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Role for the Axin-RGS domain during embryonic development: maternal vs. zygotic functions

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ROLE FOR THE AXIN-RGS DOMAIN DURING EMBRYONIC DEVELOPMENT: MATERNAL VS. ZYGOTIC FUNCTIONS

by

Patricia Neiva Coelho Schneider

An Abstract

Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
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Thesis Supervisors: Assistant Professor: Douglas W. Houston
Professor: Diane C. Slusarski

ABSTRACT

Upon sperm entry, the vertebrate egg undergoes a series of cell divisions that create a number of smaller cells without increasing the embryonic mass. This induces an elevation of intracellular calcium transient that is conserved across species. In zebrafish, fertilization occurs through an opening in the chorion, the micropyle and in *Xenopus* it can occur anywhere in the animal hemisphere. Wnt signaling activation is required during dorsal-ventral axis specification and it needs to be suppressed during the regionalization of the brain. Axin is a negative regulator of Wnt signaling and contains an RGS (Regulator of G Protein Signaling) domain. RGS domains are typical of RGS proteins, which are involved in a distinct signaling pathway, G-protein signaling. RGS proteins exert a negative effect of G-protein signaling by accelerating the GTPase activity (GAP) of the Gα subunit, thus turning off the signaling. Axin contains an RGS domain, however, it is not clear whether Axin is directly involved in G-protein signaling. We will also present a work performed using another negative regulator of the Wnt signaling network called naked cuticle (Nkd). Nkd has been shown to modulate β-catenin dependent and independent Wnt signaling. In chapter 2, we will show that the Axin-RGS like function is dispensable during the formation of the dorsal-ventral axis. We manipulated this protein by creating a point mutation in a critical aminoacid within the Axin-RGS domain, known to be detrimental for the GAP function of RGS proteins, $Axin1^{Q162A}$. Maternal depletion of Axin1 in *Xenopus* oocytes causes hyperactivation of Wnt signaling and results in dorsalization. $Axin1^{Q162A}$ is able to suppress the dorsalization of maternally depleted embryo and restore normal dorsa-ventral axis formation.

In chapter 3, we will describe the role of Axin during the patterning of the vertebrate brain. We show that the point mutant is not able to restore normal brain development in zebrafish embryos after Axin knockdown. We hypothesize that Axin-RGS like function is necessary during the patterning of the vertebrate brain that occurs after zygotic transcription has been initiated. Moreover, we show that Axin-RGS like activity may be dispensable

during this stage of development. Finally, we demonstrate that *Axin1*^{Q162A} localization differs from the wildtype Axin1 and Axin1 but not *Axin1*^{Q162A} is localized to the plasma membrane upon Gα overexpression in zebrafish embryos.

Embryonic organ laterality is preceded by molecular and physiological asymmetries. In chapter 4 we describe the role of another Wnt antagonis, Nkd cuticle, during left-right patterning. Prior to organogenesis, a group of cells called Dorsal Forerunner Cells, (DFCs), migrate ahead of the dorsal blastoderm during gastrulation to form the Kupffer's vesicle (KV). This vesicle will trigger a signaling cascade that will culminate with left-right determination. We show data that support the requirement of Nkd in organ laterality and convergence and extension movements using zebrafish and *Xenopus laevis*.

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CERTIFICATE OF APPROVAL

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PH.D. THESIS

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To my parents: Rosane Neiva and Geraldo Coelho
To my husband: Igor Schneider
To my daughter: Anna Schneider

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Upon sperm entry, the vertebrate egg undergoes a series of cell divisions that create a number of smaller cells without increasing the embryonic mass. This induces an elevation of intracellular calcium transient that is conserved across species. In zebrafish, fertilization occurs through an opening in the chorion, the micropyle and in *Xenopus* it can occur anywhere in the animal hemisphere. Wnt signaling activation is required during dorsal-ventral axis specification and it needs to be suppressed during the regionalization of the brain. Axin is a negative regulator of Wnt signaling and contains an RGS (Regulator of G Protein Signaling) domain. RGS domains are typical of RGS proteins, which are involved in a distinct signaling pathway, G-protein signaling. RGS proteins exert a negative effect of G-protein signaling by accelerating the GTPase activity (GAP) of the Gα subunit, thus turning off the signaling. Axin contains an RGS domain, however, it is not clear whether Axin is directly involved in G-protein signaling. We will also present a work performed using another negative regulator of the Wnt signaling network called naked cuticle (Nkd). Nkd has been shown to modulate β-catenin dependent and independent Wnt signaling. In chapter 2, we will show that the Axin-RGS like function is dispensable during the formation of the dorsal-ventral axis. We manipulated this protein by creating a point mutation in a critical aminoacid within the Axin-RGS domain, known to be detrimental for the GAP function of RGS proteins, $Axin1^{Q162A}$. Maternal depletion of Axin1 in *Xenopus* oocytes causes hyperactivation of Wnt signaling and results in dorsalization. $Axin1^{Q162A}$ is able to suppress the dorsalization of maternally depleted embryo and restore normal dorsa-ventral axis formation.

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Embryonic organ laterality is preceded by molecular and physiological asymmetries. In chapter 4 we describe the role of another Wnt antagonis, Nkd cuticle, during left-right patterning. Prior to organogenesis, a group of cells called Dorsal Forerunner Cells, (DFCs), migrate ahead of the dorsal blastoderm during gastrulation to form the Kupffer's vesicle (KV). This vesicle will trigger a signaling cascade that will culminate with left-right determination. We show data that support the requirement of Nkd in organ laterality and convergence and extension movements using zebrafish and *Xenopus laevis*.

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AP	Anterior-Posterior
BMP	Bone morphogenetic protein
Ca^{2+}	Calcium
CE	Convergent extension
DV	Dorsal-Ventral
Dvl	Dishevelled
GAP	GTPase actvating protein
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
LEF/TCF	Lymphoid Enhancer Factor/T-Cell Factor
LDL	Low density lipoprotein
LR	Left-Right
MAPK	Mitogen-activated protein kinase
Mbl	*Masterblind*
MO	Morpholino
PCP	Planar cell polarity
RA	Retinoic acid
RGS	Regulator of G-protein signaling
TGF	Transforming growth factor
Wg	wingless
WMISH	Whole-mount *in situ* hybridization

CHAPTER I
INTRODUCTION

This thesis is focused on the role of Axin1, a key protein of the Wnt signaling pathway. It reports a differential requirement of the Axin-RGS domain during maternal and zygotic stages of development in *Xenopus laevis* and zebrafish. In this introduction I will present a summary of the Wnt signaling pathway, maternal contribution of Axin1 and the formation of the dorsal-ventral axis, zygotic function of Axin1 during the patterning of the vertebrate brain and how G-protein signaling could be involved in these processes through the Axin-RGS domain. In the last chapter, I will present the work performed using another negative regulator of the Wnt signaling network called naked cuticle (Nkd). Nkd has been shown to modulate β-catenin dependent and independent Wnt signaling. I will show data that support its requirement in organ laterality and convergence and extension movements using zebrafish and *Xenopus laevis*.

Vertebrate embryonic axis formation

Upon fertilization the egg undergoes a series of biochemical modifications one of which is the elevation of intracellular Calcium (Ca^{+}). This Ca^{+} transient is observed in all species from sponges to mammals (Carroll, 2001; Runft and Jaffe, 2000; Swann et al., 2004). In zebrafish, the mature oocyte is radially symmetric and fertilization can only occur through an opening in the chorion (the micropyle) located near the animal pole of the oocyte (Iwamatsu et al., 1988; Lee et al., 1999; Webb et al., 1997). In contrast, *Xenopus* embryos have a distinct animal side and sperm entry point is the cue that breaks symmetry. Sperm entry can occur anywhere in the animal hemisphere and causing a loosening of the cortex (outer layer of the egg). During the initial cell divisions, the cortex begins to rotate relative to the core in a process called cortical rotation. This process continues until the end of the first cell cycle and creates a region of about 30^{o} of displacement of the vegetal cortex away from the sperm entry site. The cortical rotation

coincides with the translocation of maternal factors to the future dorsal side of the embryo (Moon and Kimelman, 1998).

As development progresses, the multicellular embryo undergoes a series of cell fate specification events and cellular rearrangements/movements during gastrulation that will give rise to the three germ layers: ectoderm, mesoderm and endoderm. After gastrulation the embryo has a recognizable anterior-posterior axis (A-P), dorsal-ventral axis (D-V) and left-right axis (L-R) (asymmetric positioning of the internal organs).

In *Xenopus*, the most dorsal and vegetal region of the embryo is called the Nieuwkoop center which gives rise to the Spemann-Mangold organizer (Gerhart, 1997; Gerhart, 1999). In *Xenopus*, transplantation experiments identified the Spemann-Mangold center or organizer to be sufficient to establish the spatial pattern formation within the early embryo (De Robertis and Kuroda, 2004; Gerhart, 1997). Organizer transplants induce dorsalization of the ventral mesoderm and induction of neuroectoderm. It is an evolutionary conserved signaling center that is present in all the chordates lineages (node in chick and mice and shield in zebrafish) and exerts its patterning activities during the onset of gastrulation. The major events that occur are in the dorsal endoderm (De Robertis et al., 2001; Harland and Gerhart, 1997; Nieto, 1999).

The position of the Spemann-Mangold organizing center in the dorsal marginal zone is maintained by a cross-regulatory network of ventralizing BMPs (bone morphogenetic proteins), Wnts and downstream proteins that repress the expression of organizer components throughout the rest of the embryo. Conversely, organizer-specific factors act as repressors of ventral genes. In summary, the overall organization of the embryonic patterning is dependent upon the balance between ventralizing and dorsalizing factors (Harland and Gerhart, 1997).

Wnt signaling network

Wnt proteins are secreted ligands that regulate many aspects of embryonic development through receptor-mediated activation of signaling pathways. The Wnt signaling network has major roles throughout development and is also involved in human diseases and stem cell biology (Alonso and Fuchs, 2003; van Es et al., 2003; Veeman et al., 2003a). Different Wnt ligand/receptor combinations can activate at least three distinct signaling pathways, one that leads to β-catenin dependent transcriptional activation, or canonical Wnt, and two non-canonical signaling, or β-catenin independent pathways.

The output of the canonical Wnt signaling is the regulation of β-catenin stability and localization. A protein complex including Axin, adenomatous polyposis coli (APC) and glycogen synthase kinase 3 (GSK-3), among others, tightly regulates Wnt signaling activity through interaction with β-catenin. In the absence of the Wnt ligand, this multiprotein complex promotes phosphorylation of β-catenin and its subsequent degradation (Figure 1). Binding of Wnt ligand to its receptor complex, composed of Frizzled receptors and one of the LDL receptor-related proteins Lrp5/6, activates Dishevelled (Dvl) protein. The cytoplasmic domain of Lrp5/6 has been shown to interact with Axin upon Wnt signaling activation (He et al., 2004; Mao et al., 2001; Tolwinski et al., 2003). Dvl is a central protein to all arms of the Wnt signaling network, it is a key regulator of the Wnt/β-catenin signaling and also plays a role in coordinating convergent and extension movements. Dvl recruits Axin to the plasma membrane inhibiting the β-catenin degradation complex, thus, preventing phosphorylation of β-catenin.

Wnt/ β-catenin independent ligands play a role in the activation of the planar cell polarity pathway (PCP) and intracellular Ca^{+} signaling (Wnt/Ca^{+}) signaling pathway. PCP signaling is involved in the regulation of the hair cells arrangement in *Drosophila* (Huang and He, 2008; Kuhl et al., 2000), but in drosophila, this process is independent of Wnt ligand. However, in vertebrates PCP is Wnt dependent and plays a critical role in

organizing and coordinating cell movements during gastrulation and neuronal outgrowth (Kohn and Moon, 2005; Solnica-Krezel, 2005). Core components of PCP, including the Frizzled receptor, the Dvl and Prickle are also necessary for proper cell movement during vertebrate gastrulation and are sufficient to modulate calcium release.

Wnt/ Ca^{2+} signaling also acts as a negative regulator or a limiting factor of Wnt/β-catenin signaling. Pharmacological or genetic reduction of the Wnt/Ca^{2+} pathway in zebrafish embryos or mouse limb buds generates ectopic accumulation of nuclear β-catenin and activation of β-catenin transcriptional targets (Schneider et al., 2008; Topol et al., 2003; Westfall et al., 2003a; Westfall et al., 2003b). On the other hand, stimulating Ca^{2+} release, via activated serotonin receptor, antagonizes β-catenin-dependent Wnt8-induced dorsalizing activity (Slusarski et al., 1997b).

G-protein-coupled receptors (GPCR)

G-proteins form a heterotrimeric protein complex comprising three subunits Gα, β and γ subunits. This complex can transmit extracellular signals initiated by the binding of hormones, neurotransmitters or other types of secreted signaling molecules to seven-transmembrane spanning G-protein-coupled receptors (Cabrera-Vera et al., 2003; Druey et al., 1996; Egger-Adam and Katanaev; Gold et al., 1997). Currently, there are 20 known Gα, 6 Gβ and 11 Gγ. Based on sequence similarity Gα subunits have been divided into four families, Gαs, Gαi/o, Gαq/11 and Gα12/13. These families can transmit signals from a large number of extracellular ligands/receptor combinations (Miller, 2008).

Gαs signals leads to activation of mitogen-activated protein kinase (MAPK) (Bhalla et al., 2002). Gαi/o activation leads to the inhibition of adenylyl cyclase. Signaling through Gαi/o is inhibited by pertussis toxin, which adenosine diphosphate (ADP)-ribosilates the Gα subunit at its C terminal region and prevents receptor

interaction (Smrcka, 2008). Gαq/11 is activated by calcium-mobilizing hormones and through PLC-β activates the production of IP3 (Pace et al., 1991; Wong et al., 1991). Gα12/13 can interact with Ras and stimulate phospholipase D, c-Src and PKC.

G protein signaling activation is achieved by the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the Gα subunit. The GαGTP and Gβγ subunits of the heterotrimeric complex dissociate and are free to propagate the signal via interactions with a wide range of effector proteins (Gilman, 1987; Hamm, 1998). G-protein signaling is terminated as a consequence of intrinsic GTPase activity of the Gα subunit, which hydrolyzes Gα-bound GTP to GDP, resulting in re-association of the G-protein heterotrimer.

RGS proteins have the potential to accelerate GTPase activity and serve as a shut off mechanism for G-protein-coupled receptor signaling in the cells (Figure 2). Each RGS protein contains a 120 amino acid domain, the RGS box, which is responsible for its GAP activity. These proteins are able to accelerate the intrinsic GAP activity of Gα subunits up to 1000 fold (Posner et al., 1999). RGS proteins are subdivided into 6 families based on the RGS domain homology that include the RZ, (Axin included), R4, R7, R12, RA and RL (Ross and Wilkie, 2000). RGS proteins are known by their function as inhibitors of G-proteins therefore, they can also serve as effectors or as scaffold proteins (Ross and Wilkie, 2000; Zheng et al., 1999). The variety of cellular function of these proteins is complex and demonstrates that RGS proteins have to be tightly regulated in the cell (Hollinger and Hepler, 2002).

Upstream of Dvl are the Frizzled receptors, seven-transmembrane receptors similar to G-protein coupled receptors. In fact, Frizzled receptors have been shown to require G-proteins (Liu et al., 1999b; Slusarski et al., 1997a). Prototypical RGS proteins have been shown to bind to G-protein Gα subunit through a key asparagine (Asn) residue. The Axin-RGS domain encodes a glutamine (Gln) at the equivalent position,

however, interaction between Gα subunit of G-proteins and Axin-RGS domain has not been fully elucidated.

Axin

A central component of the Wnt/β-catenin signaling pathway is Axin. Axin is a scaffold protein that acts as a major effector in the canonical Wnt signaling pathway, it binds directly to components of the degradation complex and leads β-catenin to degradation via ubiquitin-proteasome activation, thus serving as the principal negative regulator of the canonical Wnt signaling.

Axin has multiple roles in embryonic patterning; it was first identified as the product of the mouse *fused* locus, where mutations in this gene result in axis duplication in mouse embryos (Zeng et al., 1997). Additionally, ectopic expression of *axin* in dorsal blastomeres of *Xenopus* embryos causes ventralization (Zhang et al., 1998), shown to be the result of Axin-mediated β-catenin destabilization (Ikeda et al., 1998). In vertebrates there are two forms of *axin*, the constitutively expressed *axin1*, and a Wnt-inducible *axin2*. Although maternal depletion of both forms of *axin* in zebrafish has yet to be accomplished, it is possible that *axin* may display distinct maternal and zygotic functions.

Axin contains four critical domains: a β-catenin binding domain, a GSK3 binding domain, a Regulator of G-protein (RGS) domain and a DIX domain (protein interaction domain shared with Dvl) (Figure 3). Some of these Axin domains serve as binding sites for members of the degradation complex: GSK3β, β-catenin, Dvl and APC.

One of the Axin's functions is to promote phosphorylation of APC and GSK3β, increasing the affinity of APC for β-catenin (Figure 1). Once β-catenin is bound to APC, APC ensures targeting of N-terminal-phosphorylated β-catenin (Ser(P)-33 and Ser(P)-37) to the proteasome by protecting this βTrCP recognition epitope from the phosphatase PP2A (Hsu et al., 1999; Jho et al., 1999; Su et al., 2008).

In the absence of Wnt stimuli, Axin is localized diffusely in the cytoplasm and in the perinuclear region and can also be found forming puncta resembling Dvl localization (Schwarz-Romond et al., 2007). Wnt/β-catenin stimulation leads to activation of Dvl and recruitment of Axin through its DIX domain to the plasma membrane, where it associates with phopshorylated LRP6 and is subsequently turned over (Zeng et al., 2005). Mutations in amino acid residues within Dvl-DIX domain interfere with Dvl phospholipid binding (K68 and E69), actin localization and Dvl protein stability (K58). Previous studies have also shown that Axin-ΔDIX (deletion of Axin DIX domain) increases stability of Axin (Liu et al., 1999a; Mao et al., 2001).

RGS domains regulate the active half-life of G-protein signaling. Prototypical RGS proteins bind to Gα through a key asparagine (Asn) residue at position 229. The Axin-RGS domain is conserved among vertebrates, however, it encodes a glutamine (Gln) at the equivalent position and it is not known whether the Axin-RGS domain functions in G-protein signaling (Figure 3). The DIX domain of Dvl mediates actin and phospholipid binding, required for Dvl stability, yet it is unknown whether the conserved residues in the Axin-DIX domain also functions in actin and phospholipid binding as well as protein stability.

Dorsal-ventral axis determination: maternal factors

During oogenesis, *Xenopus* and zebrafish females place proteins and RNAs in the egg to support early development. These are referred to as maternal factors and include components of the Wnt/β-catenin signaling network. Oocytes contain an impressive number of maternally stored factors that are relatively homogeneously distributed, and inherited by al the cells of the developing embryo. One example is the forkhead family of transcription factors. Of the 22 forkhead box family described in *Xenopus*, six are maternally expressed (FoxD2, D3a/b, FoxD5a/b/c, FoxH1a and FoxK1) (Pohl and

Knochel, 2005). Before mid-blastula transition (MBT) these factors are responsible for intracellular signaling interactions in the early embryo.

The maternal components of the canonical Wnt pathway are of primary importance in establishing the anterior-posterior and dorsal-ventral axis in vertebrates. *Xenopus* embryos depleted of maternal β-catenin, GSK-3 binding protein (GBP) or *wnt11* mRNA still retain the three germ layers, but display loss of dorsal, anterior and posterior differentiation (ventralization) but still contains the three germ layers (Tao et al., 2005; Yost et al., 1998). In *Xenopus,* maternal *wnt11* mRNA becomes localized to the vegetal cortex during oogenesis and by the time the embryo reaches the 32-cell stage, *Wnt11* mRNA and protein are enriched on the dorsal side, presumably moved there by the wave of microtubule-based cortical cytoplasmic movement (cortical rotation) activated by sperm entry in the fertilized egg (Tao et al., 2005). The loss of Wnt signaling components supports a critical role for this signaling pathway in initiating the dorsal axis. In *Xenopus*, β-catenin is localized in the dorsal marginal zone and in zebrafish, is found in the nuclei of dorsal blastomeres (Heasman et al., 1994; Schneider et al., 1996).

The principal direct targets of the Wnt11/β-catenin activation include the organizer genes *Xnr3* and *siamois,* BMP and Wnt antagonists, *chordin, cerberus* and *noggin.* These genes are important during D-V axis and notochord formation (Bouwmeester et al., 1996; Piccolo et al., 1996; Smith and Harland, 1992). Eventually, maternal stores are turned over, zygotic transcription initiates and Wnt/β-catenin signaling shifts from a dorsal axis specification role to a role in patterning of the anterior-posterior A-P axis.

Anterior-posterior specification of the vertebrate forebrain: zygotic transcription

Many of the human attributes such as memories, emotions and conscience are organized within the forebrain. Despite its complexity, forebrain formation is conserved

in all vertebrate. The intricacy of functions between species is correlated with the extent to which these domains are elaborated as they form the mature brain.

The forebrain arises from anterior neuroectoderm during gastrulation, and by the end of somitogenesis it comprises the dorsally positioned telencephalon and eyes, the ventrally positioned hypothalamus and more caudally the diencephalon. The diencephalon contains the prethalamus (ventral) and the thalamus (dorsal) (Hoch et al., 2009; Varga et al., 1999). In zebrafish and *Xenopus*, neural induction begins during gastrulation, when the organizer/shield involutes and signals to the ectoderm to become neural tissue. The newly induced neural tissue is thought to have an anterior (forebrain) character. Next, the organizer produces signals that may include Wnts, FGFs and retinoic acids (RAs) that will transform anterior neural tissue to a more posterior identity.

It has been proposed that two concomitant signaling events happen during the specification of the brain. One is that the induced neural tissue possesses forebrain character and that a posteriorizing signal acts in a graded way to specify more posterior neural fates (Foley et al., 2000). A number of genetic studies have shown that mutations that affect the organizer during early embryogenesis can disturb the AP patterning of the neural plate and later affect forebrain specification (Fekany et al., 1999; Foley et al., 2000; Koos and Ho, 1999). Wnt signaling is implicated in many aspects of development, as mentioned above. During early embryogenesis the Wnt/β-catenin pathway is involved in D-V axis formation before and after the onset of zygotic transcription. The early activation of this signaling pathway is believed to be receptor-independent and has a dorsalizing function (Figure 4). These early events set up the dorsal-ventral axis formation and induce the activation of the organizer (Harland and Gerhart, 1997; Miller and Moon, 1997; Moon et al., 1997).

Later, Wnt signaling has an opposite effect and instead of inducing the organizer, it antagonizes it. Manipulation of Wnt signaling using *Xenopus* embryos have shown that overexpression of *Xwnt8* causes loss of head structures and a dominant-negative *Xwnt8*

construct injected into the embryos causes anteriorization, evidenced by the enlarged head and notochord. The loss of head structures caused by the late expression of *Xwnt8*, corroborates that Wnts are posteriorizing signals (Hoppler et al., 1996).

Another example of the posteriorizing effect of Wnt signaling during A-P brain patterning is seen in the *Masterblind (mbl)* genetic mutant (Figure 5). *Mbl* is a zebrafish recessive zygotic mutation in the GSK3 binding domain of Axin and homozygous mutant embryos display posteriorization of the nervous system with reduction of forebrain structures such as eyes and telencephalon as well as expansion of diencephalic epiphysis (Masai et al., 1997). In *mbl* mutants, the telencephalon, the anterior pituitary and the optic vesicles are absent or reduced, the epiphysis, a diencephalic structure is expanded (Heisenberg et al., 1996; Heisenberg et al., 2001). This indicates that ectopic activation of Wnt signaling in the brain region during zygotic stages leads to a fate transformation of telencephalon and eyes field to diencephalon. This theory is supported by studies in other vertebrates such as chick and frogs, where ectopic Wnt signaling leads to activation of the expression of posterior forebrain markers and suppression of the activation of anterior forebrain markers (Braun et al., 2003; Kiecker and Niehrs, 2001a; Kiecker and Niehrs, 2001b). In summary, these studies support the idea that within the forebrain, telencephalon and eyes are regions of low or no Wnt signaling activation and diencephalic fate is promoted by Wnt signaling.

NKD cuticle, left-right asymmetry and the Wnt signaling network

As vertebrate embryos progress through early development and enter gastrulation, morphological differences with regards to the anterior-posterior (AP) and dorsal-ventral (DV) axes are already evident, yet the embryo appears bilaterally symmetric. In zebrafish, LR symmetry breaking involves a group of non-involuting dorsal mesoderm

cells, the DFCs. These cells migrate ahead of the blastoderm margin during gastrulation (Cooper and D'Amico, 1996; Melby et al., 1996) and coalesce into a single rosette that differentiates into the KV (Oteiza et al., 2008). As they migrate, these cells express signaling molecules such as the Brachyury homolog *no tail*, *spade tail, polaris, pkd2* and *left-right dynein related 1 (lrdr1)*. DFC-specific knockdown of these gene products or ablation of the DFCs disrupts LR patterning (Amack and Yost, 2004; Essner et al., 2005).

After the KV is formed, cilia present in the lumen of the KV and in the mouse node generate a leftward fluid flow (Essner et al., 2005; Kramer-Zucker et al., 2005; Nonaka et al., 1998). The cilia-driven flow, referred to as nodal flow, has also been described in rabbit, medaka fish (Okada et al., 2005) and *Xenopus* embryos (Schweickert et al., 2007). KV/node cilia flow is thought to initiate molecular LR signals include the left-sided expression of the secreted TGFβ-related factor *nodal* (Reviewed in (Ahmad et al., 2004)) and downstream targets of *nodal* including *lefty1* and *pitx2* (Reviewed in (Hamada et al., 2002; Wright and Halpern, 2002). Left-sided gene expression in the LPM, in turn, relays asymmetric information to internal organs as organogenesis commences. Asymmetric expression of *nodal* and its downstream targets is regulated by Charon, a member of the Cer/Dan class of Nodal antagonists, which is produced in cells around zebrafish KV (Hashimoto et al., 2004) and Dante/Cerl-2 around the mouse node (Marques et al., 2004). However, how these proteins limit nodal expression only to the left side remains unclear. Hence, the precise signaling mechanism linking KV/node function and post-KV/node asymmetric gene expression remains uncertain.

A notable aspect of the KV formation is its apparent planar cell polarity (PCP), evidenced by the coordinated directional DFC migration and subsequent differentiation into the KV, with cilia protruding from the apical surface of the lumen epithelial cells. Noncanonical Wnts, also referred to as β-catenin-independent Wnts, are involved in determining the alignment of hairs on the wings in *Drosophila,* in coordinating CE movements during gastrulation in vertebrate species (Wnt/PCP) (Kohn and Moon, 2005;

Solnica-Krezel, 2005), and in modulation of Ca^{2+} release (Wnt/Ca^{2+}) (Westfall et al., 2003a). Recent reports indicate that proteins involved in β-catenin-independent Wnt signaling, including Vangl1 and Prickle2, play a role in establishing a polarized epithelium in the mouse, *Xenopus* (Antic et al., 2010) and chick (Zhang and Levin, 2009) nodes. In addition, Frizzled-2-mediated noncanonical Wnt signaling has been proposed to regulate ciliogenesis in the KV (Oishi et al., 2006) and Seahorse, a protein associated with cilia-mediated processes, binds Dvl and can promote Wnt/PCP pathway (Kishimoto et al., 2008). Nevertheless, the precise role of noncanonical Wnts in KV formation and LR asymmetry is yet to be determined.

Wnts of the canonical pathway (Wnt/β-catenin) function by disabling a β-catenin-degradation complex, leading to β-catenin stabilization. Subsequently, stabilized β-catenin protein translocates to the nucleus and interacts with the members of the LEF/TCF transcription factor family to promote the activation of downstream target genes (Polakis, 2000) (Figure 1 and 6). The Wnt/β-catenin pathway has also been implicated in KV formation and LR patterning, as zebrafish Wnt3 and Wnt8 morphants have reduced KV size, shorter cilia, decreased *charon* expression and LR patterning defects (Lin and Xu, 2009). In addition, in the mouse node, Wnt3a signals via β-catenin and is required for expression of Pkd1 in mechanosensory cilia and for proper LR asymmetry (Nakaya et al., 2005). Interestingly, while Wnt/β-catenin pathway has a role in KV function, it appears to be repressed earlier, during DFC migration. In fact, β-catenin activity is suppressed in the DFCs and misexpression of a constitutively active form of β-catenin in DFCs results in proper LR patterning defects (Schneider et al., 2008). Furthermore, DFC-specific knockdown of Axin, a Wnt/β-catenin antagonist, also leads to LR patterning defects (Schneider et al., 2008). Therefore, proper DFC migration, KV formation and LR patterning appears to involve both β-catenin-dependent and independent Wnt signaling may require potential mediators that can integrate signals from different arms of the Wnt network.

Among candidate mediators, Nkd displays the distinctive property of affecting both β-catenin-dependent and -independent arms of the Wnt signaling network (Figure 6). The *Drosophila nkd* has been shown to function as a Wg inducible antagonist (Zeng et al., 2000). The mechanism underlying Nkd-mediated antagonism of Wg involves binding to the basic/PDZ domain of Dvl, a scaffold protein that is central to both β-catenin-dependent and -independent Wnt pathways (Rousset et al., 2001). Mammals possess two Nkd homologs, Nkd1 and Nkd2 (Katoh, 2001; Wharton et al., 2001), which also interact with the basic/PDZ domain of Dvl and inhibit the Wnt/β–χατενιν pathway (Wharton et al., 2001; Yan et al., 2001b). A prominent feature of Nkd protein is a single EF-hand. EF hands are conserved Ca^{2+}-binding motifs that usually occur in adjacent pairs, but have been observed in other configurations (Kawasaki et al., 1998). The EF-hand motif present in Nkd is most similar to the high-affinity Ca^{2+}-binding EF-hand 3 of the recoverin family of myristoyl switch proteins (Zeng et al., 2000) (Figure 7).

Sequence analysis and functional studies reveal important differences between fly and vertebrate Nkd homologs. Fly Nkd has an unusual pair of histidine residues that introduces an extra amino acid in the EF loop and replaces an oxygen-donating residue predicted to coordinate ion binding (Fig. 7). Based on comparisons with EF hand-crystal structures, the presence of the pair of histidines may impair ion binding (Wharton et al., 2001). In fact, some characterized EF hand motifs have undergone amino acid substitutions in oxygen-donating residues that prevent ion binding (Wharton et al., 2001).

Comparison of protein sequences suggests that the EF-hand in vertebrate and fly Nkd homologs may have diverged to perform different biological roles, and functional studies appear to support this notion. In both fly and vertebrate Nkd, binding to Dvl is mediated by a region including the EF-hand motif and flanking sequences, termed EFX (Rousset et al., 2001; Wharton et al., 2001).

Various reports indicate that Nkd-mediated Wnt antagonism involves the binding of Nkd and Dvl, leading to Dvl degradation (Creyghton et al., 2005; Guo et al., 2009; Hu

et al., 2010). Interestingly, overproduction of both mammalian and fly Nkd homologs leads to defects in PCP pathway (Rousset et al., 2001; Yan et al., 2001b) and zebrafish Nkd1 and Nkd2a were shown to antagonize both canonical and noncanonical Wnt signaling (Van Raay et al., 2007). Whether Nkd proteins function as a switch between different arms of Wnt pathways (Yan et al., 2001b) or simply as antagonist of both pathways (Van Raay et al., 2007), Nkd activity may enable cells to translate converging Wnt signaling cues into a distinct output.

Goals

It has long been unclear to what extent the Axin RGS domain performs a prototypical RGS-like function with regard to Wnt signaling activation. My primary goal was to determine whether Axin-RGS domain has an RGS-like function in the context of Wnt signaling. Additionally, I was interested in elucidating the Axin-RGS domain function during the specification of the A-P axis of the brain, more specifically during the regionalization of the forebrain using *Xenopus* and zebrafish. Finally, I sought to understand the maternal function of Axin-RGS domain using *Xenopus* embryos to perform maternal depletion of Axin.

Previous observations in the lab have identified a discrete, aperiodic Ca^{2+} release activity in the DFCs during gastrulation. The Slusarski lab have also demonstrated that this aperiodic Ca^{2+} release is involved in the regulation of β-catenin nuclear localization and also played a role in the migratory behavior and ability of the DFCs to form a KV and promote LR axis determination (Schneider et al., 2008). My primary goal was to determine whether Nkd functions as a Ca^{2+} sensor to translate DFC regional Ca^{2+} fluxes into β-catenin regulation, DFC behavior and Dvl degradation and LR patterning.

Animal model systems

Xenopus laevis

Adult female *Xenopus laevis* frogs will be used as a source of oocytes and eggs for experiments. Females used as egg or oocyte donors are viable following hormone injection to induce egg-laying or minor surgery to obtain oocytes. The female frogs are rested for 2 months before further use. Male frogs will be sacrificed to obtain testis tissue as a source of sperm for fertilizations.

Danio rerio

The zebrafish (*Danio rerio)* will also be used in this project. The zebrafish are easy to maintain under laboratory conditions and amenable to genetic manipulations. They are small vertebrates with external-egg laying making for easy embryo collection. Most importantly, the eggs are transparent, allowing for investigations in the living embryo. Also, there is a genetic mutant in *axin1,* which will facilitate our analysis of the Axin1 function during embryonic development.

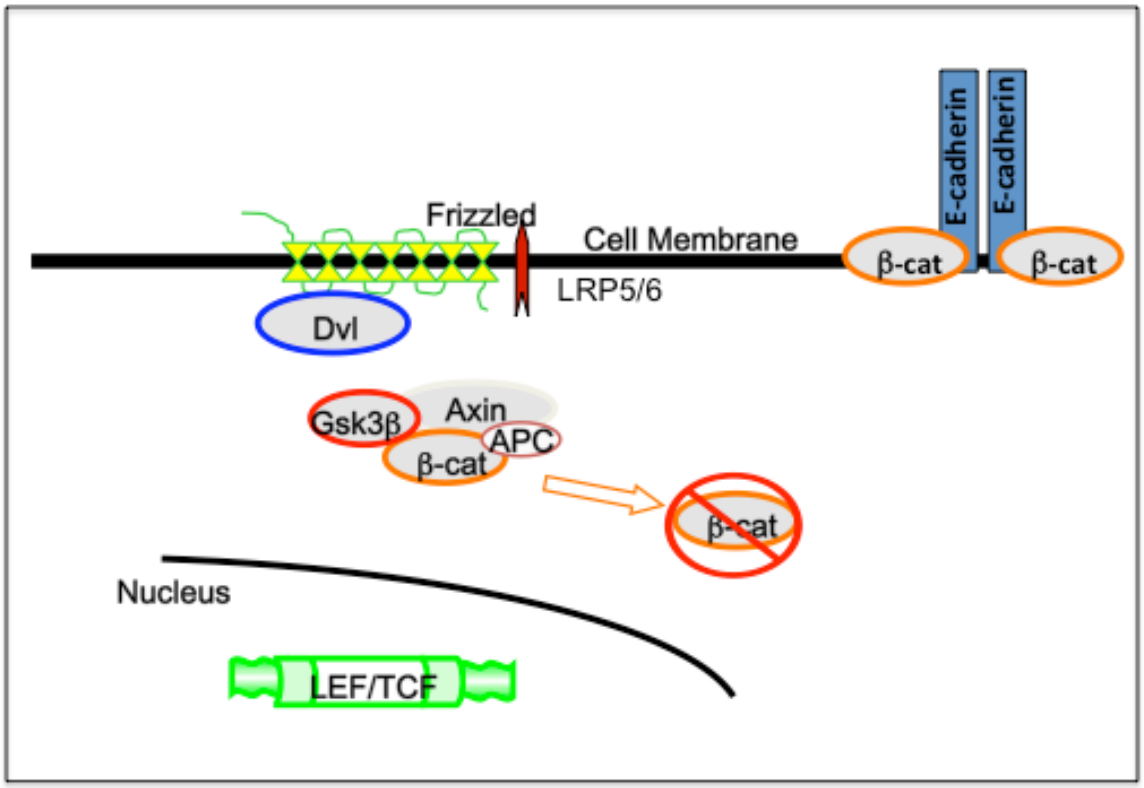

Figure 1: A simplified schematic of the Wnt/ β-catenin pathway. Axin is negative regulator of Wnt signaling pathway and is a member of the degradation complex that interacts with ß-catenin causing its phosphorylation and degradation.

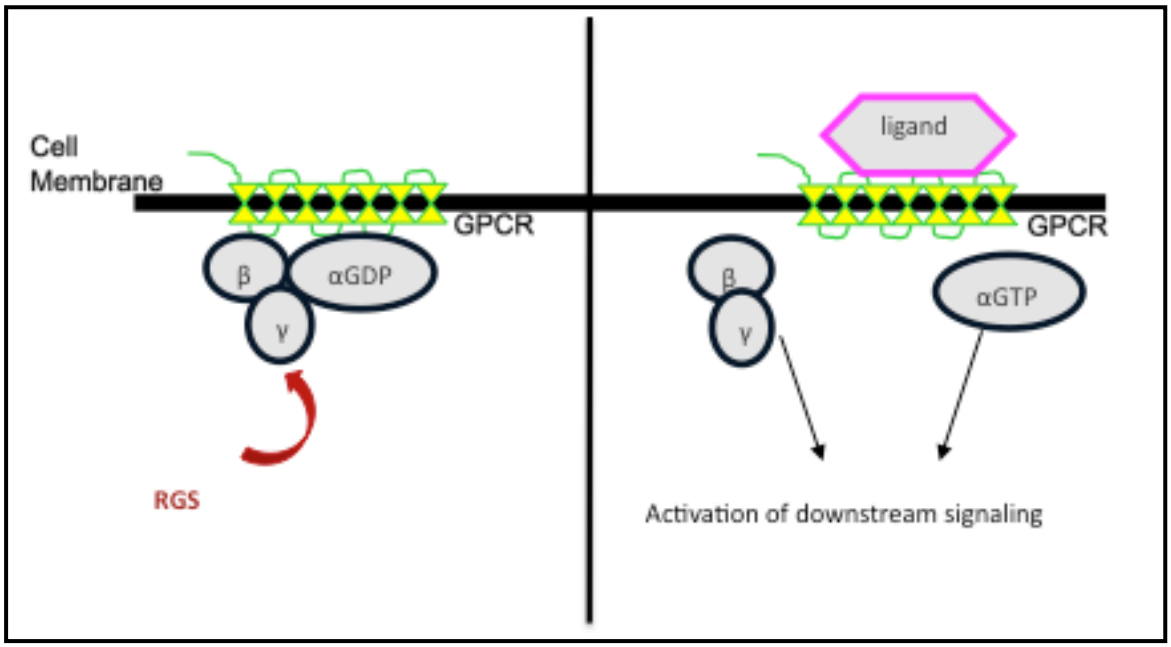

Figure 2: Simplified diagram of the G-protein signaling. Upon ligand binding to a G-protein coupled receptor, the Gα subunit dissociates from Gβγ, activating downstream effectors. This activation is achieved by catalyzing GDP–GTP exchange on the Gα subunit. RGS (regulators of G protein signaling) proteins accelerate the GTPase activity, leading to re association of the αβγ subunits turning off G-protein signaling.

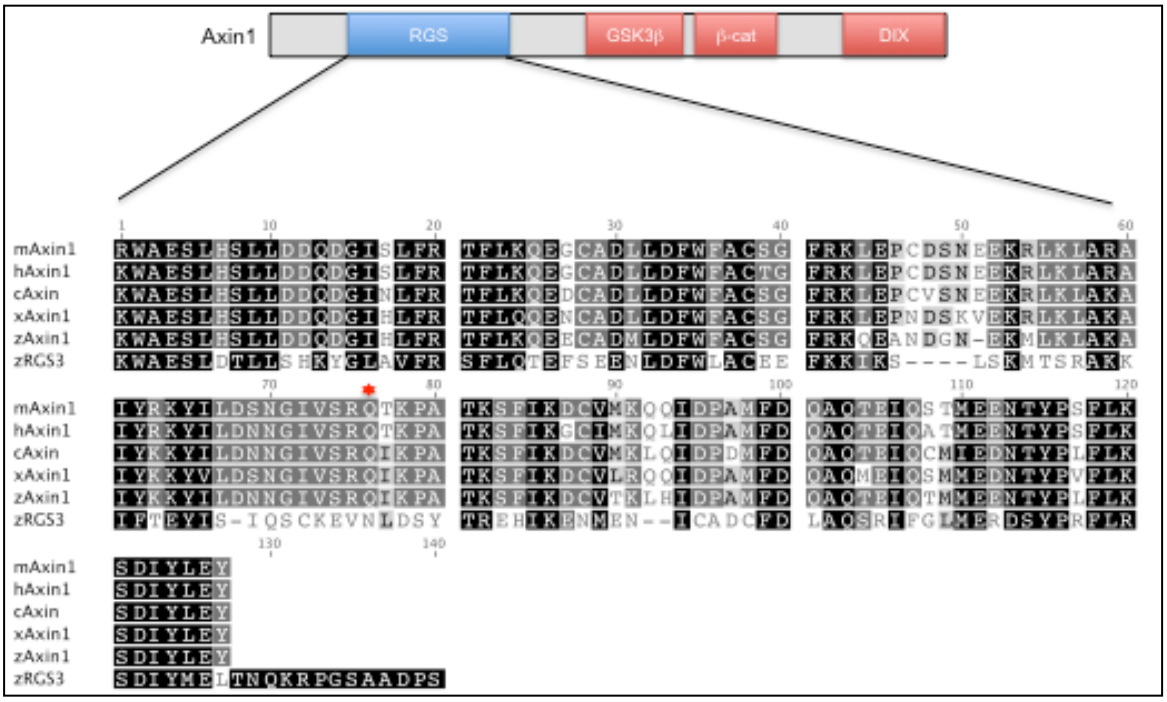

Figure 3: Alignment of Axin-RGS domains homologues; mouse Axin1 (mAxin1), human Axin1 (hAxin1), chicken Axin1 (cAxin1), zebrafish Axin1 (zAxin1) and *Xenopus* Axin1 (xAxin1). Black shadow denotes 100% similar residues, dark gray shadow denotes 80-100% similar, light gray shadow denotes 60-80% similar and white shadow denotes less than 60% similar. Red asterisk shows critical amino acid (asparagine, N, in RGS3 and glutamine, Q, in Axin) for the function of RGS proteins as GTPase Accelerating Protein (GAP).

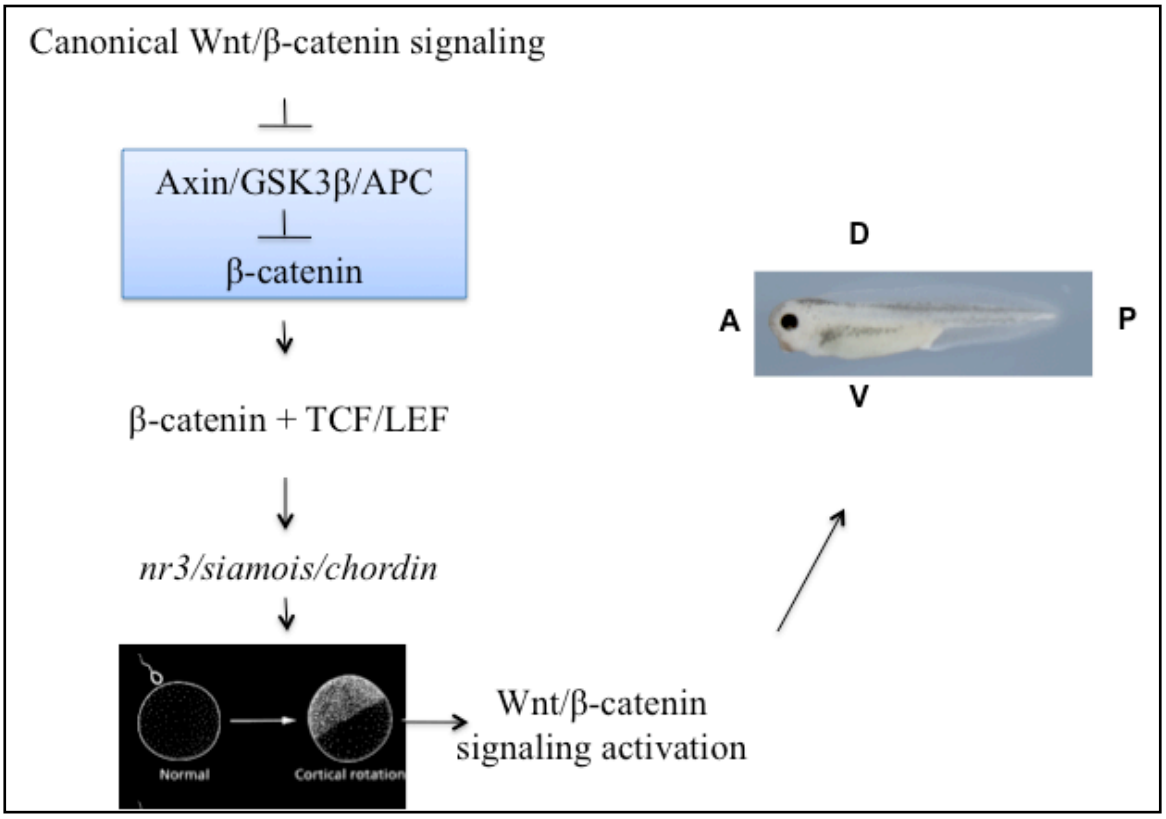

Figure 4: (A) Diagram of dorsal axis specification. Wnt signaling activation leads to Dvl recruitment to the plasma membrane and subsequent dissociation of the members of the degradation complex, Axin/APC/GSK3β. The accumulation of β-catenin in the cytoplasm leads to its translocation to the nucleus where it associates with the cofactors TCF/LEF, this association leads to the activation of Wnt target genes such as *nr3* and *siamois.* In *Xenopus* embryos the dorsal specification is dependent upon cortical rotation. The movement of determinants to the future dorsal side of the embryo activates Wnt signaling and patterns the D-V and A-P axis.

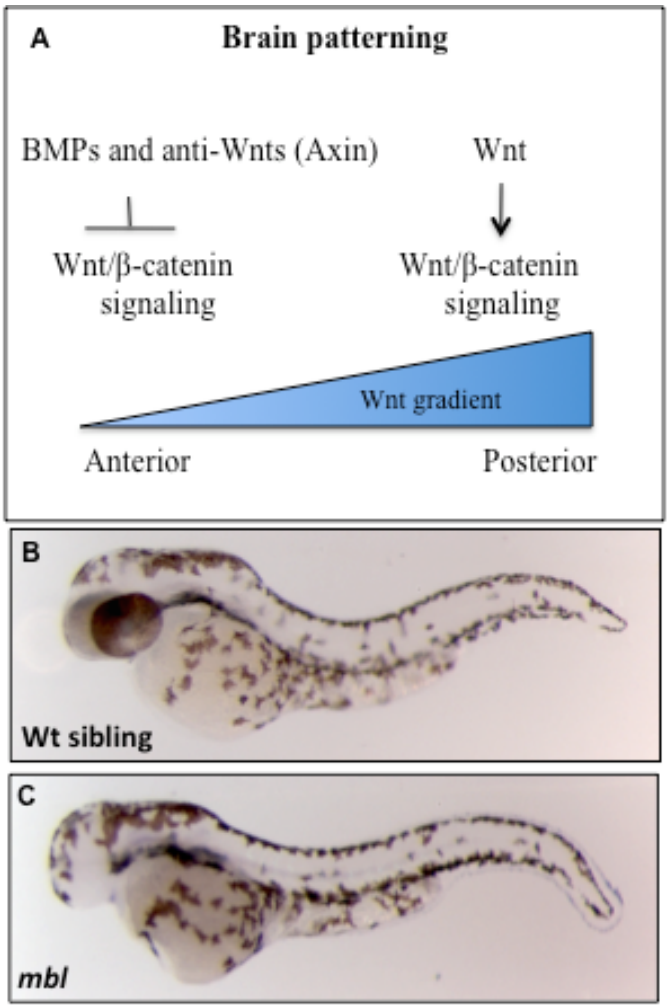

Figure 5: Wnt activity gradient during brain patterning. (A) Simplified diagram of the events that occur during brain patterning. In the anterior portion of the brain BMPs and anti-Wnts are actively repressing Wnt signaling whereas in the most posterior region, activation of Wnt signaling leads to the posteriorization of the brain. (B) wt sibling and (C) zebrafish genetic mutant, *masterblind.* The *masterblind* phenotype is caused by ectopic activation of Wnt signaling in the brain region during zygotic stages leads to a fate transformation of telencephalon and eyes field to diencephalon.

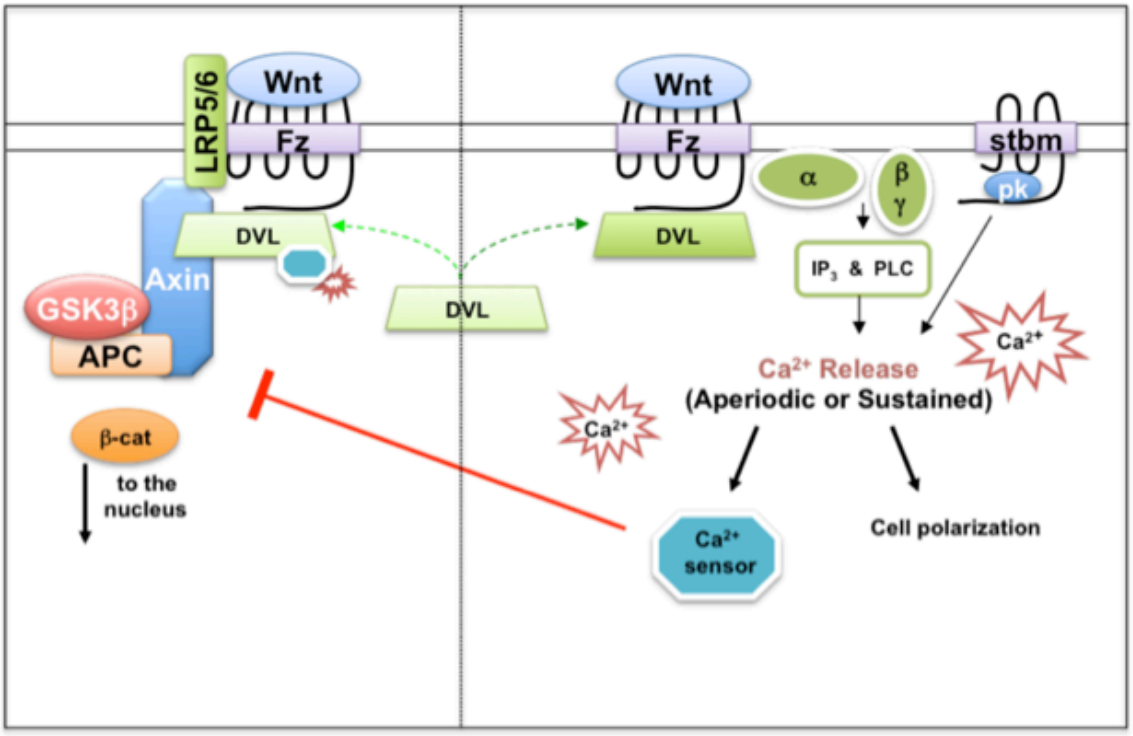

Figure 6: A simplified schematic of the Wnt signaling network. β-catenin-independent pathways to the right side and β-catenin-dependent components to the left side of the dotted line. β-cat=β-catenin. Courtesy of Dr. I. Schneider.

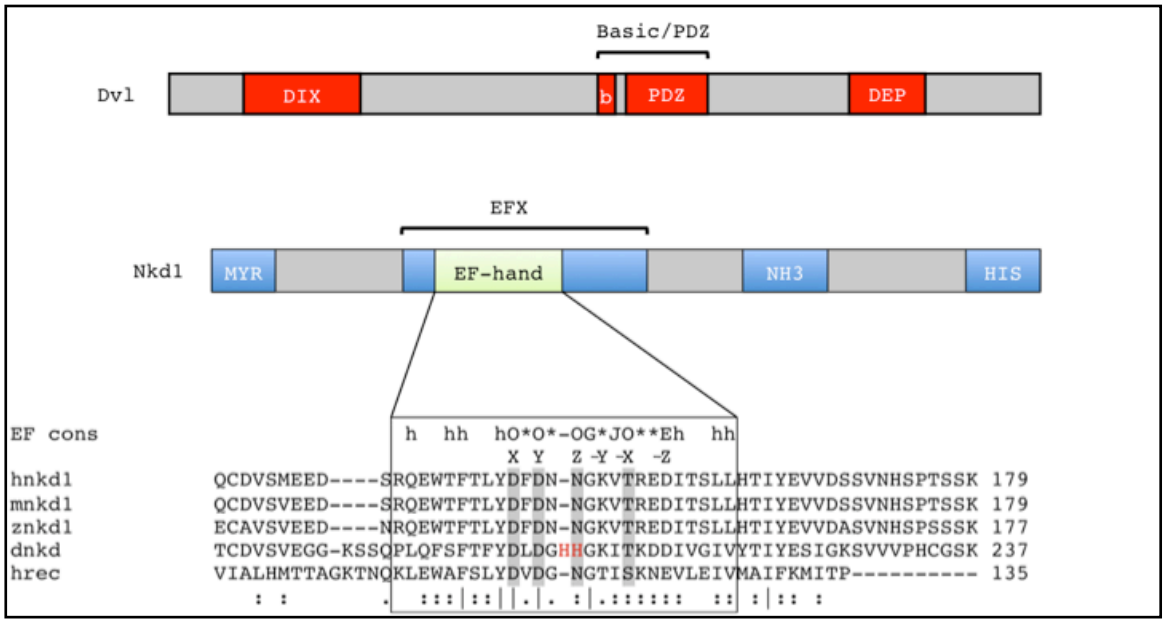

Figure 7: EF-hand residues critical for Ca^{2+} binding are conserved in vertebrate Nkd1. Schematic representation of Dvl and Nkd1; binding between Dvl and Nkd1 is mediated by Dvl basic/PDZ domain and Nkd1 EFX domain. Alignment of EF-hand similarity between Nkd and third EF hand in recoverin family of proteins. Alignment of Nkd1 homologues; human (hnkd1), mouse Nkd1 (mnkd1), zebrafish Nkd1 (znkd1), Drosophila nkd (dnkd) and human Recoverin (hrec). "|" denotes identical residues, ":" denotes conserved substitutions and "." denotes semi-conserved substitutions. Consensus EF-hand residues (EF cons): h, hydrophobic residue; E, acidic residue, usually glutamic acid; O, oxygen-donating residue that binds Ca^{2+}; G, glycine; asterisk, variable amino acid; J, hydrophobic residue; X, Y, Z, coordinates of Ca^{2+} binding in three-dimensional space. Courtesy of Dr. I. Schneider.

CHAPTER II
AXIN-RGS LIKE FUNCTION IS DISPENSABLE DURING MATERNAL STAGES OF *XENOPUS* EMBRYO

Introduction

Dorsal ventral patterning of the early embryo requires cortical rotation, a movement of determinants to the future dorsal side of the embryo, resulting in activation of the canonical Wnt signaling (Wnt/β-catenin). The stabilization of the Wnt/β-catenin leads to transcriptional activation of dorsal genes (Moon and Kimelman, 1998). β-catenin is tightly regulated by a group of proteins called, the destruction complex. Axin is a central protein in the destruction complex and it serves as a scaffold protein for the destruction of β-catenin. The asymmetry of gene expression caused by the activation of Wnt signaling in the future dorsal side of the embryo is a very well established event, however, the exact mechanism by which this occurs is still unclear. Based on molecular manipulation in *Xenopus* embryos we hypothesize that Axin-RGS like activity is dispensable during maternal stages of development when the dorsal-ventral axis is being specified. The goal is to determine whether a point mutation in the putative Gα binding site would affect the ability of Axin to rescue the loss of maternally depleted Axin function.

Materials and methods

Xenopus laevis oocytes and embryos manipulation

Oocytes were isolated from female *Xenopus* by laparotomy. The donor female was anesthetized in buffered MS222 (0.1% MS222 +0.7% sodium bicarbonate). The

oocytes were maintained in OCM (60% L-15 medium, 0.4mg/ml BSA, 1mM glutamine, 0.1 mg/ml gentamicin). Eggs were recovered from laying females, fertilized using a sperm suspension and maintained in 0.1xMMR (1XMMR: 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM EGTA, 5 mM HEPES, pH 7.7). After fertilization, egg jelly coats were removed using cysteine (2% cysteine/0.1% MMR, pH 7.8) and injected as described previously (Cuykendall and Houston, 2009). For injections of mRNAs after fertilization, embryos were dejellied and transferred to 2% Ficoll/0.5×MMR at the one-cell stage.

Host transfer

For the *axin1* maternal depletion, mature oocytes were manually defolliculated and cultured as described previously (Xanthos et al., 2001). Oligos were resuspended in sterile, filtered water and injected at doses of 3 or 4 ng into the equatorial region of oocytes. Oocytes were cultured immediately at 18°C.

axin1-myc mRNA encoding the wildtype or mutant forms was injected into the vegetal side of the oocytes. Injected oocytes were stimulated to mature by the addition of progesterone to the culture medium and were incubated overnight. After 12 hours, eggs were labeled with vital dyes and transferred into an egg-laying female to separate the experimental eggs from the host eggs. After 2-3 hrs, the female was squeezed and the eggs were fertilized with a sperm suspension (Heasman et al., 1994; Houston and King, 2000). Colored embryos were sorted from host embryos and analyzed morphologically during development. Following fertilization of the transplanted eggs, control, *axin1*-depleted and *axin1*-depleted and rescued embryos were allowed to develop to the gastrula stage in order to analyze dorsal and ventral gene expression by RT-PCR.

Real time PCR

Total RNA was prepared from oocytes and embryos using proteinase K and then treated with RNase-free DNase as described (Houston and Wylie, 2005). Real-time RT-PCR was carried out using the LightCycler 480 System (Roche Applied Science). Samples were normalized to *ornithine decarboxylase* (*odc*) levels and relative expression values were calculated against a standard curve of control cDNA. Samples lacking reverse transcriptase in the cDNA synthesis reaction failed to give specific products.

Animal cap assay

Embryos were injected at 1 cell stage with 150*ng axin1or axin1*^{Q162A} + 1pg of *wnt8* or 1pg *wnt8* alone in the animal pole and the presumptive ectodermal sheets were cut from late blastula stage and incubated in 1X MMR for 1 hr, as described previously (Ariizumi et al., 2009). Total RNA was extracted from 5 caps. Caps for RT-PCR analysis were frozen on dry ice and stored at − 80 °C. Samples were homogenized in 200 µl of RNA lysis buffer per embryo, DNase I treated, subjected to RT-PCR using *nr3* primer set and analyzed by agarose gel as described in (Houston and King, 2000).

Statistical analysis

The results of the zebrafish and *Xenopus* injections were subjected to the Fisher's exact test.

Results

axin1 and a*xin1*^{Q162A} misexpression inhibit Wnt/β-catenin signaling

Injection of both wildtype and mutant forms of Axin caused ventralization, including loss of head structures (brain and eyes) and enlargement of ventral posterior tissues, to a similar extent in zebrafish and *Xenopus* embryos (Figure 9). To examine whether these effects were related to the ability of *axin*^{Q162A} to inhibit Wnt/β-catenin signaling, we performed animal cap assays. Caps were cut from late blastulae previously injected with wnt8 (1 pg) along with *axin1* or *axin1*^{Q162A} (1 ng) and analyzed for expression of *nr3*, a Wnt target gene, by RT-PCR (Smith et al., 1995). Consistent with our observations on whole embryos, both full-length *axin1* and *axin1*^{Q162A} were sufficient to block the activation of *nr3* in animal caps and suppress dorsalization in embryos by injecting *wnt8* (Figure 10).

Axin^{Q162A} rescues the maternal function of Axin1 in *Xenopus* embryos

To determine the extent that Axin-RGS activity might be generally required for Wnt/β-catenin signaling, we next tested the requirement for Axin-RGS function in another well-characterized paradigm of Wnt signaling: maternal Wnt signaling during *Xenopus* dorsoventral axis formation (Kofron et al., 2001). Axin loss-of-function in this context results in elevated Wnt/β-catenin activity and dorsalization of the embryo (Kofron et al., 2001). We injected antisense oligonucleotides against *axin1* into stage VI oocytes, followed 24 hours by injection of 60 pg of either zebrafish *axin1* or *axin1*^{Q162A}. Embryos were derived from these oocytes by transferring them into an egg-laying host female for in vitro fertilization (Figure 8). The resulting embryos were either frozen down at the gastrula stage for RT-PCR analysis or left to develop for morphological and gene expression analysis at the tailbud stage.

axin1-depleted embryos developed normally through cleavage and blastula stages, and developed deep and hyperpigmented blastopores during gastrulation, as described (Kofron et al., 2001). These embryos developed either second axes or enlarged heads (81%; n=44), exhibiting a dorsalized appearance. Injection of *axin1* and *axin1*^{Q162A} could both partially rescue the maternal knockdown of *axin1*, reducing the incidence of dorsalized embryos to 22% (n=32) and 24% (n=21), respectively (Figure 11).

To examine the effects of maternal *axin1* depletion and rescue on Wnt signaling more directly, we analyzed levels of *chordin (chd),* a direct target of Wnt/β-catenin signaling (Wessely et al., 2001). Depletion of maternal *axin1* resulted in hyperactivation of *chd* expression, as assessed by realtime qPCR, similar to what was shown in previous studies. Consistent with our phenotypic data describe above, injection of *axin1* and *axin1*^{Q162A} could both restore Wnt target genes to near-normal levels to a similar degree. A representative analysis is shown in Figure 11; results were repeated three times with similar results. Injection of 60 pg *axin1* or *axin1*^{Q162A} alone into control oocytes had no effect on development or on Wnt target gene levels (data not shown). In contrast to our results in the zebrafish, these results suggest that Axin-mediated RGS-like activity is likely not necessary in the regulation of maternal Wnt signaling during *Xenopus* axis specification.

Discussion

Here we show that maternal *axin1* depletion causes ectopic activation of the Wnt signaling. This upregulation can be suppressed using the *axin1* and *axin1*^{Q162A} mutant constructs and the relative expression *chd* in the embryos injected with both *axin1* constructs were comparable to wildtype levels. The restoration of normal dorsal axis formation was also observed morphologically. Injection of *axin1* and *axin1*^{Q162A} could partially rescue the maternal knockdown of *axin1*, reducing the incidence of dorsalized embryos. Activation of Wnt signaling during maternal stages is critical for proper dorsal

axis specification and Axin plays a key role in the degradation complex controlling the cytoplasmic accumulation of β-catenin.

This observation confirms that Axin function as a scaffold protein is intact and the point mutation in the RGS domain predicted to decrease Gα binding does not affect its ability to act as a scaffold protein in the degradation complex.

Prototypical RGS proteins are related by the RGS domain, that is approximately 120 amino acid residues present in the N terminal of the protein and are capable of binding Gα subunits and accelerate GTP hydrolysis (Natochin et al., 1997; Posner et al., 1999). Axin as well as conductin are grouped in a family of RGS proteins that have not been shown to have GAP activity (RA family), however, Axin is capable of binding to Gα proteins but no GAP activity have been described yet suggesting that proteins that contain RGS domain may have additional functions to regulate intracellular signals in addition to or apart from having a GAP function (Castellone et al., 2005; Natochin et al., 1997; Posner et al., 1999).

In summary, using *axin1* maternal depletion we were able to assess Axin1 function maternally, and our results demonstrate Axin-mediated RGS-like activity is dispensable during the establishment of the D-V axis, and both *axin1* and *axin1* ^{Q162A} mutant construct were able to compensate for the loss of maternal Axin1 function.

Table 1: axin1 overexpression in *Xenopus*.

Groups	n	Normal	Ventralized
axin1	100	13 (13%)	87 (87%)
$axin1^{Q162A}$	62	19 (30%)	43 (70%)

Note: *n* = number of embryos.

Table 2: Maternal depletion and rescue of *axin1* in *Xenopus* embryos.

Groups	n	Normal	Dorsalized
Uninjected	40	40 (100%)	0 (0%)
axin1- depleted	44	8 (10%)	36 (90%)
axin1- depleted +*axin1**	32	25 (78%)	7 (22%)
axin1- depleted + $axin1^{Q162A}$**	21	16 (76%)	5 (24%)

Note: *n* = number of embryos. *p-values*: *1.76e-7. **9.67×10^{-6} .The results of the zebrafish injections were subjected to the Fisher's exact test.

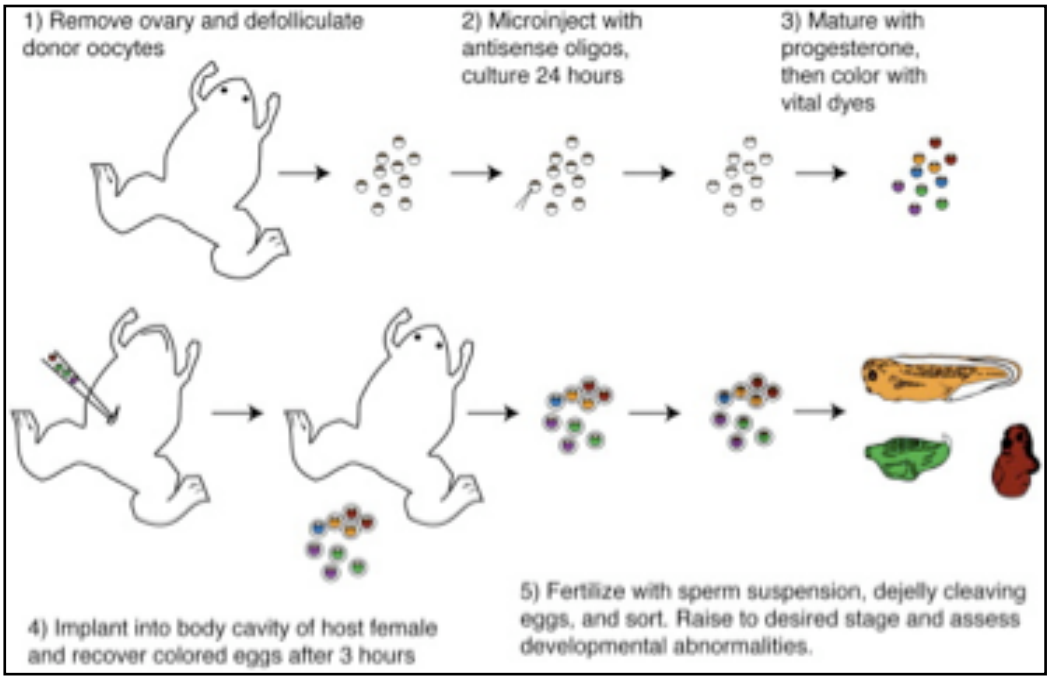

Figure 8: Diagram of the host-transfer procedure. Ovary is obtained from females by laparotomy, and full-grown stage VI oocytes are manually defolliculated and cultured in oocyte culture medium. Antisense oligonucleotides are injected at doses of 2–6 ng, depending on the gene of interest. Injected oocytes are cultured 24 h to allow mRNA and oligo degradation to occur, and are treated with 2 μM progesterone. The next day, oocytes are colored with vital dyes and transferred to ovulating host females. Eggs are obtained and fertilized by in vitro fertilization.

Source: Hulstrand, A.M., Schneider, P.N., Houston, D.W. (2010). The use of antisense oligonucleotides in *Xenopus* oocytes. Methods (Elsevier) *in press.* doi: 10.1016/j.ymeth.2009.12.015

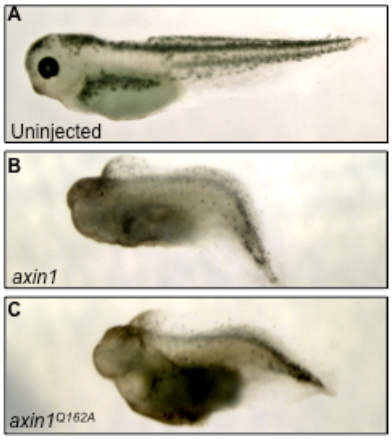

Figure 9: Axin1 overexpression causes ventralization in *Xenopus* embryos. (A) uninjected (B) *axin1* and (D) $axin1^{Q162A}$.

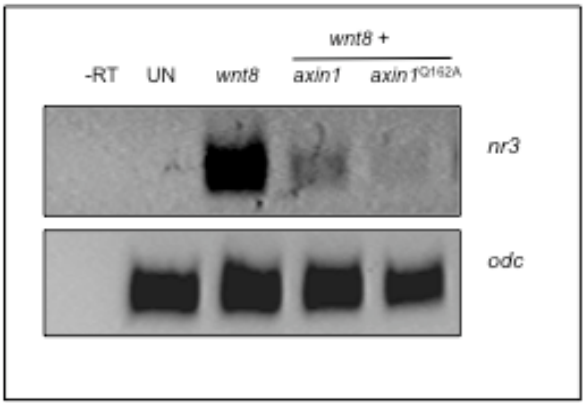

Figure 10: Animal cap assay. Caps were cut from late blastulae previously injected with wnt8 (1 pg) along with *axin1* or *axin1*^{Q162A} (1 ng) and analyzed for expression of *nr3*, a Wnt target gene, by RT-PCR. ODC was used as a loading control.

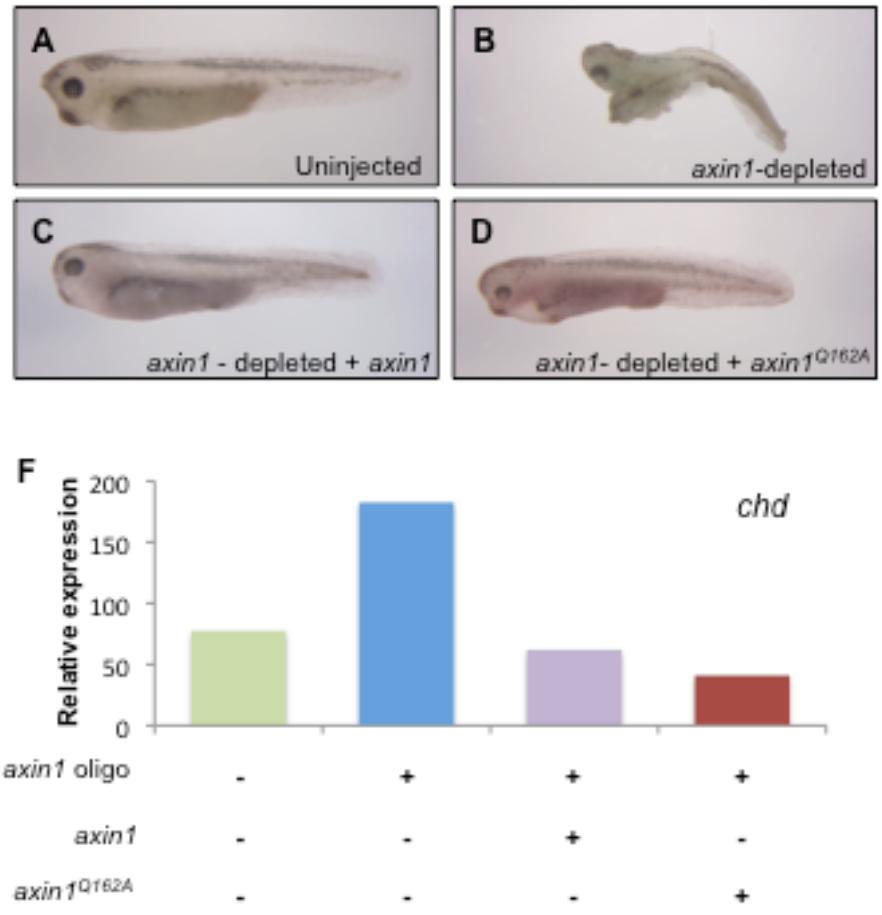

Figure 11: Depletion of maternal *axin1* caused hyperactivation of Wnt signaling. (A) uninjected; (B) *axin1*-depleted; (C) *axin1*-depleted and rescued with wildtype *axin1;* (D)) *axin1*-depleted and rescued with $axin1^{Q162A}$. *(F)* Real time PCR showing *chd* relative expression.

CHAPTER III
AXIN-RGS DOMAIN IS REQUIRED FOR PROPER BRAIN PATTERNING DURING EMBRYONIC DEVELOPMENT

Introduction

Suppression of Wnt signaling is necessary for induction of vertebrate heads. For example, the *headless* mutation abolishes the transcriptional repression function of Tcf3, resulting in ectopic expression of Wnt target genes and truncated head formation. Moreover, alterations in Wnt signaling affect dramatically the AP patterning of vertebrate brain (Heisenberg et al., 2001; Kim et al., 2000). Another example is the ma*sterblind* (*mbl*) zebrafish mutant, which harbors a null mutation in the *axin1* gene. These mutants have constitutively active Wnt/β-catenin signaling, resulting in posteriorization of the anterior brain and consequently in the absence of eyes and telencephalon (Heisenberg et al., 2001; Masai et al., 1997; Sanders and Whitlock, 2003). Axin is a major negative regulator of the Wnt signaling and thus, its regulation during brain patterning is critical for the proper formation of the vertebrate brain.

To characterize the effects of specific residues in Axin-RGS domain, I mutated a highly conserved residue in the Axin-RGS domain that has been shown to be critical for GAP activity of prototypical RGS proteins (Natochin et al., 1997). The Axin-RGS domain encodes a glutamine (Asn) in that location, thus it is unclear if the Axin has GAP and/or Gα binding activity. In this chapter, I describe the effect that the Axin-RGS point mutant has in suppressing the *mbl* phenotype or in rescuing the loss of *axin1* using *axin1* MO knockdown.

The differential roles of Axin-RGS in Wnt signaling occurring maternally in the frog, or during brain patterning in the fish, could reflect either species-specific roles in Wnt signaling or a cell type or temporal requirement in different Wnt signaling contexts

during development. To distinguish these possibilities, we examined whether *axin1* and *axin1*^{Q162A} differed in their ability to rescue inhibition of zygotic Axin1 function in *Xenopus*. In this chapter I utilized maternal depletion technique to determine whether there is a differential requirement of Axin-RGS domain during maternal and zygotic stages of *Xenopus* embryos.

Materials and methods

Danio rerio embryo manipulation

Fertilized eggs were collected from natural spawning of adult zebrafish. Embryos were rinsed with embryo medium (Westerfield, 1995) and staged according to Kimmel et al. (1995). After experiments were performed, zebrafish embryos were cultured at 28°C to the appropriate stage for dorsal-ventral assessment, forebrain development or fixed for whole-mount in situ hybridization. Forebrain development was analyzed visually under a light microscope or by whole-mount in situ hybridization with *dlx2* probe at 24 hours post-fertilization.

Xenopus laevis oocytes and embryos manipulation

Oocytes were isolated from female *Xenopus* by laparotomy. The donor female was anesthetized in buffered MS222 (0.1% MS222 +0.7% sodium bicarbonate). The oocytes were maintained in OCM (60% L-15 medium, 0.4mg/ml BSA, 1mM glutamine, 0.1 mg/ml gentamicin). Eggs were recovered from laying females, fertilized using a sperm suspension and maintained in 0.1xMMR (1XMMR: 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM EGTA, 5 mM HEPES, pH 7.7). After fertilization, egg jelly coats were removed using cysteine (2% cysteine/0.1% MMR, pH 7.8) and injected as described previously (Cuykendall and Houston, 2009). For injections of mRNAs after

fertilization, embryos were dejellied and transferred to 2% Ficoll/0.5×MMR at the one-cell stage.

Antisense modified oligonucleotides (MO) and misexpression studies

To characterize the effects of specific residues in Axin-RGS domain, I cloned zebrafish *axin1*. Utilizing sequences in the NCBI database, I designed primers to clone full-length zebrafish *axin1* and *axin2* from embryonic staged cDNA pools. The clones were sequenced confirmed and subcloned into an expression vector, which allowed efficient translation in zebrafish and *Xenopus*. Since *axin1* was the most abundant transcript, it was selected for further analysis. For structural-functional examination of the Axin-RGS domain, wildtype and mutant Axin-RGS constructs were generated using site directed mutagenesis (QuikChange site-directed mutagenesis kit from Stratagene) (see APPENDIX A for primer sequences).

Morpholinos and mRNA injections

Morpholino or in vitro transcribed *axin1* mRNAs were injected into the yolk of 1-cell zebrafish embryo. To find the sub phenotipic dose of mRNA to be used in the rescue experiments, I injected a series of doses (60, 100, 150, 200 and 300 pg) of *axin1* and *axin1*^{Q162A} in *Xenopus* and 25, 50, 75, 100 and 150pg of mRNAs in zebrafish embryos.

Masterblind crosses

An intercross between two *mbl* mutation-bearing heterozygotes will produce 25% diploid mutant offspring. *mbl* heterozygote crosses were set up and the fertilized eggs were injected at 1-4 cell stage with *axin1*-myc or *axin1*^{Q162A}-myc. The rescue of the *mbl*

phenotype was confirmed by sequencing the region around the *mbl* mutation. The primers used generated a 400bp band and each PCR product was sequenced and confirmed (see APPENDIX A for primer sequences).

Whole mount in situ hybridization in zebrafish

For all manipulations, embryos were fixed in 4% paraformaldehyde/1 X PBS. Digoxygenin-labeled RNA probes (Roche) were synthesized as follows: *dlx2 (T7/NotI*). Single-probe hybridizations were done as described in (Schneider et al., 2008). After detection, embryos were mounted and photographed.

Whole mount in situ hybridization in *Xenopus*

For all manipulations, embryos were fixed in 1X MEMFA (10XMEMFA:1M MOPS, 20mg EGTA, 10Mm MgSO4). *pax6* digoxygenin-labeled RNA probe (Roche) was synthesized using *T7 and EcoRI.* In situ was done as described in (Kerr et al., 2008).

Western blot

Zebrafish embryos were lysed in phosphoprotein buffer (80 mM ß-glycerophosphate pH 7.0, 20 mM EGTA, 15 mM MgCl2, 1 mM DTT, 1 mM PMSF, 1:50 protease inhibitor cocktail; Sigma) and cleared by centrifugation at 15,000 g. The equivalent of 10 embryos were loaded on 10% SDS-PAGE Ready Gels (BioRad) and transferred to nitrocellulose. Membranes were blocked in 5% non-fat dry milk in PBS, 0.1% Tween 20 and incubated in primary antibody (mouse anti-myc) diluted in the same buffer. Detection was performed using the Super Signal West Pico system (Pierce). Exposure times were approximately 1 minute. Antibodies and dilutions used were anti-myc (1:1000) and anti α-tubulin (1:10,000).

Coimmunoprecipitation

Embryos were coinjected at the 4-cell stage with Gαi-HA and *axin1*-FLAG or Axin1^{Q162A}-FLAG and cultured to stage 10. Samples were lysed in ice-cold lysis buffer (150mM NaCl, 20mM Tris-HCl, pH8, 1mM EDTA, 1 μg/ml aprotinin, 100 μM PMSF, 10 μg/ml leupeptin). The lysates were incubated with HA-beads. The immune complexes were washed three times with lysis buffer, resuspended in 20μl SDS-PAGE buffer and heated at 100°C for 5 min. The mixture was subjected to SDS-PAGE on 10% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes by electroblotting. Nitrocellulose membrane was blotted against Axin-FLAG (mouse anti-FLAG M2-Peroxidase) and against Gα-HA (mouse anti-HA clone 3F10, 1:500, Roche Applied Science).

Immunolocalization

Embryos were fixed in 4% paraformaldehyde/1X PBS and incubated overnight with anti-myc (company) followed by secondary antibody conjugated with a fluorescent label (Alexa 488, Molecular Probes), as described in Westfall et al. (2003). Whole mount embryos were optically sectioned using laser confocal microscope system (63X/1.2 water objective; model TCS-NT; Leica).

Statistical analysis

The results of the zebrafish and *Xenopus* injections were subjected to the Fisher's exact test.

Results

Cloning and generation of *axin1* and *axin1*^{Q162A}

To specifically address the interaction of Axin with Gα subunits in Wnt signaling, we sought to mutate amino acid residues that would disrupt GAP activity, but not APC but not APC binding. The regions of RGS3 that interact with Gα as well as the regions of Axin known to bind APC are highlighted in figure 12. The alignment of different Axin homologues with RGS3 demonstrates that the Axin-APC interaction domain is distinct from a potential Axin-Gα interaction domain. Moreover, the RGS4 three-dimensional structure shows that the asparagine residue required for binding to Gα and GAP activity is positioned opposite to the APC-binding region of the domain.

Although the overall similarity in amino acid sequence between the Axin RGS domain and prototypical RGS proteins is extensive, as has been noted previously (Dohlman and Thorner, 1997; Egger-Adam and Katanaev; Hollinger and Hepler, 2002), vertebrate Axin encode a glutamine (amino acid 162) in the position of the conserved Asn (amino acid 128 in human RGS4) (Egger-Adam and Katanaev).

Glutamine is predicted to have at least partial Gα binding, based on in vitro assays (Natochin and Artemyev, 1998). For structural-functional studies of the Axin-RGS domain, we generated a point mutation in Axin1 to replace the glutamine at position 162 with an alanine. A similar change in RGS4 severely decreased RGS4-Gα binding affinity and eliminated GAP activity (Natochin and Artemyev, 1998). Both *axin1* and *axin1*^{Q162A} are expressed when injected into zebrafish embryos.

Overexpression of *axin1* and *axin1*^{Q162A} causes loss of axial structures in zebrafish

mRNAs encoding *axin1* and *axin1*^{Q162A} were injected into zebrafish embryos and monitored for developmental defects. Expression of both wildtype and mutant forms of Axin ventralized zebrafish embryos generating loss of head structures (brain and eyes)

and enlargement of ventral posterior tissues, to a similar extent (Figure 12 and table 2). Molecular verification support that a dorsal marker and direct target of Wnt signaling chordin is reduced (Figure 13 and table 3).

axin^{Q162A} fails to suppress the *mbl* phenotype in zebrafish embryos

Overexpression of *axin1* and *axin*^{Q162A} is sufficient to limit Wnt signaling (Heisenberg et al., 2001). We next determined the role for Axin RGS domain in patterning by evaluating the extent that *axin1* and *axin*^{Q162A} suppress both the knockdown phenotype and the genetic mutant phenotype, embryos homozygous for the *masterblind (mbl*) mutation. *Mbl* mutation is an Axin loss of function mutation and embryos have reduced forebrain structures including the telencephalon, olfactory placodes and optic vesicles coupled with an expanded epiphysis (diencephalon) (Heisenberg et al., 1996; Heisenberg et al., 2001; Masai et al., 1997). Injection of an antisense Morpholino phophorodiamidate oligonucleotide directed against *axin1* (*axin1*-MO) into 1-2 cell wildtype zebrafish embryos, resulted in a high percentage of embryos showing phenotypes identical to the *mbl* mutant (89%; n=127), namely reduced telencephalon and reduced or absent eyes (Figure 14, Table 4). These phenotypes were assessed both by morphology and by in situ hybridization at at 24hpf against *distal-less homeobox gene 2a* (*dlx2a*), a marker of ventral telencephalon, diencephalon and pharyngeal arches (Thisse et al., 2004). Embryos injected with *axin1-MO* showed reduced *dlx2a* expression in the telencephalon in the majority of cases (80%; n=63), whereas the diencephalon and pharyngeal arch expression was unaffected. We compared these phenotypes with those of embryos coinjected with either *axin1* or *axin1*^{Q162A} with the MO. Injection of full-length *axin1* mRNA suppressed the MO phenotype, of reduced telencephalon by morphology (38%; n=95) and telencephalic *dlx2a* expression (22%; n=50). In contrast, *axin1*^{Q162A} did not suppress the MO-induced telencephalic defects, resulting in similar percentage of

embryos with morphologically reduced/absent telencephalon (76%; n=122) and reduced *dlx2* (65%; n=69) (Figure 15 , table 5).

As confirmation of the *axin1*-MO knockdown and 'rescue", I injected *axin1* and *axin1*^{Q162A} into progeny obtained from a cross of *mbl* heterozygotes. Such a cross is predicted to result in 25% homozygous mutant offspring genetically deficient in Axin1 activity, which would display defects in anteroposterior brain patterning.

Uninjected embryos from *mbl* heterozygote crosses showed the predicted frequency of mutant phenotypes (23%; n=150). Injection of *axin1* mRNA into 1-4 cell stage embryos from an *mbl* incross was sufficient to reduce the incidence of *mbl* phenotypes (head defects by morphology and *dlx2* expression at 24hpf as above) (11%; n=121) (Figure 15 , table 5). Whereas embryos injected with *axin1*^{Q162A} had a similar incidence of mutant phenotypes as the uninjected samples.

To analyze the genotype of the embryos, genomic DNA was isolated from selected embryos. In uninjected embryos from an *mbl* incross, embryos injected with the *mbl*-like phenotype had the expected T-to-A transversion at nucleotide 1220, while embryos that appeared wildtype did not. Sequence analysis of mbl incross embryos injected with *axin1*mRNA revealed that 3/8 embryos selected with normal *dlx2* expression harbored the homozygous *mbl* point mutation, thus providing direct verification that Axin1 RNA can suppress the phenotype of a genotypically homozygous mutant, indicating that rescue of mutant embryos had indeed occurred. Overall, these data suggest that RGS activity of the Axin-RGS domain is required to attenuate normal Wnt signaling during anteroposterior patterning of the brain.

axin^{Q162A} fails to restore normal forebrain expression of *pax6* after loss of *axin1* in *Xenopus* embryos

We designed a morpholino against *Xenpous axin1* and injected it following fertilization, at the 1-2 cell stage. Inhibition of *axin1* in this context does not affect initial formation of the dorsoventral axis, but results in embryos with anteroposterior defects, including truncated heads and small eyes (table 6). We used in situ hybridization against *pax6* to assess anteroposterior brain patterning in these embryos. In neurula stage *Xenopus* embryos, *pax6* is expressed predominantly in the forebrain and optic vesicles, and to a lesser degree in the midbrain and dorsal spinal cord (Hirsch and Harris, 1997). In embryos injected with *axin1-MO*, the forebrain and optical vesicle expression of *pax6* was greatly reduced in a majority of embryos (69%; n=45). This effect could be rescued to a large extent (36% affected; n=42) in embryos injected with *axin1* mRNA. Interestingly, *axin1*^{Q162A} failed to restore normal *pax6* expression, mimicking our results in zebrafish (Figure 16, table 7). These results show that the Axin-RGS domain is required to inhibit zygotic Wnt signaling in *Xenopus*, suggesting a differential requirement for Axin1 during maternal and zygotic stages of embryonic development.

Axin1 and Axin1^{Q162A} can bind to Gαi during gastrula stage of *Xenopus* embryos

To confirm that the Axin ^{Q162A} mutation indeed lacks RGS activity, we first test its ability to bind to Gα protein. Natochin *et al*. have demonstrated that RGS4 binding ability correlates with its GAP activity in vitro (Natochin et al., 1998). Axin-RGS domain has been shown to interact with Gα subunit in cell culture studies, but no GAP activity was observed (Castellone et al., 2005; Natochin et al., 1998; Stemmle et al., 2006). A similar mutation within the RGS domain of RGS4 causes loss of affinity of Gα and RGS4 (Natochin and Artemyev, 1998). We tested whether Axin-RGS domain can bind Gαi in whole embryos by coinjection in 2 cell *Xenopus* of Axin1-FLAG or Axin ^{Q162A}-FLAG

mRNAs with Gαi-HA mRNA and performing coimmunoprecipitation assays. Our results show that both forms of Axin are capable of binding Gαi in stage 10 *Xenopus* embryos (Figure 17) however, this is not a quantitative method and it is possible that the Axin ^{Q162A} mutant form retains some binding ability to Gαi but binds with less affinity to Gαi, as compared to Axin1. Furthermore, we also tested the ability of RGS3 and RGS3^{N-A} mutant, which contains a mutation changing the key asn to an ala shown to lack GAP activity (Natochin and Artemyev, 1998), to bind to Gαi. Our results show that Gαi binds to both RGS3 wildtype and point mutant in *Xenopus* oocytes, during gastrulation and during neurulation (Figure 18).

Axin1 but not Axin1^{Q162A} promotes the redistribution of Axin upon Gαi overexpression in zebrafish embryos

Tagged constructs for axin1 and *axin1^{Q162A}* were generated and RNA injected into embryos. Axin1 and Axin1^{Q162A} localization is mostly cytoplasmic and in a puncta (Figure: 19). To determine if the differential role of axin-RGS is reflected in this localization, I evaluated axin and Axin1^{Q162A} distribution in the presence of exogenous Gαi. If Axin is indeed interacting with Gα, endogenous levels may be limited, therefore providing exogenous Gαi, we could better discern Axin-G protein interaction. I find that Axin1 forms punta in the cytoplasm and is redistributed away from the cytoplasm possibly to the plasma membrane, whereas Axin1^{Q162A} remains diffusely expressed (Figure 19). Taken together with the functional assays, these data suggest that Axin-RGS interacts with Gαi and this is necessary for its zygotic function in zebrafish.

Discussion

Axin is a scaffold protein required for the degradation of β-catenin. Axin loss of function leads to cytoplasmic accumulation of β-catenin and upregulation of Wnt signaling (Carl et al., 2007; Huang and He, 2008; Ikeda et al., 1998; Kofron et al., 2001; Luo et al., 2005). Here we report a novel function for the Axin-RGS domain during the patterning of the vertebrate brain. Our findings after *axin1* and *axin1* ^{Q162A} point mutant overexpression suggest that the Axin function as a scaffold protein for the degradation complex is intact and Axin-RGS domain region where APC binding site is localized is not affected by the point mutation made to disrupt Axin-RGS-Gα interaction. *axin1* and *axin1* point mutant constructs are both sufficient to induce ventralization in overexpression assays.

Wnt signaling activation is important for the posteriorization of the vertebrate brain and is active in a posterior to anterior gradient (Heisenberg et al., 1996; Kiecker and Niehrs, 2001a). We used *dlx2* as a forebrain marker to analyze the ability of the Axin1^{Q162A} mutant construct to rescue the loss of *dlx2* expression in the forebrain region as a result of the Axin1 knockdown using *axin1* MO. Our data shows that the Axin-RGS domain is critical during the anteroposterior patterning of the brain. Loss of forebrain structures as well as loss of *dlx2* expression in this region was not rescued with Axin1^{Q162A} mutant construct. In contrast, Axin1 rescued the morphological and molecular head defect phenotypes; in this case, *dlx2* expression was restored. Additionally, we tested for the ability of the Axin1 ^{Q162A} mutant to suppress the loss of zygotic Axin1 function in the zebrafish genetic, *mbl* and as expected, the Axin1 ^{Q162A} mutant construct was not able to suppress the *mbl* phenotype in a statistically significant manner and the rescued fish retained the eyeless phenotype.

Axin has been shown to bind directly to Gαs and promote growth of colon cancer cells (Castellone et al., 2005), but the extent that this finding relates to the in vivo

functions of Axin during embryonic development is less clear. We propose based on our analysis that Axin-RGS like function is required during anteroposterior patterning of the vertebrate brain to regulate Wnt signaling. And given the differential localization of the Axin1 and Axin1 point mutant, is via heterotrimeric G-protein signaling.

In summary, we have shown a requirement of the Axin-RGS domain during forebrain formation in zebrafish and *Xenopus* embryos. A point mutation in the Axin-RGS domain predicted to decrease affinity of Axin to Gα and eliminate Gap activity impairs Axin function and its ability to rescue the loss of telencephalon expression of *dlx2* in the zebrafish forebrain, and restore *pax6* normal expression in *Xenopus.* These data suggest that Wnt signaling and G-protein signaling may play a role during the anteroposterior patterning of the brain.

Table 3: Summary of embryonic defects after overexpression of *axin1* in zebrafish embryos.

	N	**Normal**	**Ventralized**
Axin1	109	22 (20%)	87 (80%)
$Axin1^{Q162A}$	69	21 (30%)	48 (70%)

Note: n = number of embryos. Embryos with complete loss of dorsal structures and short anteroposterior axis were scored as ventralized.

Table 4: Summary of embryonic defects after overexpression of *axin1* in zebrafish embryos using *chordin* as a marker.

	n	**Normal**	**Reduced *chordin* expression**
Uninjected	17	17 (100%)	0 (0%)
axin1	30	11(36%)	19 (64%)
$axin1^{Q162A}$	23	9 (42%)	14 (58%)

Note: *n* = number of embryos.

Table 5: Summary of head defects after *axin1* knockdown and rescue.

Morphological analysis (%)			
	N	**Normal**	**Reduced head**
Uninjected	97	97 (100%)	0 (0%)
Axin1-MO	127	14 (11%)	113 (89%)
Axin1-MO + *axin1**	85	59 (62%)	26 (38%)
Axin1-MO + $axin1^{Q162A}$ **	122	29 (24%)	93 (76%)

Note: n = number of embryos; p-values: *7.47e-19. **9.97x10-1. The results of the zebrafish injections were subjected to the Fisher's exact test.

Table 6: Expression of forebrain marker *dlx2* in the *masterblind* mutant and in the *axin1* MO knockdown zebrafish embryo.

		***dlx2* expression in the telencephalon**	
Group	**N**	**Normal**	**Reduced/absent**
Wt x Wt	57	57 (100%)	0 (0%)
mbl-/-	150	115 (77%)	35 (23%)
axin1	121	90 (75%)	13 (11%)
$axin1^{Q162A}$	80	53 (66%)	17 (22%)
Uninjected	45	45 (100%)	0 (0%)
axin1-MO	63	13 (20%)	50 (80%)
axin1- MO + *axin1**	50	39 (78%)	11 (22%)
axin1-MO + $axin1^{Q162A}$**	69	24 (35%)	45 (65%)

Note: n = number of embryos; p-values: *1.27e-9. **8.2 x10-2. The results of the zebrafish injections were subjected to the Fisher's exact test.

Table 7: MO knockdown in *Xenopus*

Groups	**n**	**Normal**	**Head defects**
uninjected	11	11 (100%)	0 (0%)
*axin1*MO	32	8 (25%)	24 (75%)

Note: *n* = number of embryos

Table 8: MO knockdown and rescue in *Xenopus*

Groups	**N**	**Normal**	**Reduced *pax6* expression**
Uninjected	43	43 (100%)	0 (0%)
MO	45	14 (31%)	31(69%)
MO+*axin1**	42	27 (64%)	15 (36%)
MO+ $axin1^{Q162A}$**	49	19 (38%)	30 (62%)

Note: n = number of embryos. p-values: *1.85x10-3. **2.87x10-1. The results of the Xenopus injections were subjected to the Fisher's exact test.

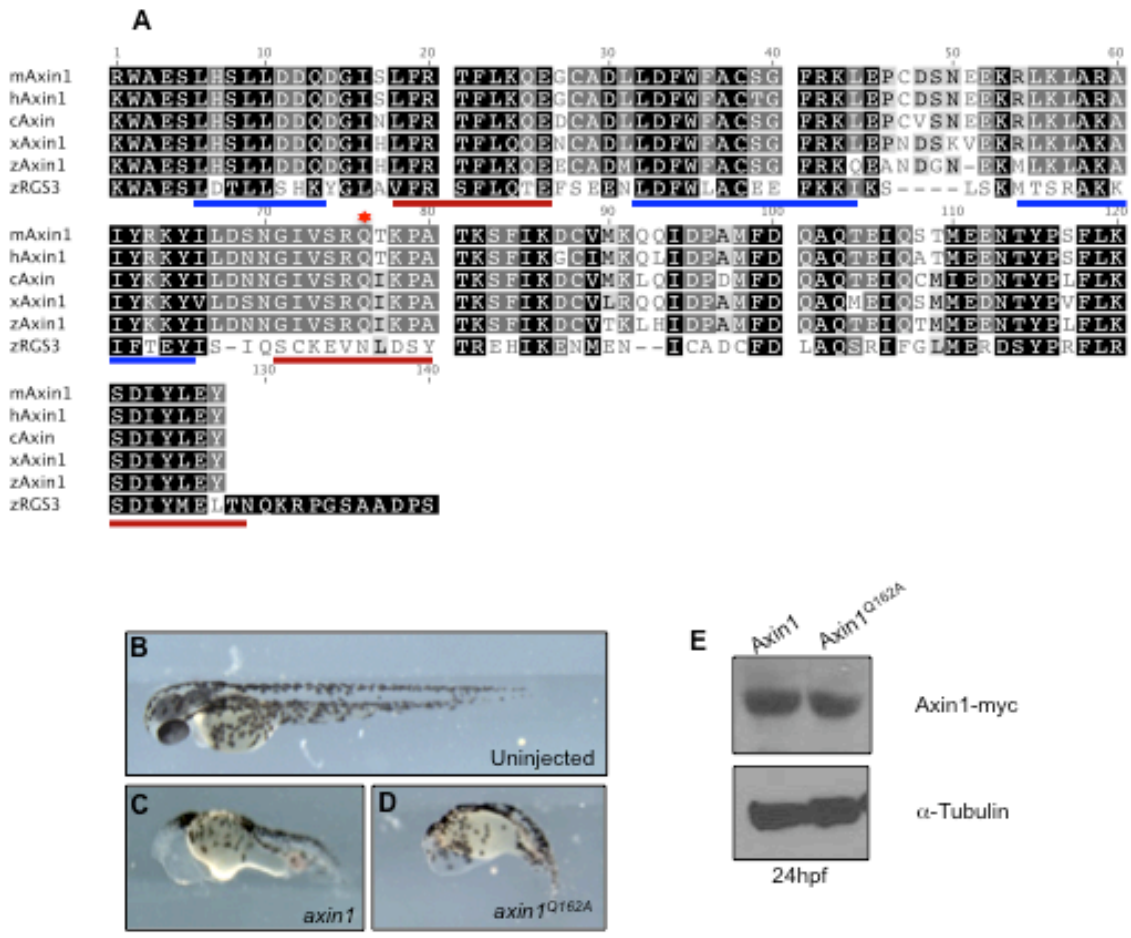

Figure 12: Axin1 overexpression causes ventralization. (A) Axin alignment showing in blue, the regions of Axin-APC interactions and in red the predicted regions for Axin-Gα interaction. (B) Western blot using anti-myc antibody detects myc-tagged recombinant *axin1* and *axin1*^{Q162A} at 24hpf in zebrafish embryos. (C-E) Axin overexpression. (C) wt, (D) *axin1* and (E) *axin1*^{Q162A}.

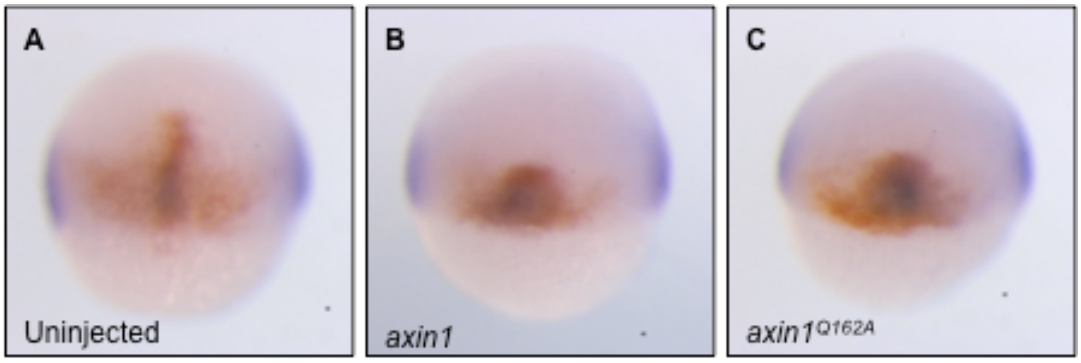

Figure 13: axin1 and axin1 axin1Q162A misexpression cause reduction of chordin expression. Lateral view 30% epiboly embryo. (A) uninjected embryo (B and C) axin1 axin1Q162A respectively.

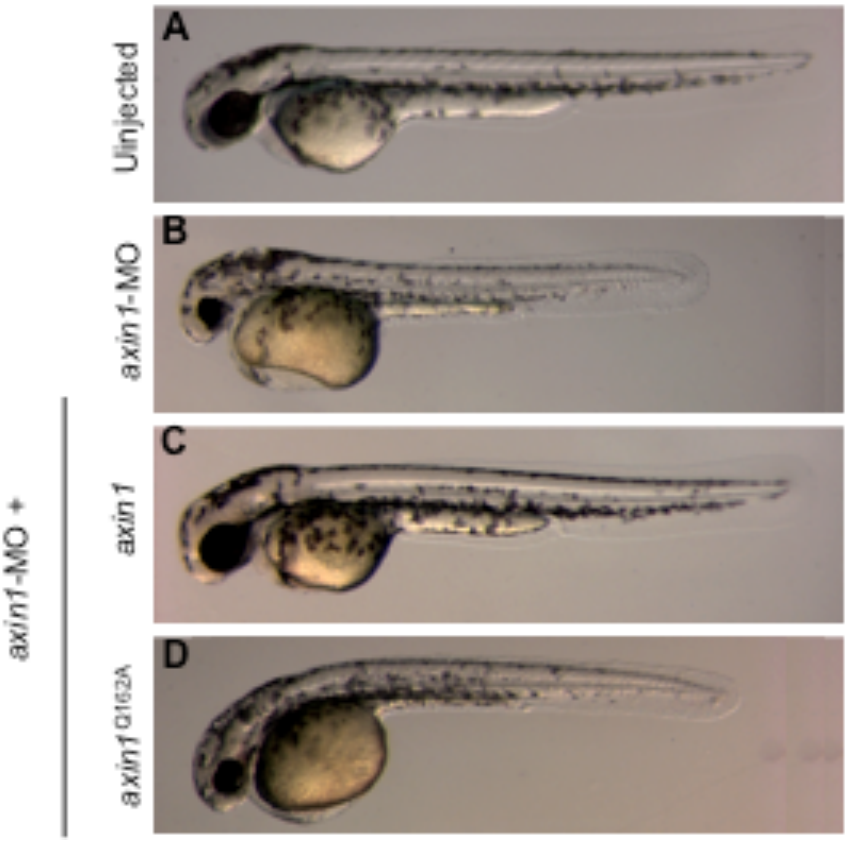

Figure 14: *axin1* knockdown causes head defects. (A-D) Lateral view, 48hpf, showing the morphology after MO knockdown. (A) uninjected control, (B) *axin1*MO knockdown, (C) Rescue of *axin1* knockdown with *axin1* mRNA and (D) Rescue of *axin1* knockdown with $axin1^{Q162A}$.

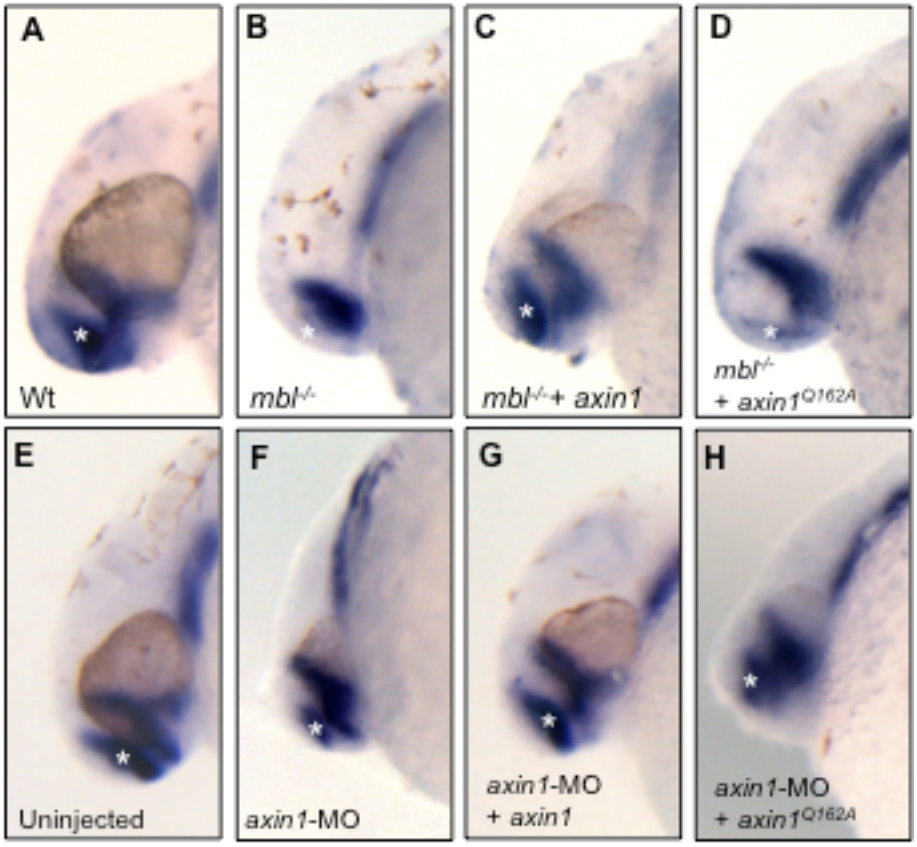

Figure 15: *axin*^{Q162A} fails to suppress the *mbl* phenotype in zebrafish embryos. (A-D) Lateral view, *mbl* fish at 24hpf, showing *dlx2* expression in the forebrain (telencephalon and prethalamus) and diencephalon regions (pharyngeal aches). (A) is a wt sibling, (B) *mbl* , *mbl* injected *axin1* (C) or *axin1*^{Q162A}(D). (E-F) lateral view, 24hpf, *axin1* MO knockdown and rescue showing *dlx2* expression in uninjected control embryo (E), axin1 MO knockdown (F), and morphant embryos injected *axin1* (G) or *axin1*^{Q162A}(H). Asterisks indicate *dlx2* expression in the telencephalon region.

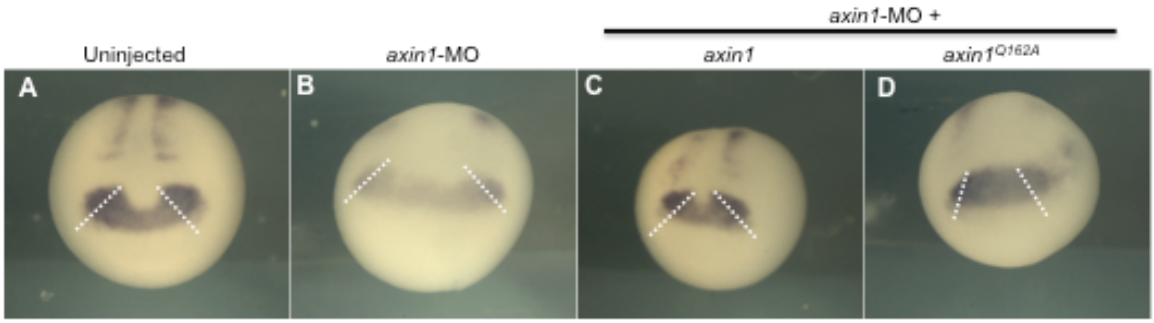

Figure 16: *axin*^{Q162A} fails to rescue the expression of *pax6 in the forebrain*. (A-D) frontal view of stage 14 *Xenopus* embryos injected with *axin1* MO and rescue with the wildtype and mutant constructs. (A) uninjected embryo (B) *axin1* MO injected (C) morphant embryos injected *axin1* (D) morphant embryos injected with *axin1*^{Q162A}. White line depicts the region of the forebrain that will become telencephalon, showing lack of rescue with the mutant construct.

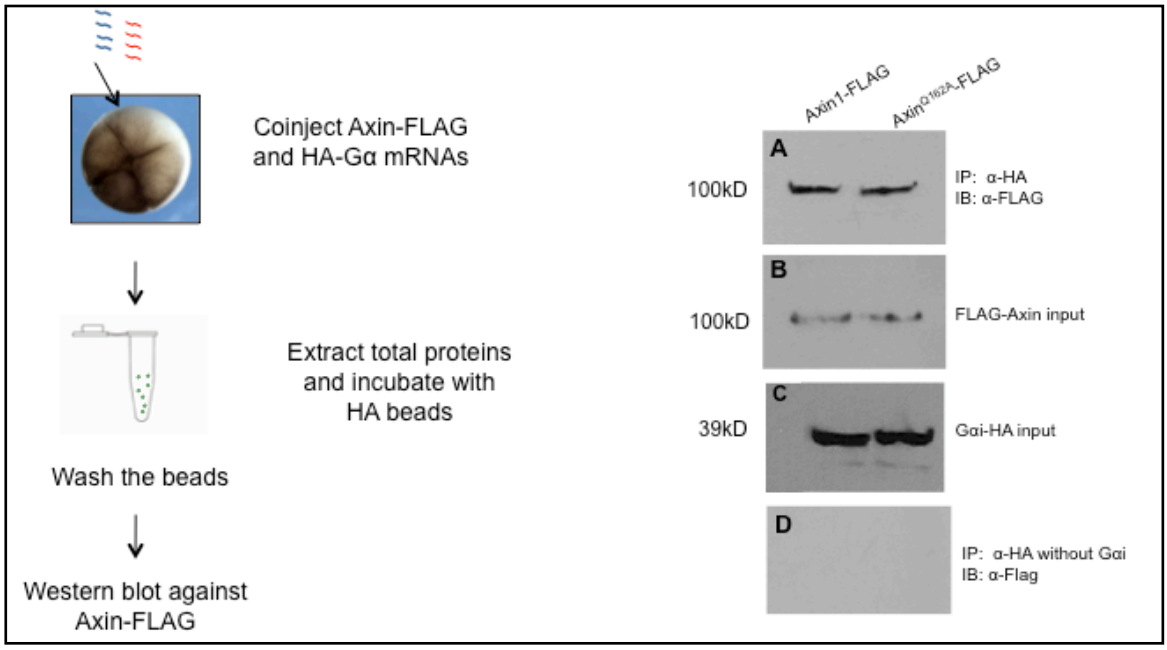

Figure 17: Both Axin1 ad Axin1Q162A can bind to Gα during maternal stages in Xenopus embryos. Coimmunoprecipitation of Gα and Axin1 or Axin1Q162A . Xenopus embryos were coinjected at the 4-cell stage with Gαi-HA and axin1-FLAG or Axin1Q162A-FLAG. HA beads were utilized to pulldown Gα and immunobloting was performed using anti-FLAG antibody.

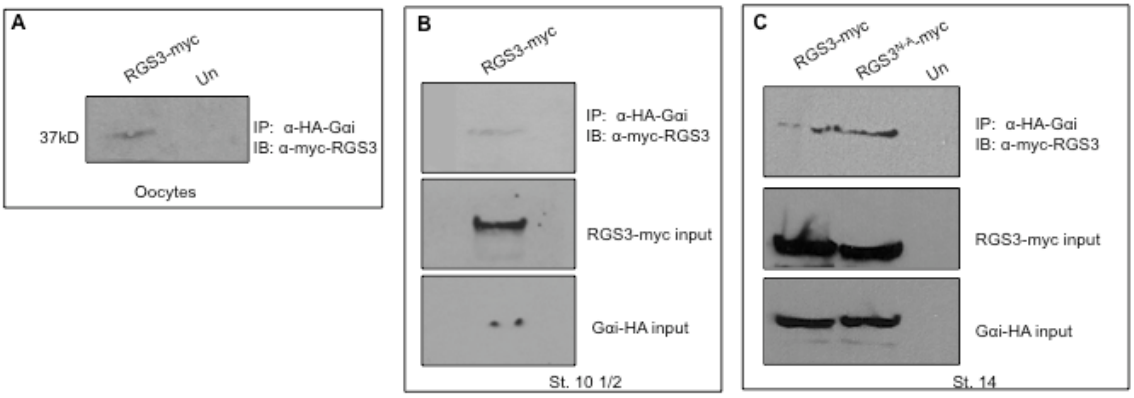

Figure 18: Coimmunoprecipitation of Gα and RGS3 (wildtype and mutant). In oocytes and embryos. (A) Xenopus oocytes were coinjected with Gαi-HA and rgs3-myc. (B) Xenopus embryos were coinjected at the 4-cell stage with Gαi-HA and rgs3-myc and embryos were frozen down at stage 10 ½ for CoIP. (C) rgs3-myc wildtype or rgs3-point mutant were coinjected with Gαi-HA. HA beads were utilized to pulldown Gα and immunobloting was performed using anti-myc antibody.

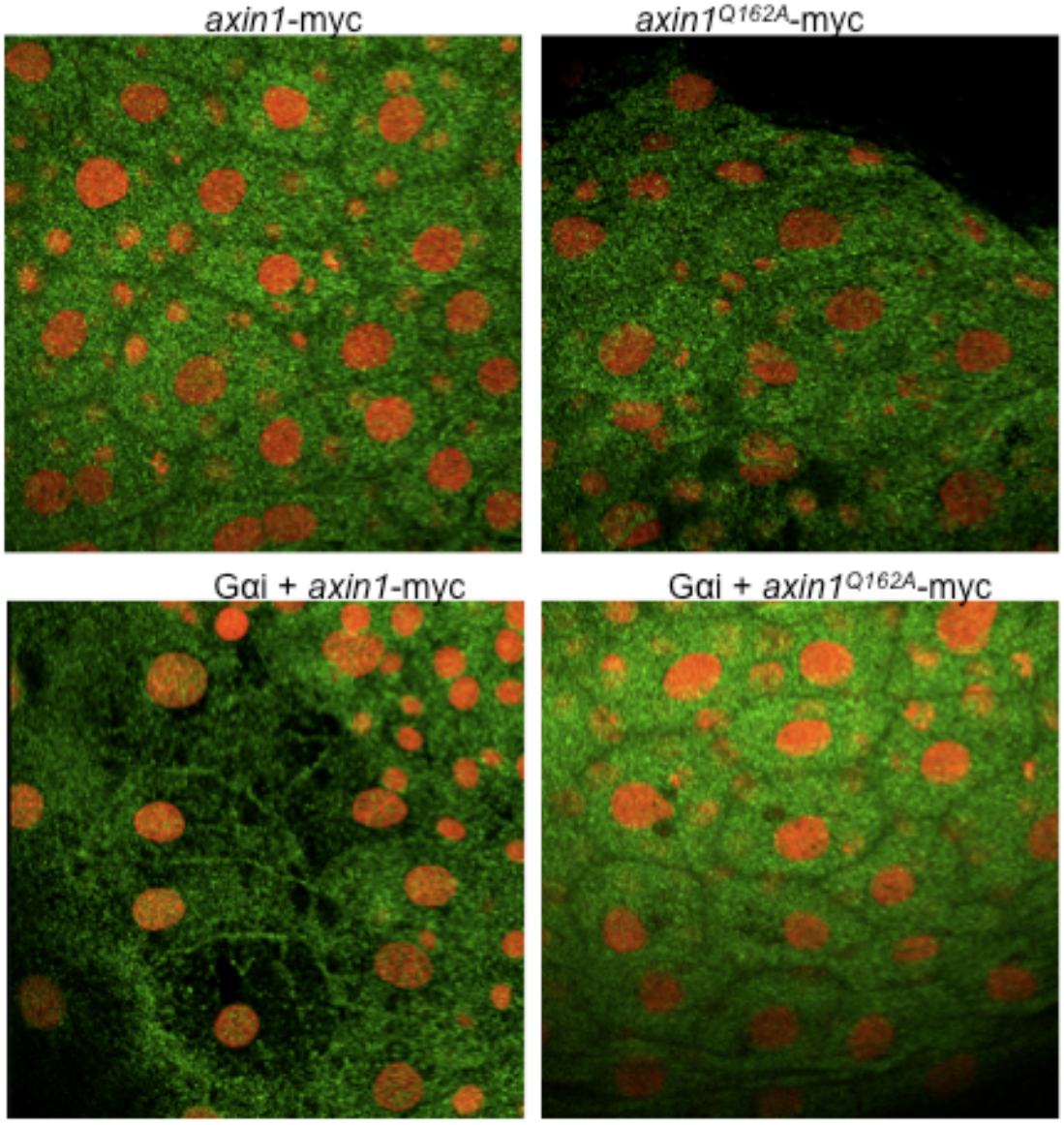

Figure 19: Overexpression of Gαi causes Axin1 but not Axin1Q162A accumulation at the plasma membrane in zebrafish embryo. This Figure shows a mounted view of zebrafish embryos at 80% epiboly. axin1-myc and axin1Q162A-myc were injected in 1-2 cell zebrafish embryo. (A) Axin1; (B) axin1Q162A. Anti-myc staining for Axin1 and axin1Q162Ain green and Topro3 staining nuclei in red. Embryos were co-injected with Gα and (C) axin1-myc or (D) Axin1Q162A-myc. Antibody staining was performed using anti-myc antibody.

CHAPTER IV
NKD1 IS REQUIRED FOR ASYMMETRIC CHARON EXPRESSION AND LEFT-RIGHT PATTERNING

Introduction

The establishment of the left-right (LR) axis in zebrafish embryos relies on signals from the dorsal forerunner cells (DFC) and the Kupffer's vesicle (KV). While the Wnt signaling network influences many aspects of embryonic development (Grigoryan et al., 2008), its precise role in LR patterning is still unclear. One arm of the Wnt network leads to stabilization of β-catenin and activation of downstream target genes (Dale, 1998). Other Wnt ligands appear to act independently of β-catenin to modulate Ca^{2+} release and influence cell polarity (Kohn and Moon, 2005; Solnica-Krezel, 2005). Central to all arms of the Wnt network is Dishevelled (Dvl) function. Naked Cuticle (Nkd) binds Dvl and has been shown to modulate β-catenin dependent and independent Wnt signaling (Van Raay et al., 2007; Yan et al., 2001b). Here, we show that knockdown of Nkd1 specifically in the DFC results in β-catenin accumulation altered DFC migration, KV formation, ciliogenesis and LR patterning defects. Furthermore, we identify asymmetric expression of the Nodal antagonist *charon* around the KV and show that Nkd1 knockdown impacts asymmetric *charon* expression. We also demonstrate a requirement for a functional Nkd1 EF-hand, a putative Ca^{2+}-binding motif, to modulate zebrafish and *Xenopus* cell movement. Moreover, our data support that a functional Nkd1 EF-hand is necessary for Nkd1 turnover and Dvl localization/stability. Our findings show that Nkd1 acts as a b-catenin antagonist in the DFCs necessary for LR patterning.

Materials and methods

Danio rerio embryo manipulation

Fertilized eggs were collected from natural spawning of adult zebrafish. Embryos were rinsed with embryo medium (Westerfield, 1995) and staged according to Kimmel et al. (1995). EGFP migration in the Dusp6:d2EGFP transgenic line was assessed by WMISH with EGFP.

Xenopus laevis embryos manipulation

Eggs were recovered from laying females, fertilized using a sperm suspension and maintained in 0.1xMMR (1XMMR: 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM EGTA, 5 mM HEPES, pH 7.7). After fertilization, egg jelly coats were removed using cysteine (2% cysteine/0.1% MMR, pH 7.8) and injected as described previously (Cuykendall and Houston, 2009). For injections of mRNAs after fertilization, embryos were dejellied and transferred to 2% Ficoll/0.5×MMR at the one-cell stage.

Whole-mount in situ hybridization (WMISH) and LR scoring

For all manipulations, embryos were fixed in 4% paraformaldehyde/1xPBS and in situ hybridization were done as described (Schneider et al., 2008). DFC migration in the Dusp6:d2EGFP transgenic line was assessed by WMISH with EGFP. Zebrafish cardiac jogging was assessed in live embryos at 28 hpf by DIC optics and by WMISH with *nkx2.5*. CE movements were ssessed by WMISH with *krox20* (*EcoRI*/T7). and *myoD* (*EcoRI*/T7). KV was analyzed in live embryos at 8-10 somite-stage by light microscopy and by WMISH with *charon* (*EcoRI*/T7). Digoxygenin-labeled antisense RNA riboprobes (Roche Applied Science) were synthesized using linearized templates and the appropriate RNA polymerase.

Western blot

Embryos at the 1-2 cell stage were injected with 8 pg of myc-tagged *nkd1* or *dvl-2* RNA. Nkd1 and Dvl were cloned into Tol2kit p3E-MTpA, by Multisite Gateway cloning, as described by the manufacturer (Invitrogen, CA). At 80% epiboly (8.5 hpf), embryos were lysed in phosphoprotein buffer (80 mM ß-glycerophosphate pH 7.0, 20 mM EGTA, 15 mM MgCl2, 1 mM DTT, 1 mM PMSF, 1:50 protease inhibitor cocktail; Sigma) and cleared by centrifugation at 15,000 g. 10ul loading buffer per embryo was added to the cell lysate. One embryo equivalent was run on a pre-cast 10% Tris HCL protein gel (Bio-Rad Laboratories) for 105 minutes at 100 V. The protein was transferred to a nitrocellulose membrane for 1 hour at 400 mA. The membrane was blocked in milk blocking buffer for 1 hour and then probed with mouse anti-Myc antibody (9E10, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1500 in blocking buffer, PBS, 0.15 molar NaCl, 0.1% Tween 20, 0.04 g/ml of dried milk) overnight at 4 ^{o}C. The membrane was washed with blocking buffer and then PBS and imaged with the Odyssey system (LI-COR Biosciences, Lincoln, NE).

Dvl localization

Multichannel confocal microscopy was used to evaluate Topro 3 and Dvl-GFP localization. Embryos were mounted and imaged on the Leica SP2 AOBS scanning laser confocal microscope system with 63x (oil lens) magnification.

Antisense oligonucleotide-mediated knockdown

MO (8 pg) or *in vitro* transcribed, Nkd1 RNA, c-terminally-tagged Dlv2-Myc, Dvl-GFP

(70-100 ng/µl) were injected into the yolk of 1-cell-stage or 512-cell-stage embryos. MO knockdown efficiency was assessed by RT-PCR of 4 hpf cDNA from 1-cell-stage injected embryos. For assessment of MO-mediated knockdown efficiency, partial Nkd1 and β-actin was amplified (see APPENDIX B for primer sequences).

Statistical analysis

The results of the zebrafish injections were subjected to the Fisher's exact test.

Results

Nkd1 function in DFCs is required for organ laterality

To evaluate if the endogenous role of Nkd1 is to translate Ca^{2+} levels into regulation of Wnt signaling, we manipulated Nkd1 function in the DFCs, a group of cells that display aperiodic Ca^{2+} fluxes associated with β-catenin activity and LR patterning (Schneider et al., 2008). During gastrulation, the zebrafish DFCs migrate into the tailbud to give rise to the ciliated KV, leading to molecular and morphological manifestations of organ asymmetry (Essner et al., 2005). The following experiments were performed in collaboration with Dr. I Schneider and S. Derry.

We utilized a splice-blocking morpholino oligonucleotide (MO) and evaluated knockdown efficiency by RT-PCR (Figure 20A). Since Nkd1 has functions in other tissues, global knockdown led to severe defects, not allowing appropriate evaluation of laterality. To this end, the DFC progenitors were targeted with a late stage MO injection (Amack and Yost, 2004) and evaluated for several aspects of LR patterning (Figure (B-C). Organ asymmetry was evaluated by cardiac jogging. In wildtype and control-injected embryos, the heart migrates to reside on the left side, whereas DFC-knockdown of Nkd1 caused aberrant cardiac phenotypes, including no jog, right jog, and *cardia*

bifida (Figure 20 D-G and Table 9). Therefore, DFC knockdown of Nkd1, similar to DFC Ca^{2+} inhibition, results in organ laterality defects.

DFC-specific Nkd1 knockdown results in β-catenin accumulation and defects in DFC migration and KV formation

Inhibition of Ca^{2+} fluxes in the DFCs results in upregulation of β-catenin activity, DFC migration defects and disrupts KV formation, culminating in LR patterning defects (Schneider et al., 2008). DFC-targeted knockdown of Nkd1 exhibited laterality defects similar to Ca^{2+} inhibition. As a result, we assessed β-catenin nuclear localization in DFCs of $Nkd1MO^{DFC}$-injected embryos. Analysis of β-catenin distribution in the DFC region revealed little or no expression (Schneider et al., 2008). In uninjected controls at 70-80% epiboly, an average of 7% of DFCs display β-catenin-positive nuclei (Figure 21A, C, E). In contrast, β-catenin-positive nuclei are readily identified in 28% of $Nkd1MO^{DFC}$-injected embryos (Figure 21B, D). To determine whether Nkd1 knockdown disrupted DFC migration, we monitored the DFCs in Dusp6:d2EGFP (Molina et al., 2007) transgenic embryos, which express EGFP specifically in DFCs. Embryos injected with $Nkd1MO^{DFC}$ were analyzed at discrete time-points between 80% epiboly to eight-somite stage. During epiboly, the DFCs appear as a tight cluster of cells in wt and in $Nkd1MO^{DFC}$ embryos (Figure 22A,B). At somite stages, wt DFCs form a compact cluster at the midline (Figure 22C,D) whereas $Nkd1MO^{DFC}$ embryos displayed a smaller cluster at the midline with other cells randomly dispersed (Figure 22E, F). At the eight-somite stage in wiltype, the EGFP-expressing DFCs form a circular KV (Figure E), while in $Nkd1MO^{DFC}$ injected embryos the DFCs formed a markedly smaller expression domain (Figure F). Morphological analysis in live wt and control embryos at 8-12 somite stages reveals the crater-like KV structure wider than the notochord (Figure G, H). Nkd1 knockdown resulted in altered KV formation, with 47% of embryos showing reduced or

absent KVs (Figure 22I). Since cilia within the KV have been shown to be critical for proper LR patterning, we evaluated the cilia content in Nkd1MO^{DFC} injected Dusp6:d2EGFP transgenic embryos. Compared to EGFP expressing cells in the wildtype KV (Figure 22 J), Nkd1MO^{DFC} KV cells (EGFP-positive) have reduced cilia content and length (Figure 22 K). In conclusion, Nkd1 knockdown in DFCs results in aberrant β-catenin accumulation, partial DFC dispersal, a reduction in KV size and cilia loss.

Asymmetric gene expression around KV requires Nkd1 function

To confirm aberrant KV formation I evaluated *charon* expression, a Cer/Dan Nodal antagonist that is expressed in cells forming a horseshoe shape around the posterior KV (Hashimoto et al., 2004). Charon inhibits expression of the Nodal related gene *spaw* and its downstream targets *lefty1* and *lefty2* (*lefty1/2*) on the right side lateral plate mesoderm (LPM) but not on the left side (Hashimoto et al., 2004). In Medaka, another teleost fish, *charon* is expressed asymmetrically across the KV (Hojo et al., 2007). At 8- to 10-somite-stage, uninjected and CtrlMO^{DFC}-injected zebrafish embryos revealed a similar bias with stronger *charon* expression on the right side of the KV (Figure 23A, B). This raises the possibility that asymmetric *charon* expression may be necessary for inhibition of Nodal on the right LPM and allow left-sided expression of spaw and its downstream components. Interestingly, upon Nkd1MO^{DFC} knockdown, *charon* expression is largely symmetric, with 62% of embryos showing no expression bias (Figure 23C). If the right-sided bias in *charon* expression accounts for the inhibition of right-sided *spaw* expression, we predicted Nkd1 knockdown to result not in loss, but in bilateral *spaw* expression and its downstream targets *lefty1/2*. In fact, *spaw* and *lefty1/2* were mostly bilateral in Nkd1MO^{DFC} embryos (data not shown). DFC-targeted co-injection of *nkd1* RNA can significantly restore asymmetric distribution of *charon* in

Nkd1MO^{DFC} injected embryos (Figure 23D). Thus, DFC-knockdown of Nkd1 disrupts asymmetric *charon* expression, resulting in bilateral *spaw* and *lefty1/2*.

The KV cilia have been postulated to create a directional fluid flow, ultimately generating an asymmetric signal (Essner et al., 2005; Kramer-Zucker et al., 2005). In Medaka, asymmetric *charon* is cilia-dependent (Hojo et al., 2007). To test whether zebrafish asymmetric *charon* expression is cilia-dependent, I targeted three ciliary proteins known to be required for correct LR patterning: Inversin (Invs), Seahorse (Tilb) and Polaris (Ift88) (Bisgrove et al., 2005; Kishimoto et al., 2008; Otto et al., 2003; Sun et al., 2004). Whereas KV formation appears unaffected, MO-knockdown of each protein led to mainly bilateral *charon* expression around the KV (Figure 24). Therefore, I conclude that asymmetric *charon* expression around the KV is cilia-dependent. Since I found that Nkd1MO^{DFC} resulted in reduced cilia number, I conclude that loss of *charon* asymmetry in Nkd1MO^{DFC} embryos is likely due to loss of KV cilia.

Nkd1 EF-hand motif is required for cell movement in *Xenopus* and zebrafish

Deletion mutants identified a requirement of the Nkd1 EF-hand for Wnt/β-catenin antagonism in *Xenopus* overexpression assays, yet specific alterations of the Ca^{2+} binding amino acids were not evaluated for β-catenin independent Wnt signaling. To evaluate if the Nkd1 EF-hand motif impacts the β-catenin independent Wnt output, we mutated oxygen-donating amino acids predicted to mediate ion binding in zebrafish Nkd (D142N/D144N; Nkd1^{mut}) (Figure 25). During gastrulation, vertebrate embryos undergo CE movements to thicken (dorsal convergence) and elongate (axis extension) the embryo (Keller, 2005). It was previously shown that mouse Nkd1 induced CE defects in *Xenopus* using overexpression assays. I tested the zebrafish Nkd1 overexpression in *Xenopus* and observed shorter curled emvryos with failure in blastopores and neural tube closure indicative of CE defects (Figure 26B). In contrast, overexpression of the Nkd1^{mut} did not

impact CE movements (Figure 26C). To verify that Nkd1^{mut} protein is formed, myc-tagged Nkd1 and Nkd1^{mut} constructs were expressed in *Xenopus* embryos. Western blot analysis detected both the Nkd1- and Nkd1^{mut} -myc tagged protein (Figure 26 J). I also tested for the ability of the mutant construct to cause CE defects in zebrafish embryos using *krox20* and *myoD* as molecular markers for rhombomeres 3 and 5 and somites, respectively. As observed in *Xenopus* embryos, the *nkd1* injection caused CE defects (26F-G) and nkd1^{mut} construct does not impact CE movements and the injected embryos (26 H,I) recapitulates the wt control embryos (26 D,E). Of note is the greater abundance of the Nkd1^{mut} -myc relative to Nkd1-myc protein. If indeed alteration of ion binding amino acids impacted Nkd1 turnover and function, then similar levels and activity would be evident in zebrafish assays.

Nkd1 EF-hand is required to modulate Dvl localization and turnover

One of the hallmarks of PCP and CE is the translocation of Dvl to the plasma membrane (Rothbacher et al., 2000; Veeman et al., 2003b; Yang-Snyder et al., 1996), however the biological relevance remains to be determined. Dvl-GFP was used to evaluate whether Nkd1 impacted Dvl subcellular localization in zebrafish. Dvl-GFP alone appears as puncta in the cytoplasm and a similar pattern was observed in Dvl-GFP coexpressed with Nkd1mut (Figure 27A). Coinjection of Dvl-GFP with Nkd1 results in membrane localization and a noticeable decrease in DVL-GFP expression. Since we observe turnover of zebrafish Nkd1, by western blot at a different stages of development, we hypothesize that Nkd1 binds Dvl leading to the turnover of both products and that this process requires a functional EF-hand. To test this, Dvl2-myc was injected in zebrafish embryos alone or coinjected with Nkd1. Injected embryos were lysed at gastrula stages for western blot analysis. Coinjection of Dvl2-Myc with wt Nkd1 results in a marked decrease in the amount of Dvl2-myc protein, while embryos co-expressing Dvl2-myc

with Nkd1mut retain Dvl2 protein (Figure 27A). To monitor in vivo, we coexpressed Dvl-GFP with Nkd RNAs in whole embryos. Injected alone, Dvl-GFP is broadly expressed as well as in Dvl-GFP coinjected with Nkd1mut (Figure 27B). Consistent with western blot results, little or no GFP expression could be detected in embryos coexpressing Dvl and Nkd1 (Figure 27C). Despite the fact that deletion of the Nkd1 EF-hand mutants can bind to Dvl (Yan et al., 2001a), our data indicates that a functional Nkd1 EF-hand is required to promote Dvl localization or activity-dependent turnover.

Discussion

Ca^{2+} modulation in the DFCs controls β-catenin levels, DFC polarized migration and KV formation, and is required for asymmetric molecular cues and organ LR patterning (Schneider et al., 2008). Nkd1 is expressed in the DFCs, contains a putative Ca^{2+}-binding motif and impacts both β-catenin-dependent and independent Wnt pathways (Van Raay et al., 2007). Therefore, we hypothesized that in the context of live embryos, Nkd1 functions as a sensor of DFC Ca^{2+} fluxes, to regulate Wnt and LR patterning. Targeted knockdown of Nkd1 in the DFCs resulted in small KVs containing little or no cilia, heart laterality defects and bilateral expression of asymmetric markers.

A subset of KV cells express the Nodal antagonist *charon*, crucial for proper LR patterning (Hashimoto et al., 2004). Our analysis revealed a right-sided *charon* expression bias in wt and control embryos, whereas $Nkd1MO^{DFC}$ embryos have reduced KV cilia and unbiased *charon* expression. By independently targeting three ciliary proteins, INVS, TILB and IFT88, we show that *charon* asymmetry around the KV is cilia-dependent. These results suggest a mechanism for loss of *charon* asymmetry in $Nkd1MO^{DFC}$ embryos, which could be, at least in part, a result of loss of KV cilia.

In *Drosophila*, Nkd has been shown to antagonize Wg by binding to the basic/PDZ domain of Dvl (Rousset et al., 2001). Dvl is a central component of both canonical and non-canonical Wnt signaling, yet the mode in which Nkd modulates Dvl function is unclear. In the present study we show that zebrafish Nkd1 overexpression results in Dvl membrane localization and degradation and CE momevent defects. We reveal that mutations in key ion-binding residues in the Nkd1 EF-hand motif prevent Nkd1-mediated impact on CE as well as Dvl localization/turnover. Our results suggest that Nkd1 exerts its effects by regulating Dvl stability and that Ca^{2+} binding may be required for this activity.

Here, we have shown the requirement for a functional Nkd1 EF-Hand to modulate CE movements and Dvl localization/stability. Our data support that Nkd1 product is turned over, possibly in an activity-dependent manner and that endogenous Nkd1 is required in DFCs for proper KV formation and LR patterning. Our results indicate that Nkd1 acts as an ion sensor to control Dvl stability and to translate DFC Ca^{2+} fluxes into proper LR patterning.

Table 9: Summary of KV defects after Nkd1 knockdown.

Kupffer's vesicle	*n*	Normal	Reduced	Absent
Wt	126	98	2	0
$CtrlMO^{DFC}$	189	92	8	0
$Nkd1MO^{DFC}$	331	53	36	11

Note: *n* = number of embryos

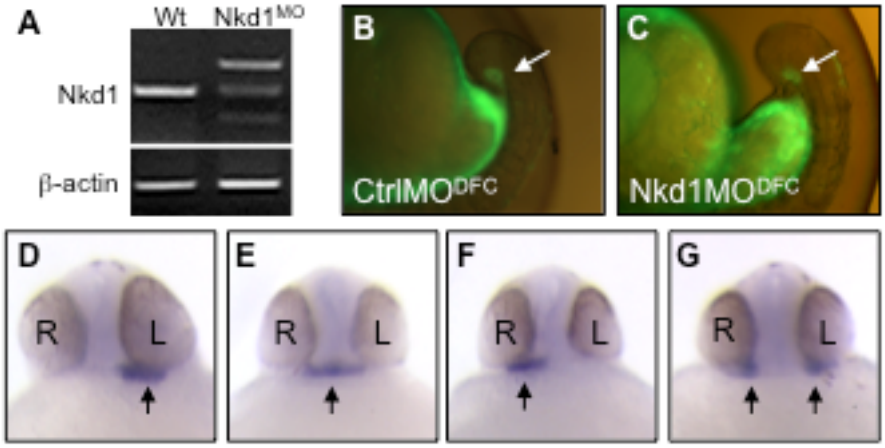

Figure 20: Heart laterality defects after Nkd1 knockdown in DFCs. (A) RT-PCR using cDNA from wt or Nkd1MO-injected embryos: β-actin or Nkd1 were amplified. Additional Nkd1 bands in Nkd1MO cDNA were confirmed by sequencing to result from both inclusion of intron 2, and exclusion of exon 3, each causing premature stops in the open reading frame. (B and C) Arrows denote efficient targeting of CtrlMO and Nkd1MO into the DFCs. (D-G) Arrows denote expression of *nkx2.5* in the heart, on the left side in wt (D) and on the middle (E), right (F) and bilaterally (G) in Nkd1MO^{DFC} embryos. (H) Summary of cardiac jogging defects. Courtesy of Dr. I Schneider.

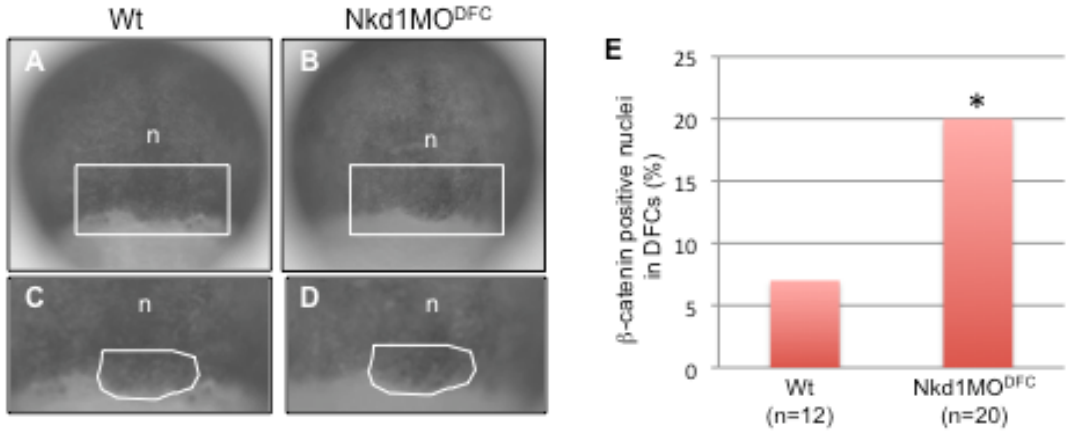

Figure 21: Nkd1 knockdown promotes nuclear β-catenin accumulation in DFCs. (A-D) Embryos at 80% epiboly stained by immunohistochemistry with anti-β-catenin antibody. In wt embryos, β-catenin is detected mostly in plasma membrane throughout gastrulation (A) and in DFCs (C). In Nkd1MO^{DFC} embryos, β-catenin is detected mostly in plasma membrane (B) but also in nuclei of cells in DFC cluster (D). (E) Graph of percent of β-catenin-positive nuclei present in DFCs of wt and Nkd1MO^{DFC} embryos. n = notochord. *$P<6.8x10^{-6}$, Fisher's exact test.

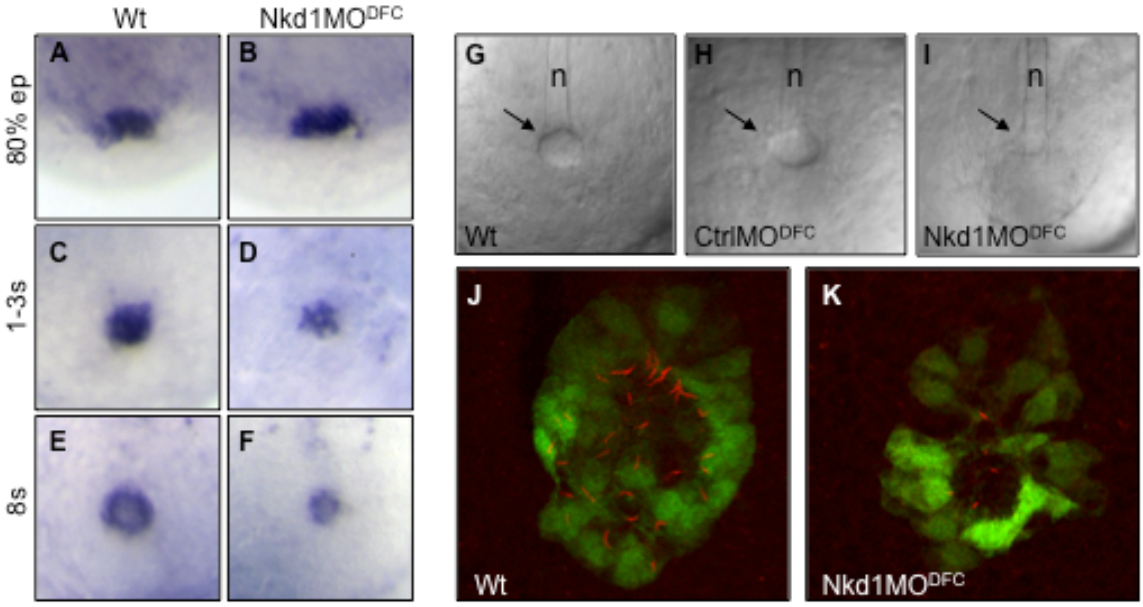

Figure 22: Nkd1 is required for proper DFC migration and KV formation**.** (A-F) EGFP (Dusp6:d2EGFP) expression in wt (A, C and E) and Nkd1MO^{DFC} (B, D and F) embryos; DFCs were assessed at 80% epiboly (A and B), tailbud to 1-somite stage (C and D) and at 8-somite stage (E and F). (G-I) Bright field image of KV in wt (G), CtrlMO^{DFC} (H) and Nkd1MO^{DFC} (I) embryos, at 10-somite stage. (J and K) Fluorescence image denoting KV cilia in wt (J) and Nkd1MO^{DFC} (K) embryos. Dusp6:d2EGFP (green) and anti-acetylated tubulin (red) indicate cilia in KV cells. Arrows indicate vesicle location; n=notochord. (F) Summary of KV defects. Courtesy of Dr. I Schneider.

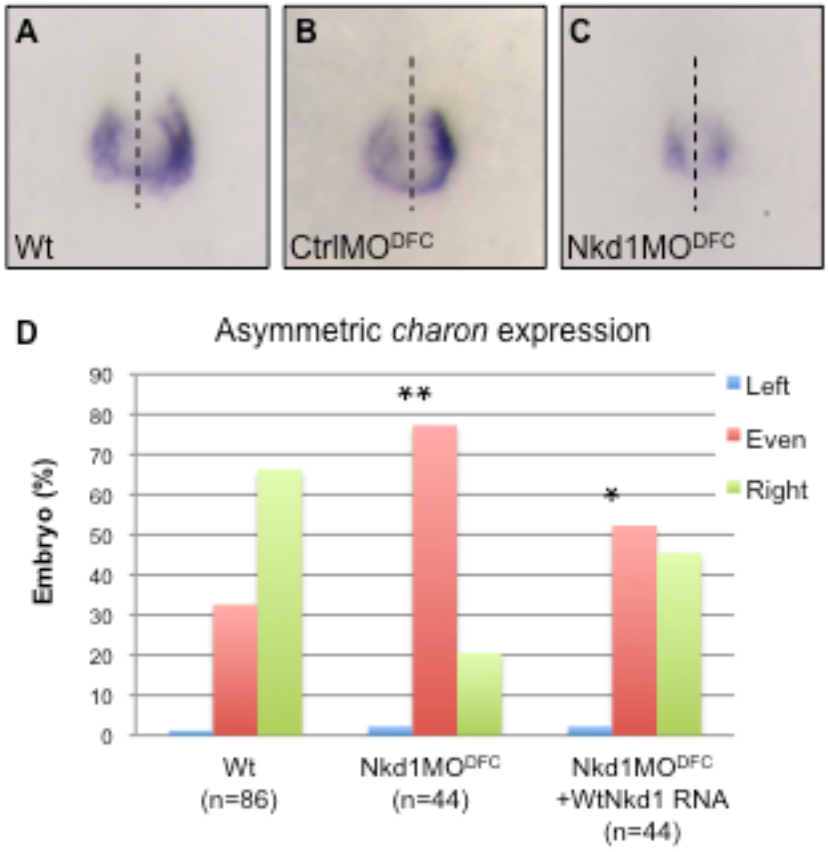

Figure 23: Nkd1 is required for asymmetric *charon* expression around the KV. (A-C) *charon* expression around the KV in in wt (A), CtrlMO^{DFC} (B) and NkdMO^{DFC}. bilateral expression in Nkd1MO^{DFC} (C). (D-F) Arrows denote left-sided *lefty1&2* expression in wt (D), CtrlMO^{DFC} (E) and bilateral expression in Nkd1MO^{DFC} (F). (G) Summary of *spaw* and *lefty1&2* asymmetric gene expression patterns.

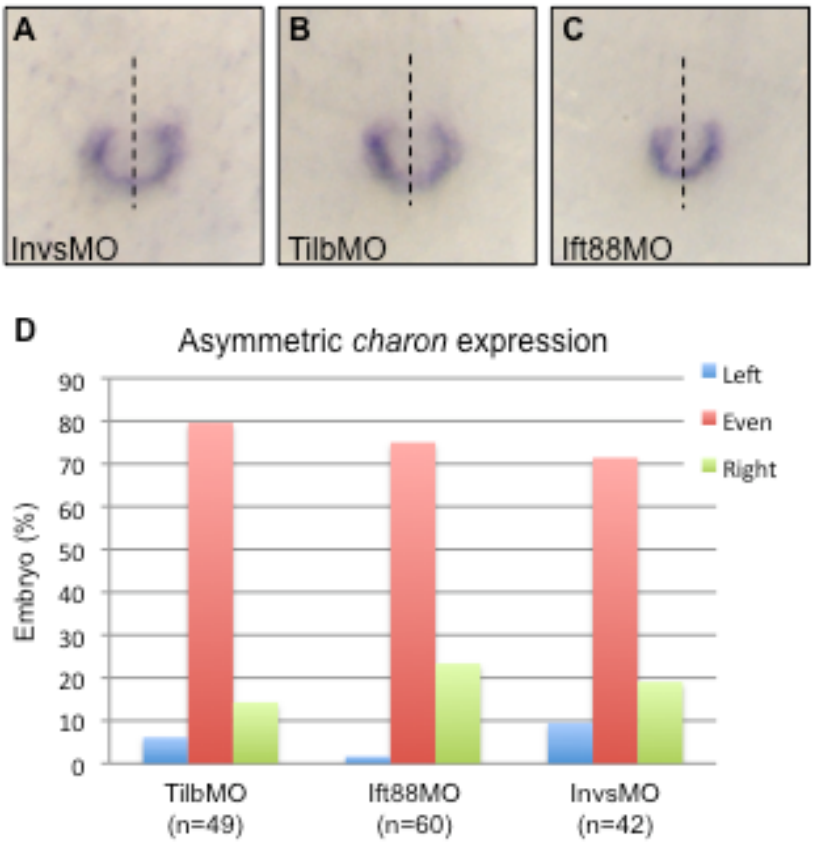

Figure 24. Asymmetric *charon* expression around the KV is dependent on cilia function. Expression of *charon* in InvsMO (A), TilbMO (B) and Ift88MO (C) embryos. Dashed line represents embryonic midline. (D) Graph of percent of embryos displaying left-sided, even, or right-sided biased *charon* expression around KV.

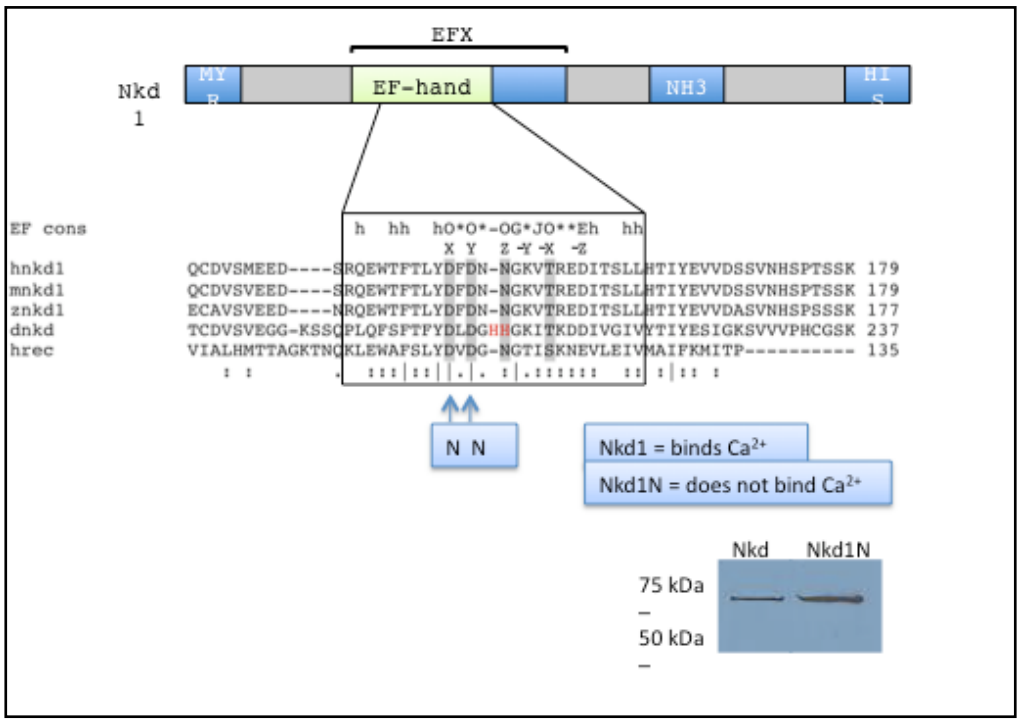

Figure 25: EF-hand residues critical for Ca^{2+} binding are conserved in vertebrate Nkd1. Schematic representation Nkd1 domains; Alignment of Nkd1 homologues; human (hnkd1), mouse Nkd1 (mnkd1), zebrafish Nkd1 (znkd1), Drosophila nkd (dnkd) and human Recoverin (hrec). "|" denotes identical residues, ":" denotes conserved substitutions and "." denotes semi-conserved substitutions. Consensus EF-hand residues (EF cons): h, hydrophobic residue; E, acidic residue, usually glutamic acid; O, oxygen-donating residue that binds Ca^{2+}; G, glycine; asterisk, variable amino acid; J, hydrophobic residue; X, Y, Z, coordinates of Ca^{2+} binding in three-dimensional space. Blue box with the "NN" denotes the aminoacids mutated. Western blot shows that Nkd1 as well as Nkd mut constructs are expressed in the cells.

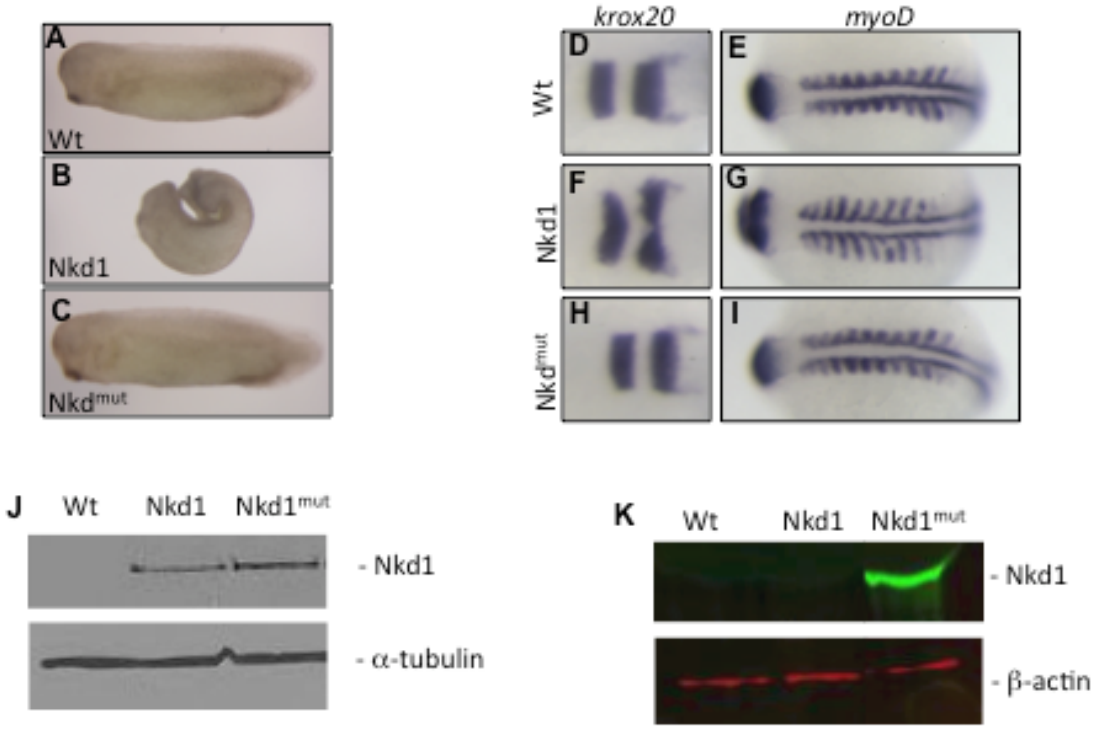

Figure 26: Nkd1 overexpression promotes C-E defects in zebrafish and *Xenopus* embryos. Xenopus embryos injected with Nkd1 and Nkd^{mut}(A-C). Molecular assessment of C-E defects in zebrafish using using *krox20* (D, F, H) and *myoD* (E, G, I) markers in uninjected (D, E), and Nkd1WT RNA-injected (F, J) and NKD mut (H, I).

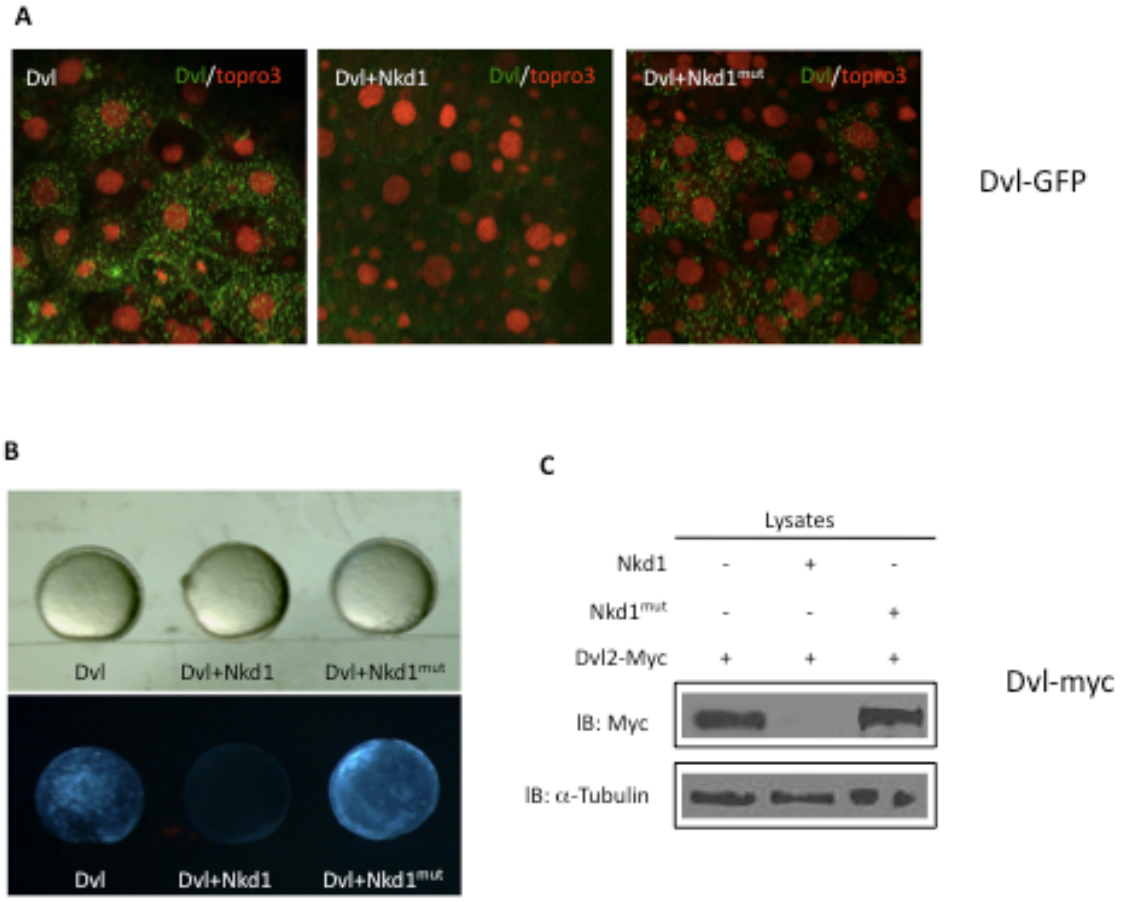

Figure 27: Nkd1 but not NKD^{mut} promotes Dvl degradation**.** (A) Dvl-GFP (green) and Topro3 nuclear staining (red). denotes Dvl localization in wt (H) and Dvl-GFP + Nkd1WT RNA (I). ((E) Western blot of zebrafish embryos injected with myc tagged Dvl2 only, or coinjected with Nkd1WT RNA, anti-α-tubulin used as loading control. Embryos were frozen at 80% epiboly and equal amounts of cell lysates were used for Western blot analysis. (F) Bright field and fluorescence (G) images of embryos at 90% epiboly, injected with Dvl-GFP only or coinjected with Nkd1WT RNA.

CHAPTER V
CONCLUSIONS AND FUTURE DIRECTIONS

Axin is a key player in the Wnt/β-catenin signaling network. Axin binds to β-catenin, GSK3β and APC, among other proteins, and antagonizes Wnt signaling by stimulating β-catenin degradation (Petersen and Reddien, 2009). Axin contains an RGS domain, but the ability of Axin to promote the GTP hydrolysis activity of Gα proteins, in the manner of an RGS protein, has not been demonstrated. Much of the work that has been done to understand the role of the Axin-RGS domain used deletion of this domain. Since deletions within the Axin-RGS domain may interfere with protein stability and also its ability to interact with other proteins in the degradation complex, these previous studies have not specifically addressed the RGS function of this domain.

Here, I demonstrated a requirement for a specific glutamine residue in the Axin-RGS domain that is predicted to be necessary for GAP function, based on homology to RGS proteins. This amino acid change is sufficient to disrupt Axin function and does not rescue the loss of forebrain structures caused by Axin1 knockdown or the *masterblind* phenotype of the Axin1 zebrafish genetic mutant, moreover, the Axin-RGS point mutant construct also failed to rescue the Axin1 loss of function in *Xenopus laevis*. Interestingly, the Axin-RGS domain point mutant construct was able to rescue the loss of maternal Axin function in *Xenopus*, suggesting that an RGS-like function is dispensable during maternal stages of *Xenopus* development.

Function of the Axin-RGS domain in Wnt/β-catenin signaling

In this thesis, we were able to make a distinction between the scaffold function and the RGS-like activity of Axin using site-directed mutagenesis to specifically abolish

the GAP function of Axin, keeping its scaffold function in the degradation complex intact. Many groups have also demonstrated the direct binding of the Axin-RGS domain to Gα proteins in vitro (Castellone et al., 2005; Katanaev et al., 2005; Stemmle et al., 2006). Previous studies have shown that members of the G12 subfamily of G-proteins are able to influence the fate of β-catenin by interacting with cadherins at the cell surface, resulting in compromised binding of cadherins to β-catenin, suggesting that Wnt signaling and G-protein signaling pathways are not acting independently of each other (Kaplan et al., 2001; Meigs et al., 2002). G-proteins can act as molecular switches, either in the GTP-bound – on, or the GDP-bound – off state. RGS proteins have a preferential binding for the activated form of Gα proteins, Gα-GTP (Barren et al., 2006; Buck and Iyengar, 2002; Cabrera-Vera et al., 2003; Kaplan et al., 2001; Meigs et al., 2002). Axin has also been shown to bind to Gα, preferentially to the activated state of Gα, though, surprisingly in these studies no GAP activity was observed in in vitro GTPases assays (Castellone et al., 2005; Stemmle et al., 2006).

In this thesis, we did not test the GAP activity of Axin; instead we tested the ability of exogenous Gαi to recruit Axin to the plasma membrane by providing excess Gαi to the embryos. We showed that, upon Gαi overexpression, Axin1 wildtype is redistributed away from the cytoplasm and to the plasma membrane; however, the Axin-RGS mutant remained cytoplasmic upon Gαi overexpression. Taken together, our results suggest the following model. Wnt ligands trigger the recruitment of Axin to the plasma membrane and this recruitment is dependent upon G protein. Axin can bind to Gα and the duration of the complex formed between Gα and Axin will dictate the potential duration of the Wnt signaling activation through frizzled receptor. This event is critical

during zygotic stages of development in which cells need to transmit the signal in a gradient fashion and a long-range activation is critical for proper Wnt signaling function. During maternal stages of development, cells are bigger and are fewer in number, thus, a possible reason for the complete rescue of the maternal Axin by the Axin-RGS point mutant is that the sustained activation of Wnt signaling via Axin-Gα interaction may be less critical during this stage and a mutation that affects Axin-RGS-like activity may not be detrimental for the proper activation of Wnt signaling.

Non canonical Wnt signaling activation of Frizzled receptors has been shown to activate intracellular calcium release through heterotrimeric G-proteins, thus activating the phosphatidyl inositol cycle among others intracellular calcium sensors (Slusarski et al., 1997a; Slusarski et al., 1997b), however, Axin was not thought to be involved in heterotrimeric G-protein signaling until 2005, when studies were published showing that Axin can bind directly to Gαs and promote growth of colon cancer cells (Castellone et al., 2005; Stemmle et al., 2006). We show that Axin is capable of binding to Gα in vivo and a point mutation within the Axin-RGS domain impairs Axin function during zygotic stages of zebrafish and *Xenopus* development, suggesting that Axin-RGS like function is required for normal brain patterning. However, whether it acts by accelerating the GTP hydrolysis of GαGTP remains to be determined in an animal model. An alternative way to test whether Axin is acting as a GAP is to replace the Axin-RGS domain with an RGS domain of a prototypical RGS protein and test for its ability to rescue Axin loss of function during maternal and zygotic stages.

Using *axin1* maternal depletion we were able to assess Axin1 function maternally, and strikingly, our results demonstrate Axin-mediated RGS-like activity is dispensable

during the establishment of the D-V axis, and both *axin1* and *axin1* ^{Q162A} mutant construct were able to compensate for the loss of maternal Axin1 function. In the future, it will be critical to determine whether Axin-RGS-like function is dispensable during maternal stages due to a differential requirement of this protein during the maternal activation of Wnt signaling or whether there are other proteins compensating for the loss of maternal Axin1 that are not capable of doing so during zygotic stages of development. Nevertheless, Axin-RGS domain appears to play crucial role during early embryonic development.

Function of NKD cuticle during left-right patterning

In vertebrates, internal organs are asymmetrically positioned across the LR axis, and the establishment of this asymmetry is key for proper development and organ function (Reviewed in (Bisgrove et al., 2003)). In zebrafish, molecular asymmetry is initiated by the KV, a ciliated structure derived from the DFCs (Cooper and D'Amico, 1996; Melby et al., 1996). In this thesis, we show that Nkd1, the zebrafish homologue of the *Drosophila* Wg antagonist *nkd* (Zeng et al., 2000), modulates Dvl stability/localization and is required for LR patterning. Our results describe a new role for Nkd1 in LR patterning and identify Nkd1 as a potential sensor of DFC Ca^{2+} fluxes.

Asymmetrical Ca^{2+} levels across the mouse and chick node have been implicated in LR axis determination (McGrath et al., 2003; Raya et al., 2004). Recent work has also postulated a distinct Ca^{2+} requirement in the regulation of KV cilia motility in zebrafish (Shu et al., 2007). Previous data from our lab identified an early Ca^{2+}-release activity that ultimately regulates β-catenin levels and promotes KV formation. While we have established a causal relationship between Ca^{2+}-inhibition and β-catenin accumulation, we recognize that a Ca^{2+} sensor would be required for translation of DFC Ca^{2+} fluxes into LR

cues. In this thesis, we show that Nkd1, a putative Ca^{2+}-binding protein and *wg* antagonist (Zeng et al., 2000), is required in the DFCs for LR patterning.

We report that zebrafish Nkd1is required in the DFCs for LR patterning. Targeted DFC knockdown of Nkd1 resulted in heart laterality defects as well as bilateral expression of asymmetric markers. This phenotype was suppressed upon coinjection with Nkd1 RNA. Nkd1 knockdown resulted in fewer DFC cells reaching the tail bud and a smaller KV, containing few or no cilia, this could be a result of inefficient Nkd1 Knockdown due to MO efficiency of targeting, or due to other Ca^{2+} sensors that may have a role in relaying DFC Ca^{2+} signals. Our analysis also revealed that wt embryos showed stronger expression of the nodal antagonist *charon* (Hashimoto et al., 2004) on the right side of the KV, and that Nkd1 knockdown in the DFCs leads to loss of cilia and to an unbiased, symmetric *charon* expression. Finally, we demonstrate that overexpression impacts CE movements and Dvl stability, whereas Nkd1 RNAs harboring mutations in predicted ion-binding residues lack the ability to produce CE defects and to impact Dvl localization/stability. Overall, our results reveal a novel role for Nkd1 in regulating Dvl stability, and suggest that Nkd1 may function as a Ca^{2+} sensor in DFCs, to regulate Dvl activity and promote proper LR patterning.

Embryonic organ laterality is preceded by molecular and physiological asymmetries. Prior to organogenesis, the DFCs migrate ahead of the dorsal blastoderm during gastrulation to form the KV. Ablation of the DFCs or mechanical disruption of the KV disrupts LR patterning (Amack and Yost, 2004; Essner et al., 2005). We show that endogenous Ca^{2+} release activity in DFCs and DFC-like cells during gastrulation correlates with β-catenin accumulation in zebrafish and *Xenopus* embryos, suggesting an antagonistic relationship between Ca^{2+} and the Wnt/β-catenin signaling in the DFCs (Schneider et al., 2008). However, the impact on DFC migration and cohesion is also consistent with deregulation of noncanonical Wnt network, which may be mediated by Wnt/Ca^{2+} antagonism of Wnt/β-catenin (Slusarski et al., 1997b; Torres et al., 1996).

Whether Wnt/β-catenin, Wnt/Ca^{2+} or both Wnt signaling networks are impacted by Ca^{2+} release inhibition, or whether a Wnt/Ca^{2+} ligand is acting upstream of DFC Ca^{2+} release requires further study.

An important structural difference between fly and vertebrate nkd homologs is the presence of a myristoyl sequence present in vertebrate Nkd (Katoh, 2001; Van Raay et al., 2007; Wharton et al., 2001; Yan et al., 2001b). Mouse Nkd2 was shown to undergo myristoylation at a specific Glycine (Li et al., 2004), whereas fly Nkd lacks a canonical myristoyl sequence (Zeng et al., 2000), is not myristoylated and localizes to cytoplasmic puncta (Rousset et al., 2002) and nucleus (Chan et al., 2007; Waldrop et al., 2006). Nkd EF-hand is most similar to that of Recoverin (Zeng et al., 2000). In recoverins, binding and dissociation of Ca^{2+} induce a conformational change in the protein that promotes movement of the myristoyl group. This so-called Ca^{2+}-myristoyl switch can allow translocation of a protein to the membrane when the myristoyl group is exposed (Burgoyne and Weiss, 2001).

It is reasonable to consider that vertebrate Nkds may function as Ca^{2+}-myristoyl switch proteins, responding to oscillations in Ca^{2+} levels by translocation to the cell membrane, to influence Dvl function or localization. Myristoylated Nkd2 has been shown to escort TGF-alpha to the basolateral plasma membrane (Li et al., 2004). In the future, it would be critical to determine whether vertebrate Nkd homologs bind to Ca^{2+} or display different subcellular localization depending on Ca^{2+} levels. In spite of functional differences observed in cell culture assays between wt Nkd and Nkd EF-hand mutants, mice with compound EFX deletion mutations in the nkd1 and nkd2 are fertile and viable (Zhang et al., 2007), undermining the importance of Nkd homologs in vertebrate embryonic development. Nevertheless, zebrafish Nkd homologs appear to play crucial role during early zebrafish development (Van Raay et al., 2007), and overproduction of Nkd homologs in *Xenopus* also produces early embryonic phenotypes (Zeng et al., 2000); Yan et al., 2001). Therefore, model organisms such as the zebrafish and frogs may be

better suited for future studies aimed on dissecting the embryonic roles for vertebrate Nkd homologs.

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APPENDIX A. OLIGONUCLEOTIDE PRIMER SEQUENCES

Zebrafish axin1 full-length primers

axin1 forward primer: ATGAGCATGAGTGTAAACGAGAAG

axin1 reverse primer: GTCGACCTTCTCCTCTTTTCCGAT

Zebrafish Gαi full-length primers

Gαi forward: GCATGTTGAATACTGTGCGTTT

Gαi reverse: TGCACATCAAAACTGGCTTC

Primers for site-directed mutagenesis

axin1^{Q162A}_forward primer: GGAATCGTATCCCGGGCGATCAAACC

axin1^{Q162A}_reverse primer: CCTTAGCATAGGGCCGTCTAGTTTGG

Zebrafish *mbl* primers:

axin1 forward primer: AGCTGGAGGTGTTTTCTCCA

axin1 reverse primer: TGGGGTATGGCATAAATTGC

Zebrafish NKD1 full-length primers

*ndk*1_forward primer: GATGCAGAGAAATGGGTAAACTTCA

*nkd*1_reverse primer: TCTTCTTCCACTGATACAGCACA

Zebrafish β -actin full-length primers

β -actin _forward primer: TCAGCCATGGATGATGAAAT

β -actin _reverse primer: GGTCAGGATCTTCATGAGGT

Oligos for RNA depletion

*Zaxin1*MO: ACTCATGCTCATAGTGTCCCTGCAC; (Schneider et al., 2008)

X*axin*oligo: T*T*C*CTCGCCAGGAA*C*T*G*G; (Kofron et al., 2001)

x*axin*MO: ACTCATTTTGATGGCGTTTGTATAC; (* indicates a phosphorothioate bond).

Nkd1: CTATCGCCTAAAACAAGAAAACACG

Nkd2a: CACTCCAGATCCTGACAGATCACAC

Nkd3 GCTTGTGTCAGAAGTTGTGCATCTC

Ift88: CAACTCCACTCACCCCATAAGCTGT

Invs: CAACGCCGTCTGCTGTAGAACAAAC

Tilb: TCAGATCCTCACTGATGCGGACCAT

APPENDIX B. PLASMID CONSTRUCTS

Vector name	Category	Antibiotic resistance	Cloning sites	Source
*Axin1*pCR8/GW/TOPO	Entry	Spectinomycin	EcoRI	Lawson lab
*Axin1*pCS2FLAG	Destination	Ampicillin	NotI/ SP6	Yan/Mei's lab
*Axin1*pCS3 myc tag	Destination	Ampicillin	NotI/ SP6	Lawson lab
*Gαi*pCS2HA	Destination	Ampicillin	NotI/ SP6	Yan/Mei's lab
*nkd1*pCS3 myc tag	Destination	Ampicillin	NotI/ SP6	Yan/Mei's lab
dvl2 pCS3 myc tag	Destination	Ampicillin	NotI/ SP6	Yan/Mei's lab