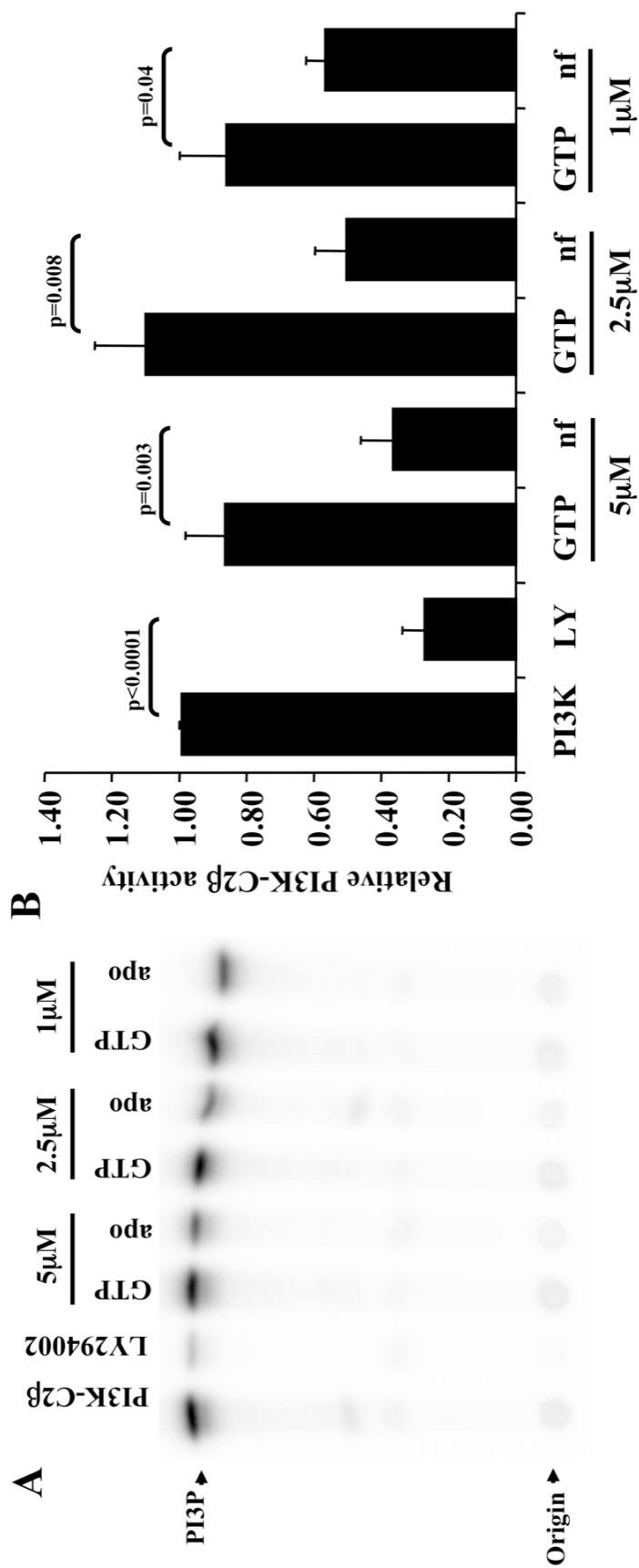
6. **Point mutations in the effector domain of Ras or the RBD of PI3K-C2β disrupt Ras-PI3K-C2β interaction.**

Effectors of activated Ras bind the switch 1 region of Ras (aa 25-45), also termed the effector domain. Point mutations (Thr35Ser, Glu37Gly, and Tyr40Cys) in this region of activated Ras (Ras12V) differentially disrupt the interaction with specific effectors (Figure 14A). I examined whether these mutations in the context of Ras17N/69N disrupted binding to PI3K-C2β. Mutation of Thr35 and Glu37 of Ras17N/69N decreased PI3K-C2β binding whereas Tyr40 mutation had no appreciable effect (Figure 14B-D). These mutations in the context of activated Ras (Ras12V) have a similar effect on interaction with Class I PI3Ks (193) suggesting that PI3K-C2β interacts with the same region of Ras as Class I PI3Ks but only when Ras is nucleotide-free. Conversely, specific mutations in the RBD of Class I PI3Ks (Thr208 of PI3K-C1α) block Ras binding both *in vitro* and *in vivo* (57, 194). Although the RBD of PI3K-C2β shares only 18% identity with PI3K-C1α–RBD, mutation of the analogous Thr in PI3K-C2β to Asp (Thr392 to Asp) resulted in a significant decrease in Ras binding (Figure 14E). Furthermore, mutation of Lys379 in the PI3K-C2β-RBD also resulted in significant impairment in Ras binding. These

Figure 13. Nucleotide free Ras inhibits PI3K-C2β activity. (A) The *in vitro* kinase activity of purified PI3K-C2β was assayed in the presence of nucleotide-free Ras or Ras loaded with GTPγS (5μM, 2.5μM, and 1μM). LY294002 (10μM) was used as a negative control. The radioactive phospholipid products were extracted and eluted on a TLC plate. **(B)** Nucleotide-free (nf) Ras dose dependently inhibits PI3K-C2**β**. LY294002 (10μM) inhibits PI3K-C2β activity while Ras-GTP (5μM, 2.5μM, and 1μM) does not. The graph represents relative kinase activity normalized to PI3K-C2β alone ±S.E.M. from 4-6 independent experiments.

Figure 13. Nucleotide free Ras inhibits PI3K-C2β activity.
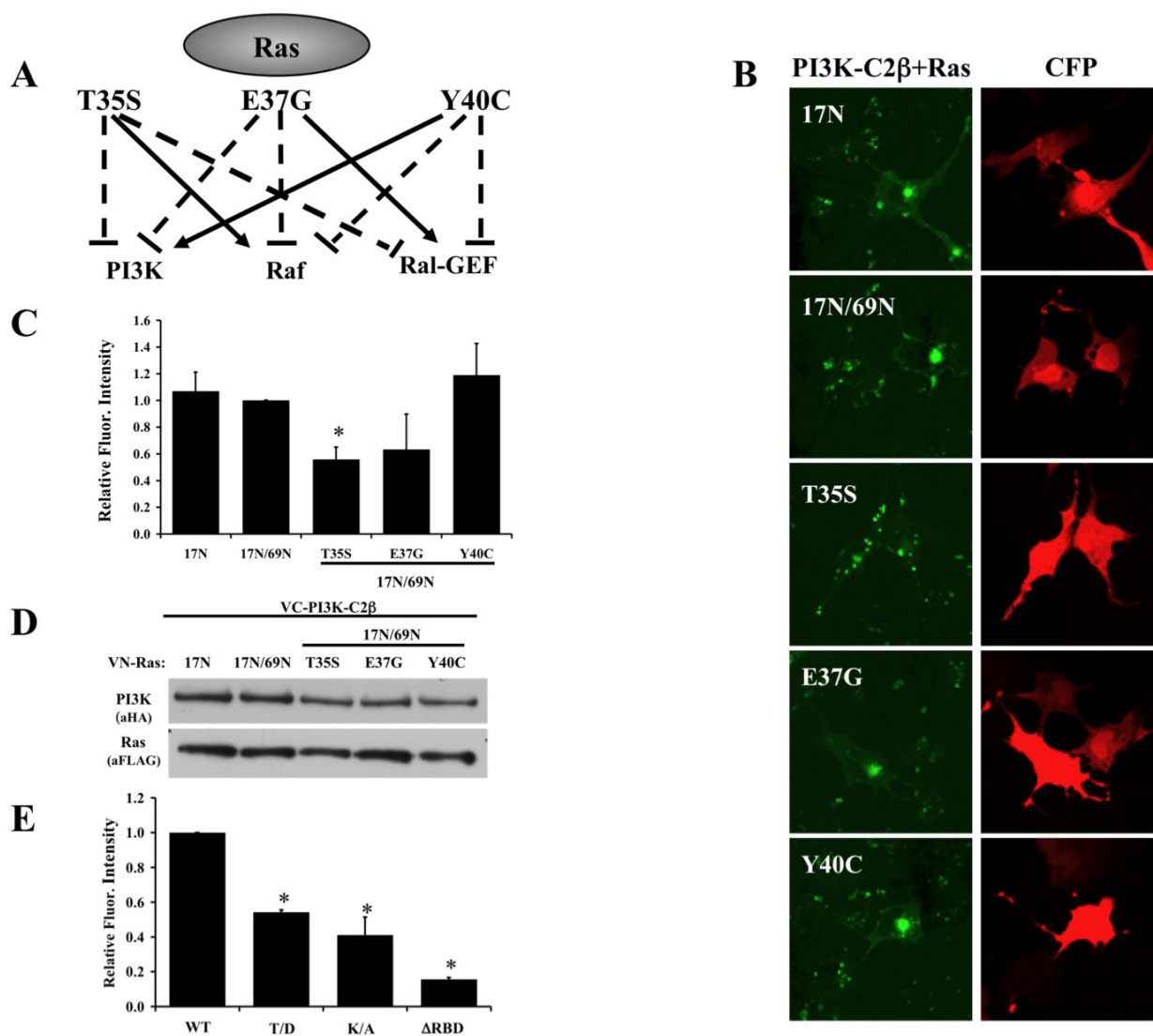
Figure 14. Mutations in the effector region of Ras disrupt PI3K-C2β binding. (A) Point mutations in the effector region of Ras12V disrupt interactions with specific Ras targets. **(B)** VC-tagged PI3K-C2β was co-transfected with VN-tagged Ras17N, 17N/69N, or one the effector mutants in the background of Ras17N/69N to prevent exchange factor interference. BiFC signal is pseudo-colored green. Effector mutations that disrupt Class I PI3K binding to Ras12V disrupt Class II PI3K binding to Ras17N/69N. CFP (red) was used as a transfection control. **(C)** Graph represents the average fluorescence intensity per cell ±S.E.M. from at least three independent experiments (27-51 cells were counted for each condition, *p=0.02). **(D)** Western blot analysis demonstrates equal expression of all constructs. **(E)** Mutation of Thr392 to Asp or Lys379 to Ala in full-length PI3K-C2β disrupts interaction with Ras17N. The ΔRBD mutant was also included as a negative control. Graph represents the average of three independent experiments (52-69 cells were counted for each condition, *p<0.05).

results indicate that the PI3K-C2β-RBD utilizes a similar molecular surface as Class 1 PI3K

RBD in the recognition of the switch 1 region of Ras. However, PI3K-C2β binding is specific to

nucleotide-free Ras and must be mediated by distinct structural differences.

7.   **PI3K-C2β alters Ras signaling.**

Our results predict that the RBD of Raf, but not PI3K-C2β, should block Ras-

dependent signaling. Indeed, expression of the Raf-RBD dramatically decreased Elk-1-

dependent transcription by >80%, whereas PI3K-C2β-RBD expressing cells were not inhibited

(Figure 15A), further supporting the model that the PI3K-C2β-RBD does not interact with Ras-

GTP.

To determine whether the PI3K-C2β-RBD binds targets *in vivo*, I tested the

ability of the PI3K-C2β-RBD to block the inhibitory activity of Ras17N (Figure 15B).

The transforming activity of oncogenic Src (SrcY527F) was reversed in the presence of Ras17N

consistent with the requirement for Ras in Src-mediated transformation (179, 195). Expression of

PI3K-C2β–RBD dose-dependently blocked Ras17N inhibition of Src transformation consistent

with the binding of nucleotide-free Ras by PI3K-C2β-RBD (Figure 11). In contrast, expression

of the Raf-RBD did not affect Ras17N inhibition of Src transformation. However, like Ras17N,

expression of only the Raf-RBD with oncogenic Src inhibited transformation consistent with the

role of active Ras in Src transformation (179, 195) and with the ability of this RBD to bind and

inhibit activated Ras. In contrast, expression of the PI3K-C2β-RBD alone did not inhibit Src

transformation consistent with its inability to bind RasGTP (Figure 14B)

Figure 15. Biological activity of PI3K-C2β-RBD versus Raf-RBD. (A) Expression of the Raf-RBD but not the PI3K-C2β-RBD or GST alone inhibited EGF-stimulation of a Gal-Elk reporter assay . GST-RBDs were expressed equally. GST alone migrated at a faster rate and was not visible in this image. Results represent the average relative activation ±S.E.M. from at least three independent experiments. (*p< 0.05 compared to unstimulated GST, **p<0.01 compared to EGF stimulated GST). **(B)**. PI3K-C2β-RBD dose-dependently inhibits the effect of Ras17N on Src-mediated transformation. NIH/3T3 cells were transfected with 100 ng of SrcY527F expression construct in the presence or absence of Ras17N. Co-expression of the PI3K-C2β-RBD reverses the inhibitory effect of Ras17N on Src transformation whereas the Raf-RBD does not. In contrast, expression of the Raf-RBD alone, but not the PI3K-C2β-RBD, significantly inhibited Src-mediated transformation. The results represent the average relative focus forming activity ±S.E.M. from three independent experiments performed in triplicate. Asterisks denote samples that were significantly different from Src alone (*p<0.05). (Transformation assay performed by Xeurong Wang)

Figure 15. Biological activity of PI3K-C2β-RBD versus Raf-RBD.
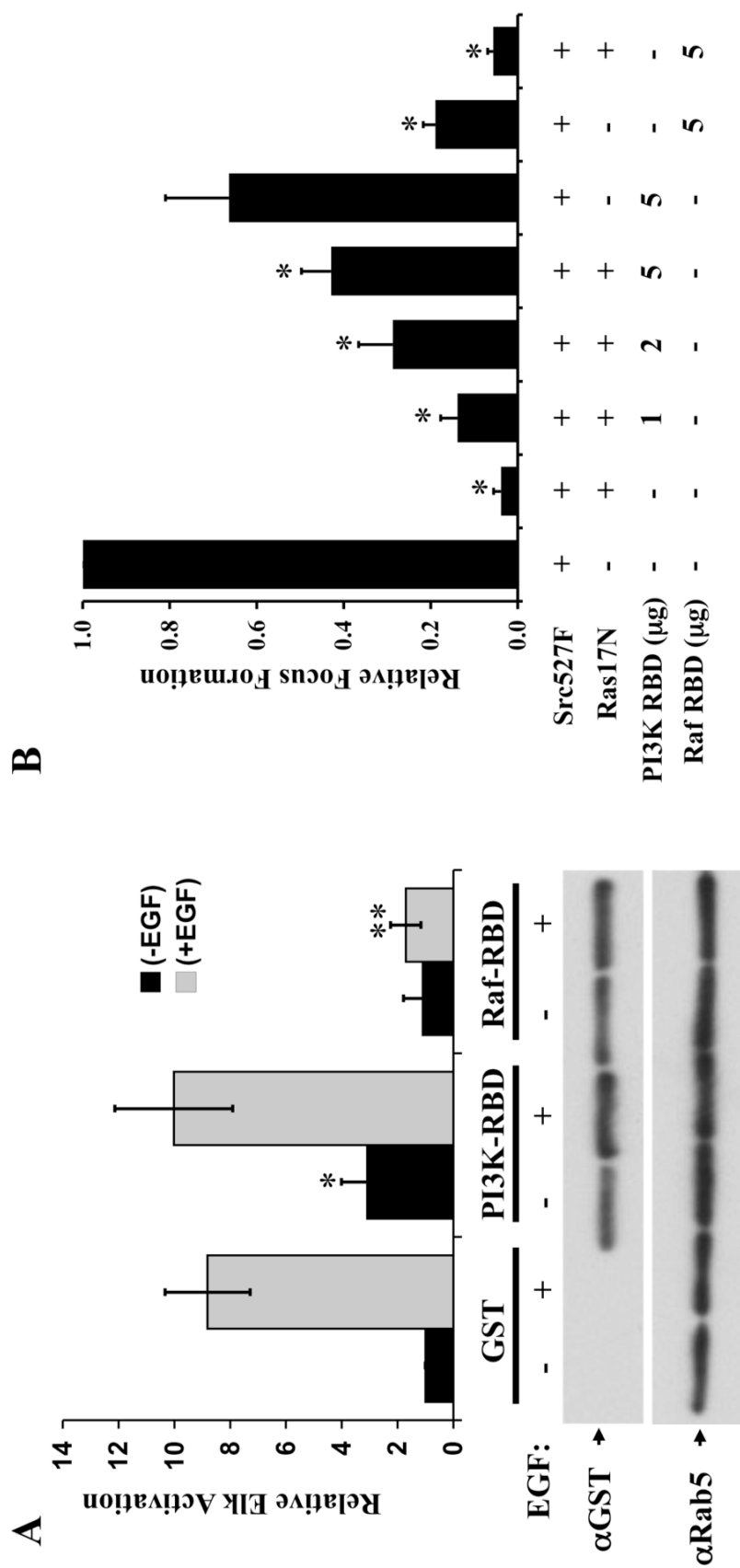
D.   Discussion

Ras, like all GTPases, cycles between an inactive GDP-bound state and an active GTP-bound state. The transition from the inactive to active state requires formation of nucleotide-free Ras through the action of exchange factors. However, this state is considered to be a short-lived intermediate *in vivo* (109) based on the relatively high GTP:GDP ratio *in vivo* , the ability of GTP to dissociate the GEF-Ras complex *in vitro* (191), and the assumption that there are no proteins *in vivo* that might stabilize nucleotide-free Ras and prevent GTP loading. My results provide the first direct evidence for a protein that may stabilize nucleotide-free Ras *in vivo*. I demonstrate that the RBD of PI3K-C2β binds nucleotide-free Ras *in vitro* (Figure 12). In contrast to the GEF-Ras complex, which is disrupted by addition of guanine nucleotides, the PI3K-C2β RBD-Ras complex is stable even in the presence of high concentrations of GTP or GDP. These data suggest that nucleotide-free Ras may exist in cells while complexed with PI3K-C2β. Although current methods do not allow for detection of nucleotide-free GTPases, endogenous Ras and PI3K-C2β co-localize on a subset of intracellular vesicles. Furthermore, our BiFC results provide additional support for our model. PI3K-C2β preferentially interacts with Ras17N, which has a 30-fold lower affinity for nucleotide compared to wild type Ras and therefore should exist for longer periods in the nucleotide-free state. As a result, BiFC traps this form of Ras resulting in greater fluorescence complementation for Ras17N (and Ras17N/69N) compared to wild type or constitutively activated Ras (61L or 12V).

My findings suggest that nucleotide-free Ras may function in regulating cell signaling. Addition of nucleotide-free Ras to PI3K-C2β inhibited its *in vitro* lipid kinase activity compared to addition of RasGTP. This result is consistent with our observation that ITSN activation of AKT is blocked by expression of Ras17N (Figure 7) and dependent on PI3K activity (33). Thus,

PI3K-C2β represents the first identified biochemical target of nucleotide-free Ras and challenges the long held assumption that nucleotide-free Ras is a short-lived intermediate *in vivo* in the generation of RasGTP (109).

Based on these observations, I propose that once nucleotide-free Ras is generated through GEF-stimulated nucleotide release, PI3K-C2β binding displaces the GEF and stabilizes nucleotide-free Ras. This interaction has two consequences: inhibition of GTP loading on Ras bound to PI3K-C2β and inhibition of PI3K-C2β lipid kinase activity. Such a mechanism might allow for the transport of nucleotide-free Ras to distal cellular compartments where PI3K-C2β dissociates from Ras upon stimulation, *e.g.*, ITSN binding to PI3K-C2β. This dissociation of nucleotide-free Ras would then lead to immediate GTP loading, i.e., Ras activation and derepression of PI3K-C2β (Figure 16). Indeed, the PI3K-C2β RBD-Ras complex is refractory to dissociation by high nucleotide concentrations (Figure 12D), and ITSN activates both Ras and PI3K-C2β (1, 33). Furthermore, RasGTP could then couple to Class 1 PI3Ks (or other Ras effectors) resulting in simultaneous activation of both Class I and Class II PI3K isoforms. While there are clear examples for compartmentalized activation of Ras at intracellular sites by specific GEFs (196), my model suggests a GEF-independent activation of Ras at such intracellular sites. Such a mechanism would decouple GEF localization from GTPase activation while allowing for integration of multiple signaling pathways.

It has been long accepted that Class I PI3Ks are effectors of Ras-GTP. However, our data demonstrates for the first time a role for Ras in Class II PI3K regulation. Point mutations in the effector region of Ras that disrupt Class I PI3K binding also disrupt Class II PI3K binding. In addition, concomitant point mutations in the RBDs of Class I and Class II PI3Ks disrupt Ras association (Figure 14). Taken together these data suggest that Class I and Class II PI3Ks
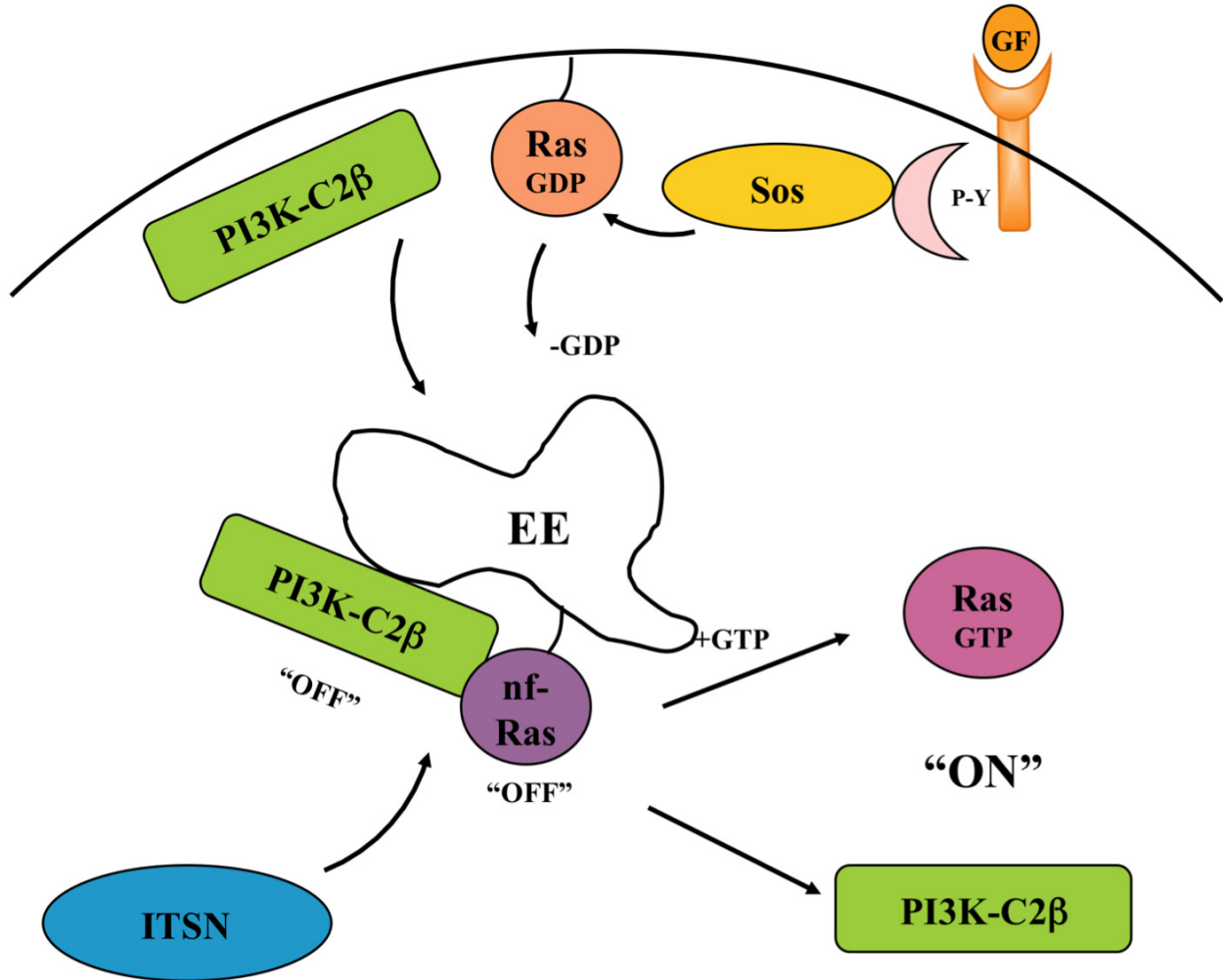
Figure 16. Model for ITSN1-Ras-PI3K-C2β pathway. Growth factor stimulation of receptor tyrosine kinase leads to the recruitment of the Grb2-Sos complex resulting in dissociation of GDP from Ras. We propose that PI3K-C2β binds to this transient apo-Ras trapping it in the nucleotide free state. The PI3K-C2β-nucleotide-free-Ras complex then translocates to distal sites such as early endosomes (EE) where ITSN1 then binds to PI3K-C2β leading to the release of nucleotide-free Ras. Once released from the ITSN-PI3K-C2β complex, Ras binds GTP resulting in its activation. The dissociation of nucleotide-free Ras from PI3K-C2β also leads to activation of its lipid kinase activity.

associate with the same region of Ras, but that this association is mediated by distinct structural differences in the PI3Ks as well as Ras-GTP vs nucleotide-free Ras.

While RasGDP and RasGTP have been thought of as the predominant forms of Ras involved in cell signaling, our results suggest that nucleotide-free Ras may also play a role in cell signaling. These results demonstrate that *in vitro* nucleotide-free Ras is stabilized by the binding of PI3K-C2β. In addition, nucleotide-free Ras inhibits the lipid kinase activity of PI3K-C2β. My findings suggest a novel model for compartmentalized signaling in which PI3K-C2β results in the redistribution of Ras to intracellular vesicles leading to localized Ras activation and engagement of specific Ras effectors resulting compartmentalized activation of these targets.

III. COMPARTMENTALIZED SIGNALING: THE RIGHT PLACE AT THE RIGHT TIME

A.   Introduction

Membrane trafficking is an important component to intracellular signaling. One way that cells ensure signal specificity is compartmentalization. Sorting and restriction of proteins to specialized compartments limits the pool of possible interacting partners to which a molecule is exposed. In general, receptors are stimulated at the PM, the activated receptor recruits effectors, and the complex is internalized. There is evidence that internalization does not only terminate signaling, but is also necessary for full activation of these signaling cascades. Additionally, signaling occurs on these endomembrane compartments, thus creating compartmentalized signaling.

B.   Compartmentalized Signaling

EGFR stimulation leads to cell proliferation, adhesion, survival, and migration. Upon ligand stimulation the EGFR dimerizes and becomes autophosphorylated. These p-Tyr sites recruit multiple proteins with SH2 and phosphotyrosine binding domains (PTB), including Grb2 and SHC. Organization of the EGFR and downstream effectors is necessary to differentially activate signaling pathways. One way this is accomplished is signaling from endomembrane compartments. After dimerization and phosphorylation, the EGFR is internalized into endosomes. This internalization and entry in to the endocytic pathway is termed down-regulation, although there is evidence that the EGFR continues to signal from these compartments. Indeed inhibition of EGFR endocytosis differentially affects activation of

downstream effectors. ERK1/2 and the p85 adaptor subunit of PI3K have decreased

phosphorylation while the phosphorylation of SHC and PLC-γ are increased under conditions

where EGFR endocytosis is blocked (197). Evidence from our lab demonstrates that transient

ITSN silencing decreases EGFR internalization and activation of ERK and Elk-1 (31). These

data suggest that internalization and trafficking of the EGFR is necessary for full activation of

downstream signaling pathways. Isolated vesicles have been shown to be positive for kinase

active, phosphorylated EGFR (p-EGFR) (198-200). Active EGFR is also found on isolated

endosomes from rat livers long after stimulation and removal from the PM (201). Additionally,

internalized EGFR remains bound to its ligand, EGF, suggesting that after internalization p-

EGFR can still recruit effectors(202). Indeed a complex of p-EGFR, SHC, Grb2, and Sos have

been found on endosomes and this complex is able to activate Raf (201). The Ras GAP, p120 is

also internalized with activated EGFR and remains bound to EGFR on endosomes (203).

Additionally, endosomal localized EGFR can activate Ras, ERK1/2, and AKT with little effects

on PLC-γ, suggesting that endosomes are platforms facilitating specific signaling pathways

(204). The internalization and trafficking of the EGFR to these various endosomal compartments

is mediated by the Rab family of GTPases.

C.   <u>Rabs</u>

The Rab family is the largest member of the Ras superfamily of GTPases with over 60

members in humans (205). Rabs insure that specific cargo is trafficked between distinct

intracellular organelles and compartments. Rabs are necessary in order to maintain the

appropriate membrane components of these compartments. Rabs are involved at every level of

membrane trafficking including, vesicle budding, tethering, fusion, and delivery (205). In order

for Rab proteins to exert spatial and temporal control over vesicle trafficking, they cycle between an inactive GDP and an active GTP bound state. The loading of GTP is facilitated by guanine nucleotide exchange factors (GEFs) while GTPase activating proteins (GAPs) increase the intrinsic GTPase activity of the Rabs to increase the rate of GTP hydrolysis back to GDP (206). Once activated, the Rab proteins recruit effectors allowing for downstream signaling to occur (207). Two major components of the Rab trafficking pathway are Rab5 and Rab7 that mediate early and late endosomal trafficking, respectively (Figure 17).

1.   <u>Rab5</u>

Rab5, one of the most studied Rab proteins, regulates the early endocytic pathway and is involved in formation of clathrin-coated pits, clathrin-coated vesicle (CCV) to early endosome (EE) fusion, EE to EE fusion, and endosome motility (205, 208-210). Early endosomes coordinate plasma membrane and intracellular transport and are an important step in receptor sorting. The current model for Rab5 signal transduction is the formation of a Rab5 signalosome, which is a localized signaling complex driven by activated Rab5 (205). The formation of the signaling complex begins when Rab5-GDP is converted to Rab5-GTP by a GEF, such as Rabex5 (211). In the cytosol, Rabex5 is in a complex with the Rab5 effector Rabaptin5 (211). Rab5-GTP recruits the Rabex5:Rabaptin5 complex creating a positive feedback loop and allowing for a localized increase in Rab5-GTP (212). The activated Rab5 can then recruit other effectors such as the Class III PI3K, hVPS34, leading to an increase in PI_3P (213). Once PI_3P is generated proteins with PI_3P binding domains, such as the FYVE (<u>F</u>ab1p, <u>Y</u>OTB, <u>V</u>ac1p, <u>E</u>arly Endosome Antigen1) domain of early endosomal antigen 1 (EEA1), are recruited
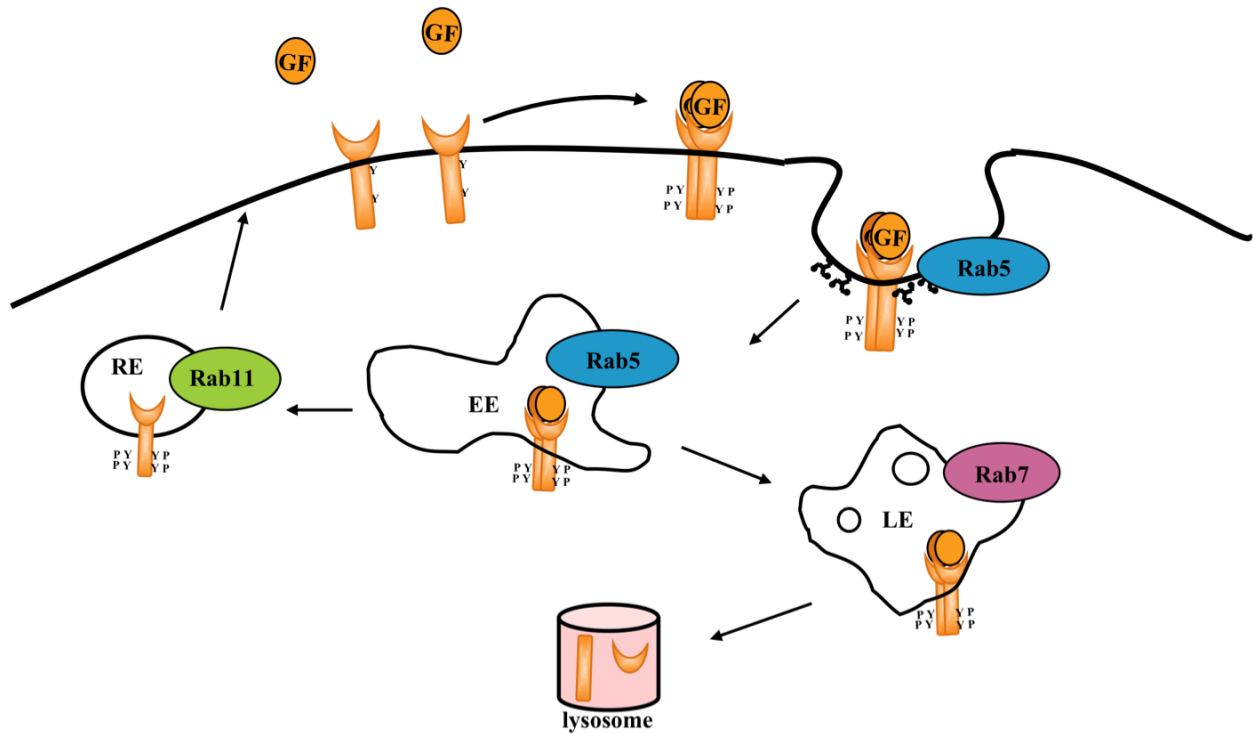
Figure 17. Rab mediated trafficking. Upon growth factor stimulation RTKs dimerize, autophosphorylate, and are internalized in clathrin coated pits. Rab5 mediates internalization and early endosomal fusion. From this point the receptor can be trafficked from to a late endosomal compartment and on to the lysosome, mediate by Rab7, or recycled back to the PM, mediated by Rab11.

to endosomes (214). Currently, only one Rab5 signalsome consisting of Rab5, Rabex5, Rabaptin5, hVPS34, and EEA1 has been well characterized, but data suggests that the system is much more complex. Over 20 proteins from bovine brain that bind activated Rab5 have been identified by affinity chromatography, and analysis has also shown that there is a large pool of Rab5:Rabaptin5 that interacts independently of EEA1 suggesting the formation of a Rab5 signaling complex independent of EEA1 (215, 216).

2.   **Rab7**

Rab7 regulates late endosomal trafficking and lysosomal degradation (217). Rab7 has been shown to play a role in the conversion of EE to late endosomes (LE) as well as the transport of cargo from LE/multi-vesicular bodies (MVBs) to the lysosome (218). The specific control of Rab7 over later trafficking events is evident in experiments utilizing dominant negative Rab7 (Rab7 DN). Over expression of Rab7 DN does not disrupt internalization, trafficking of vesicles to EEA1-positive EE, or recycling (219-222). The Rab7 DN defect is specifically observed as an accumulation of cargo in LE/MVBs and decrease in cargo degradation (220-222); conversely, expression of constitutively active Rab7 increases transport of cargo to the lysosome (223, 224). Additionally, silencing of Rab7 resulted in a defect in EGFR degradation, but not in transferrin recycling or transition of EGFR from the PM to EE; indicating that Rab7 mediates degradation, not transition from EE to LE (220). Rab7 activity is also necessary for maintenance of the lysosome. Rab7 silencing or expression of Rab7 DN disrupts the structure of the lysosome and causes the mis-localization of lysosomal cargo(210). In cells, activated Rab7 recruits RILP (Rab-interacting lysosomal protein) (225). RILP then

complexes with the ESCRT-II (Endosomal Sorting Complex Required for Transport II) to sort cargo in multi-vesicular bodies (226).

D.   ITSN's Role in Rab Trafficking

ITSN has been linked to the Rab trafficking pathway through a high-throughput yeast two-hybrid screen that identified Rabaptin5 and KIF16B as ITSN interacting partners (48). In addition, ITSN enhances the ubiquitylation and degradation of the EGFR (31). Interestingly, transient silencing of ITSN inhibits internalization of the EGFR and downstream signaling from the receptor (31). These data indicate that ligand stimulation and trafficking of the receptor are necessary for full activation of downstream signaling. Interestingly, PI3K activity is also necessary for proper targeting of intracellular vesicles(227-229), and ITSN activates PI3K-C2β suggesting a role for this Class of PI3K in vesicle trafficking (33).

E.   PI3Ks Regulate Membrane Trafficking

Phosphoinositides (PIs) are necessary for proper targeting and fusion of intracellular vesicles. PI3Ks generate 3' phosphorylated PIs. The production of these lipids on intracelluar compartments is an important component to compartmentalized signaling. These PI's recruit proteins with lipid binding domains. Three major lipid binding domains include PH, FYVE, and PX domains. PH domains recognize $PI_{3,4}P_2$ and $PI_{3,4,5}P_3$, FYVE domains bind PI_3P, and PX domains recognize PI_3P and $PI_{3,4}P_2$ (230). These domains have been used to show that $PI_{3,4,5}P_3$ is produced transiently in response to stimulation while PI_3P is constitutively present on intracellular vesicles (231). A typical example of lipid recruitment is the activation of AKT at the plasma membrane. Class I PI3Ks generate $PI_{3,4,5}P_3$ at the plasma membrane to recruit the PH

domain of AKT. Here AKT is then dually phosphorylated to become fully activated. Another example is the recruitment of KIF16B to PI$_3$P on early endosomes through its PX domain (232). Studies in yeast first revealed a role for PI3K in trafficking. Yeast protein VPS34 is homologus to mammalian Class III PI3K, and is required for sorting into the vacuole in yeast (233). Vps34 and Class I PI3K, p110β, directly interact with Rab5-GTPγS (213). Additionally, p85α serves as a GAP for Rab5 decreasing levels of activated Rab5 (234). Rab7 also forms a complex with Vps34 that produces PI$_3$P on late endosomes (235), and Vps34 activity is necessary for formation of multivesicular bodies (228). However, a large portion of the data supporting the role of PI3K in receptor trafficking utilizes PI3K inhibitors that block activity of all classes of PI3K. Indeed, wortmannin and LY294002 block endosomal fusion (227, 229, 236). Recent evidence suggests that Class II PI3Ks might also participate in receptor trafficking. The Class II PI3Ks are recruited to EGFR after stimulation, are localized to intracellular vesicles, and are activated by clathrin (65, 72, 237, 238).

IV. INTERSECTIN IS LOCATED ON MULTIPLE INTRACELLULAR COMAPARMENTS

A. <u>Introduction</u>

Subcellular trafficking and localization of receptors and signaling complexes is important to understanding spatio-temporal cell signaling. ITSN was originally discovered as a regulator of endocytosis, but recent evidence has demonstrated a role for ITSN as a platform for intracellular signaling. ITSN increases the ubiquitylation of the EGFR and has been linked to the trafficking of the EGFR through a yeast-two-hybrid screen that identified Rabaptin5 and KIF16B as potential ITSN binding partners. These two proteins are essential regulators of EGFR trafficking through interaction with the monomeric G-protein Rab5 (211, 212, 232, 239). These data lead me to examine whether distinct pools of ITSN localized to early and late endosomes may exist. In addition, ITSN regulates PI3K-C2β and given the importance of PI3K activity in membrane trafficking, I also examined whether PI3K-C2β might associate with Rab family members in a compartmentalized-specific manner.

B. <u>Materials and Methods</u>

1. <u>Cell lines, Transfection and Reagents</u>

COS cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. COS Cells were transfected with Lipofectamine (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. HeLa cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum.

HeLa Cells were transfected with Lipofectamine2000 (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. Antibodies used include anti-hemagglutinin (HA) (Covance, Emeryville, CA), anti-FlagM2 (Sigma, St. Louis, Missouri), anti-Rab5 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-EEA1 (Santa Cruz Biotechnology, Santa Cruz, CA). The fluorescent secondary antibodies conjugated to FITC, TRITC, and Cy5 used were from Jackson ImmunoResearch, (West Grove, PA.)

2.   DNA Constructs

The BiFC vectors pFlag-VN-173N and pHA-VC155N were gifts from Dr. Chang-Deng Hu (Purdue University, West Lafayette, IN). The Rab constructs were gifts from Dr. Richard Pagano (Mayo Clinic, Rochester, MN)).

3.   Western Blot

Cells were treated and analyzed by Western blot as described (31).

4.   Immunofluorescene and Imaging

Confocal and imaging was performed as described (186). Briefly, cells were fixed in 3.7% formaldehyde and imaged on a Zeiss LSM 510 confocal microscope. For immunofluoresecne, after fixing cells were blocked (1xPBS, 3% BSA, 0.1% Tx-100) for 1hr. Primary antibody was added for 2hrs at room temperature, rinsed 3x with PBS. Secondary antibody was added for 1hr at room temperature and rinsed 3x with PBS.

5.   Calculation of Co-localization

The Zeiss LSM 510 co-localization tool was used to calculate the co-localization coefficients. The co-localization coefficient is the number of co-localized pixels in channel 1 or 2 compared to the total number of pixels above background in that channel. A coefficient of 0 is no co-localization and 1 is complete co-localization. Coefficients were converted to percentages by multiplying by 100.

C.   Results

1.   ITSN is localized to an early endosomal compartment.

Although ITSN was initially characterized as an endocytic adaptor protein, data suggest that ITSN may also function in the trafficking of receptors (48) (31, 240). Rabaptin5 is a Rab5 effector that was identified as an ITSN binding partner in the yeast two-hybrid screen(48). To verify this interaction, I immunostained cells for endogenous ITSN and myc-Rabaptin5. Unfortunately, the antibodies for endogenous Rabaptin5 are not suited for immunostaining. Indeed the two proteins co-localize on intracellular vesicles (Figure 18). To determine if there are distinct pools of ITSN that interact with components of the Rab trafficking pathway, I examined the interaction of ITSN with components of the Rab pathway using immunofluorescence and bimolecular fluorescence complementation (BiFC) approaches. First I examined the localization of endogenous ITSN with Rab5 and Rab7 (Figure 19, 20). I observed significant co-localization of endogenous ITSN with Rab5 and Rab7 (Figure 19, 20). Using imaging software (see Materials and Methods) I calculated the percent of co-localized pixels in each channel. Approximately 20% of endogenous ITSN and Rab5 co-localize together (Table III). Using BiFC, I demonstrate that ITSN and Rab5 physically interact on vesicles as well. The
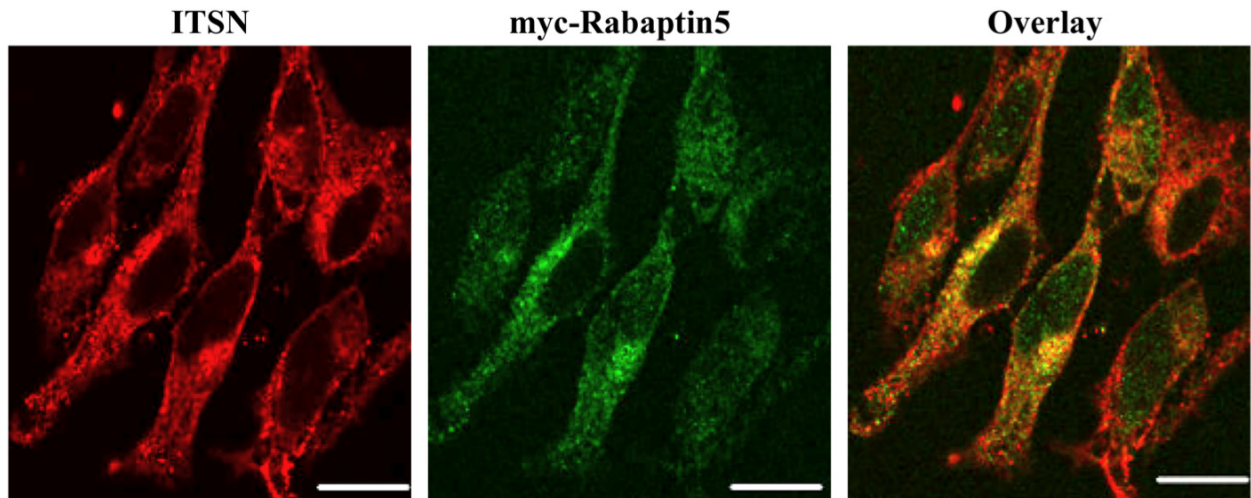
Figure 18. myc-Rabaptin and endogenous ITSN1 co-localize. HeLa cells were immunostained for myc-Rabaptin5 (green) and endogenous ITSN (red). Myc-Rabaptin5 and ITSN co-localize (yellow) (48). (size bar=20μm)
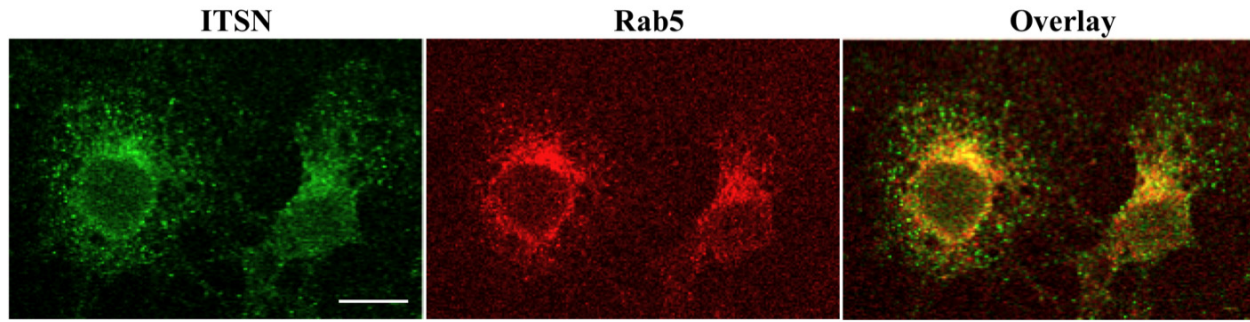
Figure 19. Endogenous Rab5 and endogenous ITSN co-localize. COS cells immunostained for endogenous ITSN (green) and Rab5 red). Significant co-localization was seen in the perinuclear region (yellow). (size bar=20μm)
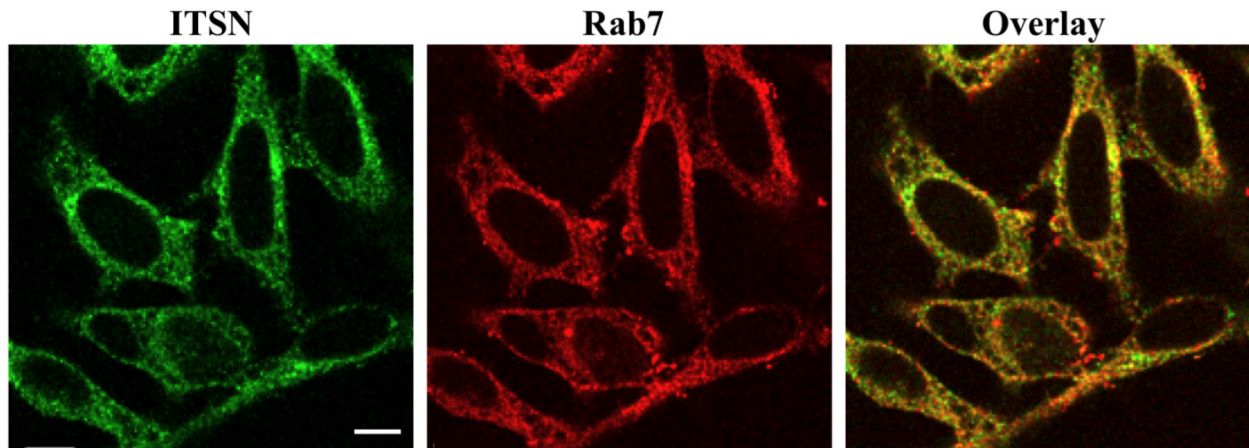
Figure 20. Endogenous Rab7 and endogenous ITSN co-localize. HeLa cells were immunostained for endogenous ITSN (green) and Rab7 (red). ITSN and Rab7 co-localize (yellow). (size bar=20µm)

TABLE III. PERCENT CO-LOCALIZATION

Ch1	Ch2	Percent of Ch1 co-localized with Ch2[a]	Percent of Ch2 co-localized with Ch1	Cells counted
ITSN	Rab5	21	16	24
ITSN	Rab7	29	60	43
VC-ITSN+VN-Rab5	EEA1	42	51	41
VC-PI3K-C2β+VN-Rab5	EEA1	74	8	33
VC-PI3K-C2β+VN-ITSN	EEA1	80	1	75
VC-PI3K-C2β+VN-Ras	ITSN	51	40	61
VC-PI3K-C2β+VN-ITSN	Ras	85	8	37

[a]The percent of co-localized pixels were calculated as described in the Materials and Methods.

Channel 1 (Ch1): Endogenous ITSN was indirectly labeled with FITC conjugated secondary antibody. VN and VC tagged proteins result in Venus fluorescence.

Channel 2 (Ch2): Endogenous Rab5 and Rab7 were indirectly labeled with TRITC conjugated secondary antibody. Endogenous EEA1 was indirectly labeled with Cy5 conjugated secondary antibody. Ras and ITSN were tagged with Cerulean.

BiFC signal between ITSN and Rab5 is greatly reduced when co-expressing a dominant-negative mutant of Rab5 (S34N) suggesting that ITSN interacts with Rab5 when it is GTP-bound (Figure 21). This is consistent with the localization of Rabaptin5 with ITSN and the role of Rabaptin5 in Rab5 activation (211, 212). Interestingly, ITSN and Rab7 association is significantly less compared to Rab5 (Figure 21). Although the endogenous proteins co-localize, the BiFC suggests that ITSN and Rab7 are not in complex. EEA1 is a common Rab5 effector and often used as a marker for early endosomes. Indeed, about 40% of the ITSN:Rab5 complex co-localizes with EEA1 (Figure 21D, Table III). Thus, ITSN interacts with Rab5 and co-localizes with two Rab5 effector proteins on early endosomes.

2.   PI3K-C2β localizes to an early endosomal compartment.

Given the interaction between ITSN and PI3K-C2β (33) and the association of PI3K-C2β with vesicles (72, 91), I next tested whether PI3K-C2β interacts with Rab GTPases. PI3K-C2β interacts with Rab5 WT on intracellular vesicles; however, Rab5 S34N shows reduced interaction suggesting that PI3K-C2β associates with activated Rab5 (Figure 22). PI3K-C2β also forms a complex with Rab7. Interestingly, very little EEA1 co-localizes with the PI3K-C2β:Rab5 BiFC complex (Figure 22D, Table III) suggesting that PI3K-C2β is not producing PI$_3$P on this population of vesicles.

3.   EEA1 is not recruited to ITSN:PI3K-C2β positive vesicles.

I next examined the population of vesicles positive for ITSN and PI3K-C2β (33). VN-ITSN and VC-PI3K-C2β were co-transfected into COS cells and then immunostained for endogenous EEA1. Interestingly, a low percentage of EEA1 co-localizes with the
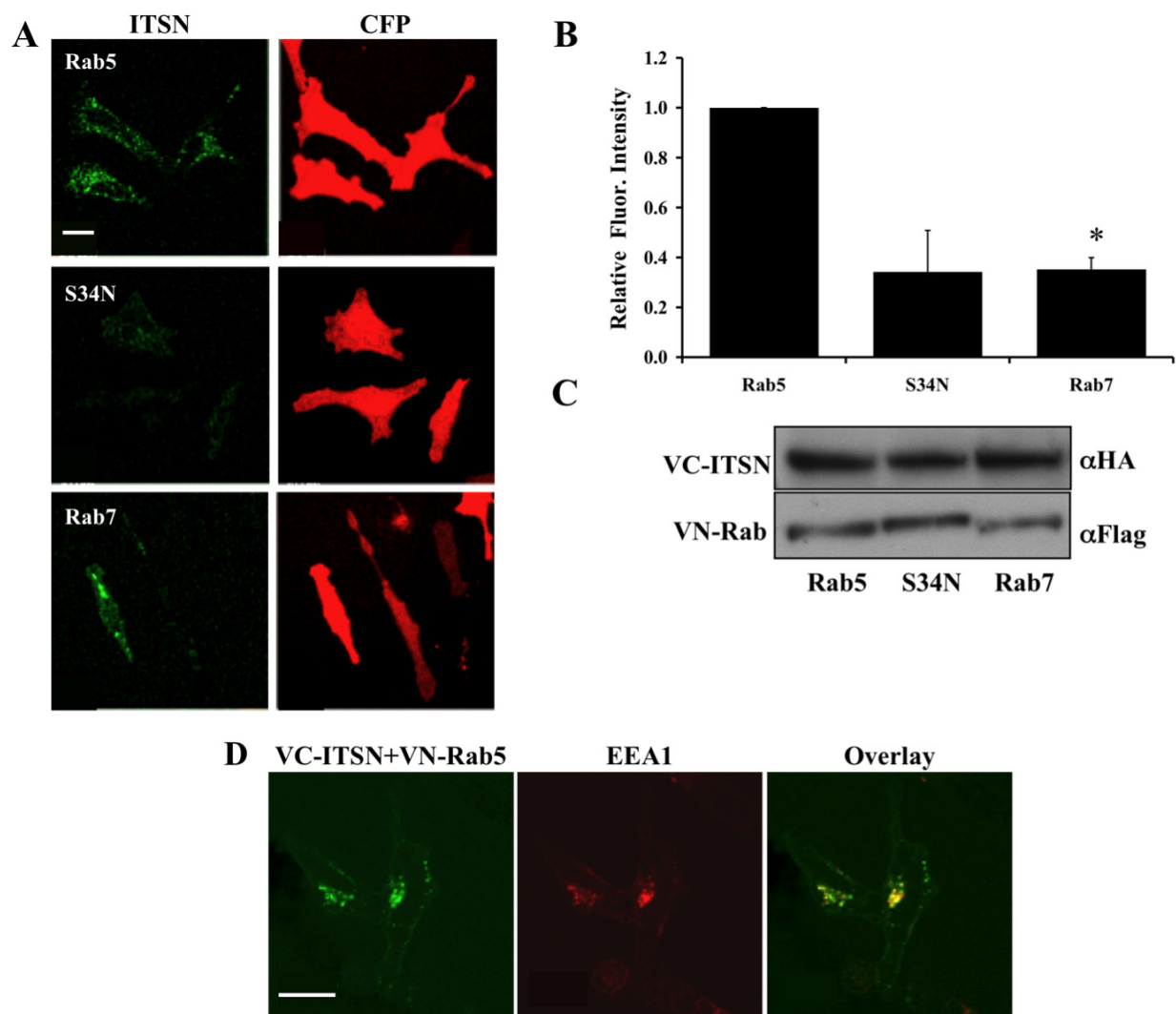
Figure 21. A pool of ITSN is on early endosomes. (A). VC-ITSN was co-transfected with VN-Rab5, VN-Rab5 S34N, or VN-Rab7 with CFP (red) as a transfection control. VN-Rab5 and VC-ITSN form a BiFC complex (green) while VN-Rab5 S34N and VN-Rab7 resulted in a decrease in BiFC signal with VC-ITSN. **(B).** The graph represents the average fluorescence intensity per cell ±S.E.M. from two independent experiments (39-48 cells were counted for each condition, * p<0.05) **(C)** Western blot analysis demonstrates equivalent expression of all constructs. **(D)** HeLa cells transfected with VN-Rab5 and VC-ITSN (green) were immunostained for EEA1 (red). Co-localization between the VN-Rab5/VC-ITSN BiFC complex and EEA1 shows the the BiFC complex is localized to functional early endosomes (48). (size bar=20μm)
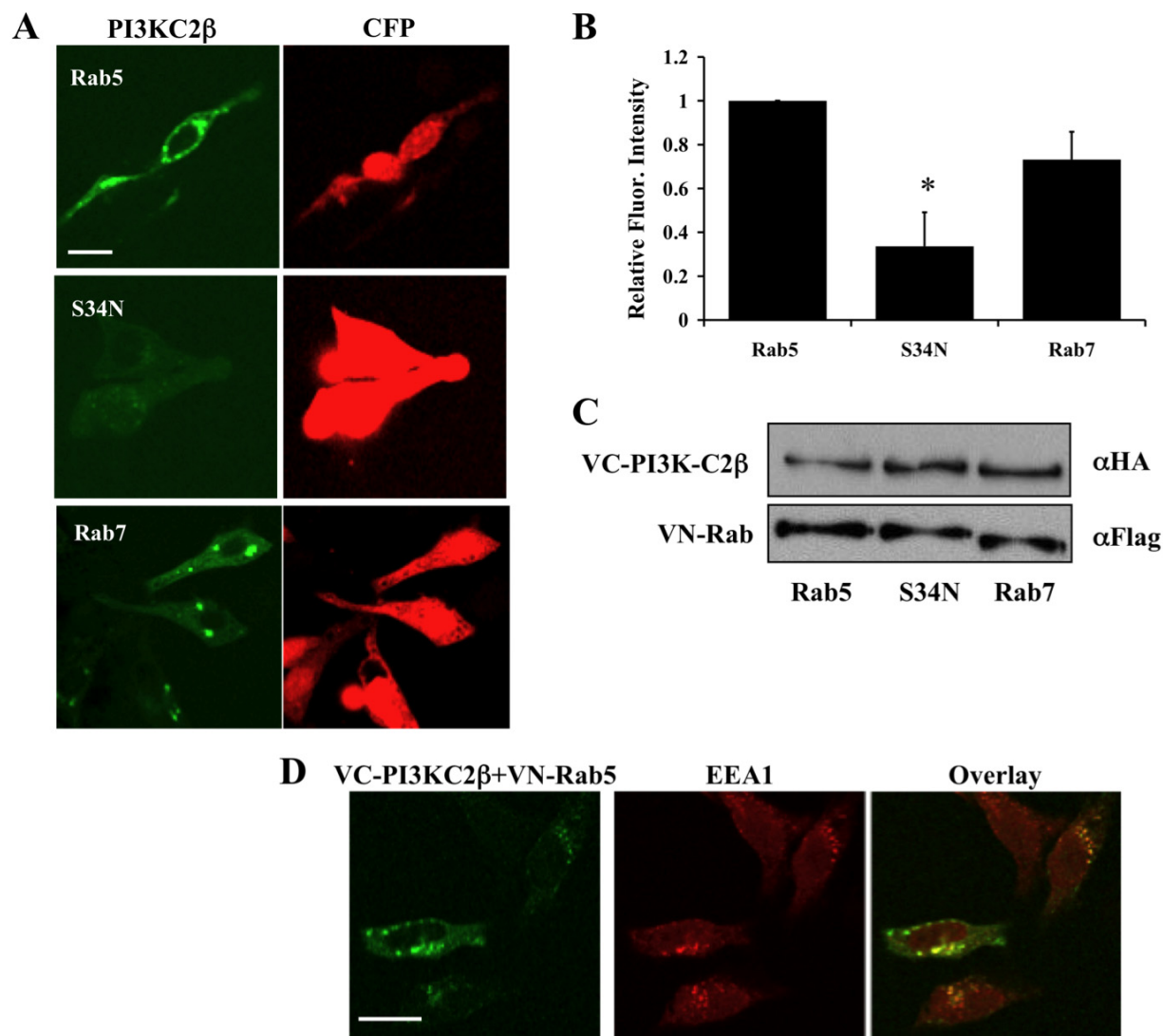
Figure 22. PI3K-C2β localizes with early and late endosomes. (A). VC-PI3K-C2β was co-transfected with VN-Rab5, VN-Rab5 S34N, or VN-Rab7 with CFP (red) as a transfection control. VC-PI3K-C2β form sa BiFC complex (green) with VN-Rab5 and VN-Rab7 while VN-Rab5 S34N resulted in a decrease in BiFC signal. **(B).** The graph represents the average fluorescence intensity per cell ±S.E.M. from at least three independent experiments (33-39 cells were counted for each condition, * p< 0.01) **(C)** Western blot analysis demonstrates equivalent expression of all constructs. (**D)** HeLa cells transfected with VN-Rab5 and VC-PI3K-C2β (green) were immunostained for EEA1 (red). The overlay shows low levels of co-localization (yellow). (size bar=20μm)
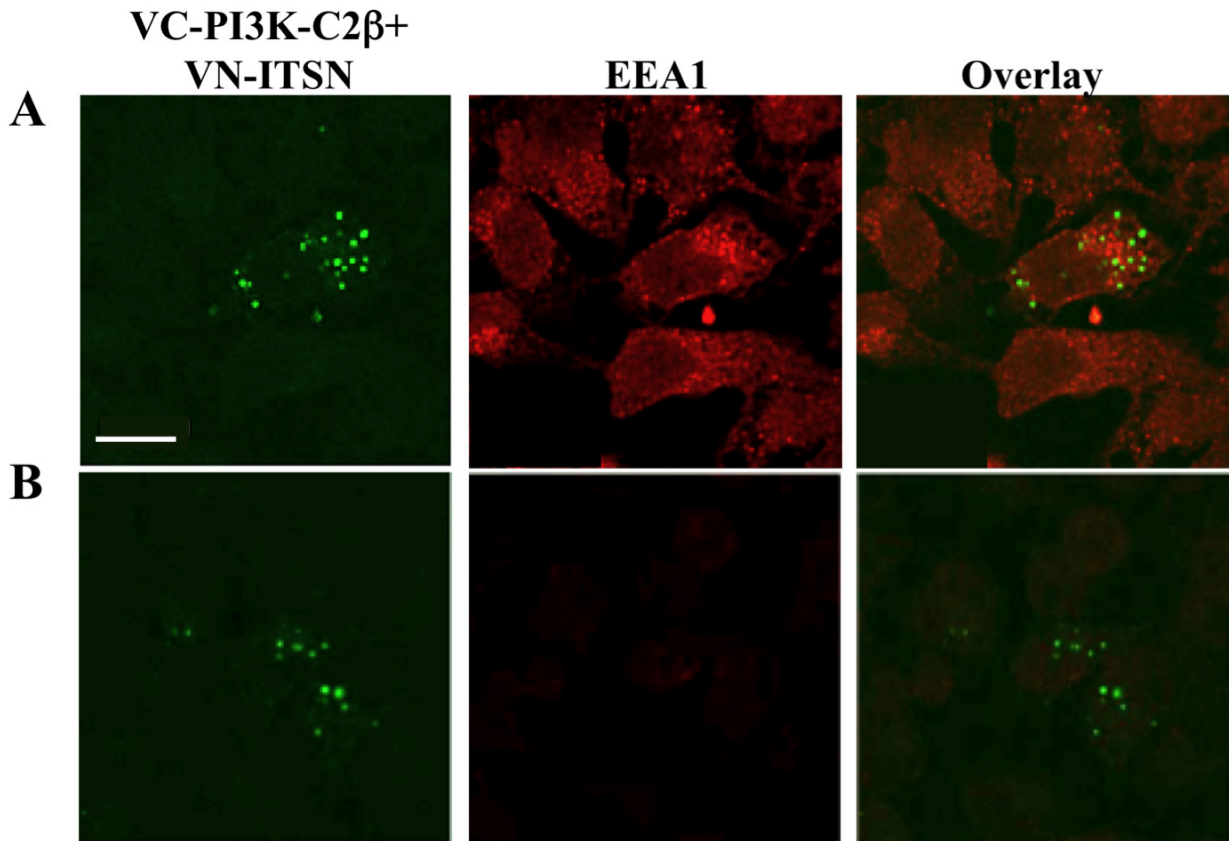
Figure 23. ITSN and PI3K-C2β complex does not localize with EEA1. **(A)** HeLa cells transfected with VN-ITSN and VC-PI3K-C2β (green) were immunostained for EEA1 (red). The overlay shows low levels of co-localization (yellow). (B) Panel 1: The YFP signal does not bleed into the EEA1 channel. Panel 2: There is no non-specific binding of the EEA1 antibody. (size bar=20μm)

ITSN:PI3KC2β BiFC complex, suggesting that PI3K-C2β is not producing PI$_3$P and therefore not recruiting EEA1 (Figure 23, Table III).

4. Different pools of ITSN interact with Ras and PI3K-C2β.

In addition to Rabs, ITSN interacts with Ras to induce GTP loading (1). Utilization of BiFC allows for the visualization of proteins that are in a complex, allowing for the isolation of the different ITSN pools. Indeed, I observed two different pools of ITSN. One pool of ITSN was found in a complex with PI3K-C2β alone, and a second pool of ITSN was observed in a trimolecular complex with Ras and PI3K-C2β. First, a BiFC complex of VC-PI3K-C2β and VN-Ras was co-transfected with Cerulean-ITSN. Under these conditions approximately 50% of the Ras-PI3K-C2β BiFC complex co-localizes with Cerulean-ITSN. Similarly, about 40% of the Cerulean-ITSN co-localizes with VC-PI3K-C2β:VN-Ras BiFC complex (Figure 24A, Table III). Next VC-PI3K-C2β and VN-ITSN are fixed in a BiFC complex and co-transfected with Cerulean-Ras. . Under these conditions, only 8% of Cerulean-Ras co-localized with the VC-PI3K-C2β:VN-ITSN (Figure 24B, Table III).

D. Discussion

Compartmentalization of proteins allows a single protein to regulate multiple biochemical pathways. ITSN is one such protein. We have observed ITSN and PI3K-C2β localization with the early and late endosomal pathways. My data demonstrates that ITSN localizes to distinct pools of Rab5, Rab7, and PI3K-C2β. Such compartmentalization may explain how a scaffolding protein, such as ITSN can organize subcelluar signaling. ITSN:Rab5 complex has a higher

A
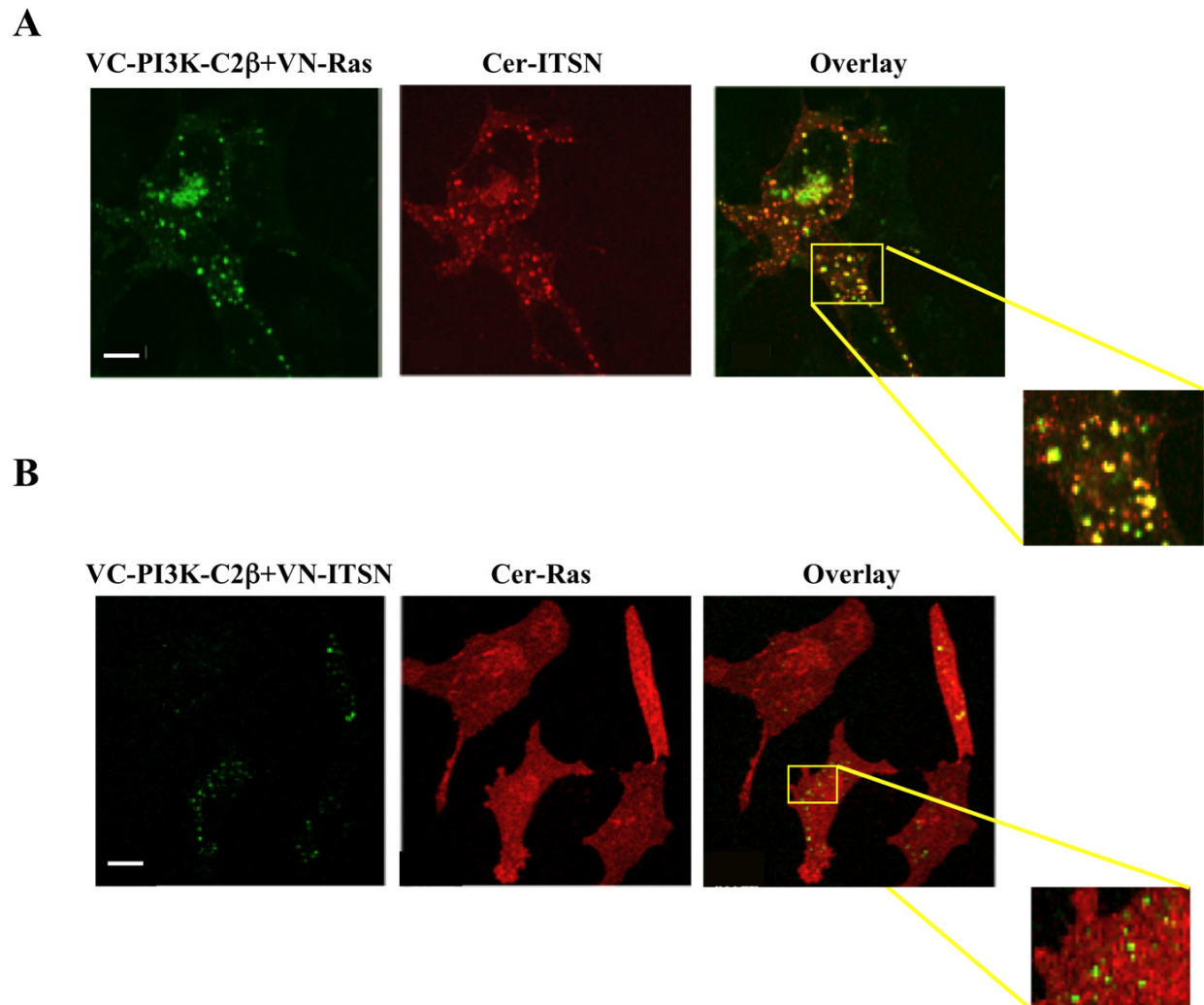

B

Figure 24. Different pools of ITSN, PI3K-C2β, and Ras. A. VC-PI3K-C2β and VN-Ras BiFC complex (green) was co-transfected with cerulean-ITSN (red). The overlay represents co-localization (yellow). **B.** VC-PI3K-C2β and VN-ITSN BiFC complex (green) was co-transfected with Cerulean-Ras (red). The overlay represents co-localization (yellow). (size bar=20μm)

fluorescence intensity compared to ITSN:Rab7, suggesting that ITSN preferentially interacts with the early endosomal pathway. ITSN co-localizes with Rab7, but has reduced levels of BiFC fluorescence, suggesting that ITSN is present on late endosomes, but not in a complex with Rab7 (Figure 21). In addition, ITSN:Rab5 positive vesicles recruit EEA1, while PI3K-C2β:Rab5 vesicles do not suggesting that PI3K-C2β does not produce PI$_3$P *in vivo* and that Rab5 is active on ITSN positive vesicles. Furthermore, the ITSN:PI3K-C2β BiFC complex also does not recruit EEA1 again suggesting that PI3K-C2β does not produce PI$_3$P. There is a pool of ITSN on EEA1 positive vesicles and a pool of ITSN on EEA1 negative vesicles, suggesting that ITSN regulates a Rab5-EEA1 trafficking pathway as well as a PI3K-C2β pathway independent of EEA1 and PI$_3$P.

My BiFC Ras analysis also indicates there are multiple pools of ITSN. Ras has been identified on the PM, Golgi, ER, early endosomes, and recycling endosomes (122, 133, 162, 165-167). Due to this broad subcellular distribution, 85% of the VC-PI3K-C2β:VN-ITSN complex is seen localized with Cerulean Ras, while only a low percentage (8%) of Cerulean Ras is localized with the BiFC complex. This suggests that there is a pool of PI3K-C2β and ITSN that interacts independent of Ras. Interestingly, using the same proteins in a different configuration reveals an endomembrane compartment that consists of all three proteins. By visualizing the pool of Ras that is in a complex with PI3K-C2β (i.e. VC-PI3K-C2β:VN-Ras), co-localization with ITSN is observed. 51% of the VC-PI3K-C2β:VN-Ras complex localizes with Cerulean ITSN, a six fold increase compared to when Ras is Cerulean tagged (Table III).

The temporal and spatial aspect of cell signaling is becoming more important as research continues to discover new pathways and new functions of proteins. The subcellular localization of a protein leads to the activation of specific effectors. In the case of ITSN, a pool of ITSN

complexes with Rab5 while a second pool regulates PI3K-C2β. Scaffolds serve as a platform for

proteins to come together to signal more efficiently. Understanding where these scaffolds are

located in the cell is essential to understanding cell signaling.

V. FUTURE DIRECTIONS

Cellular miscommunication is the basis for disease development. Understanding the basics of cell signaling is imperative to developing new targets for therapeutics. My dissertation has outlined two novel signaling pathways each discovered through the laboratory's interest in ITSN. Due to the scaffolding properties of ITSN and the role ITSN plays in multiple signal transduction pathways, ITSN is not a strong candidate for a drug target. Despite this, ITSN is a useful tool in deciphering cell signaling.

ITSN can be found on multiple intracellular compartments (Chapter 3). Where ITSN is located in the cell determines what downstream pathways will be activated. My work has shown that there are different pools of ITSN on early endosomes, in a complex with PI3K-C2β, and with Ras, and I have confirmed yeast-two hybrid data demonstrating that ITSN is localized with the Rab5 effector, Rabaptin5. Yet, the role of ITSN in Rab5 activation on early endosomes has not been determined. In addition, temporal measurements of ITSN mediated EGFR trafficking need to be performed to determine the effects of ITSN on the rate of EGFR trafficking. Interestingly, the effectiveness of scaffolds follows a bell shaped curve: low expression of a scaffold prevents complexes from forming, too high expression leads to the formation of dead-end complexes with both instances leading to inefficient signaling (35). Indeed we have observed this phenomenon with ITSN. High overexpression of ITSN inhibits internalization of the EGFR, while a moderate increase in ITSN levels enhances degradation (31). Different levels of ITSN expression need to be analyzed when examining the role of ITSN in EGFR trafficking. ITSN has been linked to the early endosomal pathway, degradation, and recycling of the EGFR. Different

levels of ITSN may result in various distributions of ITSN and therefore different signal regulation.

The role of PI3K-C2β in trafficking also needs to be further explored. Work with collaborators has shown that loss of PI3K-C2β leads to defects in early and late endosomes as well as delayed EGFR trafficking, but the downstream targets of PI3K-C2β have yet to be determined. I did not observe recruitment of EEA1 to Rab5:PI3K-C2β positive vesicles suggesting that PI3K-C2β is not producing PI_3P or is not active on these vesicles. Although the latter option is less likely to be true due to the fact that ITSN:PI3K-C2β positive vesicles also do not recruit EEA1 , and ITSN is know to activate PI3K-C2β [33]. The idea that PI3K-C2β and Rab5 are forming a novel Rab5-signaling domain is intriguing. Currently the only Rab5-signalosome characterized is composed of Rab5-GTP, Rabaptin5/Rabex5, hVps34, and EEA1. Determination of the specific proteins recruited to the Rab5:PI3K-C2β complex may define a new platform for Rab5 signaling.

My dissertation work has also explored the role of ITSN and PI3K-C2β in Ras signaling. Ras is the most frequently mutated gene in human cancers, yet there are no therapies to target Ras-related tumors, underlying the importance of the need to further understand Ras signaling. ITSN overexpression leads to an increase in activated Ras on intracellular vesicles (1). In addition, ITSN interacts with and activates PI3K-C2β (33). These observations lead to the initial hypothesis that the pool of ITSN that activated Ras was interacting with and activating PI3K-C2β as is seen with Class I PI3Ks and Ras at the plasma membrane. My results as presented in Chapter 2 demonstrate that nucleotide-free Ras interacts with and inhibits PI3K-C2β. This result leads back to the initial question of, what are the downstream effects of ITSN activated Ras?

Overexpression of ITSN does not lead to an increase in MAPK activation, but other classic Ras effectors such as Class I PI3K and Ral-GDS have yet to be tested.

The interaction between nucleotide-free Ras and PI3K-C2β is a completely novel observation which challenges the current mechanism for GTPase regulation. Traditionally, the nucleotide-free Ras intermediate is thought to be short-lived and unstable, yet my data would suggest that nucleotide-free Ras may be stabilized by PI3K-C2β. The trafficking of nucleotide-free Ras accounts for the delayed activation of Ras on the Golgi as well as the lack of GEFs found on the Golgi (139, 140, 163). The role of nucleotide-free Ras in cell signaling, i.e. PI3K-C2β inhibition, is a novel discovery that warrants further exploration. The next step is to demonstrate the presence nucleotide-free Ras *in vivo*. Antibodies that specifically recognize the nucleotide-free state would be beneficial for monitoring this entity. Furthermore, the PI3K-C2β-RBD could be used to purify nucleotide-free Ras from cell lysates. Subsequent loading of the purified Ras with nucleotide would demonstrate the presence of this entity *in vivo*. In addition, FRET probe assays may be performed to analyze the trafficking of nucleotide-free Ras by PI3K-C2β. A Ras FRET probe would consist of a donor (CFP), an acceptor (YFP), Ras, and the RBD of Raf. This Ras-FRET probe would generate a FRET signal when bound to GTP, but no FRET when nucleotide-free or GDP bound (Figure 25). Using the probe one should observe FRET (i.e. Ras activation) at the plasma membrane in the absence of PI3K-C2β. Co-transfection of PI3K-C2β with the probe should result in delayed and re-localized FRET signal to intracellular compartments. In addition, other Ras mutants defective in nucleotide binding (Ras16N, Ras119A, Ras116/K/Y, and Ras146V) should be evaluated for PI3K-C2β interaction. Furthermore, these mutations in the background of an activating mutation have low affinity for nucleotide, but are still transforming. It would be interesting to see if the PI3K-C2β-RBD can
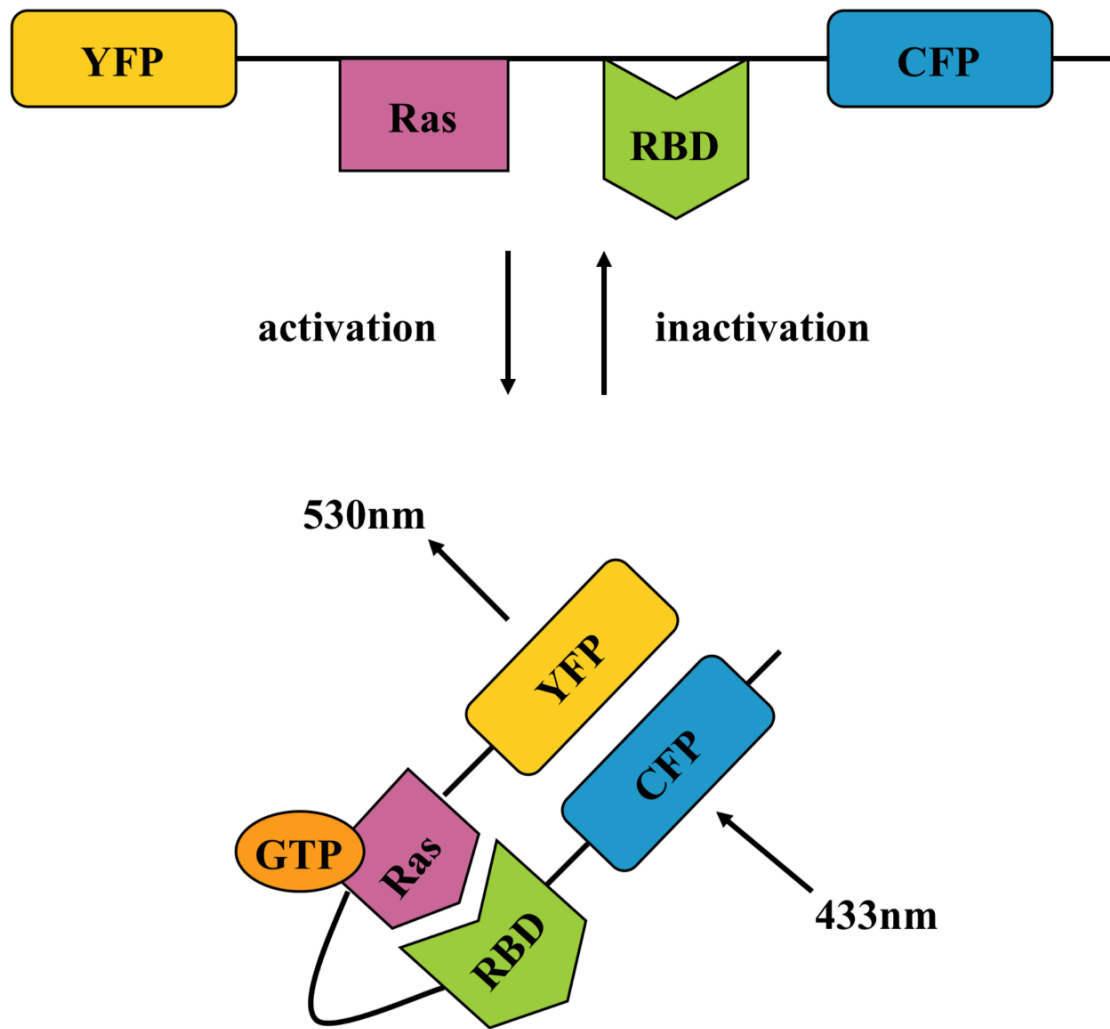
Figure 25. Ras FRET probe. When Ras is activated it will interact with the Raf-RBD bringing together the CFP donor and YFP acceptor, resulting in a FRET signal.

inhibit the transforming activity of these mutants by binding to the nucleotide-free mutant and preventing downstream signaling.

According to my data ITSN binding disrupts the PI3K-C2β:nucleotide-free Ras complex, allowing for nucleotide to bind (Figure 16), but this mechanism has yet to be tested. It would be interesting to determine if ITSN can compete off nucleotide-free Ras from PI3K-C2β, and if this Ras is subsequently loaded with nucleotide.

While my studies have been limited to H-Ras, both N- and K-Ras isoforms exhibit compartmentalized signaling activity as well (196) suggesting that the nucleotide-free forms of these Ras isoforms may also play an active role in regulating signaling. In addition there are other members of the Ras subfamily including, R-Ras (R-Ras2/TC21, R-Ras3/M-Ras), Ral (A and B), Rap (1A, 1B, 2A, 2b), Rheb, Rin, and Rit. Although interaction between PI3K-C2β and Ras-GDP or Ras-GTP was not detected, the nucleotide dependence for interaction with other members of the Ras family of monomeric GTPase should be analyzed. For example, PI3K-C2β interacts with wild-type Rab5, but not dominant negative Rab5, suggesting a GTP dependent interaction; however, this has yet to be tested with purified proteins.

In addition to PI3Ks many other proteins have been identified that contain RBDs or Ras-Association (RA) domains (241). Although these domains have little sequence homology they adopt a similar ubiquitin-fold structure (242). Interestingly, a group of these domains including Myr5, Tiam-1, Rain, and PLCε do not bind nucleotide-loaded Ras raising the question of whether additional RBDs may share a similar activity in binding nucleotide-free Ras (242-244). In addition, Class II PI3K alpha (PI3K-C2α) also contains an RBD that is 53% similar to PI3K-C2β-RBD warranting further investigation into the Ras-binding properties of this PI3K.

Much less is understood about Class II PI3Ks compared to Class I PI3Ks. One major pitfall in the area of PI3K-C2β research is that the *in vivo* lipid product is unknown. Detection of the *in vivo* lipid product is difficult due to the transient production and low concentration of these lipids. Determining the *in vivo* lipid product of PI3K-C2β is imperative for defining the downstream effectors of PI3K-C2β.

Although there are still many questions to be addressed and future experiments to be performed, the discoveries made in my dissertation have redefined monomeric G-protein signaling. Previously, it was understood that nucleotide-free monomeric G-proteins do not exist *in vivo*, yet in order for a GTPase to convert from a GDP-bound state to a GTP-bound state, a nucleotide-free intermediate must be generated. The stabilization of this intermediate by PI3K-C2β and subsequent trafficking of nucleotide-free-Ras generates a novel model for GTPase activation. These discoveries should lead to novel research into the role of nucleotide-free GTPases in cell signaling and GTPase regulation.

VI. APPENDIX

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Title: Regulation of Neuron Survival through an Intersectin-Phosphoinositide 3'-Kinase C2β-AKT Pathway

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Publication: Molecular and Cellular Biology

Publisher: American Society for Microbiology

Date: Nov 15, 2007

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ABSTRACTS: **Wong, K.A**, Chen, Y-J., O'Bryan, J.P. (2008) ITSN Regulation of Rab Family GTPases. Experimental Biology.

Wong, K.A, Chen, Y-J., O'Bryan, J.P. (2009) Ras regulates PI3K-C2β through a novel mechanism. Cold Spring Harbor Phosphorylation, Signaling and Disease.

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