THE ROLE OF SRGAP2
IN VERTEBRATE GASTRULATION

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by
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ABSTRACT

During vertebrate gastrulation, an elaborate series of cellular motility events occur that define the three germ layers, and establishes the primary body axis. While it is known that non-canonical Wnt signaling plays a crucial role during this period of development, a complete picture of the molecular mechanisms controlling this pathway has yet to be established. Previous studies has shown that one essential component of the pathway linking the Wnt ligands to the cytoskeletal changes that occur during vertebrate development is Dishevelled-associated activator of morphogenesis (Daam1). Daam1 bridges the gap between Dishevelled (Dvl) and the small GTPase RhoA and is required for Wnt-dependent RhoA activation, but the biochemical details of this process are to date still undefined. To identify additional factors that might be involved in this process, a yeast two-hybrid screen using a C-terminal region of Daam1 was performed. During this screen SLIT-ROBO Rho GTPase-activating protein 2 (**SRGAP2**), was identified.

The studies presented here were designed to establish a functional interaction between SRGAP2 and Daam1 during non-canonical Wnt signaling and to characterize the function of SRGAP2 during early vertebrate development. My studies uncover that SRGAP2 and Daam1 do indeed interact and that this interaction is positively influenced by Wnt stimulation. I also uncover via immunocytochemistry, that these two proteins share common sub-cellular localization patterns in HeLa cells that is responsive to Wnt stimulation. Further, I show that ectopic expression of SRGAP2 in HeLa cells has adverse effects on stress fiber formation in Wnt5a treated cells. And lastly, micro-injection

experiments in Xenopus *laevis* show that over- or under-expression of SRGAP2 produces severe gastrulation defects in the developing embryos.

These studies together demonstrate that SRGAP2 plays a critical role in regulating non-canonical Wnt signaling through its interaction with Daam1 and through regulation of the monomeric GTPases Rho. It also shows that SRGAP2 plays a functional role for gastrulation during early vertebrate development.

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CHAPTER 1
INTRODUCTION

During vertebrate embryogenesis, cells undergo a complex and extensive series of migratory events that produce morphological changes for establishing the three primary germ layers and forming the basic body plan of the organism (Smith 2009; Keller 2002; Heasman 1997; Smith & R 2006). One major cellular rearrangement undertaken by cells during this process is termed convergent extension. During convergent extension, cells begin a process of narrowing medio-laterally within the dorsal region of the embryo (convergence) and elongating along the anterior-posterior axis (extension) (Wallingford et al. 2002; Wallingford 2012; Heasman 2006). These cellular movements occur in a highly organized and precise manner, and while it has been shown that a Wnt signaling pathway involving Disheveled, Daam1, and RhoA plays a vital role in this process, there is still a large gap in our knowledge as to the underlying molecular mechanisms that function to regulate this pathway.

1.1 Vertebrate Gastrulation

Vertebrate gastrulation is arguably the most important morphogenetic event during vertebrate development. It consists of a series of complex cellular processes that transform the embryo in its entirety. Gastrulation involves a set of evolutionarily conserved cell movements that work together to help establish and shape the overall body plan of the developing organism (Smith & R 2006; Keller 2002; Yin et al. 2009). Included in this process is the migration of involuting cells toward the animal pole, and highly polarized movements of cells within the marginal zone. As this process progresses, it generates the archenteron, gives rise to the three primary germ layers, and establishes the initial anterior-posterior body axis (Fig. 2)(Keller et al. 1985; Ewald et al. 2004). The highly coordinated polarized cell movements that occur during this period are known, as "convergence and extension or convergent extension" movements (Keller 2002; Solnica-Krezel 2005). During convergent extension, mesodermal cells move toward the future dorsal side of the embryo and then intercalate between neighboring cells, resulting in an overall mediolateral narrowing (convergence) and anteroposterior lengthening (extension) of the embryo (Tada & Heisenberg 2012). The combination of collective cell movement and cell intercalation narrow the body of the organism mediolaterally and elongate it along the anterior-posterior axis (Tada & Heisenberg 2012)(Fig. 1)

While the cellular behavior involved in convergent extension have been known for some time, fairly little was known about the molecular cues governing these movements, until it was found that non-canonical Wnt signaling via the Dishevelled protein could regulate these movements (Sokol 1996). The non-canonical Wnt signaling pathway is the principle regulator of cell polarity during vertebrate gastrulation. Interference with non-canonical Wnt signaling results in round cell morphology, randomized protrusions, and consequently impaired C&E movements (Yin et al. 2009).

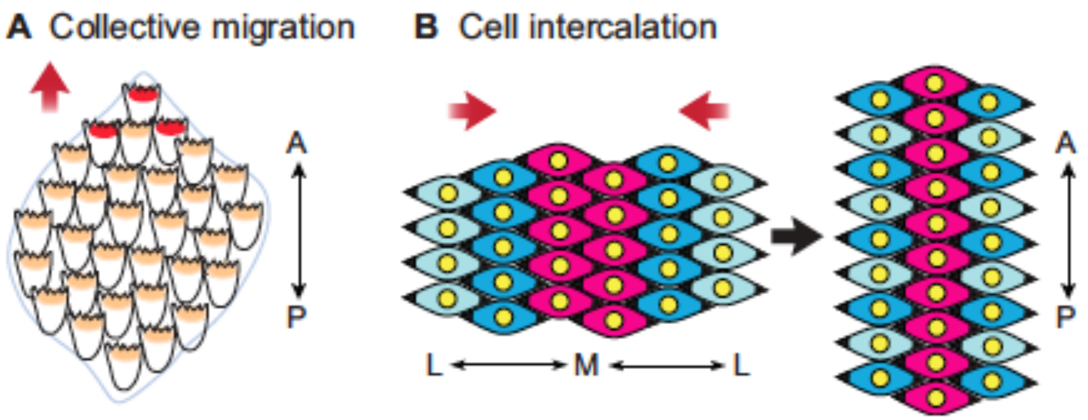


Figure 1: Convergent extension includes two types of cell movement.

A. During collective migration, cells migrate as a cohesive sheet. The cells at the leading edge are highly polarized and produce protrusions, such as lamellipodia. **B.** During medial-lateral cell intercalation, cells redistribute their positions in the anterior-posterior axis of the tissue. The red arrows indicate directions of cell movement (Tada & Heisenberg 2012)

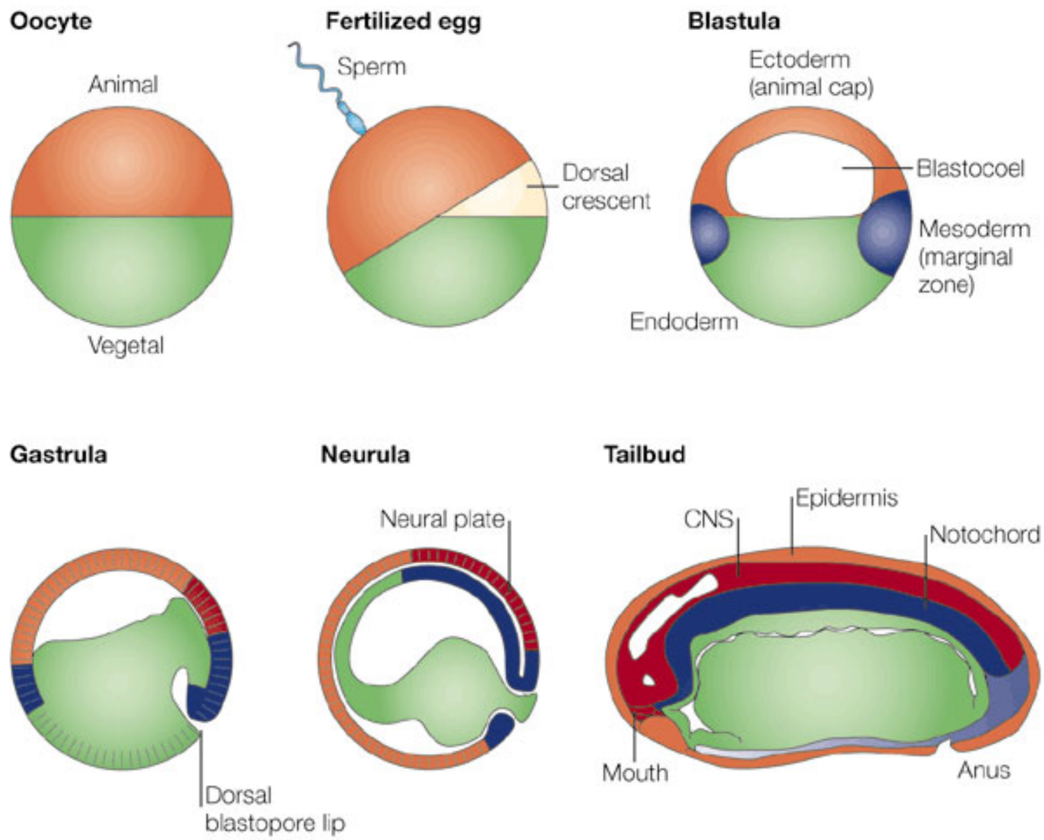


Figure 2: Early Xenopus development

After fertilization**,** the zygote undergoes multiple rapid rounds of cell division that produces a ball of cells, with a fluid-filled cavity called a blastocoel. Immediately afterwards, the cells in the developing embryo begin to involute during the process of gastrulation. Gastrulation forms the three primary germ layers and the archenteron. The three germ layers give rise to unique tissue types during the neurula and tail-bud stages (Robertis et al. 2000).

1.2 Wnt signaling

Wnt ligands are a family of evolutionarily conserved palmitoylated glycoproteins that function as concentration-dependent short and long-range signals. Within all Wnt proteins is a conserved pattern of 23–24 cysteine residues, along with other invariant amino acids (van Amerongen et al. 2009; Mikels & Nusse 2006). Study of the Wnt signaling began over thirty years ago, when it was realized that not only were the gene products of the Drosophila wingless (wg) and mouse Int1 were homologous, but that they were also part of a large evolutionarily conserved family. The name Wnt is a portmanteau, reflecting the contributions made these two foundational studies (Nusse et al. 1991).

Since its initial discovery, much work has been performed in an attempt to delineate the molecular mechanisms of this crucial pathway and the findings of many laboratories have contributed to our knowledge thus far. Wnt signaling is a tightly controlled process and expression patterns of the Wnt ligands are regulated both spatially and temporally during development and adulthood (MacDonald et al. 2009). Intracellularly Wnt signaling is transduced through multiple molecular pathways that are usually divided into two categories, canonical and non-canonical. These are also often referred to as the **β**-catenin dependent and **β**-catenin independent pathways respectively (Semenov et al. 2007). Together the canonical and non-canonical pathways mediate a large number of diverse cellular behaviors, such as cell fate decisions, axis specification, gastrulation, organogenesis, and nervous system development and maintenance (Gao & Chen 2010; Logan & Nusse 2004; Rao & Kühl 2010).

The complexity and dynamic nature of Wnt signaling cannot be understated, and a wide array of molecules has been shown to play roles in this pathway. While Wnt ligands have been shown to function in multiple signaling pathways (Semenov et al. 2007), in the three best characterized pathways, two molecules act as the common core. The first of these is Frizzled (Fz). Frizzled proteins are the primary receptors for the Wnt ligands (Komiya & Habas 2008; van Amerongen et al. 2009; Cadigan & Liu 2006). They possess seven transmembrane domains and share other structural characteristics with G-protein coupled receptors (GPCR). They also contain a cysteine rich domain (CRD) that acts as a binding site for Wnt proteins (Komiya & Habas 2008; van Amerongen et al. 2009). Directly downstream of Frizzled lies Dishevelled, the last shared link between the pathways. Dishevelled is a fairly large protein (~700 a.a.) and acts as a branching point between the canonical and non-canonical pathways. It contains three highly conserved domains that are essential for the differential routing of the Wnt signal. Its C-terminal DEP domain (Dishevelled, Egl-10 Pleckstrin) and the centrally located PDZ domain (Post synaptic density-95, *Discs*-large and Zonula occludens-1) are involved in β-catenin independent signaling, while β-catenin dependent signaling employs the PDZ domain along with the N-terminal DIX domain (for DIshevelled and Axin)) (R Habas et al. 2001; Raymond Habas et al. 2001b). Recently however, work has been performed that suggests a role for the DEP domain in both signaling pathways (Gammons et al. 2016). At present, in humans, there are nineteen known Wnt ligands, ten Frizzled (Fz) family members and three Dishevelled genes (van Amerongen et al. 2009; Komiya & Habas 2008). The large number of Wnt and Wnt effector variants along with its evolutionary longevity illustrate the importance and complexity of this signaling pathway (Niehrs 2012; Cadigan & Liu 2006). Studies of Wnt signaling began as a serendipitous union between the fields of developmental biology and oncology, as the study of this ancient pathway progresses, novel insights in both fields are continually revealed.

1.2.1 Canonical Wnt Signaling

Canonical Wnt signaling acts to regulate a transcriptional program through the degradation (unstimulated) or accumulation (stimulated) of β-catenin. In the absence of Wnt stimulation, a group of proteins that include two ser/thr kinases, GSK-3 and CKI, and two scaffold proteins, Axin and APC, promotes the ubiquitination and subsequent degradation of β-catenin. This group of proteins has been dubbed the "destruction complex" and upon stimulation by a Wnt ligand, this complex of proteins separates and ceases to ubiquinitate β-catenin, allowing for the levels of β-catenin to increase within the cytoplasm. The continued accumulation of β-catenin within the cytoplasm eventually leads to nuclear translocation of the molecule where by acting along with TCF/LEF transcription factor triggers the transcription of its target genes (MacDonald et al. 2009; Komiya & Habas 2008; van Amerongen et al. 2009). (Fig. 3)

1.2.2 Non-Canonical Wnt Signaling

Non-canonical Wnt signaling encompasses those pathways that do not employ the β-catenin as modes of downstream signaling (Niehrs 2012). For this reason, they are also referred to as β-catenin independent pathways. While there are numerous pathways believed to be regulated via Wnt ligands, the two best characterized non-canonical Wnt pathways are the Wnt-planar cell polarity (PCP) pathway and the Wnt/Ca^{2+} pathway (Veeman et al. 2003; Rosso et al. 2005; Cadigan & Peifer 2009; Kühl et al. 2000; Range et al. 2013; Rao & Kühl 2010).

Wnt-Planar Cell Polarity Pathway

The Wnt-Planar Cell Polarity pathway, which is also referred to as β-catenin-independent or simply the non-canonical Wnt signaling pathway, uses the core Wnt-signalling components Frizzled (Fz) and Dishevelled (Dvl), but the shared components and similarity between canonical and non-canonical Wnt signaling ends with Disheveled (Segalen & Bellaïche 2009; Tada & Kai 2009; Raymond Habas et al. 2001a). Unlike the canonical Wnt pathway, Wnt-PCP signaling does not appear to lead to any transcription-based regulation of cellular behavior (John B Wallingford & Habas 2005). Signaling through this pathway involves downstream components such as Daam1, Rho and Rac, that are all known regulators of the actin cytoskeleton (MacDonald et al. 2009; MacDonald et al. 2007; Komiya & Habas 2008). The PCP pathway components therefore impinge on modulation of the actin cytoskeleton that is required during the convergent extension movements gastrulation (Raymond Habas et al. 2001b; Habas et al. 2003b; Sato et al. 2006; Habas et al. 2003a).

During Wnt-PCP signaling, at the level of Dishevelled there is a bifurcation in signal propagation, splitting between Rho- and Rac-mediated events (Komiya & Habas 2008; van Amerongen et al. 2009). These small monomeric GTPases work in parallel, to modify the actin cytoskeleton during gastrulation. Signaling through Rho is mediated by the Formin molecule Daam1 (Dishevelled associated activator of morphogenesis 1) (Raymond Habas et al. 2001b). This Rho pathway leads to the activation of the Rho-associated kinase ROCK. Also included in the Rho portion of this pathway is weak-similarity GEF (WGEF). WGEF is a RhoGEF that has been shown to interact with both Daam1 and Disheveled and regulate localized RhoA activation (Tanegashima et al. 2008). Whereas signaling through Rac stimulates JNK activity (John B Wallingford & Habas 2005). Rac and Rho together work to translate the extracellular signal into morphological changes in cell shape and migration patterns.

Wnt/Ca^{2+}

While the Wnt/Ca2+ pathway is still in its infancy, much work is being done to demonstrate its importance during early vertebrate development. Early work in Xenopus demonstrated that maternal Xwnt-5A and Xwnt-11 promote ventral cell fate through both PKC as well as CamKII (Sheldahl et al. 1999; Kuhl et al. 2000). Further studies in Zebrafish, also demonstrated that genetic depletion of Wnt 5 (pipetail), both maternally and zygotically, led to a hyper-dorsalized embryonic phenotype which could be partially rescued through injection of a constitutively active form of CAMKII. This series of experiments also demonstrated that that Wnt 5 (pipetail), acting with the Wnt/ $Ca2^{+}$ can function in the dorsal/ventral patterning in early development and that pipetail can have an antagonistic effect on the Wnt/ β-Catenin pathway. (Westfall et al. 2003)

Activation of the Wnt/Ca^{2+} also involve the binding of the Wnt ligand to Frizzled, but unlike the Wnt-PCP pathway and the canonical Wnt signaling pathway, does not require Dishevelled. Instead, Frizzled acts as a typical G-protein coupled receptor (GPCR) and leads to a temporary increase in inositol 1,4,5-triphosphate (IP_3) and 1,2 diacylglycerol (DAG) derived from phosphatidyl inositol 4,5 bisphosphate (PIP2) through a heterotrimeric g-protein intermediate (Rao & Kühl 2010). The build up of IP_3 leads to the diffusion of it through the cytoplasm where it interacts with calcium channels present on the endoplasmic reticulum, which results in the release of intracellular calcium ions. (De 2011) The released cytosolic calcium binds to calmodulin, a Ca^{2+} switch protein that mediates many Ca^{2+} signaling events, which in turn can activate Ca^{2+} -calmodulin dependent protein kinase II (CaMKII) (Kühl et al. 2000). Another primary target of of calmodulin is calcineurin. Calcineurin is a protein phosphatase that activates the nuclear factor of activated T cells (NFAT)(Slusarskir, Diane C. and Pelegri 2009).

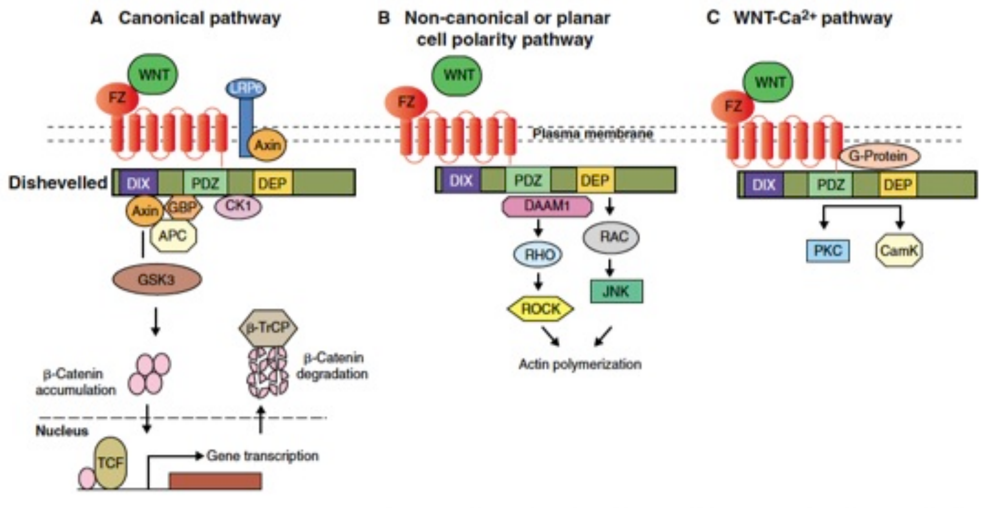


Figure 3: Wnt Signaling Pathways

A. The canonical Wnt pathway. Wnt ligands bind to Frizzled (Fz) receptor and a LRP co-receptor. The signal is transduced through Dishevelled, leading eventually to the stabilization of β-Catenin, which then acts through LEF/TCF to control the transcription of target genes. **B.** The non-canonical or planar cell polarity (PCP) cascade. A signal is received by a Fz and transduced via Dishevelled to Rac and Rho, which then activate downstream targets to modulate the actin cytoskeleton. **C.** The Wnt/Ca2+ pathway. Wnt signals through the Fz receptors and Dishevelled and G-proteins leading to the release of intracellular calcium (John B Wallingford & Habas 2005).

1.3 Daam1 and the Formin Family

Daam1 is a highly conserved protein that belongs to a family of Formin proteins. It marks the departure from the common core Wnt signaling components of Frizzled and Dishevelled and is an immediate downstream target of Disheveled in the Wnt-PCP pathway. Discovered during a yeast two-hybrid screen that employed the PDZ domain of Dishevelled, Daam1 was then found to be directly responsible for linking Dishevelled to Rho activation. Further examination showed that Daam1 played a vital role during Xenopus gastrulation and convergent extension through its ability to regulate Rho activation and through direct association with filamentous actin (R Habas et al. 2001). Daam1 like other diaphanous formin family members is a multi-domain protein, and contains two Formin Homology domains, FH1 and FH2. It also contains an N-terminal GTPase binding domain (GBD), Diaphanous inhibitory domain (DID), and a C-terminal Diaphanous auto-regulatory domain (DAD). (Figure 8)

The Formin family is composed of large multi-domain proteins that function in cytoskeletal rearrangements. Primarily, these rearrangements consist of modification of and polymerization of actin, but a small number of formins have also been shown to act on microtubules. As a family, the formins are defined by the presence of Formin homology (FH) domains. There are three FH domains, FH1 and FH2, and a loosely defined FH3 (Wallar & Alberts 2003). As a family, they are highly conserved and are found in species ranging from slime molds to humans. In mammals, there are at 15 known formins. These 15 molecules have been divided into seven distinct sub-families based on their sequences and domain architectures (Chesarone et al. 2010;

Breitsprecher & Goode 2013; Copeland et al. 2007; Evangelista et al. 2003). The sub-families are termed: Diaphanous, Dishevelled-associated activator of morphogenesis (DAAM), Delphilin, FH1/FH2 domain-containing protein (FHOD), formin (FMN), Formin related (FRL) which is also referred to sometimes as Formin-like (FMNL), and inverted formin (INF). Formin's ability to directly polymerize actin within the cell places them at the center of a wide array of cellular activities. Cell motility, cell polarity, endocytosis and endosome motility, and cytokinesis all rely upon members of this family (Campellone & Welch 2010). The best-studied formins are diaphanous, DAAM, and FRL, which are collectively referred to as the diaphanous related formins (DRF) (Bruce L Goode & Eck 2007). The DRFs all share similar domain architecture and with each domain playing a distinct, but no less critical role in the regulation of the actin cytoskeleton. The two Formin defining domains FH1 and FH2 are both centrally located within the protein. FHl domains are approximately 100 amino acids in length, and are extremely proline rich (Wasserman 1998). To date, the principal role of the FH1 domain in regards to cytoskeletal regulation has been to act as a docking site for actin binding proteins (ABPs). Profilin, the best characterized of these ABPs in relation to DRFs, is a major sequester of monomeric actin. Profilin also functions as a nucleotide exchange factor and promotes the exchange of ADP to ATP on the actin monomer (Aspenström 2010). The sequestration of monomeric actin by Profilin quells spontaneous filament formation, allows monomer addition to the barbed ends of actin filaments. As such, Profilin bound actin will continuously promote barbed end growth of actin filaments until growth is obstructed via a capping protein (Bruce L Goode & Eck 2007). The role of the FH2 domain is to directly nucleate or promote actin filament assembly. The FH2 domain is about 400 a.a. in length and functions in a dimeric fashion, each monomer can be seen as a semicircle that when combined, act to encircle actin. This domain functions in two ways to promote non- branched actin filament assembly. The first is through its ability to act as a direct actin nucleator. The second, is through its ability to prevent the addition of capping proteins from interacting with the growing barbed end of the actin filament via direct binding to and continued interaction with the progressively growing barbed end of the filament (Bruce L Goode & Eck 2007; Aspenström 2010; Chesarone et al. 2010; Evangelista et al. 2003; Aspenstrom et al. 2006). The DRFs also share common regulatory domains unique to the DRFs at the N- and C-terminal ends. At the far C-terminal end of the proteins is the Diaphanous-autoregulatory domain (DAD), this region composed of approximately 20 amino acids. When DRFs are in their resting, or inactive state, they reside as auto-inhibited proteins. The auto-inhibited state is maintained by intramolecular interactions between the DAD domain, and an N-terminally located Diaphanous-inhibitory domain (DID).

Daam1, like other formin molecules exists in an auto-inhibited state. Relief from auto-inhibition of the molecule is triggered through the binding of the far C-terminal region of Daam1 to the PDZ domain of Disheveled (Liu et al. 2008). Daam1 is believed to regulate actin cytoskeletal changes in two ways. First, through direct interaction with actin, working to prevent the addition of capping proteins allowing the continue growth of the actin filament. Second, once activated, Daam1 is then able to activate Rho GTPase that in turn leads to the activation of Rho associated kinase a known regulator of cytoskeletal changes. Past studies with Daam1 has shown it to interact with the actin binding protein Profilin. These studies demonstrated that during Wnt activation, Daam1 and Profilin collaborate to mediate effects on the actin cytoskeleton (Sato et al. 2006). Other work has shown Daam1 to partner with MIM (Missing in Metastasis). This work showed that the two proteins work together to regulate neural tube closure by affecting changes to the actin cytoskeleton (Liu et al. 2011). It is quite likely that Daam1 partners with many proteins that work to influence the actin cytoskeleton during the varied morphogenetic cellular movements required during convergent extension and neural tube closure. Therefore further identification and characterization of potential binding partners of Daam1 are needed to discern the role of Daam1 in vertebrate embryogenesis.

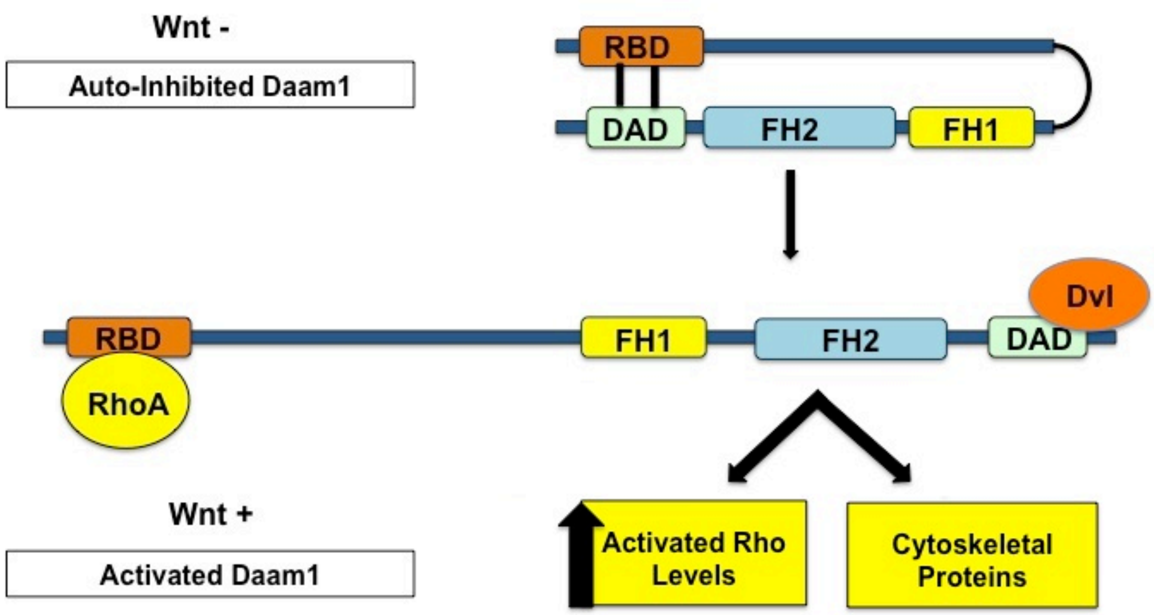


Figure 4: Activation of Daam1. In the absence of Wnt stimulation, Daam1 resides in the cytoplasm in an auto-inhibited stated via an intramolecular interaction between the C-terminally located Diaphanous Auto-Inhibitory Domain (DAD) and the N-terminal region. Wnt stimulation induces the binding of Disheveled (Dvl) to Daam1, relieving the auto-inhibited state. In the activated Daam1 not only directly interacts with GTP-bound RhoA at its Rho Binding Domain (RBD), but also causes an increase in overall intercellular activated RhoA levels. This event is believed to be mediated by interaction between Daam1 and Rho regulatory molecules.

1.4 RhoGTPases and the RhoGTPase Cycle

Rho (Ras-homology) GTPases constitute a distinct family within the superfamily of Ras-monomeric GTPases and are present in all eukaryotic cells. There are twenty-two mammalian Rho GTPases that have been described to date, three Rho isoforms A, B, and C; three Rac iso- forms 1, 2, and 3; Cdc42, RhoD, Rnd1, Rnd2, RhoE/Rnd3, RhoG, TC10, and TCL; RhoH/TTF; Chp and Wrch-1; Rif, RhoBTB1, and 2; and Miro-1 and 2 (Jaffe & Hall 2005). Members of the Rho family of GTPases play an essential role in regulating many key biological functions (Hall 2012a). In humans, 18 genes encoding members of the RhoGTPase family have been identified. Of these 18, RhoA, Rac1 and Cdc42 are by far the best characterized (Hall 2012b). Rho family GTPases act as molecular switches that are turned on and off in response to a variety of extracellular stimuli. In their active (on) state, Rho proteins are GTP-bound and can transduce signals through interaction with numerous downstream effectors. They help to govern such diverse cellular processes as cytoskeletal rearrangements, gene transcriptions, cell cycle regulation, control of apoptosis and membrane trafficking. The hydrolysis of GTP to GDP returns the protein to an inactive (off) state (Moon & Zheng 2003; Donovan et al. 2002).

Three distinct classes of proteins regulate RhoGTPases. Guanine nucleotide exchange factors (GEFs) catalyze the exchange of GDP for GTP, thereby activating the GTPase. GTPase-activating proteins (GAPs) increase the intrinsic GTP hydrolysis rate of the GTPase, returning it to the inactive state. Guanine nucleotide dissociation inhibitors (GDIs) sequester the GDP-bound form of some GTPases in the cytosol and prevent them from localizing to membranes or being activated by GEFs (Hodge & Ridley 2016; Jaffe &

Hall 2005). Rho GTPases are post-translationally modified by lipid modification. This lipid modification targets them to the plasma membrane and can affect their interactions with specific RhoGEFs or downstream signaling partners (Hodge & Ridley 2016).

From the earliest work, these molecules have been inexplicably linked to specific cytoskeletal rearrangements when activated by extracellular signaling events. Rho was originally shown to promote the formation of contractile actomyosin structures (stress fibers) upon treatment with LPA (lysophosphatidic acid) and Rac demonstrated the ability to induce the formation of lamellipodia and membrane ruffles in cells treated with PDGF. Later it was demonstrated that activated Cdc42 helped generate filopodia (Ridley & Hall 1992; Hall 2012b; Etienne-Manneville & Hall 2002; Hall 1998). These early discoveries showcased the crucial roles of these monomeric GTPases in actin dynamics, but only hinted at the diversity of cellular functions that the RhoGTPase family would later be found to play a role in.

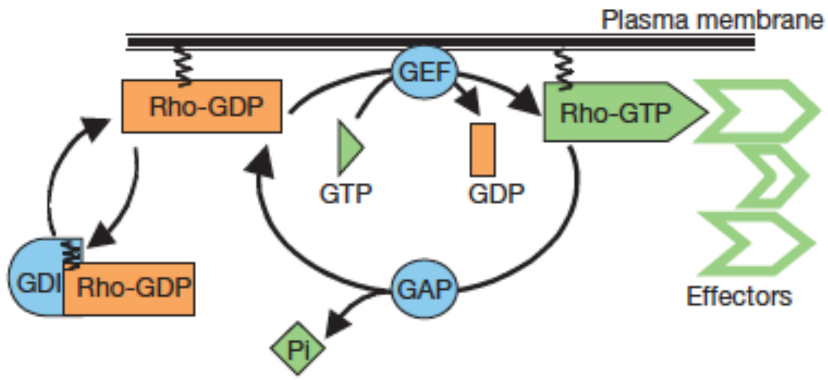


Figure 5: The RhoGTPase Cycle

RhoGTPases cycle between an active (GTP-bound) and an inactive (GDP-bound) conformation. The cycle is highly regulated by three classes of protein: guanine nucleotide exchange factors (GEFs) catalyze nucleotide exchange and mediate activation; GTPase-activating proteins (GAPs) stimulate GTP hydrolysis, leading to inactivation; and guanine nucleotide exchange inhibitors (GDIs) extract the inactive GTPase from membranes. (Etienne-Manneville & Hall 2002)

1.4.1 RhoGEFs

The release of GDP from RhoGTPase is a very slow process, a process that must be accelerated by Guanine Exchange Factors (RhoGEfs) to yield efficient activation of small GTPases. RhoGEF assisted nucleotide exchange is a multistep reaction. The exchange reaction is initiated by the formation of a low-affinity complex between the GDP-bound GTPase and the GEF, which converts into a high-affinity nucleotide-free GTPase/GEF complex after dissociation of GDP. Binding of GTP eventually displaces the GEF, yielding the active form of the GTPase (Cherfils & Zeghouf 2013).

The first RhoGEF , DBL was identified initially as an oncogene in mammalian cells. DNA isolated from a human **D**iffuse **B**-cell **L**ymphoma resulted in the discovery of the DBL oncogene that encoded an N-terminally truncated and activated protein (Rossman & Der 2013). Dbl contained a region of approximately 180 amino acids that shared homology with Cdc24, a known activator of Cdc42 (Schmidt & Hall 2002). This region has since been dubbed the Dbl homology (DH) domain and has been shown to be the catalyzing domain present in all RhoGEF molecules. Almost all known RhoGEFs also contain a pleckstrin homology (PH) domain and this domain is always positioned C-terminally to the Dbl domain. These domains work in unison to promote GDP-GTP exchange and in most studies have shown them to be the minimal required portion of the RhoGEF that can still function in nucleotide exchange. (Schmidt & Hall 2002)

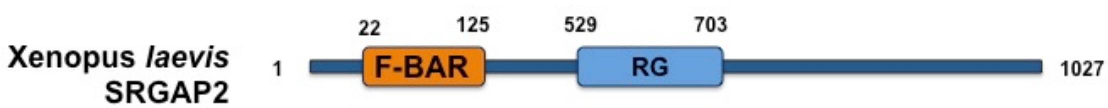
1.4.2 RhoGAPs

A common feature of small GTPases is their very slow intrinsic GTPase activity. Their intrinsically slow rate of hydrolysis, generally does not match the time scale required for their cellular functions and thus require the action of a GAP to assist in this function (Cherfils & Zeghouf 2013) RhoGAP proteins function in enhancing the GTPase activity of RhoGTPases, catalyzing the hydrolysis of the γ-phosphate group and returning the GTPase to an inactive state (Gamblin & Smerdon 1998). The defining feature of RhoGAP proteins, is a conserved RhoGAP domain that consists of about 150 amino acids (Moon & Zheng 2003). To date, 70 RhoGAPs have been reported in eukaryotes and human genome analysis has put a limit of around 80 on the number of RhoGAPs in humans. RhoGAPs outnumber the Rho GTPases they regulate by 2- to 3-fold (Tcherkezian & Lamarche-vane 2007). There are explanations for this unbalanced relationship between RhpGTPases and RhoGAPs. First, like Rho GTPases, some GAPs show preferential tissue expression and seem to have tissue- specific functions. Also, Rho GTPases are implicated in a large number of biological responses, each GAP may specifically regulate a distinct Rho GTPase signaling pathway (Tcherkezian & Lamarche-Vane 2007; Jacobs & Hall 1937; Lamarche & Hall 1994).

1.5 SRGAP2

The SRGAP family represents a small group of RhoGTPase activating proteins. There are four members of the SRGAP family. SRGAP1, SRGAP2, and SRGAP3 were all discovered and initially characterized during a study by Wong et al. that was interested in finding binding partners for the Robo family of cell surface receptors (Wong et al. 2001). The fourth member of the family ARHGAP4 was later characterized by Vogt et al. (Vogt et al. 2008). Human SRGAP2 is a large complex protein containing 1071 a.a (Fig. 6). Xenopus *laevis* SRGAP2 is slightly smaller than its human homolog at 1027 a.a. and shares 74% sequence identity to human SRGAP2. Xenopus SRGAP2 has at least two distinct domains. An N-terminal region of the protein possesses an F-BAR domain and a RhoGAP domain. The F-BAR domain is a lipid and protein-binding region that has the ability to induce membrane deformations. Proteins containing a F-BAR domain are able to sense membrane curvature and, by associating with the membrane as pairs of arcing/ banana shaped dimers, they further sculpt the plasma membrane (de Kreuk & Hordijk

2012; Safari & Suetsugu 2012). The RhoGAP domain is the catalytic domain of the protein. Its function is to aid in the hydrolysis of the γ-phosphate group of GTP bound to a Rho GTPase, converting it to GDP and effectively inactivating the Rho GTPase. A key difference between Xenopus SRGAP2 and almost all of its homologs is the absence of an SH3 domain. The SH3 domain in SRGAP2 has been shown during multiple studies to be responsible for it protein-protein interactions (Chen et al. 2012; Wong et al. 2001; Mason et al. 2011a). This discrepancy between *Xenopus* SRGAP2 and other forms of SRGAP2 may play a substantial role in determining its function. However, in the interactions seen between *Xenopus* SRGAP2 and Daam1 only the regions of SRGAP2 containing the F-Bar domain and the RhoGAP domain were required, making it plausible that human SRGAP2 interacts with Daam1 the same way.



Identity Values

Human	**74%**
Mouse	**74%**
Zebrafish	**66%**

Figure 6: Xenopus laevis SRGAP2 is a highly conserved protein

The body of work on SRGAP2 to date is limited, but there has been there have been several papers focused on its role in the nervous system, along with studies showing its importance other areas of cell biology. The early work on SRGAP2 has focused on its expression in the nervous system. Beginning with Wong et al., who in their initial characterization of SRGAP proteins, showed high levels of SRGAP2 mRNA could be detected in the lungs and brains of embryonic mice by making use of Northern Blot analyses. They also demonstrated by way of in-situ hybridization, that SRGAP2 mRNA transcripts could be detected in the ganglion eminence (GE), the septum (SP), and in the cortical plate (CP) of the neocortex (Wong et al. 2001). Chen et al, examined the expression of the SRGAP protein in dorsal root ganglia by RT-PCR and western blot and found that while SRGAP1 and 3 were detectable, expression of SRGAP2 was missing (Chen et al. 2012). The most extensive examination into SRGAP2 expression in the CNS was performed by Bacon et al. They found, that SRGAP2 was strikingly absent from sites of neurogenesis, but was often strongly detected in regions of neuronal migration and differentiation. This pattern contrasted the expression patterns of SRGAP1 and SRGAP3 (Bacon et al. 2009).

Other studies on SRGAP2 have focused on the function of SRGAP2 at a more cellular and molecular level. For instance, an investigation by Guerrier et al. showed that SRGAP2 negatively impacts neuronal migration and that it could induce neurite outgrowth and branching. This study showed that function of SRGAP2 could be attributed to its the F-BAR domain. They demonstrated a loss of SRGAP2 via SRGAP2 knockdown siRNA reduces leading process branching and increases the rate of neuronal migration and that overexpression of srGAP2 or its F-BAR domain alone exerted opposite effects (Guerrier, Coutinho-Budd, Sassa, Gresset, Jordan, Chen, W.-L. Jin, et al. 2009). A series of experiments by Mason et al. showed that human SRGAP2 could interact with FML1, a member of the formin family known to have actin severing capabilities. The complex formed between the two molecules disrupted the ability of FML1 to affectively perform its function, by not only interacting and inactivating Rac1 upstream of FML1, but also through a potential allosteric hindrance between the FH1 domain of the formin molecule and the profilin associated actin molecule (Mason et al. 2011b). Bao et al. Demonstrated that SRGAP2 is auto-inhibited and that inhibition is relinquished through methylation of a C-terminal arginine residue. This paper showed that the inactivated form of SRGAP2 resides in the cytoplasm while the activated form localizes to the plasma membrane. This study also explored the effect that SRGAP2 has on cell migration and cell spreading. These studies also found that a loss of SRGAP2 negatively affects both cell migration and cell spreading, presumably through the inhibition of actin polymerization (Guo & Bao 2010). SRGAP2 has also been found to regulate contact-dependent cell migration in fibroblast cells. Fritz et al. showed in a recent paper, that SRGAP2 dynamically associates with the cell edge at the tip of cellular protrusions migrating fibroblast and that after the protrusion comes into contact with another cell, it dissociates from the membrane as the protrusion retracts (Fritz et al. 2015).

One key study showed that exclusively in humans, SRGAP2 underwent three duplication events. These duplication events produced three new paralogs of the original SRGAP2; SRGAP2B, SRGAP2C, and SRGAP2D. This study showed that all paralogs are expressed, with SRGAP2C showing the highest level of mRNA expression and SRGAP2B/D both being expressed at relatively low levels. They also found that both the ancestral SRGAP2A and the derived SRGAP2C copy are fixed at diploid copy number two across all humans assayed. However, the SRGAP2B and SRGAP2D copies varied from 0–4 copies among the individuals. There most interesting finding however is that the timing of the potentially functional copies, SRGAP2B and SRGAP2C, corresponds to the emergence of the genus Homo from Australopithecus (2–3 mya). This period of human evolution has been associated with the expansion of the neocortex and the use of stone tools. They suggest that the duplication events played a role in the neocortical expansion that occurred at this time (Dennis et al. 2012). A companion article to this study investigated the role of the SRGAP2 paralogs role in the developing neocortex more extensively. They found that both SRGAP2B and SRGAP2C contain a truncated F-BAR domain. Their findings also indicate, that SRGAP2C can dimerize with the ancestral SRGAP2 in the developing nervous system to inhibit its function. They show that in the mouse neocortex, SRGAP2 promotes spine maturation and limits spine density, but when SRGAP2C is expressed, inhibits this function, it leads to neoteny during spine maturation and increased density of longer spines. The authors suggested that it is the combination of these two paralogs that exist together only in humans, that is responsible for the delayed development and higher complexity of the human neocortex.

Taken together, past studies of SRGAP2 demonstrate that it plays an important role in shaping the vertebrate nervous system by affecting neuronal morphology and migration. It shows that SRGAP2 can influence membrane curvature and regulate the dynamic membrane protrusions of migrating cells. However, no research to date has demonstrated a role for SRGAP2 in non-canonical Wnt signaling during the convergent extension movements of early vertebrate development.

CHAPTER 2
RATIONALE

Gastrulation is a key process that occurs during early embryogenesis and is essential for the establishment of the vertebrate primary body plan. The cell movements that occur during gastrulation result from the formation and stabilization of protrusions (lamellipodia) on the mediolateral surfaces. Non-canonical Wnt signaling establishes the cellular polarity that informs these cells where to form the lamellipodia and in which direction to migrate (Wallingford & Harland 2002; John B Wallingford & Habas 2005). What is lacking, however, is a clear picture of how non-canonical Wnt signaling mediates changes to Rho activation levels and the actin cytoskeleton in order to bring about such polarized cell behavior and migration.

The Y2H screen performed to identify novel Daam1 binding partners uncovered SRGAP2 as a candidate Daam1 binding partner. As SRGAP2 is a known RhoGAP, and that to date, no other RhoGAP has been shown to act in the non-canonical Wnt signaling pathway, it was chosen as the focus of my studies. In this thesis, I describe the studies that I have performed to characterize the role of SRGAP2 as a Daam1 interacting protein and a RhoGAP protein able to modulate RhoGTPase activity in the non-canonical Wnt signaling pathway during early vertebrate development.

CHAPTER 3
METHODS AND MATERIALS

Yeast two-hybrid screen

SRGAP2 was identified as a potential DAAM1 binding partner during a previously performed yeast two-hybrid screen. In brief, a rat brain cDNA library (Clontech, Mountain View, CA) was screened using the far C-terminal region of hDAAM1 (A.A. 934-1078) as bait. 3.9 million independent clones were screened and two overlapping fragments of SRGAP2 was found along with other potential DAAM1 binding partners (Nusse et al. 1991).

Cloning of Xenopus *laevis* SRGAP2

An NCBI search for the Xenopus laevis homolog of SRGAP2 showed that the gene had already been sequenced and using the sequence cataloged as: Xenopus laevis SLIT-ROBO Rho GTPase activating protein 2 (srgap2), mRNA, NM_001094430.1, primers were designed to amplify and clone SRGAP2 from *Xenopus* cDNA stage 18. Full length SRGAP2 was cloned into PCS2-MT and PCS2-GFP-N3 vectors. Truncated versions of SRGAP2 and ΔN-SRGAP2 were cloned into PCS2-MT vectors. Cloning was verified by DNA sequencing using SP6 and T7 primers along with the primers listed in table 1. Full length SRGAP2 and ΔN-SRGAP2 were used as templates to generate RNA for *Xenopus* microinjection studies using mMessage mMachine SP6 purchased from Ambion (Ausin TX). In situ constructs were generated by PCR and cloned into the pGEM-Teasy Vector (Promega). After PCR reactions, inserts were first resolved on agarose gels, excised, and purified.

DNA was isolated by either Qiaquick PCR Purification Kit (Qiagen, Valencia CA), or phenol/chlorophorm/isopropanol extraction. DH5α cells were used for all bacterial transformations. For isolation of vectors after amplification, Qiagen Miniprep or Maxiprep kits were used (Qiagen, Valencia CA).

Primers and PCR reactions

Table 1 contains the list of primers used and the experiments that employed them. All primers were purchased from Integrated DNA Technologies (Coralville IA) and optimized according to melting temperature and length of construct. PCR reactions were performed as follows: 95^{o} for 5 minutes; 25-30 cycles of 95^{o} for 30 seconds, 53-57^{o} for 30 seconds. RNA for RT-PCR reactions was extracted for Xenopus laevis embryos homogenized in homogenization buffer (1M Tris pH 7.5, 5M NaCl, 0.5M EDTA pH 8, 10% SDS, and 400μg/mL Proteinase K) using the phenol/chloroform method of RNA extraction and 8M LiCl. Reverse transcription was performed using two different kits. For amplification of larger DNA sequences, SuperScript II Reverse Transciptase First Strand DNA Synthesis Kit (Invitrogen, Carlsbad CA) was used. For Shorter sequences, iScript cDNA Synthesis Kit was used (Bio-Rad, Hercules CA).

Experiment	Gene	Primer Sequence (5'-3')	Rest Sites
RT-PCR	**SRGAP2**	**FWD**:CCGCTCGAGATGACATCGCCG **REV:** GCGCTCGAGTCACGGAGAC	**XhoI**
ΔN- Rescue	**SRGAP2**	**FWD:** CAAGGCCTCTCGAGATGCCGGCGAA **REV:** GCGCTCGAGTCACGGAGAC	**XhoI**
SRGAP2 Truncates	**SRGAP2=F-BAR**	**FWD**:CCGCTCGAGATGACATCGCCG **REV:** GCGCTCGAGTCATAGAAATTCTTTTA	**XhoI**
	SRGAP2-RG	**FWD**: GCGCTCGAGCCGGCGAAATTTAAAA **REV:** CCGCTCGAGATGCAGAATGATGATG	**XhoI**
	C-SRGAP2	**FWD:** CCGCTCGAGTCACACCGGACAAAA **REV:** GCGCTCGAGTCACGGAGAC	**XhoI**
Sequencing	**1-400**	**REV:** TTTCCTAGCTTGGTTGCTACA	
	400-800	**FWD:** CATGGACTGACAGACGGAAT **REV:** TGATCTGACCAAATCGTGGA	
	800-1200	**FWD:** CCTGCTTCTCACACAGGTAAAG **REV:** CAGATGCATTTGTTGCTTCC	
	1200-1600	**FWD:** CCAGGCCAAATACACAGAAAA **REV:** CTTGGATGGTCTGCAGCGT	
	1600-2000	**FWD:** CAGCAGCTACAGTGTCGTTTG **REV:** GAGAACCTGACACCCGAAAT	
	2000-2400	**FWD:** CCAAGCAACCCATTCCACTA **REV:** ATGTGATTGGCAGGACACCT	
	2400-2800	**FWD:** ATCCCTACAATCTGGCCATC **REV:** CTGGCCTCTTCCTCTGTTTG	
	2800-3200	**FWD:** CACAGCAAGAGCCACTCTCA **REV:** AAGGTATCCAGCACCACGTC	
	3200-	**FWD:** GCTACCATGAACTCTGCCCT **REV:** TTAACCTCTCCCCTGCCTTT	
RT-PCR	**Xbra**	**FWD:** GCTGGAAGTATGTGAATGGAG **REV:** TTAAGTGCTGTAATCTCTTCA	
	GSC	**FWD:** CACACAAAGTCGCAGAGTCTC **REV:** GGAGAGCAGAAGTTGGGGCCA	
	Chordin	**FWD:** CCTCCAATCCAAGACTCCAGCAG **REV:** GGAGGAGGAGGAGCTTTGGGACAAG	
	EFIα	**FWD:** CCTGAACCACCCAGGCCAGATTGGTG **REV:** GAGGGTAGTCAGAGAAGCTCTCCACG	

Table 1: List of Primers used for indicated experiments. FWD: Forward primer; REV: Reverse primer

Antibodies

Mouse monoclonal anti-Myc antibody (c-Myc (9E10): sc-40, Santa Cruz) was used for immunoprecipitation, immunoblotting, and immunocytochemistry. Rabbit poyclonal anti-Myc antibody (A-14, sc-789, Santa Cruz) was employed in some immunoprecipitation reactions. Mouse anti-GFP antibody (Roche) was used as a capture antibody in immunoprecipitation reactions. Roche Anti-GFP is a mixture of two monoclonal mouse clones (7.1 and 13.1). Rabbit monoclonal anti-GFP ((D5.1)XP, Cell Signaling) was used for Immunoblotting, All antibodies were used at optimized concentration for each method, with appropriate secondary antibodies. Alexa Fluor secondary antibodies against mouse Abs were used in immunocytochemistry experiments, along with Texas Red or Oregon Green Phalloidin, purchased from Molecular Probes (Invitrogen, Eugene OR)

Immunoprecipitation, Immocytochemistry, and Immunoblotting Experiments

HEK293T and Hela cells were seeded in 6-well plates. At 60-80% confluence, cells were transfected using Polyfect reagent (Qiagen) according to the manufacturers protocol using 1–2 μg of each indicated plasmid. Post-transfection, cells were allowed to recover for 12-24 hours in DMEM containing 10% FCS and then washed 2X with PBS before being incubated for an additional 24 hours in media containing 0.5% FCS. For experiments involving stimulation with Wnt conditioned media, after 12 hours recovery time, cells were washed twice with PBS, then incubated for an additional 24 hours under low serum conditions before application of the conditioned media. Treatment with conditioned media lasted between 4 and 5 hours. Cells were lysed using 0.5% NP-40 buffer.

For immunoprecipitation reactions, cells were lysed in 600 μL of 0.5% NP-40 buffer, 50 μL of the original lysate was saved and used for Western Blot analysis to examine expression levels of the expressed fusion proteins. The remaining sample was then incubated with 20 μL prewashed Protein G-PLUS Agarose beads (sc-202, Santa Cruz, Santa Cruz CA). After the initial pre-clearing step, 250 μL of each sample was incubated overnight with 3 μL of anti-Myc, or anti-GFP antibody over at 4^o on a nutator. The following morning, 10 μL of prewashed Protein G-PLUS Agarose beads (sc-202, Santa Cruz, Santa Cruz CA) was added to each sample and again incubated at 4^o on a nutator for an hour. Following this, samples were spun down and washed 5X in 0.5% NP-40 lysis buffer. After the final step, 20-30 μL Laemmli buffer with 10% β-mercaptoethanol was added to each sample and the sample were placed in a hot plate at 98^o for 5 minutes before being placed in ice.

Western Blotting was performed as follows: 10% SDS-PAGE gels were made, and between 10 μL and 20 μL of samples were added to each lane. The gel was then run at 110 volts until finished. Gels were then transferred to nitrocellulose membranes at 40-60 volts for two hours, and the membranes were then blocked for one hour in 5% non-fat dried milk in PBST. Following the blocking step, membranes were washed in PBST and then incubated on the nutator with primary antibody for one hour at room temperature or at 4^o overnight. Before application of the secondary antibody, membranes were washed 3X with 5% milk in PBST. Secondary antibodies were diluted in either 1% newborn calf serum in PBST or 5% milk in PBST. Incubation with the secondary hour was always for two hours. Following this, membranes were washed 3X with 5% milk in PBST, followed 3X washes with PBST. Blots were then developed using homemade ECL reagent and standard methods.

GST-Pull Down Assays

GST-pull down assay was performed using whole cell lysates of 293T cells transfected with Myc-tagged SRGAP2. Briefly, equal amounts of GST fusion proteins and GST were incubated at 30°C for 30 min in 50 μl of nucleotide exchange buffer [50 mM HEPES (pH 7.08), 5 mM EDTA, 0.1 mMEGTA, 50 mM NaCl, 0.1 mM DTT] with 0.5 mM of GDP or GTPγS, and the reaction was terminated by addition of $MgCl_2$ to a final concentration of 20 mM. Then, pre-cleared lysates were incubated with the GST–GTPases (GDP or GTPγS bound) at 4°C for 1 hour before the beads were washed three times in wash buffer [20 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 0.1% Triton X-100] and analyzed by SDS-PAGE, followed by immunoblotting with anti-Myc monoclonal antibody.

Co-expression of constitutively active forms of Rho, Rac, and Cdc42 with SRGAP2

Myc tagged constructs containing RhoA (L^{63}RhoA), Rac1 (V^{12}Rac1), and Cdc42 (V^{12}Cdc42) were used. These constructs were obtained from Dr.Karen Symes, Boston University.

Morpholinos

Morpholino oligonucleotides (MOs) purchased from Gene Tools, LLC (Philomath, OR) were designed to block the translational start site of SRGAP2. The oligonucleotide sequences are listed in Table 2.

Gene	Morpholino Sequence
xSRGAP2	**5' GATGTCATTTTCCTAGCTTGGTTGC 3'**
Standard Control Morpholino	**5' CCTCTTACCTCAGTTACAATTTATA 3'**

Table 2: Morpholinos used in *Xenopus* loss-of-function studies

Micro-Injection studies in *Xenopus laevis*

Priming of female *Xenopus laevis* frogs was performed using 200-300 μL of diluted human chorionic gonadotropin hormone (2000 U/mL) (Sigma) by sub-cutaneous injection the evening before experiments were performed. The morning of experiments, the primed frogs were squeezed to release eggs. Eggs were then incubated with 1/4-1/2 dissected testis homogenized in 500μL 0.1% MMR. After a 5-10 minute period to ensure that the fertilized eggs had properly adhered to the petri dish, they were covered with 0.1% MMR for 20 minutes. Embryos were then removed from the petri dish and placed into a beaker were they were treated with 3% cysteine in 0.1%MMR and vigorously swirled in the beaker to de-jelly embryos. Following de-jellying, embryos were washed 10X times with 0.1% MMR. Embryos were then separated into three separate petri dishes and incubated at temperatures of 14^{o}C, 18^{o}C, and room temperature to allow for staggered developmental times.

Needles used for injections were calibrated according to length and pressure to allow for a 20nL injection per embryo, (10nL per blastomere). Embryos were injected at the four-cell stage in either the dorsal or ventral blastomeres. mRNA for the injections was made using the mMessage mMachine kit (Ambion) as previously described. During injections, embryos for placed small petri dishes that contained a mesh bottom to allow for proper positioning of the embryos. The dishes also contained 3% Ficoll and 5% BSA in 0.1% MMR. Two hours after injection, embryos placed in 0.1% MMR buffer once again and allowed to develop until the appropriate time. Embryos were then transferred to MEMFA before scoring and visualization occurred, and then moved to vials of methanol for long-term storage. Embryos were visualized using the Nikon SMZ800 stereomicroscope.

CHAPTER 4
RESULTS

4.1 SRGAP2 is a Daam1-Interacting Protein

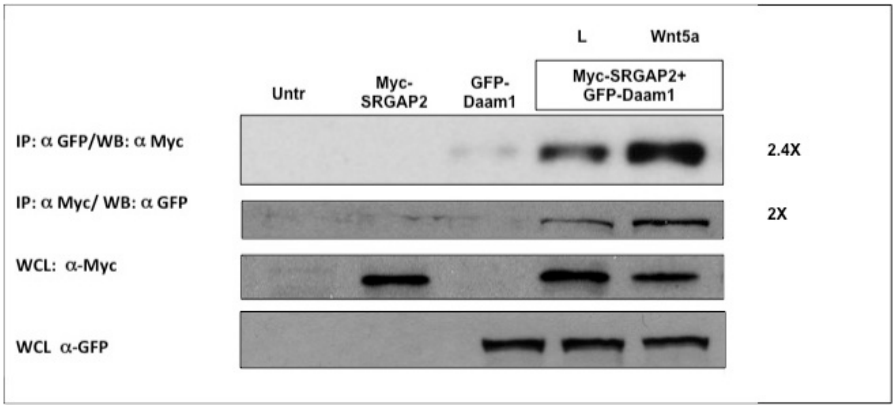
4.1.1 SRGAP2 interacts with DAAM1

The yeast two-hybrid screen revealed an interaction between SRGAP2 and Daam1 and suggested that SRGAP2 may be a functional partner for Daam1 function in non-canonical Wnt signaling. In order to verify that this interaction can occur outside of yeast, we employed mammalian cells to validate potential interaction and to further test whether the interaction was influenced by Wnt stimulation. Two constructs were generated for these studies: a Myc-tagged SRGAP2 and a GFP-tagged hDAAM1. These constructs were transfected into HEK293T cells and twenty-four hours post-transfection, the cells were then incubated in low serum media for an additional 24 hours before being treated with either control media, or Wnt conditioned media for 4 hours. Following the incubation period, an immunoprecipitation experiment was performed using commercially available Myc-and GFP-antibodies. The results demonstrate that an interaction between SRGAP2 and Daam1 did occur and validated the yeast two-hybrid experiment, and importantly that the interaction is increased in the presence of Wnt conditioned media. (Fig. 7A)

4.1.2 SRGAP2 interacts preferentially with activated Daam1

Daam1 like most other members of the Formin family is an auto-inhibited protein. In the inactive state, Intramolecular interactions between a C-terminally located **D**iaphanous **A**uto-inhibitory **D**omain (DAD) and a N-terminally located **D**iaphanous **I**nhibitory **D**omain maintain the auto-inhibited state until Daam1 is relieved from this condition through interaction with Dishevelled via the DAD domain of Daam1 (Bruce L. Goode & Eck 2007; Liu et al. 2008). Due to this phenomenon, both C-Daam1 and Daam1ΔDAD that abrogates this interaction, act as activated forms of Daam1. Taking advantage of the fact that GFP tagged constructs of Daam1 and Daam1ΔDAD (Fig. 8a) are almost the same size and are expressed roughly at the same levels, a co-immunoprecipitation experiment was performed to determine if SRGAP2 interacted preferentially with the inactive, or active form of Daam1. The results show that SRGAP2 interacts preferentially with Daam1ΔDAD. These results reiterate the results seen during the co-immunoprecipitation reaction using cells treated with Wnt conditioned media, and further suggest that SRGAP2 and Daam1 interaction is increased upon activation of the non-canonical Wnt signaling pathway (Fig. 7B).

A.



B.

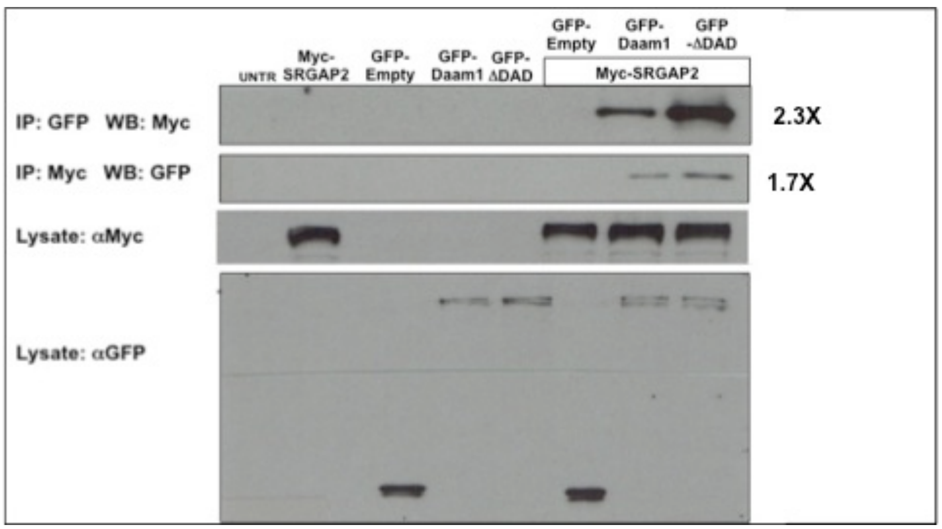


Figure 7: SRGAP2 interacts with Daam1 and the interaction is positively influenced by upon activation of the non-canonical Wnt signaling pathway.

A. Co-immunoprecipitation experiment showing that SRGAP2 and Daam1 can interact, and that the interaction increases after cells are incubated in either Wnt5a or Wnt3a conditioned media. **B**. SRGAP2 interacts preferentially with activated Daam1 in the form of Daam1ΔAD.

4.1.3 SRGAP2 Interacts with the N- and C-Terminal Regions of Daam1

Having determined that SRGAP2 and Daam1 can indeed interact as predicted by the yeast two-hybrid results, the next step was to more closely examine the interaction by determining what region(s) of the proteins are responsible for the interaction. For these studies, co-immunoprecipitation experiments were performed which utilized two GFP-tagged truncated forms of Daam1 along with full length Myc-SRGAP2 (Fig. 8A). The truncated versions of Daam1 used for this experiment were the N-terminal region of Daam1 (N-Daam1) and the C-terminal region of Daam1 (C-Daam1). A GFP empty vector was used as a negative control for the experiment. The truncated versions of Daam1, and the GFP empty vector were co-transfected into 293T cells along with Myc-tagged SRGAP2. The results of this experiment show that SGAP2 can physically interact with both N-Daam1 and C-Daam1 (Fig 8B).

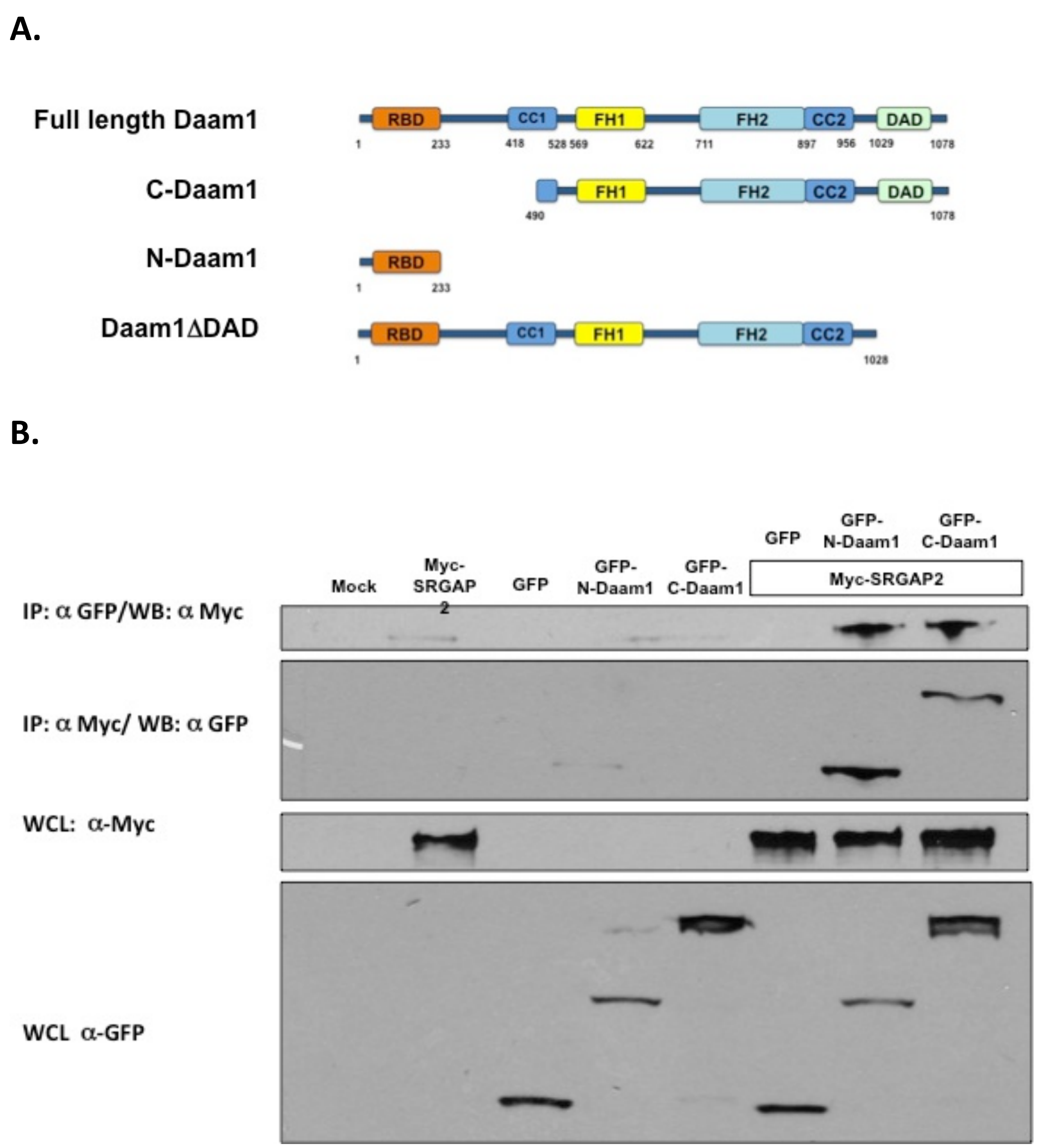


Figure 8: SRGAP2 interacts with both the N-terminal region and the C-terminal region

A. Protein domain structure of Daam1 and its truncated mutant constructs**. B.** Co-immunoprecipitation experiment showing that full length SRGAP2 can interact with both N-Daam1 and C-Daam1, but does not interact with the GFP empty vector that was used as a control.

4.1.4 Multiple Regions of SRGAP2 Interact with Daam1

To gain additional insight into the interaction between SRGAP2 and DAAM1, truncated versions of SRGAP2 were generated (Fig 9) and used in conjunction with the truncated forms of Daam1 in a series of co-immunoprecipitation experiments to determine what region(s) of SRGAP2 was involved in the interaction (Fig 8A). As SRGAP2 contains at least two distinct domains, three Myc-tagged constructs were generated. A construct containing the N-terminally located F-Bar domain (Myc-F-Bar), another containing the RhoGAP domain (Myc-RG), and finally a construct containing the C-terminal region of the protein (Myc-C-S). For the first experiment, the three SRGAP2 constructs were co-transfected into 293T cells along with C-Daam1, the cells were allowed a 24 hour recovery time in full serum media, before being incubated in low serum conditions for an additional 24 hours. The same procedure was then repeated, but using N-Daam1 along with the SRGAP2 truncates. The results of these two experiments indicate that Myc-RG, the middle region of SRGAP2 that contains the RhoGAP domain interacts with N-Daam1, while the N-terminal region of SRGAP2 that contains the F-Bar domain, interacts with C-Daam1. (Fig. 10)

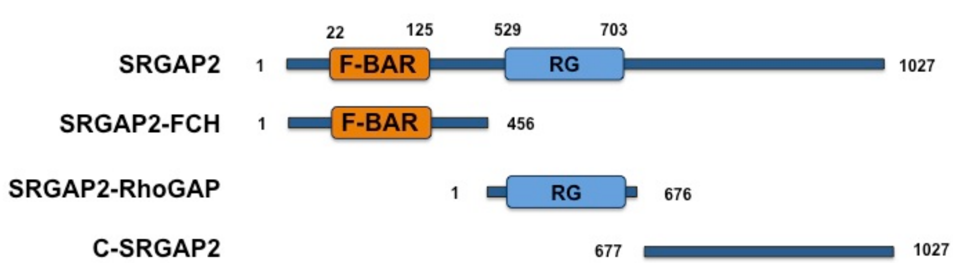
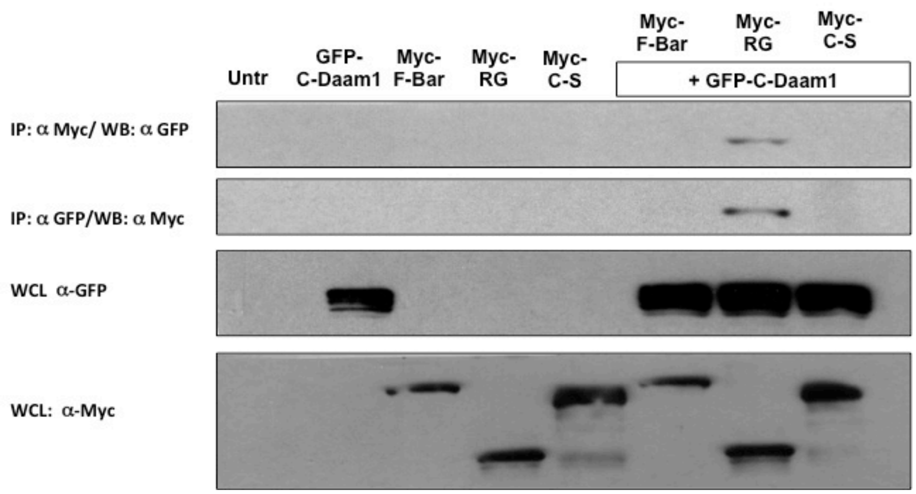


Figure 9: Diagram depicting SRGAP2 domain composition and truncated versions

A.



B.

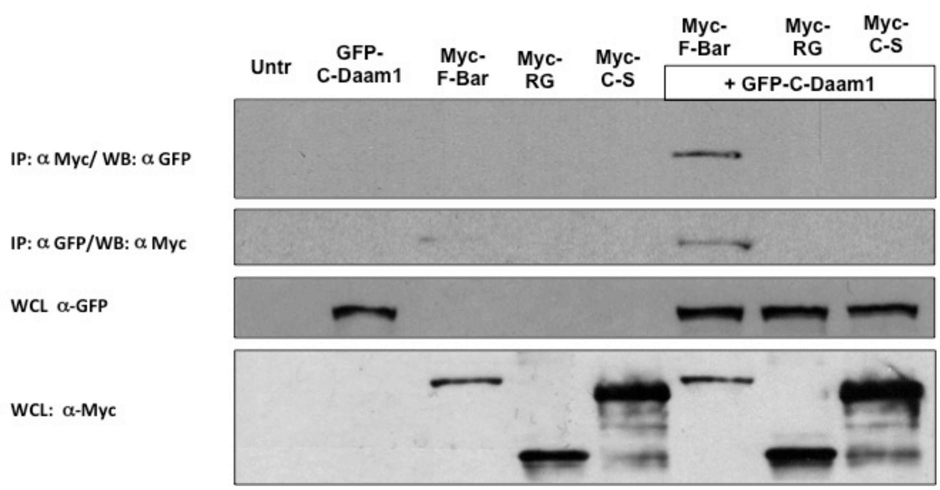


Figure 10: Multiple regions of SRGAP2 interact with Daam1

A. The mid region of SRGAP2 that houses the RhoGAP physically interacts with the N-Daam1, the N-terminal region of Daam1 that contains the RBD domain. **B** The N-terminal region of SRGAP2 that contains the F-Bar domain interacts with C-Daam1, the C-terminal region of Daam1.

4.2 SRGAP2 is a RhoGAP specifically for RhoGTPase

4.2.1 SRGAP2 Interacts with RhoA

To determine if SRGAP2 was a RhoGAP specifically for Rho, Rac, or Cdc42, a GST pull-down assay was performed using GST-tagged constructs of the GTPases and cell lysates from HEK293T cells transfected with Myc-tagged SRGAP2. In this assay, the GST-tagged constructs are incubated with either 10 μM of the non-hydrolysable GTP-γ-S or GDP and act as the activated or non-activated form of RhoGTPase. The results show weak binding between SRGAP2 and all constructs containing a RhoGTPase fusion protein when compared to the empty vector, but SRGAP2 preferentially bound to the activated form of RhoA, suggesting that it is a RhoGAP specifically for RhoA. (Fig. 11)

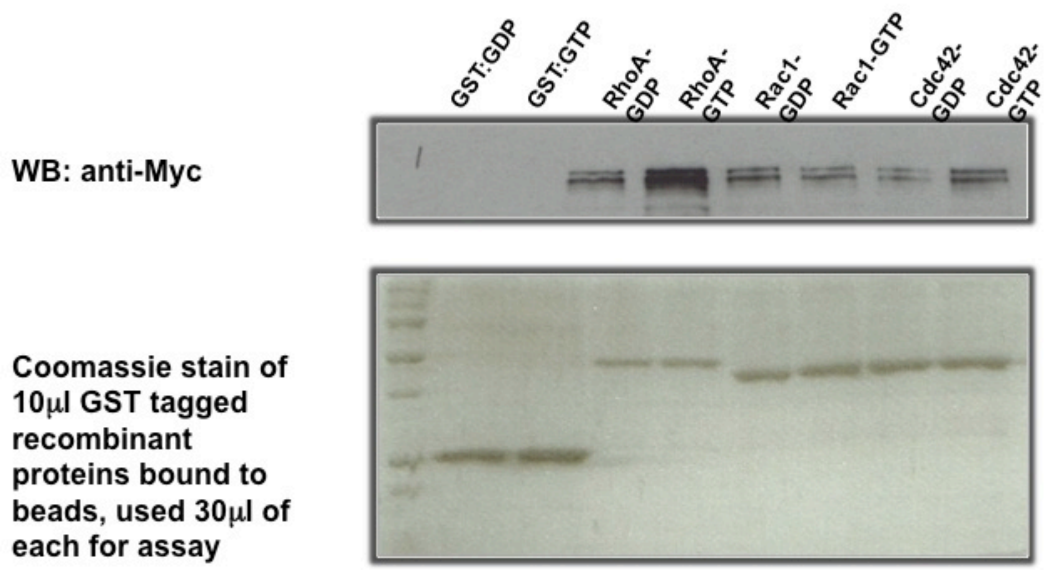


Figure 11: SRGAP2 binds preferentially to GTP-bound RhoA.

The results of a pull-down assay using GST fusion versions of Rho, Rac, and Cdc42 that had been incubated with either GDP, or GTP-γ-S, a non-hydrolysable form of GTP show that SRGAP2 binds preferentially with GTP-bound Rho when compared to both GTP or GDP bound forms of the other GTPases.

4.2.2 SRGAP2 decreases the levels of GTP-bound Rho in HEK293T cells

To determine whether SRGAP2 could influence the levels of GTP-bound Rho, Rac, or Cdc42 within cells, an RBD/PBD assay was performed. The RBD assay exploits the fact that effector proteins interact solely with the GTP-bound forms of RhoGTPases. The RBD assay utilizes a fusion of Rho-binding domain of Rhotekin a well-established Rho effector molecule with Glutathione S-transferase (GST) coupled to glutathione beads to affinity-precipitate GTP-bound RhoA (Mezzacappa et al. 2012). The PBD assay functions identically to the RBD assay, but uses the p21-binding domain of p21 activated kinase (PAK) allowing affinity-precipitation of the activated forms of Rac and Cdc42 (Benard et al. 1999; Ren et al. 1999). The results show, that SRGAP2 has a substantial impact on the levels of activated RhoA, but little to no affect on the levels of GTP-bound forms of Rac1, or Cdc42. These results are in agreement with the previous experiment and reinforce the conclusion that SRGAP2 is a RhoGAP specifically for Rho. (Fig. 12)

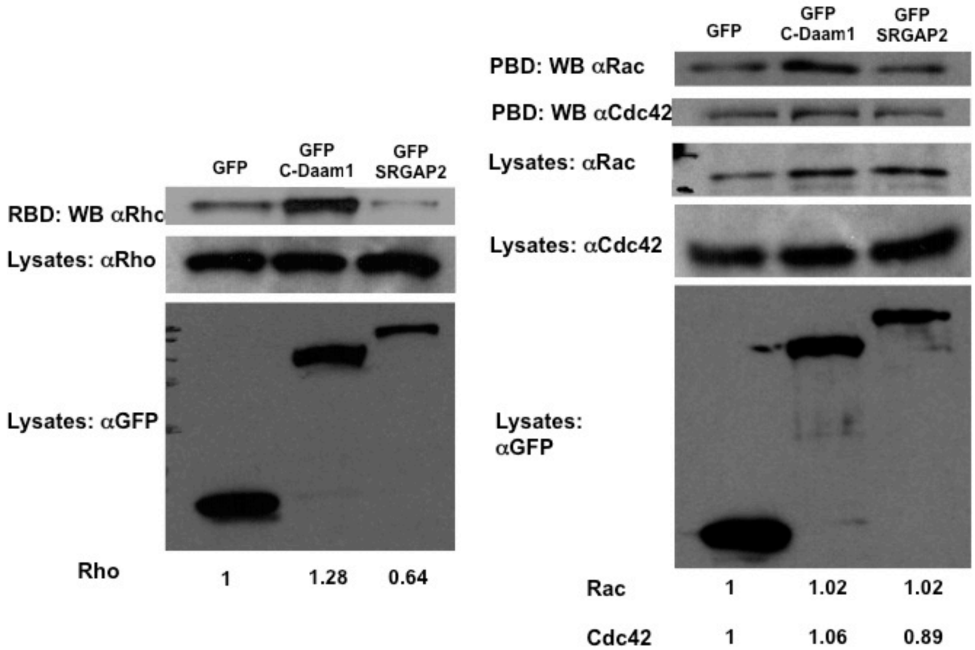


Figure 12: Ectopic expression of SRGAP2 lowers levels of activated Rho in 293T cells

The RBD/PBD assay examining levels of activated Rho, Rac, and Cdc42 in 293T cells transfected with GFP, as a negative control, GFP-C-Daam1, which has previously been shown to increase levels of GTP-bound Rho, as a positive control and GFP-SRGAP2. The results show that that GFP-SRGAP2 decreases the levels of activated Rho GTPase in cells. Little difference was seen in levels of activated Rac or Cdc42.

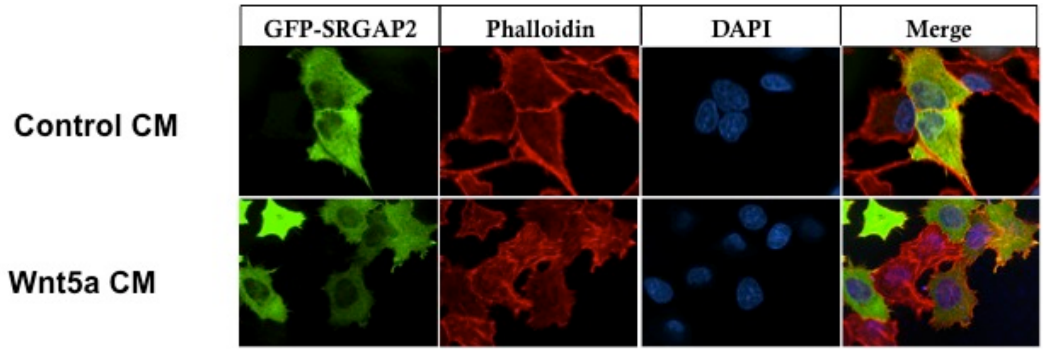
4.3 Subcellular Localization Pattern of SRGAP2

Having determined the SRGAP2 and DAAM1 biochemically interacted; the investigation was next continued on a subcellular level. These experiments were designed to garner insight into the localization and function of SRGAP2 through the examination of its subcellular localization pattern using immunofluorescent microscopy. If SRGAP2 plays a role in regulating Daam1 during non-canonical Wnt signaling, it would be expected that these two proteins would co-localize in response to Wnt treatment. Past work on Daam1 has already demonstrated that its subcellular localization pattern can be modulated by Wnt stimulation (Liu et al. 2008). These experiments showed that in the absence of Wnt stimulation, Daam1 primary resides in the cytoplasm, but upon stimulation, can be seen co-localized with actin stress fibers and along the plasma membrane (Liu et al. 2008). I first examined the cellular localization pattern of SRGAP2 alone, then in relation to Daam1, and finally if and how the pattern of these two proteins change in response to Wnt stimulation. Also, as actin stress fiber formation is a hallmark of Wnt stimulation, a secondary goal was to examine the affect that over-expression of SRGAP2 has on cell morphology and the actin cytoskeleton (Liu et al. 2011).

4.3.1 SRGAP2 is found throughout the Cytoplasm

To examine the subcellular localization pattern of SRGAP2, HeLa cells were transfected with GFP tagged SRGAP2 and then incubated with either Wnt5a conditioned media, or L-Control media (Fig. 13). This experiment was performed with a dual purpose; first, to observe if the presence or absence of Wnt had any affect on the subcellular localization pattern of SRGAP2, and secondly, to examine if SRGAP2 could influence the ability of Wnt5a to induce actin stress fibers in stimulated cells. The results of these studies demonstrate, that in the absence of Wnt, SRGAP2 is localized throughout the cytoplasmic region, and that upon treatment with Wnt5a CM, SRGAP2 translocated to the plasma membrane, specifically to areas of cellular protrusions (Fig. 13B and Graph 1). These studies also show that over-expression of SRGAP2 negatively affects stress fiber formation in Wnt treated cells (Fig. 14 and Graph 2). These results demonstrate that the localization pattern of SRGAP2 in cultured cells is altered upon Wnt stimulation and that SRGAP2 can directly affect Wnt mediated cytoskeletal changes. Taken together, these studies demonstrate that SRGAP2 functions within the non-canonical Wnt signaling pathway as a regulator of the actin cytoskeleton.

A.



B.

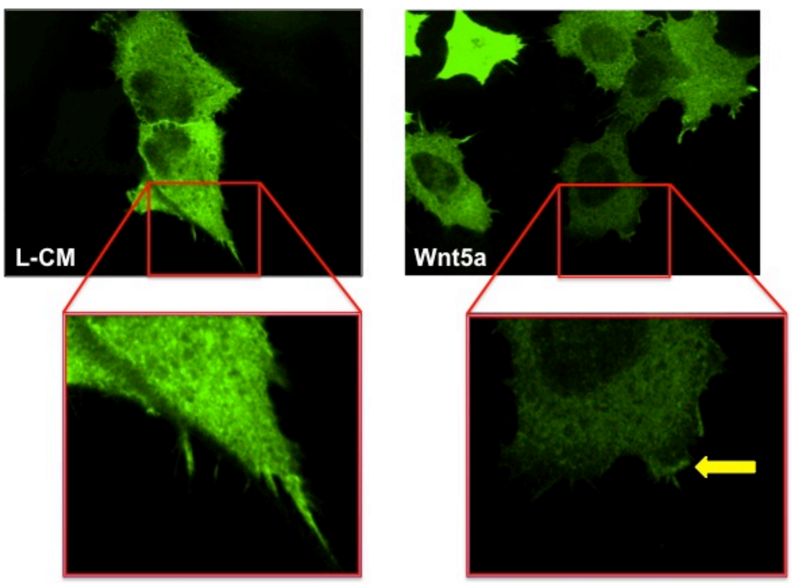
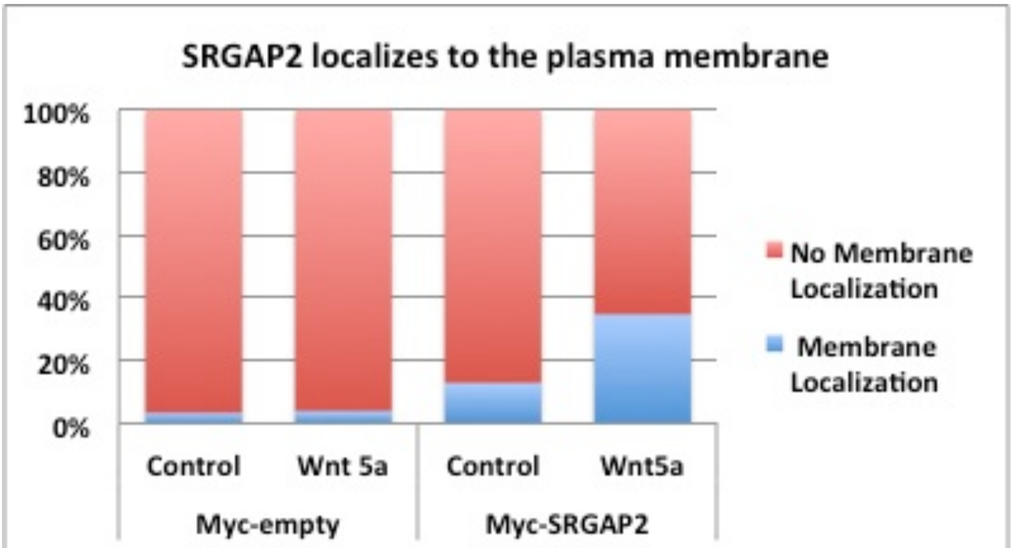


Figure 13: Subcellular localization pattern of SRGAP2

A. Hela cells transfected with GFP-SRGAP2 and incubated in control conditioned media show that SRGAP2 is found throughout the cytoplasm **B.** When cells are incubated in Wnt5a conditioned media, SRGAP2 translocates to the plasma membrane particularly to areas of cellular protrusion, such as lamellipodia and filopodia.



Graph 1: SRGAP2 localizes to the plasma membrane

Myc- SRGAP2 localizes to the plasma membrane under Wnt5a stimulated conditions. No difference was seen in control cells expressing an empty Myc vector.

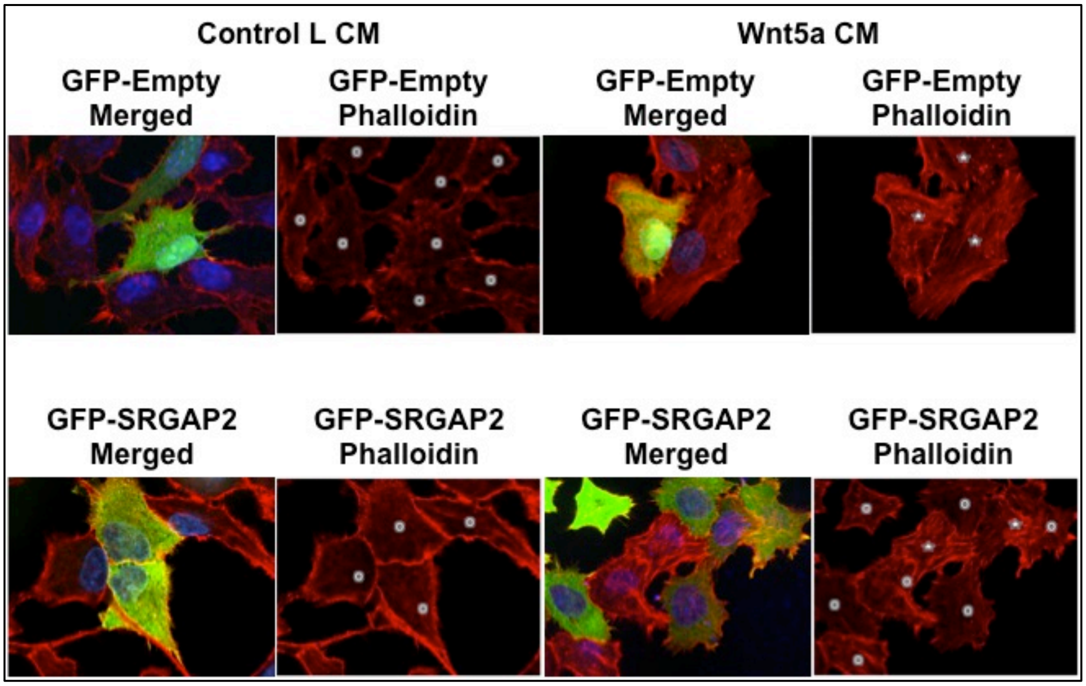
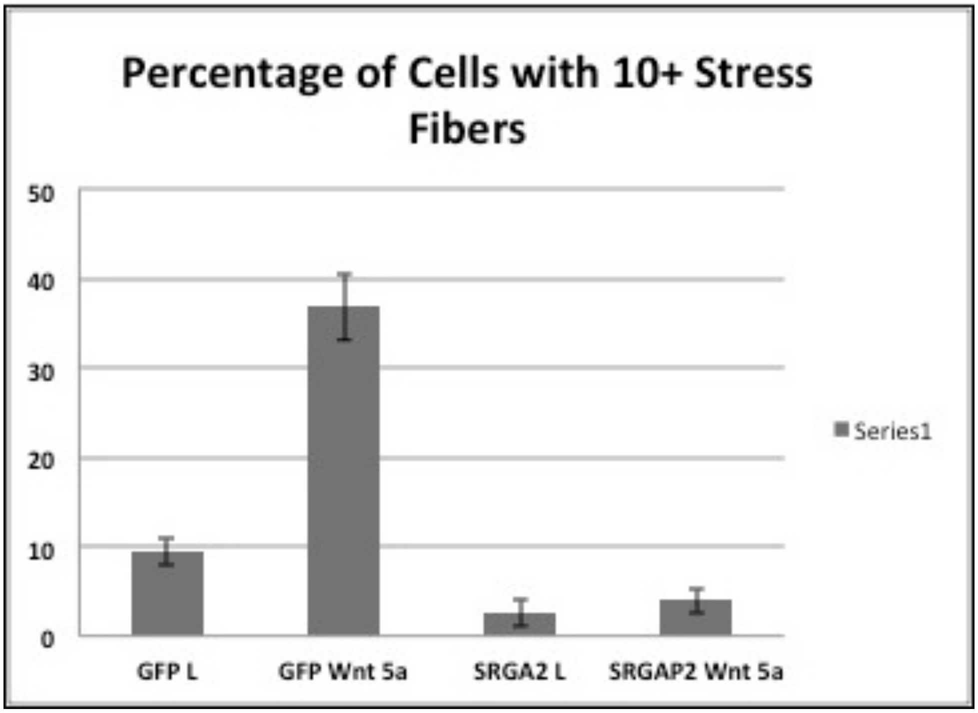


Figure 14: Actin stress fiber assay

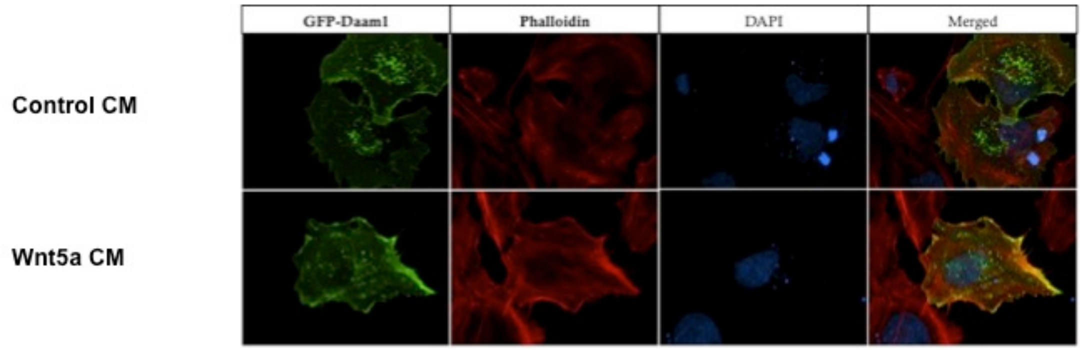
HeLa cells transfected with GFP-SRGAP2 of GFP-Empty vector were cultured in Wnt5a conditioned media, or L-control conditioned media for 4 hours. When incubated in Wnt5a conditioned media, cells containing GFP-SRGAP2 show a severe decrease in actin stress fiber number when compared with cells expressing the GFP empty vector. Actin here is labeled with Texas Red phalloidin. Cells in image are marked with either (**O**) for negative stress fiber formation, or (**+**) for stress fiber formation.



Graph 2: SRGAP2 reduces Wnt5a induced actin stress fiber

Cultures of Hela cells transfected with GFP-empty vector experience an approximately 35% increase in the number of cells containing ten or more stress fibers per cell when incubated with Wnt5a conditioned media for a period of 4 hours. Cultures of Hela cells transfected with GFP-SRGAP2 exhibit no increase in stress fiber formation. Cells were counted as positive for stress fibers if ten or stress fibers were present within the cell.

A.



B.

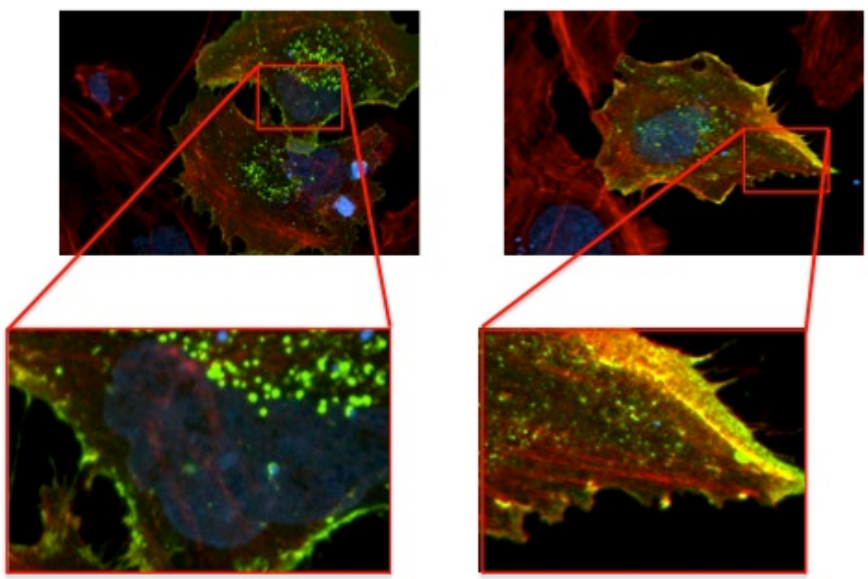
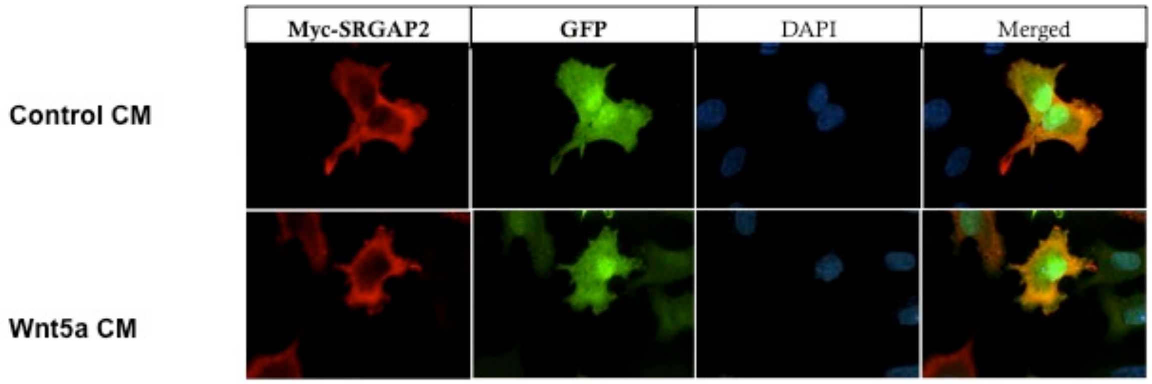


Figure 15: Subcellular localization pattern of Daam1

A: HeLa cells were transfected with GFP-tagged Daam1 and incubated with control media or Wnt5a conditioned media. When incubated in control media, Daam1 is found diffusely throughout the cytoplasm and to a lesser degree at the plasma membrane. It can also be found in distinct puncta in the peri-nuclear area. However, after Wnt5a treatment, Daam1 can be found co-localizing with actin filaments, seen here labeled with phalloidin. **B.** Enlarged images of Daam1 subcellular localization patterns.

A.



B.

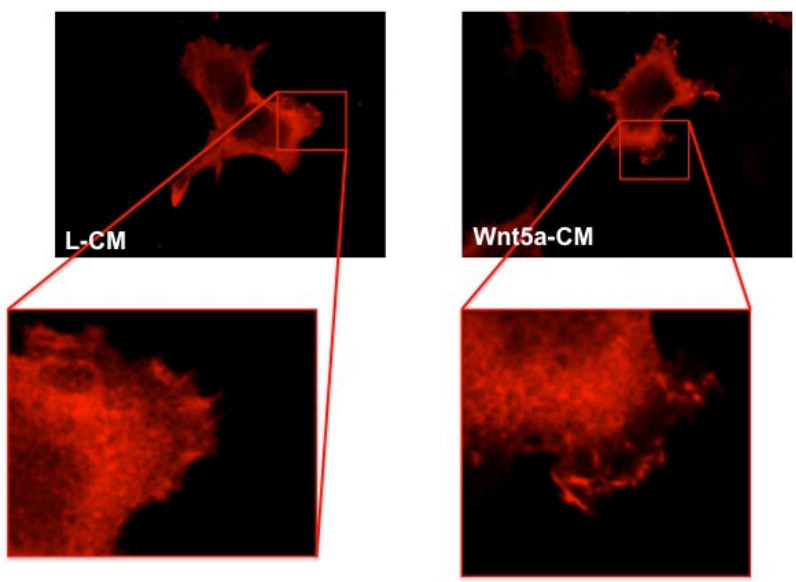


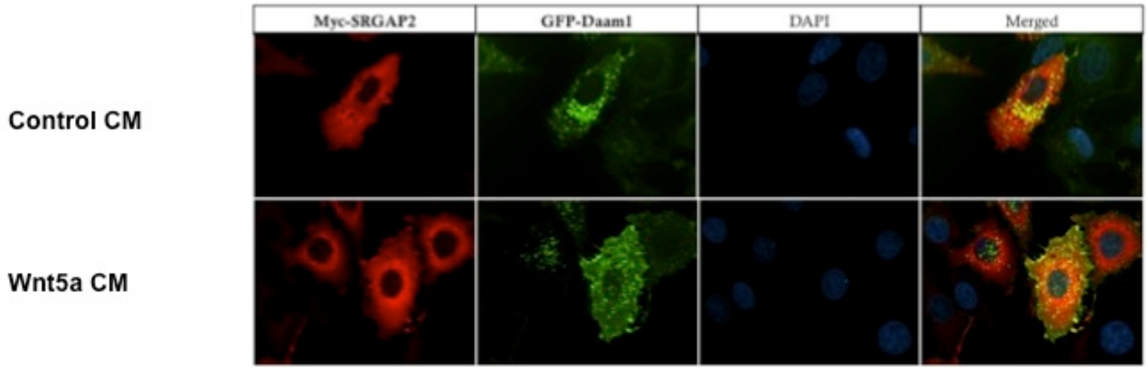
Figure 16: Subcellular localization pattern of Myc- Srgap2 and GFP

A. Upon treatment with Wnt5a conditioned media, SRGAP2 translocates to the cell periphery into areas of membrane protrusions such as lamellipodia **B.** Enlarged images of SRGAP2 localization patterns.

4.3.3 SRGAP2 and DAAM1 Co-Localize in response to Wnt Conditioned Media

Having determined that the subcellular localization pattern of SRGAP2 is influenced in cells exposed to Wnt5a conditioned media and that over-expression of SRGAP2 negatively affects Wnt5a induced actin stress fiber formation. The co-immunoprecipitation experiments performed showed that SRGAP2 and Daam1 interacted, and that the interaction increases when cells were incubated in Wnt conditioned media. To reaffirm these results and gain further insight into where this interaction occurs on a cellular level, a series of immunofluorescence studies were performed. For these studies, HeLa cells were co-transfected using epitope tagged SRGAP2 and Daam1 constructs. The cells were once again incubated with either Wnt5a conditioned media or control media for four hours before the cells were fixed and examined using immunofluoresent microscopy. The results indicate that in the absence of Wnt signaling, both proteins are found primarily within cytoplasm; SRGAP2 localization patterns mirrors the pattern found when during the single transfection experiments, while Daam1 is found primarily within distinct puncta within the cytoplasm, it is also found along the cell membrane. Limited co-localization of the two proteins was witnessed. When cells co-transfected with the two proteins are treated with Wnt5a conditioned there is an increase in co-localization, and the two proteins can be seen together along the plasma membrane and in areas of cellular protrusion. Under Wnt stimulated conditions, cells co-transfected with SRGAP2 and Daam1 also show and increase in cellular protrusions, the edges appear jagged and there is an increase in membrane ruffles.

A.



B.

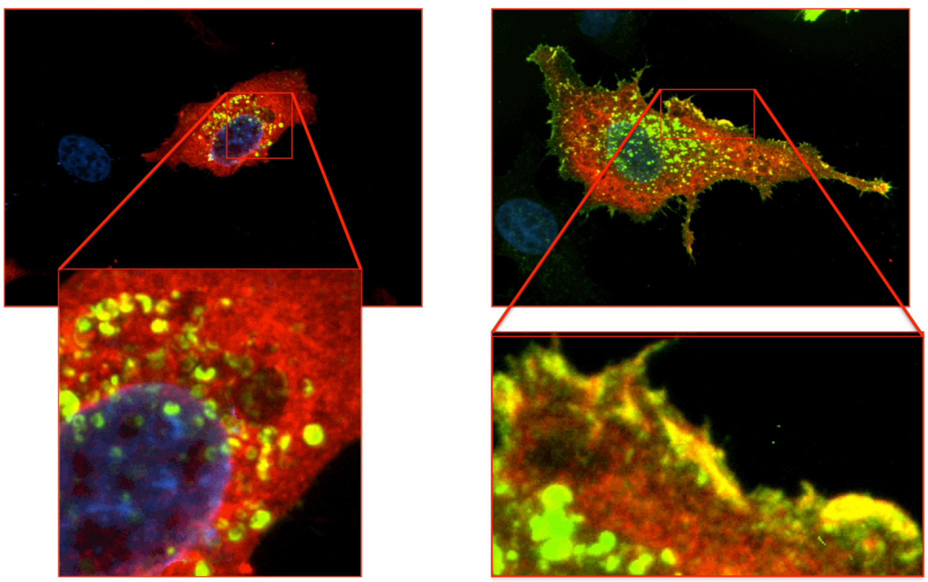


Figure 17: Co-localization pattern of Myc-SRGAP2 with GFP-Daam1

A. When Hela cells are co-transfected with Myc-SRGAP2 and GFP-Daam1 and then incubated in Wnt5a conditioned media, they co-localize along the plasma **B**. Enlarged image showing SRGAP2 and Daam1 co-localization patterns in response to Wnt stimulation

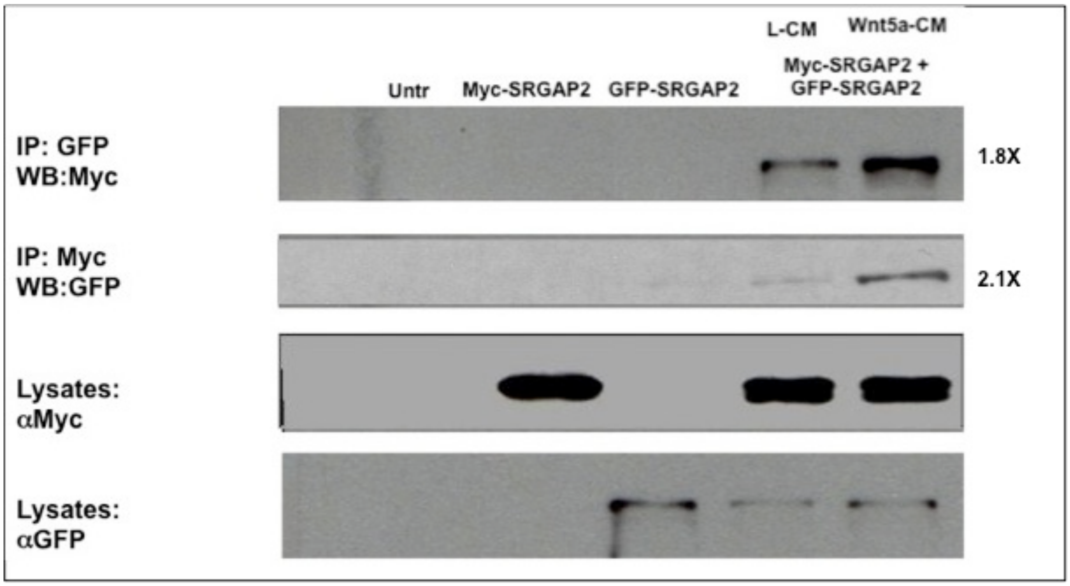


Figure 18: Wnt stimulation leads to SRGAP2-SRGAP2 interaction

A co-immunoprecipitation reveals an increased interaction between Myc-SRGAP2 and GFP-SRGAP2. This suggests that Wnt stimulation can lead to dimerization of SRGAP2 proteins.

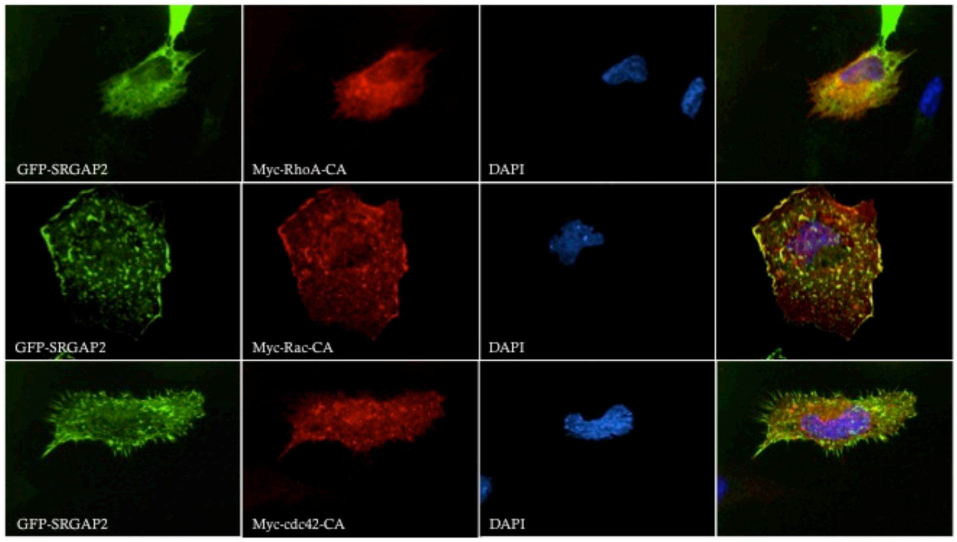
4.3.4 Wnt stimulation leads to SRGAP2-SRGAP2 interaction

SRGAP2 contains an N-terminally located F-BAR domain. These domains are known to function as dimerized pairs and localizes to the plasma membrane and also cause membrane curvature (Frost et al. 2009; Dawson et al. 2006; Roberts-Galbraith & Gould 2010). Having observed SRGAP2 relocalized to the plasma membrane when cells were stimulated with Wnt5a conditioned media, I tested whether Wnt stimulation could influence dimerization of SRGAP2 molecules. Co-transfection of 293T cells with both Myc and GFP-tagged versions of SRGAP2 followed by a co-immunoprecipitation experiment was used to determine if Wnt treatment did in fact lead to increased complex formation between the two tagged proteins. The results show that there is an increase interaction when cells were incubated in Wnt5a conditioned media as compared to cells cultured in control conditioned media and suggest that Wnt stimulation may indeed lead to dimerization of SRGAP2 molecules (Fig. 18).

4.3.5 SRGAP2 co-localizes with constitutively activate forms of Rho and Rac, but not Cdc42.

To gain a better understanding into SRGAP2's cellular localization pattern, series of immunofluorescent studies was performed to examine if and how SRGAP2 may change its sub-cellular localization in the presence of activated forms of RhoA, Rac1, and Cdc42. HeLa cells were co-transfected with GFP-SRGAP2 and a Myc-tagged version of constitutively activated forms of the RhoGTPases (**RhoA-CA, Rac1-CA, and Cdc42-CA**). The results indicate, that SRGAP2 does co-localize with RhoA-CA. They can be seen along actin fibers near the cell periphery. The results also show that SRGAP2 and Rac1-CA have a high level of co-localization. While this may appear contradictory to the data obtained through biochemical investigations, that showed no interaction between SRGAP2 and Rac, it may be explained in two ways; SRGAP2 is an F-Bar domain protein, and the F-Bar has been shown to respond to curvature within the cell membrane (Frost et al. 2009; Safari & Suetsugu 2012; de Kreuk & Hordijk 2012) and since activated Rac has the ability to induce the formation of lamellipodia and membrane ruffles (Hall 2012b). it is possible that SRGAP2 translocates to the membrane in response to the induced curvature (Fig. 19).

A.



B.

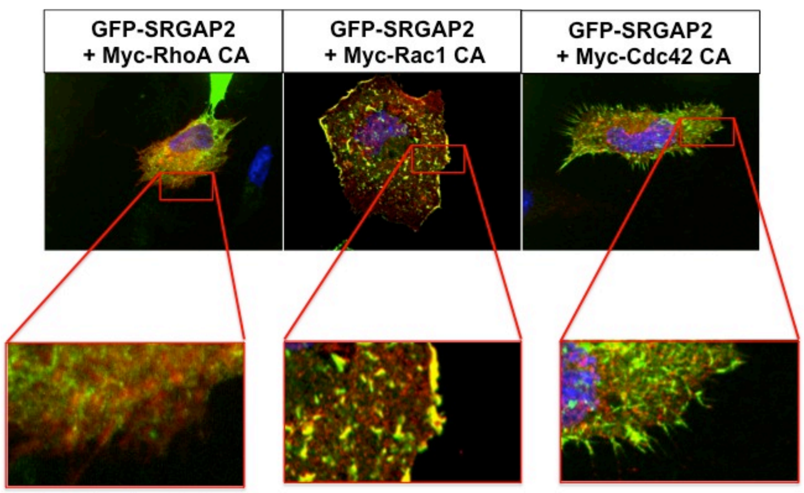


Figure 19: Co-localization pattern of SRGAP2 with constitutively active forms of RhoA, Rac1, and Cdc42.

A. Hela cells co-transfected with GFP-SRGAP and Myc-tagged constitutively active of RhoA, Rac1, and Cdc42 **B.** Enlarged regions of Figure A show both that SRGAP2 co-localizes with both RhoA and Rac1 but not CDC42.

4.4 Effects of SRGAP2 on *Xenopus* development.

4.4.1 SRGAP2 is expressed maternally and throughout development

If SRGAP2 is functional in Wnt signal transduction, its mRNA should be expressed in a temporal and spatial manner indicative of where Wnt signaling normally occurs. Therefore I first examined the temporal expression pattern of SRGAP2 during stages of *Xenopus* development starting from the egg stage to the tadpole stage by RT-PCR (Fig 20). These studies revealed that SRGAP2 is expressed both maternally and throughout development. It also reveals that levels are relatively constant as the embryo develops.

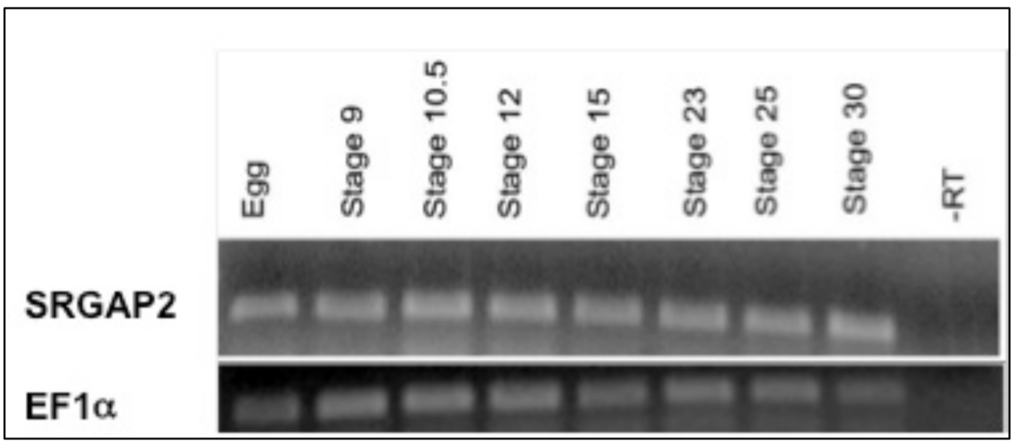


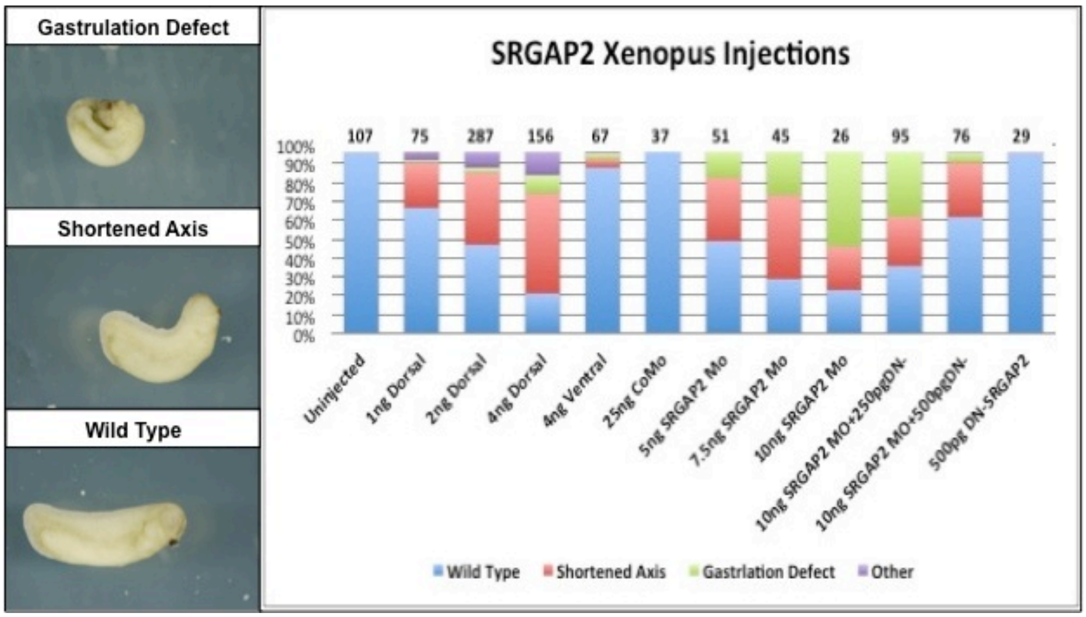
Figure 20: Expression of SRGAP2 at various stages of Xenopus development

RT-PCR revealed that SRGAP2 mRNA is expressed maternally and maintains a consistent expression level throughout the various stages of development. EF1a is used as a loading control, -RT; no reverse transcriptase.

4.4.2 Gain-of-function and loss-of-function of SRGAP2 produces severe gastrulation defects

To elucidate the function of SRGAP2, we examined its role in early development. It is well established that non-canonical Wnt signaling is required for the cell movements involved in the directed cell movements of early development, and over-expression or depletion of functional components of this pathway in dorsal-marginal blastomeres of the four-cell embryo will interfere with such cell movements. Experiments were performed to determine the effects of over-expression or depletion of SRGAP2 on the developing *Xenopus laevis* embryo. For loss-of-function studies, an SRGAP2 MO was designed (Fig 22A). Additionally, a ΔN-SRGAP2 construct was generated that lacked the MO binding region to be used as a rescue construct. To first test the efficacy of the MO, I injected a reporter construct that was composed of SRGAP2 along with a region the 5'UTR directly upstream of the start codon. The Morpholinos effectively decreased the expression of this construct in a dose dependent manner. (need figure). The results show that the MO was effective at knocking down SRGAP2 expression and that the rescue construct was not affected by the SRGAP2 MO.

Dorsal injection of embryos of with SRGAP2 RNA at doses ranging from between 1 and 4ng produced a dose-dependent increase in the number of *Xenopus* embryos exhibiting gastrulation defects, or a milder phenotype of a shortened body axis, while ventral injection of embryos with 4μg SRGAP2 RNA produced no noticeable phenotypes. Dorsal injection of antisense SRGAP2 Morpholino resulted in morphant phenotypes that possess shortened dorsal-ventral axis, or that failed to properly gastrulate. Equivalent injections of control Morpholinos failed to produce a noticeable phenotype. When Morpholinos were co-injected with SRGAP2 rescue mRNA a partial rescue of the morphant phenotype was observed. This phenotype is suggestive of a role of SRGAP2 in gastrulation, as interference with this process results in such phenotypes. (Fig. 22B and Graph 3)



Graph 3: Results of *Xenopus* micro-injection experiments

Embryos were injected at the four-cell stage with RNA, SRGAP2 MO, or SRGAP2 MO + rescue RNA. Embryos injected with RNA or SRGAP2 MO exhibited severe gastrulation defects with partial or totally open neural tubes and curved body axis. Embryos injected with SRGAP2 MO + rescue RNA showed a dose dependent suppression of the morphant phenotypes. Gastrulation defects caused by over- or under expression of SRGAP2 are dose dependent.

A.

B.

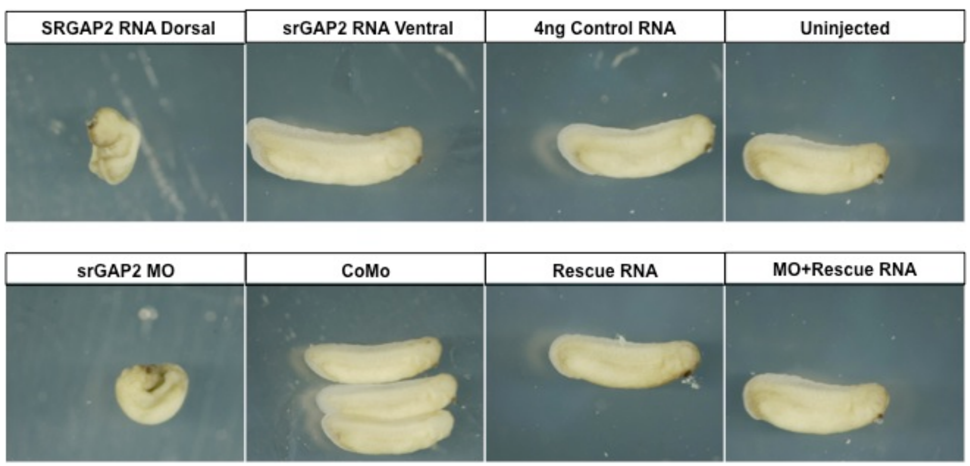


Figure 21: SRGAP2 regulates vertebrate gastrulation.

A. Diagram showing Morpholino sequence, targeted region, and ΔN-SRGAP2 rescue construct. **B.** Dorsal injection of both SRGAP2 RNA and SRGAP2 MO lead to severe gastrulation defects in *Xenopus* embryos, whereas control RNA (GFP-empty vector) or ventral injection of SRGAP2 RNA show no noticeable phenotype. The same is true of embryos injected with control MO. Embryos injected with SRGAP2 MO can be rescued using low doses of rescue RNA.

4.4.3 SRGAP2 does not affect mesoderm induction

The gastrulation phenotypes produced by the over expression or knockdown of SRGAP2 could result either from defective cell movement or be indirect due to disrupted mesodermal induction. To distinguish these possibilities, expression of the organizer mesodermal marker genes goosecoid (gsc), brachyury (Xbra) and xWnt 8 was examined by RT-PCR in SRGAP2 RNA or SRGAP2 MO injected embryos (Artinger et al. 1997; Smith & R 2006; Saint-Jeannet et al. 1994). Expression levels for these genes in embryos injected with SRGAP2 RNA, or SRGAP2 MO were indistinguishable from those in control embryos indicating that SRGAP2 does not affect mesoderm induction. (Fig 23)

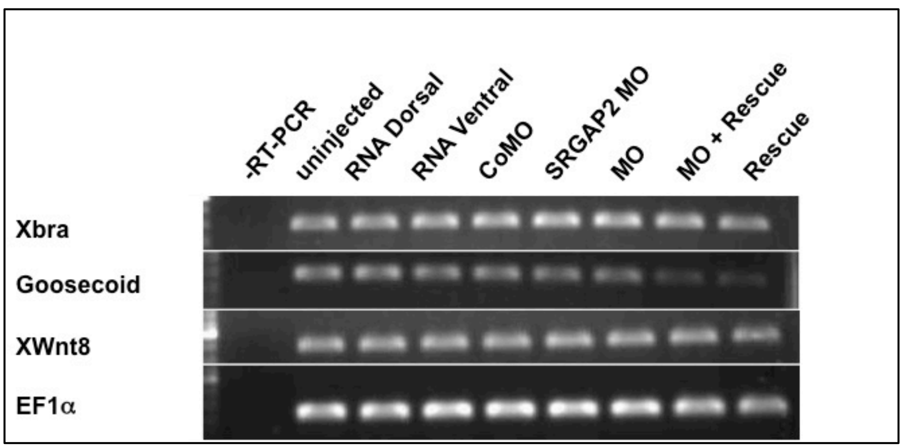


Figure 22: SRGAP2 over- or under-expression does not affect mesoderm specification

RT-PCR was used to check expression levels of known mesodermal markers; Xbra, Goosecoid, and XWnt8, in Xenopus stage 10.5 embryos. EF1-α was used as loading control. RNA injects used 4ng SRGAP2, CoMo injects used 25μg, SRGAP2 MO used 10μg, and 500 pg of rescue construct was used. Results indicate that levels of the mesodermal markers remain unchanged.

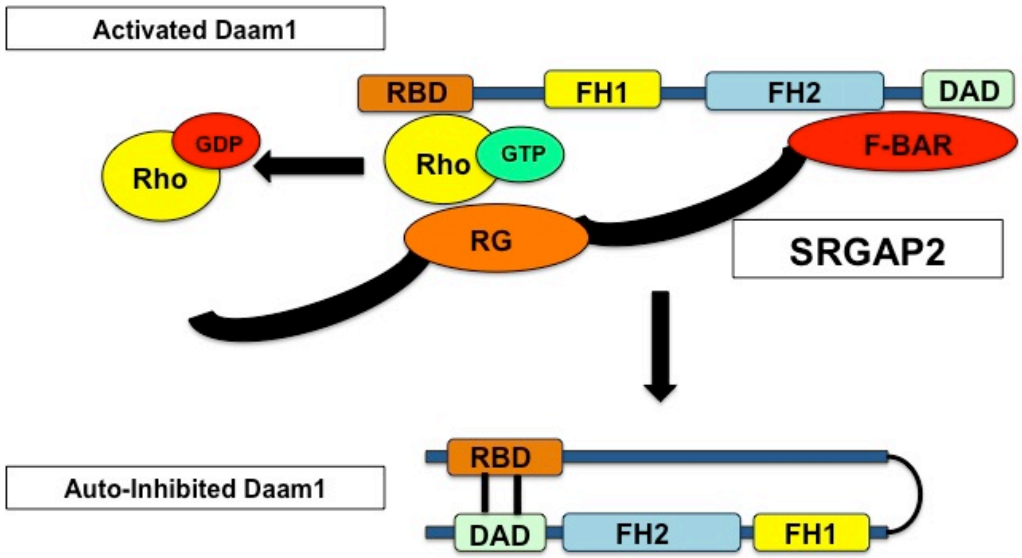


Figure 23: Model for SRGAP2/ Daam1 interaction

SRGAP2 interacts with Daam1 through two distinct regions, the N-terminal region of SRGAP2 interacts with the C-terminal region of Daam1, while the RhoGAP region of SRGAP2 interacts with the N-Terminal region of Daam1 that contains the Rho Binding Domain.

CHAPTER 5
DISCUSSION

Study of the Wnt signaling began over thirty years ago when it was realized that not only were the gene products of the Drosophila wingless (wg) and mouse Int1 were homologous, but that they were also part of a large evolutionarily conserved family of signaling proteins (Rijsewijk et al. 1987). Wnt signaling pathways participate in multiple developmental events during embryogenesis and have also been implicated in adult tissue and stem cell homeostasis (Logan & Nusse 2004). Aberrant signaling by both canonical and non-canonical Wnt signaling have been shown to be involved in multiple diseases and developmental defects. During development, mis-regulation or mutations in molecules involved in proper signaling can have severe repercussions such as tetra-amelia, a rare genetic disorder characterized by absence of limbs (Logan & Nusse 2004). In adults, Wnt signaling has also long been associated with various forms of cancer. The non-canonical Wnt-PCP has been shown to be involved in cancer metastasis and is linked to several developmental defects such as neural tube closure disorders (Mulligan & Cheyette 2013; Wallingford & Harland 2002). Due to the large number of pathologies, it becomes clear that the Wnt pathways regulate a high number of biological activities and that a complete understanding of the molecules involved would be a crucial step in hoping to one day prevent or cure these malignancies. Therefore finding new components of the Wnt signaling pathways becomes a worthy and important goal in the area of biomedical research.

In this thesis, I have identified SRGAP2 as an effector molecule for Daam1 in the non-canonical Wnt signaling pathway. SRGAP2 works to regulate Daam1 activity through direct interaction, and its ability to lower levels of GTP-bound Rho within a cell. Through the use of biochemical, cell biological, and embryological techniques, I have demonstrated that SRGAP2 further plays a crucial functional role in early vertebrate development.

SRGAP2 is a Daam1 interacting protein

Daam1 has been demonstrated to play a critical role in non-canoical Wnt signaling during early vertebrate development. Functioning directly downstream of Dishevelled to mediate cytoskeletal changes during the cellular migrations witnessed during gastrulation and neurulation (Raymond Habas et al. 2001b). Activation of Daam1 has also been shown to lead to an increase in intracellular levels of activated RhoA (Raymond Habas et al. 2001b). One important question that remained was: What are the molecular mechanisms involved in Daam1 mediated Rho activation? Since the initial findings regarding Daam1, it has been found that this activity is mediated by at least in part by one RhoGEF, WGEF, a RhoGEF that interacts with both Dishevelled and Daam1 during convergent extension (Tanegashima et al. 2008). In this thesis, I provide evidence that SRGAP2, a known RhoGAP, also plays a vital role in Daam1-Rho dynamics.

I have found that SRGAP2 prefers interacting with the activated or non-autoinhibited form of Daam1 whether the activation is due to the presence of Wnt, or through the removal of the DAD domain involved in maintaining Daam1 in an auto-inhibited state (Fig. 7). Also, this thesis shows that SRGAP2 and Daam1 interact in two distinct regions. First, the middle region of SRGAP2 that contains the RhoGAP domain interacts with the N-terminal region of Daam1 that contains the Rho binding domain. Second, the region of SRGAP2 that contains the F-BAR domain interacts with the C-Daam1, the C-terminal half of Daam1.

Knowing that Wnt stimulation leads to the alleviation of Daam1's auto-inhibited state and the subsequent binding of activated Rho to the RBD domain, it may be that the conformational changes that occur in Daam1 after being activated are necessary to expose the sites required for SRGAP2 interaction.

SRGAP2 binds preferentially to activated RhoA

It has been established, that Daam1 is necessary for the activation of RhoA in non-canonical Wnt signaling (Raymond Habas et al. 2001b; Liu et al. 2008). Having found that SRGAP2, a known RhoGAP, can interact with Daam1 lead to the natural question of whether SRGAP2 can regulate Rho activity. Past research performed on human SRGAP2 has shown that hSRGAP2 is a RhoGAP specifically for the small monomeric GTPase Rac (Wong et al. 2001; Guerrier, Coutinho-Budd, Sassa, Gresset, Jordan, Chen, W. L. Jin, et al. 2009; Mason et al. 2011a). Understanding that *Xenopus* SRGAP2 may function differently than its human homolog, due to the lack of the SH3 domain, a GST-pull-down experiment utilizing the three best characterized RhoGTPases, RhoA, Rac1, and Cdc42 was performed. Surprisingly, the results of this experiment indicate that contrary to human SRGAP2, *Xenopus* SRGAP2 bound preferentially to activated RhoA and not Rac1.

It is possible that *Xenopus* SRGAP2 behaves differently than the human version due to the lack of a SH3 binding domain that is found in most other species.

To gain further insight into the specificity of SRGAP2, an RBD/PBD assay was utilized to analyze the affect the SRGAP2 had on levels of activated Rho, Rac, or Cdc42 in 293T cells. The findings of this experiment show that cells transfected with SRGAP2 contain lower levels of activated Rho. No significant change was witnessed in levels of activated Rac or Cdc42. These two experiments reinforce the fact that *Xenopus* SRGAP2 may act specifically on Rho.

Subcellular Localization Pattern of SRGAP2

To gain further insight into the role of SRGAP2 in Wnt signaling, studies examining the subcellular localization of SRGAP2 and Daam1 were performed using either Myc-tagged or GFP-tagged versions of the two proteins. The results show that both SRGAP2 and Daam1 have distinct subcellular localization patterns that change upon Wnt stimulation. In non-stimulated cells, SRGAP2 is found throughout the cytoplasm, but when cell are incubated in Wnt5a conditioned media, there is increased localization to the plasma membrane. When incubated under non-stimulated conditions, Daam1 is also found throughout the cytoplasm and to a lesser degree along the plasma membrane. It also localizes to punctate spots within the cytoplasm. Upon incubation with Wnt conditioned media, Daam1 increases its membrane localization and can be seen co-localized with actin fibers. When SRGAP2 and Daam1 are co-transfected, little co-localization is seen within cells unless the cells have been incubate in Wnt conditioned media, at that time, SRGAP2 and Daam1 can be seen co-localizing along the plasma membrane. These results are in agreement with the earlier co-immunoprecipitation reactions that showed an increase in SRGAP2 and Daaam1 interaction when the Wnt signaling pathway is activated. As Wnt signaling is known to regulate polarized cell movements, it would be of interest to determine if the subcellular location patterns are affected by cellular migration. In the studies performed for this thesis, the immunofluorescence experiments hinted at the idea that SRGAP2 localizes to the leading edge of migrating cells. In order to confirm this, and to examine if gain or loss of SRGAP2 influences cell migration rates or patterns, additional studies are required.

SRGAP2 suppresses Wnt5a induced stress fibers

Wnt stimulation can induce actin stress fiber formation which phenocopies that of activated RhoA. (Liu et al. 2011; Ridley 1999; Pellegrin & Mellor 2007). If SRGAP2 does act in the non-canonical Wnt signaling pathway as a regulator of Daam1 and Rho activation, it should abrogate Wnt induced stress fibers. In HeLa cells transfected with either GFP=SRGAP2 or GFP, cultures containing the empty control vector showed an approximately 35% increase in the number of cells containing 10 or more actin stress fibers when compared to cells containing GFP-SRGAP2. This indicates that SRGAP2 can negatively affect Wnt stimulated stress fiber formation and may function in regulating the rapid changes to the actin cytoskeleton needed during the polarized cell movements of CE. These results compliment the results seen during the biochemical investigations, and suggest that SRGAP2 functions as a regulator of Wnt activity.

Expression pattern of SRGAP2 in Xenopus laevis

For SRGAP2 to function as an effector of Daam1 in non-canonical Wnt signaling, it would need to be expressed at similar times and in similar regions as Daam1. In Xenopus laevis, RT-PCR revealed that Daam1 was found to be temporally expressed maternally and throughout development. In-situ hybridization showed Daam1 to be ubiquitously expressed into the blastopore stage; the expression pattern then became confined to the mesodermal and ectodermal tissue during the gastrulation stage. During neurulation, Daam1 showed a strong expression pattern in the cranial neural crest regions in the neural tube, and in late stages, was confined to the midbrain, hindbrain, spinal coed, cement gland, and somites (Nakaya et al. 2004). I have shown that SRGAP2 is express maternally and throughout development and that the levels remain consistent for all stages examined. This expression pattern is similar to that of Daam1 and shows that temporally, SRGAP2 is expressed at the right time to be a Daam1 interacting partner (Raymond Habas et al. 2001b). What is lacking is the spatial expression pattern of SRGAP2, this can be rectified through an in-situ hybridization study of SRGAP2 in the Xenopus laevis. Until the spatial expression pattern is known, a definitive conclusion cannot be drawn regarding SRGAP2 ability to regulate Daam1 function during early vertebrate development.

SRGAP2 regulates vertebrate gastrulation

Non-canonical Wnt signaling is an essential regulator of the convergent extension movements that cells undergo during vertebrate gastrulation. Disruption of this pathway has been shown numerous times to inhibit this process and cause developing embryos to display morphant phenotypes exhibiting a shortened body axis or gastrulation defects (Tada & Heisenberg 2012; Tada & Kai 2009; J. B. Wallingford & Habas 2005). The results presented in this thesis show that both over- and –under expression of SRGAP2 leads to embryos that possess a shortened anterior-posterior body axis, or that display severe gastrulation defects. While the results show a role for SRGAP2 during vertebrate gastrulation, additional studies will be required to better clarify its role. A key question that needs to be asks is can a constitutively active form of Daam1, such as C-Daam1 or Daam1ΔDAD rescue the morphant phenotypes seen during the gain-of-function/loss of function studies. It is also necessary to examine how SRGAP2 specifically affects convergence extension. In order to accomplish this, an animal pole explant assay will need to be performed. In this assay, the animal pole explants represent dorsal tissue and undergoes the morphogenetic characteristic of convergent extension (Raymond Habas et al. 2001b).

Although many questions remain, the results shown in this thesis suggest a model of Daam1/SRGAP2 interaction in which Wnt stimulation triggers the relief of both Daam1 and SRGAP2 from auto-inhibited states. Following this, Daam1 works to promote actin filament assembly and RhoA activation. SRGAP2 on the other hand moves to the cell membrane where it self-dimerizes via its F-BAR domains, essential establishing a restrictive environment that disallows Daam1 activity.

CHAPTER 6
SUMMARY AND CONCLUSIONS

In this thesis, I have shown that *Xenopus* SRGAP2 is a Daam1 interacting partner and that SRGAP2 plays a role in the non-canonical Wnt signaling pathway through a series of biochemical, cell biological, and embryological experiments. I have demonstrated, that SRGAP2 can regulate the actin cytoskeleton and is a critical regulator of cell migration during vertebrate gastrulation. The characterization of SRGAP2 during the course of my graduate work has further delineated the non-canonical Wnt signaling pathway and provided addition insight into the molecular

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