RECEPTOR-MEDIATED ACTIVATION OF CANONICAL WNT SIGNALING

By

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For my family

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Ala, alanine

Asp, aspartic acid

APC, adenomatous polyposis coli

ATP, adenosine triphosphate

C-terminal, carboxy-terminal

DAPI, 4',6-diamidino-2-phenylindole

DNA, deoxyribonucleic acid

EDTA, ethylene diamine tetraacetic acid

EGTA, ethylene glycol tetraacetic acid

E.T., Emilios Tahinci

GDP, guanosine diphosphate

GTP, guanosine triphosphate

HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

hr, hour

K.K.J., Kristin Kalie Jernigan

MES, 2-(N-morpholino)ethanesulfonic acid

min, minute

MO, Morpholino oligonucleotide

mRNA, messenger ribonucleic acid

N-terminal, amino-terminal

PCR, polymerase chain reaction

PMSF, phenylmethanesulphonylfluoride

P, phosphorylated

p, plasmid

Pro, proline

Ser, serine

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

Thr, threonine

Trp, tryptophan

Tyr, tyrosine

UTR, untranslated region

BOOK 1: RECEPTOR-MEDIATED ACTIVATION OF CANONICAL WNT SIGNALING

CHAPTER 1: INTRODUCTION TO CANONICAL WNT SIGNALING

Introduction

Canonical Wnt, or Wnt/β-catenin, signaling controls various cell fates in metazoan development and is misregulated in several cancers and developmental disorders. Binding of a Wnt ligand to its transmembrane co-receptors Frizzled (Fz) and low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6) inhibits phosphorylation and degradation of the transcriptional co-activator β-catenin, which then translocates to the nucleus to regulate target gene expression (Figure 1.1). In the work described in Chapters 2 and 3, I performed studies to determine the mechanism by which the Wnt co-receptors Fz and LRP5/6 activate canonical Wnt signaling. As an introduction to these studies, I summarize the discovery and history of canonical Wnt signaling as well as our current understanding of the roles and mechanisms of Wnt signaling.

Discovery of Canonical Wnt Signaling

Development of a multicellular organism with complex tissues and organs requires extensive communication between cells (reviewed in Gerhart, 1999). Secretion from one cell of a protein ligand that binds a protein receptor on the plasma membrane of another cell and promotes a molecular response in that receptor cell is one strategy by

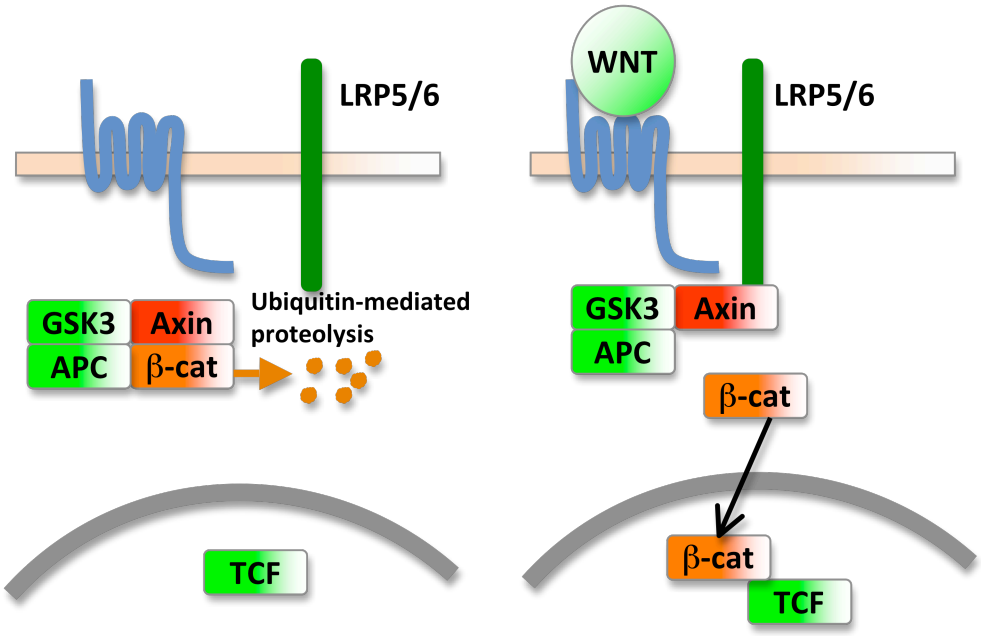


Figure 1.1: Schematic of Canonical Wnt Signaling. In the absence of a Wnt ligand, β-catenin concentrations are kept low in the cytoplasm. Cytosolic β-catenin becomes bound by a destruction complex consisting of two scaffold proteins, Axin and the adenomatous polyposis coli (APC) protein, as well as a kinase, glycogen synthase kinase 3 (GSK3). GSK3 within the destruction complex phosphorylates β-catenin leading to its recognition by an E3 ubiquitin ligase and its subsequent polyubiquitination and proteasome-mediated degradation. However, in the presence of a Wnt ligand, the ligand binds its co-receptors Fz and LRP5/6, which leads to inhibition of destruction complex formation or activity. As a result, β-catenin escapes degradation and accumulates in the cytoplasm. β-catenin then translocates to the nucleus where it binds transcription factors of the T cell factor/lymphoid enhancer factor (TCF/LEF) family and activates a cell-type-specific transcriptional program. Figure adapted from (Tolwinski and Wieschaus, 2004).

which metazoa achieve cell-cell communication. In some cases, the ligand (such as a steroid hormone) can traverse the plasma membrane and bind an intracellular receptor. In addition, when ligand and receptor are both plasma membrane-bound proteins (as in Notch signaling), neighboring cells can communicate in a manner that leads to transduction of signals within both the ligand- and receptor-containing cells. Despite the vast diversity of morphology and function throughout metazoa, relatively few signaling pathways exist; and their components and mechanisms have been remarkably well-conserved throughout evolution. For example, it has been proposed that only 17 different types of intercellular signaling pathways exist (Gerhart, 1999). Some of these pathways play roles in early developmental events as well as adult physiology, while other pathways are thought only to have roles in adult physiology. Importantly, mutations in humans that affect these signaling pathways cause numerous developmental disorders and adult diseases. Because relatively few signaling pathways control so many biological events throughout metazoa, intense study of the roles and mechanisms of these signaling pathways offers profound insight into biological regulation and disease.

The Wnt signaling pathway comprises one class of cell-cell communication that is conserved throughout metazoa, required in development and adult physiology, and misregulated in a number of diseases (reviewed in Clevers, 2006; Klaus and Birchmeier, 2008; Logan and Nusse, 2004). This signaling pathway was named for its protein ligands, called Wnts; and the historical discoveries that led to this name reflect the importance of this pathway in development and disease. In 1976, a *Drosophila* mutant was identified in which wing development was drastically inhibited; and this mutant was named *wingless* (Sharma and Chopra, 1976). The *wingless* gene was later found to also

affect segment polarity in the *Drosophila* early embryonic cuticle (Nusslein-Volhard and Wieschaus, 1980). These studies suggested developmental roles for the Wingless protein. In 1982, it was discovered that the Mouse Mammary Tumor Virus in mice promoted oncogenesis by increasing expression of a gene called Integration 1 (*Int1*) (Nusse and Varmus, 1982). In 1987, an intriguing connection between the *wingless* and *Int1* genes was discovered: *wingless* was the *Drosophila* ortholog of *Int1* in the mouse (Cabrera et al., 1987; Rijsewijk et al., 1987). Thus, a gene that was required for multiple aspects of fly development was also an oncogene. As a result, the word "Wnt" was coined to combine *wingless* and *Int1* (Nusse et al., 1991), reflecting its roles in development and cancer in flies and mammals. This finding spurred a great deal of interest in the role of Wnt proteins in biology and cancer. In addition, questions concerning the connections between development and cancer have culminated in investigation of the role of Wnts in stem cell biology (Reya and Clevers, 2005). Thus, the discovery and naming of Wnts exemplify the conservation of cell-cell signaling pathways throughout metazoa and their diverse roles in biology and disease.

The discovery, cloning, and naming of Wnts led to further elucidation of their developmental roles. In 1967, a mutant called *swaying* was shown to affect balance in mice (Lane, 1967). Mutations in this gene inhibited proper cerebellar development. Cloning of the *swaying* gene revealed that the phenotype was caused by a loss-of-function mutation in the Wnt-1 gene (Thomas and Capecchi, 1990; Thomas et al., 1991). Thus, after identification of a role for Wnts in cancer in mice, a developmental role for Wnt-1 was also found in mice. Wnts also provided a molecular explanation for the work of Spemann and Mangold who demonstrated that transplantation of dorsal tissue to

ventral regions of the amphibian embryo induced formation of an ectopic trunk and head (Spemann and Mangold, 1924). Part of the molecular basis for these transplantation results was revealed when it was shown that injection of Wnt mRNA was sufficient to induce formation of an ectopic head and trunk in *Xenopus laevis* embryos (McMahon and Moon, 1989). As was the case for *wingless* in flies, Wnts were found to regulate development in vertebrates.

In addition to affecting development and oncogenesis in model organisms, the Wnt pathway was found to play major roles in human disease. C-terminal truncation of the adenomatous polyposis coli (APC) protein, a cytoplasmic negative regulator of Wnt signaling, promotes constituitive activation of Wnt signaling and is found in over 85% of colorectal cancers (Ashton-Rickardt et al., 1989; de Lau et al., 2007; Groden et al., 1991). Thus, misregulation of Wnt signaling lies at the heart of one of the most common forms of human cancers. Mutations in several other Wnt components have been found in colorectal cancers. Although studies identifying a role for Wnts in colorectal cancers have been most compelling and comprehensive, evidence suggests that misregulation of Wnt signaling also contributes to many other cancers including hepatocellular carcinoma, breast cancer, skin cancer, lung cancer, Wilms' tumors, and prostate cancer (Klaus and Birchmeier, 2008). With regard to the roles of the Wnt co-receptors Fz and LRP5/6 in oncogenesis, Fz7 is overexpressed in hepatocellular carcinoma (Merle et al., 2004), and mutations that cause deletions within LRP5 can promote parathyroid cancers (Bjorklund et al., 2007). Indeed, it is likely that aberrant Wnt activation is involved in multiple aspects of tumorigenesis in numerous cancers. In addition to its roles in cancer, misregulation of Wnt signaling promotes several other types of human disease. Loss-of-

function mutations in Wnt-3 have been shown to cause tetra-amelia in which patients fail to develop arms and legs (Niemann et al., 2004). Loss-of-function mutations in the Wnt co-receptor Fz4 can cause Familial Exudative Vitreoretinopathy, in which retinal angiogenesis is defective (Robitaille et al., 2002). In addition, loss-of-function mutations in the Wnt co-receptor LRP5 cause loss-of-bone-density diseases such as osteoarthritis, while gain-of-function mutations in LRP5 (or loss-of-function mutations in LRP5/6 antagonists such as Sclerostin) promote diseases characterized by increased bone density (reviewed in Balemans and Van Hul, 2007). Thus, Wnt signaling is tightly regulated to promote proper levels of osteogenesis. These disorders reflect roles for Wnt signaling in multiple tissues during human development as well as in adult physiology. Given such a diversity of diseases caused by misregulation of Wnt signaling, it is likely that polymorphisms in Wnt signaling components contribute in significant but less drastic ways to numerous other prominent diseases such as cardiovascular disease (Mani et al., 2007), diabetes (Cauchi and Froguel, 2008), and neurodegenerative disease (De Ferrari et al., 2007). Further study of the roles of Wnt signaling in these diseases promises to lead to improvement in diagnosis, management, and treatment of a great number of human diseases.

The mechanisms by which loss of Wnt signaling prevents important developmental processes and by which gain of Wnt signaling promotes oncogenesis may be unified through an understanding of the role of Wnt signaling in stem cell biology. Stem cells have dual ability to self-renew and to differentiate into specialized cell-types. As it has been shown that Wnt signaling is required for maintenance of stem cell niches in development, certain Wnt-mediated developmental disorders may arise from loss of

stem cell populations (Reya and Clevers, 2005). In adult physiology, the role of Wnt signaling in stem cell maintenance has been partially characterized in colonic crypts. Epithelial cells in intestinal crypts form a continuous sheet that is completely regenerated about every 7 days (reviewed in Reya and Clevers, 2005). Stem cells reside near the bottom of these crypts where they self-regenerate or engender transit-amplifying progenitor cells that differentiate into specific epithelial cell-types. Homeostasis is achieved as cells at the tops of the crypts are shed or undergo apoptosis. Importantly, mice with mutations that inhibit Wnt signaling display loss of the stem cell compartment of the crypt (Korinek et al., 1998). It is also suggested that a gradient of Wnt signaling that is strongest at the bottom of crypts is required for stem cell and/or progenitor cell activity and maintenance (Hendriksen et al., 2008). These data suggest Wnts may be required for stem cell and/or progenitor cell maintenance in the crypt. Importantly, colorectal-cancer-associated mutations that overactivate Wnt signaling lead to crypts with increased populations of progenitor cells and enhanced cell proliferation (Kim et al., 2004). Overall, these data suggest that Wnt signaling may regulate colonic crypt maintenance by its effects on stem cell and/or progenitor cell populations in the colon, and colorectal cancer may arise from over-activation of Wnt signaling that leads to expansion of colonic stem cell populations. Activation of stem cell populations may also lie at the heart of observations that Wnt signaling is involved in regenerative biology, including regeneration of zebrafish tail fins (Stoick-Cooper et al., 2007), *Xenopus* limb buds (Yokoyama et al., 2007a), and deer antlers (Mount et al., 2006). As a result, a greater understanding of the role of Wnts in stem cell biology may uncover fundamental disease mechanisms and lead to development of therapies for Wnt-related diseases.

Historical Elucidation of Canonical Wnt Pathway Components

Early work with Wnts revealed that they play major roles in development of experimentally amenable model organisms. Genetic mutations in Wnts affect segment polarity of the *Drosophila* embryonic cuticle, and Wnt mRNA injection promotes duplication of the primary body axis in *Xenopus*. In addition, mutations in Wnts alter various aspects of mouse development. These discoveries provided a theoretical foundation by which use of these whole organism experimental systems could uncover new genes in Wnt signaling.

In *Drosophila*, screens for genes that affected patterning of the early embryonic cuticle identified a number of genes that would later be shown to be intimately involved in transduction of a Wnt signal. In the early 1990's, genes named for their altered cuticle denticle patterns such as *armadillo* (the *Drosophila* ortholog of β-catenin, a protein previously shown to localize to and function in adherens junctions) (Riggleman et al., 1990; Riggleman et al., 1989), *dishevelled* (Dsh) (Klingensmith et al., 1994; Noordermeer et al., 1994), *shaggy* (the Drosohila ortholog of glycogen synthase kinase 3 (GSK3)) (Siegfried et al., 1992), and *frizzled* (Fz) (Bhanot et al., 1996) were shown to genetically interact with *wingless* mutations. Epistasis and biochemical studies in *Drosophila* provided a basic understanding of how a Wnt signal is transduced: Wnt ligand binds the plasma membrane-bound Fz receptor and signals through Dsh to inhibit GSK3-mediated phosphorylation and degradation of β-catenin (Peifer et al., 1994a; Peifer et al., 1994b; Siegfried et al., 1994). Multiple aspects of this model were confirmed in vertebrates with mRNA injection-mediated axis duplication in *Xenopus*. In

unrelated work to determine the role of the APC gene mutated in familial adenomatous polyposis, it was found that APC bound β-catenin and was required for its degradation (Rubinfeld et al., 1993; Su et al., 1993). Studies of the mouse *Fused* gene in *Xenopus* development led to the discovery of another conserved Wnt component Axin that, like GSK3 and APC, is required for β-catenin degradation (Zeng et al., 1997). Biochemical studies in cultured cells revealed that GSK3, APC, and Axin physically associate in a complex (called the β-catenin destruction complex) that constituitively promotes β-catenin degradation (Behrens et al., 1998). Thus, the combination of work in *Drosophila*, *Xenopus*, mouse, and, later, in cultured mammalian cells suggested that Wnt binds Fz and signals through Dsh to inhibit destruction complex activity and allow β-catenin levels to rise in the cytoplasm (Figure 1.1).

Through work initiated in cultured cells and confirmed in *Xenopus* and *Drosophila*, the ultimate function of Wnt signaling was revealed to be activation of a transcriptional program. In 1991, the transcription factors T cell factor (TCF) and lymphoid enhancer factor (LEF) were cloned from cultured cells (Travis et al., 1991; van de Wetering et al., 1991). In studies to determine their function, it was discovered in 1996 that these proteins bind β-catenin in the nucleus (Behrens et al., 1996; Molenaar et al., 1996). Importantly, interaction of β-catenin with TCF/LEF was shown to be required for Wnt signaling responses in *Drosophila*, *Xenopus*, and cultured mammalian cells (Behrens et al., 1996; Molenaar et al., 1996; van de Wetering et al., 1997). Thus, it was determined that the role of Wnt-mediated stabilization of β-catenin is to allow its nuclear translocation and TCF/LEF-dependent transcriptional activity. As a result of this work, a major tool for the study of Wnt signaling was invented: researchers developed a TCF

Optimal Promoter (TOPFlash) reporter plasmid in which TCF binding sites were placed upstream of a luciferase gene, allowing a quantifiable readout of Wnt-mediated transcription in cultured cells and whole organisms (Molenaar et al., 1996). In addition, this work led to discovery of Wnt target genes such as the homeobox gene *ultrabithorax* that provided clues for the role of Wnt signaling in development and the oncogene c-Myc that provided clues to the role of Wnt signaling in cancer (He et al., 1998; Riese et al., 1997).

Many more genes involved in Wnt signaling, including the functionally redundant Wnt co-receptors LRP5/6 that were independently identified in 2000 through *Drosophila* and mouse genetic screens (Pinson et al., 2000; Wehrli et al., 2000), have been discovered through genetic and biochemical approaches. While major mechanistic questions remain, a great deal of progress has been made in understanding Wnt signal transduction since the discovery that the oncogene *Int1* was the mammalian ortholog of the *Drosophila wingless* gene.

Current Molecular Models for Canonical Wnt Signaling

Although many aspects of the mechanism of Wnt signaling are controversial and currently under study, here I describe a general mechanism by which Wnt signaling is likely transduced. In cells secreting a Wnt ligand, Wnts undergo several post-translational modifications. In the endoplasmic reticulum (ER) and Golgi apparatus, Wnts are N-glycoyslated and acylated with palmitic acid and palmitoleic acid (Smolich et al., 1993; Takada et al., 2006; Willert et al., 2003). These modifications are thought to affect the folding, trafficking, and stability of Wnt proteins. Upon secretion, Wnts may

travel numerous cell diameters as monomers, protein complexes, on liposomes, or by transcytosis to reach their intended receptor cell (Gallet et al., 2008; Panakova et al., 2005). In the extracellular matrix, certain proteoglycans may be important in regulation of Wnt trafficking (Reichsman et al., 1996). In addition, several secreted molecules have been reported to bind Wnts and inhibit their interaction with receptors. For example, Wnt inhibitory factor (WIF) and secreted Frizzled-related protein (sFRP) are secreted molecules that bind Wnts and inhibit their interaction with Fz (Hsieh et al., 1999; Rattner et al., 1997). Other secreted molecules such as Dickkopf (Dkk), Wise, and Sclerostin (SOST) bind the Wnt co-receptor LRP5/6 and likely act to prevent its Wnt-mediated activation (Glinka et al., 1998; Itasaki et al., 2003; Li et al., 2005). However, these LRP5/6 binding molecules may activate Wnt signaling in certain experimental contexts (reviewed in Cselenyi and Lee, 2008). In addition to secreted inhibitors, secreted Wnt agonists such as Norrin and R-spondin have been reported to synergize with Wnts or activate Wnt signaling independently of Wnts (Kamata et al., 2004; Xu et al., 2004). Nonetheless, when a Wnt ligand arrives at its target cell, it binds and forms a complex with both of its co-receptors, Fz and LRP5/6. It has been suggested that Wnts serve to bring Fz and LRP5/6 in close association, as synthetic association of these proteins is sufficient to activate Wnt signaling (Baig-Lewis et al., 2007; Zeng et al., 2008).

Upon binding of a Wnt ligand to its receptors, a signal is transduced that culminates in inhibition of β-catenin degradation. As a major question in Wnt signaling is how a signal is transduced from the plasma membrane, an understanding of the topology of Wnt co-receptors may provide clues to their roles in pathway activation. Fz is a seven-pass transmembrane protein with a topology similar to G protein coupled

receptors (GPCRs) (Malbon, 2004). As a result, it has been suggested that Fz may activate Wnt signaling by activation and dissociation of heterotrimeric G proteins (Wang and Malbon, 2004). Such a mechanism would have profound implications for the mechanism of Wnt signaling and for development of therapeutics that target Wnt signaling. Although work has suggested that G proteins may affect Wnt signaling, it is not clear whether Fz acts as a GPCR in this process. The role of G proteins in Wnt signaling is discussed more extensively in Chapter 3. In the ER, Fz is retained by the inhibitory Wnt pathway component Shisa, suggesting trafficking of Fz may be tightly regulated (Yamamoto et al., 2005). LRP5 and LRP6 are two type I single-span transmembrane proteins that likely have identical biochemical activities (Figure 1.2) (reviewed in He et al., 2004). The extracellular domain of these proteins contains four Tyr-Trp-Thr-Asp (YWTD) β-propeller domains each followed C-terminally by an epidermal growth factor- (EGF) like domain (Herz and Bock, 2002). The two N-terminal pairs of YWTD β-propeller and EGF-like domains are likely involved in binding Wnts (Itasaki et al., 2003), whereas the two C-terminal pairs of YWTD β-propeller and EGF-like domains are likely involved in binding the LRP5/6 antagonists Dkks (Mao et al., 2001a). Towards the membrane, three low-density lipoprotein receptor (LDLR) type A domains on the extracellular domain are found at the C-terminus of the four YWTD β-propeller and EGF-like domains (Herz and Bock, 2002). In response to Wnt signaling, the intracellular domain of LRP5/6 becomes phosphorylated by GSK3 on the Ser residue of each of five Pro-Pro-Pro-Ser-Pro (PPPSP) motifs (Zeng et al., 2005). In addition,

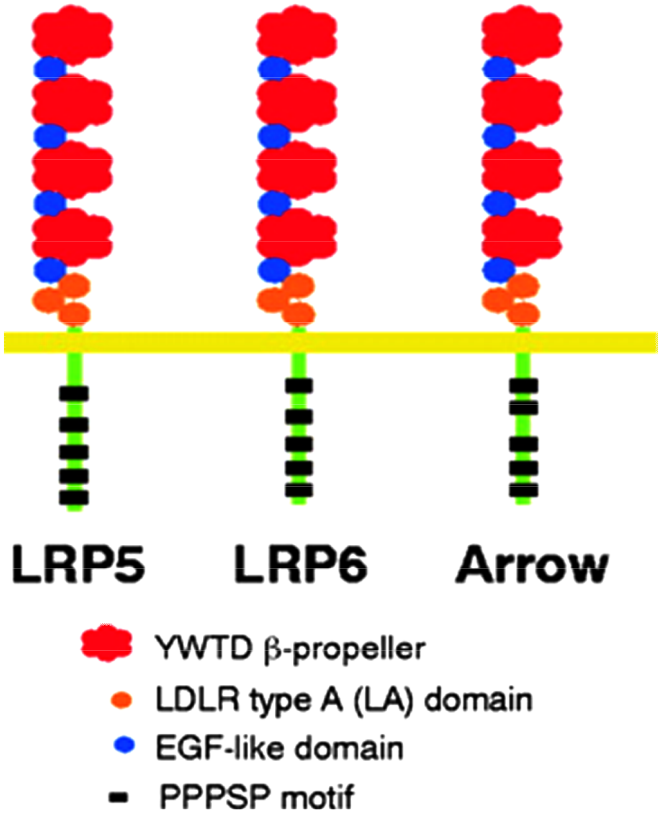


Figure 1.2: Schematic of LRP5/6 Protein Domains. LRP5, LRP6, and Arrow are depicted with their extracellular YWTD β-propellers, EGF-like domains, and LDLR type A domains as well their intracellular PPPSP motifs. Schematic adapted from (He, et al., 2004).

numerous Ser and Thr residues flanking the PPPSP motif are phosphorylated by casein kinase 1 gamma (CK1γ) in response to a Wnt signal (Davidson et al., 2005). Phosphorylation of the LRP5/6 intracellular domain by GSK3 and CK1γ is required for Wnt signaling. Of note, expression of the extracelluar domain of LRP5/6 can be used to inhibit Wnt signaling presumably by sequestering Wnts or preventing Fz activation (Tamai et al., 2000). Also, expression of the intracellular domain of LRP5/6 constituitively activates LRP5/6 mediate signaling (Mao et al., 2001b). Unlike the overexpression of the LRP5/6 intracellular domain, overexpression of full-length LRP5/6 is not sufficient to activate signaling in the absence of Wnts (Tamai et al., 2000). Thus, it has been suggested that Wnt ligands may mechanistically activate signaling by relieving the LRP5/6 extraceulluar domain's constituitive inhibition of the LRP5/6 intracellular domain. As with Fz, trafficking and maturation of LRP5/6 is likely a tightly regulated process. In the ER, proper maturation of LRP5/6 is regulated by the chaperone MesD (Hsieh et al., 2003). In addition, glycosylation and palmitoylation of LRP5/6 are required for its proper maturation and trafficking to the plasma membrane (Abrami et al., 2008).

Upon binding of a Wnt ligand to Fz and LRP5/6, the cytoplasmic protein Dsh becomes phosphorylated and activated (Yanagawa et al., 1995). Activation of Dsh has been shown to require certain amino acids on Fz, suggesting that Fz is involved in activation of Dsh (Zeng et al., 2008). Dsh binds a number of proteins including kinases, phosphatases, and G proteins that potentially regulate Wnt signaling (Malbon and Wang, 2006). While some roles for Dsh are still somewhat enigmatic, Dsh is required for Wnt-mediated oligomerization and phosphorylation of LRP5/6 by GSK3 and CK1γ of Ser and Thr resides on and around PPPSP motifs of LRP5/6 (Bilic et al., 2007; Davidson et al.,

2005; Zeng et al., 2008; Zeng et al., 2005). Phosphorylated PPPSP motifs on LRP5/6 directly bind Axin (Tamai et al., 2004). Thus, Wnt- and Dsh-activated LRP5/6 recruits and binds the destruction complex likely containing Axin, APC, β-catenin, GSK3, and casein kinase 1 alpha (CK1α) (Bilic et al., 2007; Yamamoto et al., 2006). Axin-bound GSK3 enhances phosphorylation of PPPSP motifs on LRP5/6, potentiating its activation and destruction complex recruitment (Zeng et al., 2008). Significant recruitment of the β-catenin destruction complex to LRP5/6 has been shown to occur within 5 minutes of addition of Wnt ligand (Mao et al., 2001b), and this interaction is likely a relatively stable one (Bilic et al., 2007; Yamamoto et al., 2006).

The β-catenin destruction complex that maintains low concentrations of β-catenin in the cytosol and is recruited to LRP5/6 in response to a Wnt signal is composed of two scaffold proteins (Axin and APC), two kinases (GSK3 and CK1α), the protein phosphatase 2A (PP2A), the E3 ubiquitin ligase beta-transducin repeat-containing protein (β-TRCP), the Wilms tumor suppressor gene (WTX), and β-catenin (Behrens et al., 1998; Gao et al., 2002; Liu et al., 1999a; Major et al., 2007; Seeling et al., 1999). There may be additional key components in this complex, and the stoichiometry of the complex has not been determined. Concentrations of Axin, and in some cases APC, are likely limiting for destruction complex formation (Benchabane et al., 2008; Lee et al., 2003). Thus, regulation of the levels of these proteins is critical for proper regulation of β-catenin degradation. Within the destruction complex, the kinase CK1α phosphorylates β-catenin at Ser45, which primes β-catenin for subsequent GSK3 phosphorylation at Ser33/Ser37/Thr41 (Liu et al., 2002). These GSK3 phosphorylation sites on β-catenin are subsequently recognized by the E3 ubiquitin ligase, β-TrCP (Liu et al., 1999a). This

interaction allows for Skp1-Cullin-F-box- (SCF) mediated poly-ubiquitination and proteasome-mediated degradation of β-catenin (Latres et al., 1999). Like the other proteins in this complex, WTX is required for optimal degradation of β-catenin, though its precise role has not been elucidated (Major et al., 2007). PP2A within the destruction complex also likely acts to promote efficient degradation of β-catenin (Seeling et al., 1999). Thus, this destruction complex leads to efficient, constituitive degradation of β-catenin, keeping levels of β-catenin low in the cytoplasm. Inhibition of the destruction complex by Wnt signaling or by mutation (such as truncating mutations of APC) leads to higher levels of β-catenin in the cytoplasm and increased β-catenin-mediated transcription.

Several distinct mechanisms are commonly cited to explain how Wnt-activated co-receptors inhibit β-catenin destruction complex formation or activity. In each of the following mechanisms, β-catenin stabilization occurs by inhibition of GSK3's phosphorylation of β-catenin, which is required for its polyubiquitination and degradation. In one such mechanism, Dsh bound to the Dsh homologous (DIX) domain of Axin in response to a Wnt signal recruits GSK3 binding protein (GBP) which directly inhibits GSK3's phosphorylation of β-catenin within the destruction complex (Farr et al., 2000; Yost et al., 1998). Although this mechanism has been confirmed in *Xenopus* embryos, a GBP ortholog has not been identified in *Drosophila* and genetic knockout of the three identified GBP proteins in mouse does not disrupt its development (van Amerongen et al., 2005). Thus, while GBP may play some role in Wnt signaling, it is likely not the sole mechanism by which a signal is transduced. Similarly, it has been shown that Wnt signaling may induce inhibitory Ser9/Ser21 phosphorylation of

GSK3β/GSK3α, inhibiting GSK3's ability to phosphorylate β-catenin (Yokoyama et al., 2007b). However, mice with mutation of these amino acids on GSK3 do not display developmental abnormalities (McManus et al., 2005). In another often-cited mechanism, Wnt signaling promotes dissociation of the β-catenin destruction complex (Liu et al., 2005; Nusse, 2005). Importantly, this study did not show that destruction complex dissociation correlates with Wnt-mediated transcription (Liu et al., 2005). Also, several studies have not been able to reproduce results showing Wnt-mediated destruction complex dissociation (Bilic et al., 2007; Hendriksen et al., 2008; Yamamoto et al., 2006). Thus, it seems unlikely that a Wnt signal is transduced primarily via GBP, inhibitory phosphorylation of GSK3, or destruction complex dissociation.

It has also been suggested that Wnts inhibit destruction complex activity by inhibiting GSK3's phosphorylation of β-catenin within the destruction complex at activated LRP5/6. In support of this possibility, recruitment of the β-catenin destruction complex to LRP5/6 temporally coincides with inhibition of β-catenin phosphorylation (Bryja et al., 2007; Mao et al., 2001b). Both events are detected beginning around five minutes after Wnt stimulation. In contrast to the destruction complex dissociation model, it has been shown that the entire destruction complex stably localizes at LRP5/6 in response to a Wnt signal (Bilic et al., 2007; Hendriksen et al., 2008; Yamamoto et al., 2006). Finally, immunofluorescence studies have revealed that dephosphorylated β-catenin accumulates on Axin at GSK3-phosphorylated LRP6 in response to a Wnt signal (Hendriksen et al., 2008). In addition to these studies, our work in egg extract and *in vitro* kinase assays reveals that GSK3-phosphorylated LRP6 can directly inhibit GSK3's phosphorylation of β-catenin within the destruction complex (Cselenyi et al., 2008).

Thus, several lines of evidence support a mechanism by which GSK3's phosphorylation of β-catenin is inhibited within the β-catenin destruction complex upon its recruitment to Wnt-activated LRP5/6.

Evidence in mammalian cultured cells, *Xenopus* embryos and egg extract, and *Drosophila* embryos also suggests that a Wnt signal may be transduced via degradation of Axin (Cselenyi et al., 2008; Kofron et al., 2007; Mao et al., 2001b; Tolwinski et al., 2003; Yamamoto et al., 1999). In all four systems, it has been shown that Wnt-activated LRP6 promotes degradation of Axin. Because Axin concentrations are limiting for destruction complex formation, degradation of Axin would be predicted to efficiently promote stabilization of β-catenin (Lee et al., 2003). Despite the correlation between Wnt signaling and Axin degradation, it has not been shown that Wnt-mediated decreases in Axin levels actually plays a role in β-catenin stabilization. Importantly, experiments in cultured cells have suggested that Wnts stabilize β-catenin hours before Axin degradation can be detected (Hino et al., 2005; Liu et al., 2005; Willert et al., 1999). Thus, it is possible that other mechanisms such as direct inhibition of GSK3's phosphorylation of β-catenin are responsible for initial transduction of a Wnt signal while degradation of Axin plays a role in potentiation of a signal. Thus, the precise mechanism by which a Wnt signal is transduced from the Wnt receptor complex is not known, but degradation of Axin and direct inhibition of GSK3's phosphorylation of β-catenin within the destruction complex at LRP5/6 are two potential means by which signaling may be achieved.

Although the mechanism by which β-catenin phosphorylation and degradation becomes inhibited is controversial, it has been well-documented that β-catenin levels in the cytoplasm begin to rise within approximately one hour after incubation with Wnt

ligand (Bryja et al., 2007; Liu et al., 2005). This rise in β-catenin levels might be explained simply by destruction complex inhibition in the context of constituitively translated endogenous β-catenin. It has been suggested that upon Wnt signaling, LRP5/6 bound to the destruction complex is rapidly endocytosed and that this internalization event is required for Wnt signaling (Yamamoto et al., 2006). However, the mechanistic basis for the role of endocytosis in Wnt signaling is not understood. By a mechanism that has not been well-defined, β-catenin is transported to the nucleus. Indeed, detection of nuclear β-catenin is a common cell biological assay for activation of Wnt signaling. Transport of β-catenin to the nucleus might require Rac1 GTPase activity and might involve nuclear shuttling by known destruction complex components Dsh, APC, and Axin (Cong and Varmus, 2004; Gan et al., 2008; Henderson, 2000; Wu et al., 2008). In the nucleus, β-catenin binds and relieves Groucho-mediated constituitive inhibition of TCF/LEF (Levanon et al., 1998; Roose et al., 1998). The β-catenin/TCF/LEF complex also binds a number of transcriptional co-activators with specificity towards Wnt-mediated transcription including as Pygopus, Legless, and Parafibromin (Kramps et al., 2002; Mosimann et al., 2006). This Wnt-dependent nuclear complex then promotes an extensive cell-type specific transcriptional program that depends on the epigenetic status of the cell in which signaling occurs. Increases in concentrations of proteins encoded by these genes are often detected after about 4 hours of incubation with Wnt ligand (Jho et al., 2002). Some common genes transcribed in Wnt-activated cells include the mitogenic oncogene c-Myc, which drives cell proliferation and growth (He et al., 1998). Cyclin D1 is also activated, albeit indirectly, in cells undergoing Wnt signaling (Inoki et al., 2006). Other Wnt transcriptional targets include the inhibitors of Wnt signal transduction Dkk

(an extracellular inhibitor of LRP5/6), Naked (an antagonist of Dsh activity), and Axin2, which provide negative feedback loops that downregulate signaling to return β-catenin levels to their basal state (Gonzalez-Sancho et al., 2005; Jho et al., 2002; Zeng et al., 2000). Thus, an organism can coordinate the activity and identity of certain groups of cells via Wnt-mediated activation of cell-type-specific transcriptional programs.

Non-Canonical Wnt Signaling

Wnt signaling that promotes β-catenin-mediated transcription is called canonical Wnt, or Wnt/β-catenin, signaling. Wnts have also been noted to signal through pathways that do not involve β-catenin. These β-catenin-independent Wnt signaling pathways are collectively called non-canonical Wnt signaling pathways (Semenov et al., 2007; Veeman et al., 2003). These non-canonical pathways have diverse biological roles and molecular mechanisms. Notably, none of these pathways has been characterized as completely as canonical Wnt signaling. Importantly, non-canonical Wnt signaling increases the diversity of Wnt-mediated events, allowing Wnts to assume an even greater number of roles in development and disease.

The best characterized non-canonical Wnt signaling event is known as planar cell polarity (PCP) signaling. In PCP signaling, a Wnt ligand mediates cytoskeletal rearrangement within a cell, often mediating shifts in cell polarity during development (reviewed in Zallen, 2007). During vertebrate gastrulation, Wnt/PCP signaling is involved in a process called convergence and extension whereby medial migration and intercalation of mesodermal cells causes elongation of the embryo's primary body axis (Zallen, 2007). This pathway has been studied in *Xenopus* and zebrafish where it has

been shown to involve certain Wnt ligands, Fz, LRP5/6, and the cytoplasmic Dsh protein (Tahinci et al., 2007; Theisen et al., 1994; Zallen, 2007). Consistent with Fz's potential role as a GPCR, it has been shown in *Drosophila* that this signaling pathway likely involves Fz-dependent heterotrimeric G protein dissociation (Katanaev et al., 2005). Activation of the PCP pathway affects cell polarity by signaling through multiple proteins that affect the actin cytoskeleton (Kaltschmidt et al., 2002). Notably, the PCP pathway in *Drosophila* may not utilize Wnt ligands; instead, signaling may occur through interaction between neighboring cells of plasma-membrane-bound proteins including Fz (Chen et al., 2008). In addition to PCP signaling, cell movements during vertebrate gastrulation involve another non-canonical Wnt signaling pathway whereby Wnt5a and Fz2 activate calcium flux (Slusarski et al., 1997b). As with PCP signaling, data from experiments in zebrafish suggest a role for G proteins in Wnt/Ca^{++} signaling (Slusarski et al., 1997a).

In addition to Fz and LRP5/6, two receptor tyrosine kinases (RTKs) have been identified as Wnt receptors that activate β-catenin-independent signaling events. The RTK Ryk binds Wnts and activates a signaling pathway that affects axon guidance in mice (Lu et al., 2004). Another RTK Ror2 also binds Wnts and acts through jun N-terminal kinase (JNK) to affect convergence and extension movements in vertebrate gastrulation (Oishi et al., 2003). Thus, the diversity of Wnt-mediated biological events may be increased by addition of these recently discovered Wnt receptors.

In addition to non-canonical Wnt signaling that affects the cytoskeleton, it has been shown that Wnts can mediate β-catenin-independent transcriptional events. In studies of myogenesis in the mouse, it has been shown that non-canonical Wnt signaling can activate cAMP response element-binding factor- (CREB) mediated transcription in a

pathway that involves adenylyl cyclase and protein kinase A (PKA) activation (Chen et al., 2005). In osteoblastogenesis, Wnt3a signals through Gα proteins to activate protein kinase C delta (PKCδ) (Tu et al., 2007). This signaling event occurs independently of β-catenin and plays a role in transcription of certain genes required for osteogenesis. Thus, non-canonical Wnt-signaling can mediate important transcriptional events that occur independently of β-catenin regulation.

Adding another layer of complexity to Wnt signaling, canonical Wnt signaling can have profound effects on other cell signaling pathways. This type of regulation in which one signaling event affects another is commonly referred to as crosstalk. Crosstalk between Wnt signaling and the mammalian target of rapamycin (mTOR) pathway as well as the bone morphogenetic protein (BMP) pathway may allow for crossregulation of these ubiquitous signaling pathways. Specifically, it has been shown that canonical Wnt signaling inhibits GSK3's phosphorylation of the tuberous sclerosis complex 2 (TSC2) protein, possibly within the β-catenin destruction complex (Inoki et al., 2006). This event causes activation of the mTOR pathway. The relationship between canonical Wnt and mTOR signaling may be rather intimate, as the gene Cyclin D1 thought to be a common and direct target of Wnt/β-catenin signaling, has been shown instead to be a target of Wnt-mediated mTOR activation (Inoki et al., 2006). Similarly in BMP signaling, canonical Wnt signaling prevents GSK3 phosphorylation of the Smad1 protein within the β-catenin destruction complex, enhancing Smad1-mediated signaling (Fuentealba et al., 2007). These authors discovered that certain developmental events regulated by Wnt signaling were actually mediated by Smad1 activity and not, as had been previously presumed, β-catenin activity (Fuentealba et al., 2007). Importantly,

many proteins involved in Wnt signaling have multiple biological functions, and Wnts may exert their effects in part by modulating their β-catenin-independent activities. It should also be noted that TCF-dependent transcription can be activated by processes independent of Wnts or even β-catenin (Yi and Merrill, 2007). Thus, one clue to the diverse roles of Wnt signaling in biology may come from the realization that Wnt/β-catenin signaling is only one of a great number of Wnt-mediated events in biology. Moreover, organisms likely utilize crosstalk between these pathways to increase the diversity and specificity of responses to Wnt signals.

Analysis of Receptor-Mediated Activation of Canonical Wnt Signaling in *Xenopus* Egg Extract

Given the importance of the Wnt co-receptors in development and disease, I sought to further characterize the molecular mechanisms by which Fz and LRP5/6 activate Wnt signaling. Mutations in both Fz and LRP5/6 lead to a certain developmental disorders and cancers. In addition, signaling receptors are often good candidates for the development of novel molecular therapies. As a result, I hypothesize that mechanistic elucidation of receptor-mediated activation of Wnt/β-catenin signaling will promote a better understanding of the role of Wnt signaling in disease and lead to development of novel therapies to treat these diseases.

Specifically, I sought to define the mechanism by which LRP6 inhibits β-catenin degradation and to determine the role of G proteins downstream of Fz activation in β-catenin regulation. I primarily utilized biochemically tractable, cell-free *Xenopus* egg extract to address these questions. *Xenopus* egg extract has been used extensively to

reveal fundamental aspects of cell cycle and cytoskeletal regulation (Gard and Kirschner, 1987; Miake-Lye and Kirschner, 1985). In egg extract, the molecular determinants and kinetics of β-catenin regulation are identical to those described for β-catenin in intact cells (Lee et al., 2001; Lee et al., 2003; Salic et al., 2000). By utilizing *Xenopus* egg extract to determine the roles of LRP6 and G proteins in Wnt signaling, I was able to perform a number of experimental procedures that would have been very difficult or impossible to achieve in intact cells. For example, I was able to add precise concentrations of proteins and quantify their effects; and, I was able to immunodeplete endogenous proteins and replace them with certain mutants that allowed me to test specific hypotheses. In addition to using *Xenopus* egg extract, I reconstituted activity of LRP6 and G proteins in assays containing only purified, recombinant components in order to test my hypotheses with even greater biochemical detail and control. Finally, to confirm the conclusions gleaned from my biochemical studies in egg extract and in assays containing purified components, I tested predictions from these models in intact cells and whole organisms. I draw the following major conclusions from my work in these systems: LRP6 activates canonical Wnt signaling independently of Axin degradation by inhibiting GSK3's phosphorylation of β-catenin; and Gαo, Gαi, Gαq, and Gβγ promote β-catenin stabilization by inhibiting GSK3's phosphorylation of β-catenin.

BOOK 1: RECEPTOR-MEDIATED ACTIVATION OF CANONICAL WNT SIGNALING

CHAPTER 2: LRP6 TRANSDUCES A CANONICAL WNT SIGNAL INDEPENDENTLY OF AXIN DEGRADATION BY INHIBITING GSK3'S PHOSPHORYLATION OF β-CATENIN

Most work described in this chapter has been published (Cselenyi et al., 2008).

Introduction

The best-characterized form of Wnt signaling is the Wnt/β-catenin, or canonical Wnt, pathway. During Wnt/β-catenin signaling, a Wnt ligand binds transmembrane co-receptors Frizzled (Fz) and low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6) and initiates a process that leads to stabilization and nuclear translocation of β-catenin. In the nucleus, β-catenin binds transcription factors of the T cell factor/lymphoid enhancer factor (TCF/LEF) family and activates a Wnt/β-catenin transcriptional program.

Although the mechanism by which a Wnt ligand mediates β-catenin stabilization is poorly understood, regulation of β-catenin levels in the absence of Wnt signaling has been well-characterized. In the absence of a Wnt ligand, β-catenin is marked for degradation through its interaction with a destruction complex consisting of two scaffold proteins, Axin and adenomatous polyposis coli protein (APC), and two kinases, glycogen synthase kinase 3 (GSK3) and casein kinase 1a (CK1α) (Logan and Nusse, 2004). CK1α

phosphorylation of β-catenin primes it for subsequent phosphorylation by GSK3, which targets β-catenin for ubiquitin-mediated proteolysis (Logan and Nusse, 2004). It is hypothesized that Wnt signal transduction stabilizes β-catenin by inhibiting destruction complex formation or activity.

The Wnt co-receptor LRP5/6 is required for Wnt/β-catenin signaling (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000). Although LRP6 is more potent than LRP5 in certain assays, experiments have not revealed qualitative differences in their mechanisms of action (Mi and Johnson, 2005). Wnt signaling through LRP5/6 has been proposed to inhibit destruction complex formation by promoting degradation of the destruction complex scaffold Axin. LRP5 overexpression was initially shown to promote Axin degradation in cultured mammalian cells (Mao et al., 2001b). Genetic studies in *Drosophila* indicate that activation of the Wnt pathway by Arrow, the LRP5/6 ortholog, decreases steady-state Axin levels (Tolwinski et al., 2003). Wnt signaling through LRP6 also promotes degradation of endogenous Axin in *Xenopus* oocytes and embryos (Kofron et al., 2007). Because the concentration of Axin is significantly lower than that of other destruction complex components, decreases in Axin concentrations represent a potentially robust mechanism for β-catenin stabilization (Lee et al., 2003). As a result, LRP5/6-mediated Axin degradation has been proposed to be a critical event in transduction of a Wnt signal (Tolwinski and Wieschaus, 2004).

Although there is strong evidence that signaling by LRP5/6 reduces Axin levels, Wnt-mediated stabilization of β-catenin in cultured mammalian cells occurs approximately two hours before substantial changes in Axin levels are detected (Hino et al., 2005; Liu et al., 2005; Willert et al., 1999). These data suggest that Axin degradation

may not be required for initial signal transmission; alternatively, turnover of a small, localized pool of Axin may be necessary for signaling but may be undetected in these experiments. In fact, such a mechanism has been described for β-catenin: the vast majority of β-catenin is associated with cadherins at cellular membranes, and only the small, cytoplasmic pool of β-catenin protein is stabilized in response to Wnt signaling (Heasman et al., 1994; Peifer et al., 1994b). Here, we reconstitute LRP6 signaling in biochemically tractable *Xenopus* egg extract, which has been used to accurately reconstitute cytoplasmic aspects of Wnt signal transduction (Lee et al., 2001; Major et al., 2007; Salic et al., 2000). Using this system, we test current models positing that LRP6 stabilizes β-catenin via degradation of Axin or sequestration of Axin (Figure 2.1). Notably, we find that LRP6 can promote β-catenin stabilization in the absence of Axin degradation by directly inhibiting GSK3's phosphorylation of β-catenin.

Methods

Plasmids and Recombinant Proteins

Axin truncation mutants were made by PCR from full length, Myc-tagged mouse Axin and subcloned in pCS2. AxinΔ298-437 and AxinΔ437-506 have been previously described (Salic et al., 2000). AxinSA harbors Ser-Ala mutations at the following predicted GSK3 phosphorylation sites: SANDSEQQSLS. We refer to mouse Axin (GenBank Accession: XM_914907) amino acid (aa) 126 as "start methionine" (CSLMQSP). Mouse and human LRP6ICD (aa1397-1614) (GenBank Accessions:

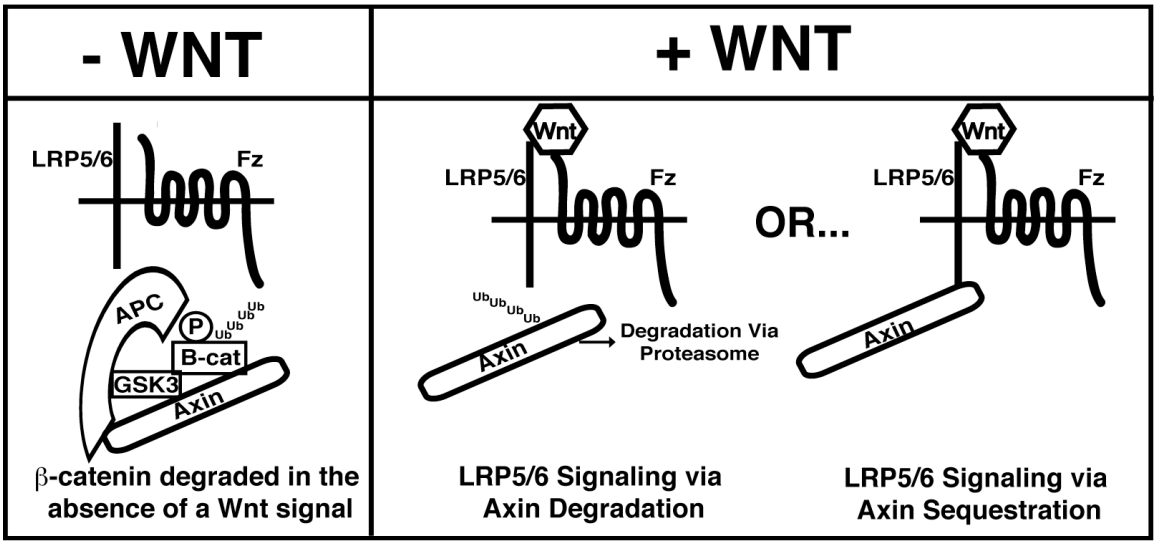


Figure 2.1: Previously Published Models for the Mechanism by which LRP6 Stabilizes β-catenin. In the absence of a Wnt ligand, the destruction complex (Axin, APC, and GSK3) promotes phosphorylates of β-catenin, leading to its ubiquitin-mediated degradation. In the presence of a Wnt ligand, LRP6 promotes degradation of Axin, which may promote β-catenin stabilization by inhibiting destruction complex formation (Tolwinski and Wieschaus, 2004). Alternatively, Wnt-activated LRP6 recruits Axin and may sequester it away from other destruction complex components (Nusse, 2005a).

NM_008514 and NM_002336, respectively) were subcloned into pET11-D or pCS2 using a PCR-based approach. For Lrp6ICD mRNA (Figure 2.2 only), the intracellular domain of mouse LRP6 with an N-terminal myristoylation target sequence was engineered into pCS2. For BiFC, YN (YFP1-154), YC (YFP155-238), LRP6ICD with an N-myristoylation sequence, and human β-catenin were cloned into pCS2. Fusions for BiFC were separated by a Ser-Gly-Gly-Gly-Gly-Ser linker. All oligonucleotide primer sequences are available upon request.

For LRP6ICD purification, BL21 cells harboring LRP6ICD-pET11-D were grown to OD_{600} of 0.3 at 37°C and induced with IPTG (0.3 μg/ml) for 9 hr. Induced bacteria were harvested and protein was purified on Nickel NTA-beads (Qiagen). Eluted protein was concentrated to 1 mg/ml, flash frozen, and stored at –80°C. For GST-ubiquitin purification, protein was expressed and purified as above but induced with IPTG (1 μg/ml) for 4 hr and purified on glutathione resin.

mRNA Synthesis and RT-PCR

Capped RNA for embryo injection was synthesized from linearized plasmid DNA templates using mMessage mMachine (Ambion). Animal caps were cut from stage 9 embryos and cultured in 75% MMR until stage 11. RT-PCR for siamois, Xnr3, and ODC were performed using primers and conditions previously described (Tahinci et al., 2007).

Xenopus Egg Extract Degradation Assay and Depletion

Xenopus egg extract was prepared and degradation assays were performed as described (Salic et al., 2000). Extract was incubated with LRP6ICD at a concentration of 1.6 μM unless otherwise noted. In Axin depletion experiments, IVT proteins were made in wheat germ lysate (Promega). Dsh and Axin immunodepletions were performed and confirmed as described (Salic et al., 2000) with modifications. *Xenopus* egg extract was incubated with an equal volume of Protein A-Affiprep beads (BioRad) bound to either Dsh or Axin polyclonal antibodies. Incubation was performed at 4°C for 2 hr with inversion every 10 min. In Figure 2.7, Axin antibody was covalently conjugated to Protein A magnetic beads (NEB) for depletion.

λ Phosphatase Treament

Samples (0.8 ml) from a *Xenopus* egg extract degradation assay were added to λ phosphatase buffer (12.5 ml) and 400 U λ phosphatase (NEB), incubated for 30 min at 30°C, and then diluted in sample buffer. Samples were then processed for SDS-PAGE and autoradiography.

Ubiquitination Assay

Radiolabeled, IVT Axin (1 μl) was incubated at RT with 17.5 μl egg extract supplemented with GST-ubiquitin (50 μg/ml) in the presence or absence of LRP6ICD.

At indicated times, the reaction was diluted with 100 μl Buffer A (50 mM Tris pH 8, 200 mM NaCl, 0.1% Tween-20, and 0.1 mM PMSF) and applied to 5 μl glutathione-Sepharose beads. After 2 hr shaking at 4°C, the beads were washed with 3 ml Buffer A, 1 ml Buffer B (50 mM Tris pH 8, 50 mM NaCl, and 0.1 mM PMSF), and eluted with sample buffer and analyzed by SDS-PAGE and autoradiography.

Trypsin Digest

Xenopus egg extract (3 μl) was incubated with IVT, radioloabeled Axin (0.5 μl) and GSK3 (15 μg/ml) for 30 min. Bovine pancreatic trypsin (0.38 mg/ml) (Sigma) was added and samples were incubated at RT for 80 sec. Soybean trypsin inhibitor (0.8 mg/ml) (Sigma) and sample buffer were then added for analysis by SDS-PAGE and autoradiography.

Axin/LRP6ICD Binding Assay

For Axin pull down, Nickel-NTA beads (10 μl) (Qiagen), LRP6ICD (10 μg), egg extract (20 μl), and radiolabeled, wheat germ IVT Axin (2 μl) were combined. Sample volume was adjusted to 40 μl with Buffer A, and samples were incubated at RT for 30 min. Buffer A (200 μl) was then added followed by 2 hr shaking at 4°C. Beads were washed with 9 ml Buffer A, and protein was eluted from beads with sample buffer and analyzed by SDS-PAGE and autoradiography.

Immunoprecipitation

For IP, egg extract (50 μl) was incubated with or without LRP6ICD and IVT β-catenin (3 μl) for 2 hr. Buffer A (700 μl) was added to extract with Protein A beads covalently conjugated to myc or Axin antibody followed by 2 hr shaking at 4°C. Beads were washed with Buffer A (4 ml), eluted with sample buffer, and analyzed by SDS-PAGE and immunoblot.

Tau Phosphorylation

Recombinant Tau (rPeptide, Tau-441) was added to egg extract (40 μg/ml) supplemented with GSK3 (NEB) (5 μg/ml). After 2 hr incubation at RT, extracts were immunoblotted.

Kinase Assay

LRP6ICD or LRP6ICD(PPPAPX5) (4.1 μM), MBP-Axin (0.1 μM) (Salic et al., 2000), GSK3 (0.79 μM) (NEB), and CK1 (1.37 μM) (NEB) were pre-incubated with 500 μM ATP and kinase buffer (20 mM HEPES (pH 7.5), 300 mM NaCl, 2 mM DTT, 1 mM EDTA, 10 mM $MgCl_2$, and 0.2% Tween 20) for 10 min at RT. His_6-β-catenin (0.22 μM) (Salic et al., 2000) and Tau (0.34 μM) (rPeptide Tau-441) were then added and samples were removed for immunoblotting after 45 min at RT.

Immunoblotting

Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted. Bands were visualized using horseradish peroxidase-conjugated secondary antibodies and SuperSignal West Pico or Femto Chemiluminescent Substrate

(Pierce). For reblotting, membranes were stripped by incubation in NaOH (0.4 M) for 15 min followed by 15 min in H_2O and reblocking. αTubulin was blotted with DM1α (Sigma) [1:5,000 dilution]. β-catenin P33/37/41 antibody was purchased from Cell Signaling [1:1,000 kinase assay] [1:250 egg extract]. β-catenin P45 antibody was purchased from Cell Signaling [1:500]. N-termal *Xenopus*-β-catenin antibody was a generous gift from Barry Gumbiner [1:3,000]. Antibodies for Axin immunodepletion and immunoprecipitation were described previously (Salic et al., 2000). Axin antibody for immunoblot was purchased from R & D (Anti-human/mouse/rat Axin 1) [1:100]. Antibodies to Dsh were described previously (Salic et al., 2000) [1:100]. GSK3 was blotted with IH8 (Affinity Bioreagents) [1:500]. 6XHistidine tag was blotted with the MCA1396 antibody (Serotec). Phospho-LRP6 (Ser1490) was purchased from Cell Signaling [1:500]. Myc was blotted with 9E10 (Sigma) [1:500]. Total Tau was blotted with T-1308-1 (rPeptide) [1:15,000]. Antibody to Tau P396 was purchased from Cell Signaling [1:500].

Cell Culture, Transfection, and Bimolecular Fluorescence Complementation (BiFC)

HEK293 cells were transfected with Lipofectamine 2000 (Invitrogen) and maintained in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics. Cells were grown at 37°C in 5% CO_2 for 24 hr and then incubated at 30°C for 20 hr. Cells were fixed at RT for 20 min on fibronectin-coated coverslips with 4% formaldehyde in CB buffer (10 mM MES pH 6.1, 138 mM KCl, 3 mM MgCl, 2 mM EGTA) supplemented with 11.66% w/v Sucrose. Slides were mounted with VectorShield containing DAPI stain and imaged using Nikon Eclipse 80i

fluorescence microscope with a Nikon 60xA objective and a Cool Snap ES camera. YFP signal was measured by excitation at 515 nm and emission at 555 nm. All images were taken under identical settings.

Results

Recombinant LRP6 Intracellular Domain Protein Activates Wnt/β-catenin Signaling in *Xenopus* Embryos.

LRP5/6 is a single-span transmembrane Wnt co-receptor. Expression of the LRP5/6 intracellular domain in cultured mammalian cells accurately recapitulates LRP5/6 signal transduction, promoting β-catenin stabilization and regulating Wnt/β-catenin target gene expression (Mi et al., 2006; Mi and Johnson, 2005). Notably, the intracellular domain of LRP6 was recently reported to be generated by a Wnt3a-regulated, γ-secretase-mediated event, suggesting that the LRP6 intracellular domain may play a physiological role in Wnt signaling (Mi and Johnson, 2007). To obtain soluble LRP6 for analysis in biochemically tractable *Xenopus* egg extract, we bacterially expressed and purified recombinant polypeptide encoding the LRP6 intracellular domain lacking its transmembrane domain (LRP6ICD; Figure 2.2A and B).

We first tested whether LRP6ICD activates Wnt/β-catenin signaling *in vivo*. Ventral injection of LRP6ICD protein into *Xenopus* embryos at a concentration similar to that of other pathway components (Lee et al., 2003) induces complete axis duplication and promotes transcription of Wnt/β-catenin targets, *siamois* and *Xnr3*, in ectodermal

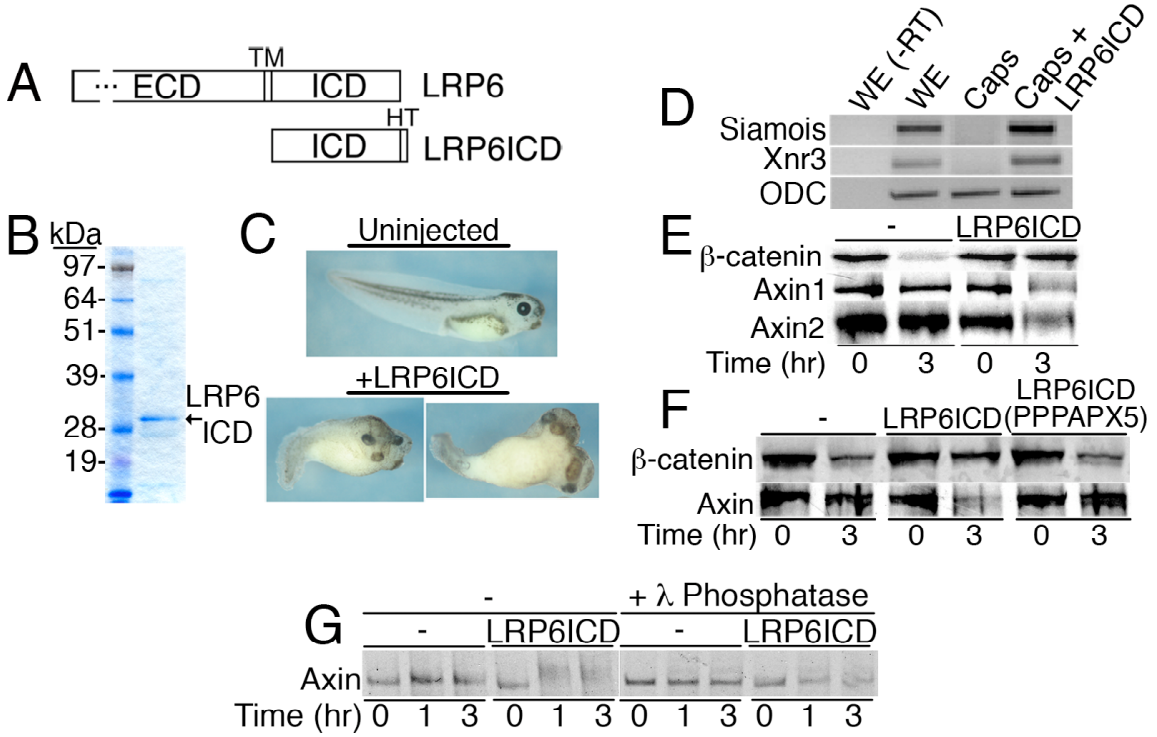


Figure 2.2: Recombinant LRP6ICD Activates Wnt Signaling *In Vivo* and in *Xenopus* Egg Extract. (A) LRP6ICD spans the intracellular domain of mouse LRP6 (aa1397-1614) and does not include its transmembrane domain. ECD, extracellular domain; ICD, intracellular domain; TM, transmembrane domain; HT, 6Xhistidine tag. (B) Coomassie-stained gel of recombinant LRP6ICD (1 μg) purified from bacteria. This purification was performed by Kristin Kalie Jernigan (K.K.J.) and Christopher Stephen Cselenyi (C.S.C.). (C) Injection of LRP6ICD protein (33 nM) into each ventral blastomere of 4-cell *Xenopus* embryos promotes development of a complete ectopic axis (bottom left panel, embryo side view; bottom right panel, embryo ventral view) in 73% of embryos (n=15). A lower dose of LRP6ICD protein (20 nM) promotes axis duplication in 46% of embryos (n=15). This experiment was performed by Emilios Tahinci (E.T.). (D) Injection of LRP6ICD (33 nM) at the 4-cell stage promotes ectopic transcription of Wnt/β-catenin targets *Xnr3* and *siamois* in animal caps as assayed by RT-PCR. WE, whole embryos; Caps, animal caps; WE-RT, no reverse transcriptase added; ODC, ornithine decarboxylase (loading control). This experiment was performed by E.T. (E) Addition of LRP6ICD (1.6 μM) to *Xenopus* egg extract prevents degradation of radiolabeled, IVT β-catenin and promotes degradation of radiolabeled, IVT Axin and Axin2. This experiment was performed by K.K.J. and C.S.C. (F) Unlike LRP6ICD, LRP6ICD(PPPAPX5) (1.6 μM) does not inhibit β-catenin degradation or promote Axin degradation. (G) LRP6ICD promotes a reduced mobility of IVT, radiolabeled Axin on SDS-PAGE in addition to Axin degradation. Treatment with λ phosphatase reverses the LRP6ICD-induced Axin mobility shift.

explants (Figure 2.2C and D). Our results provide phenotypic and transcriptional evidence that recombinant LRP6ICD protein purified from bacteria promotes Wnt/β-catenin signaling *in vivo*.

LRP6ICD Promotes β-catenin Stabilization and Axin Degradation in *Xenopus* Egg Extract.

To establish a cell-free system that would facilitate biochemical analysis of LRP6 signaling, we tested whether recombinant LRP6ICD, which activates Wnt signaling *in vivo*, prevents degradation of β-catenin in *Xenopus* egg extract. We find that LRP6ICD protein prevents degradation of radiolabeled, *in vitro*-translated (IVT) β-catenin in *Xenopus* egg extract (Fig. 2E). Consistent with a proposed mechanism for LRP6 signaling, we demonstrate that LRP6ICD also stimulates degradation of IVT Axin and Axin2 (Figure 2.2E). We also tested whether LRP6ICD induces phosphorylation of Axin. We find λ phosphatase reverses the LRP6ICD-mediated upward mobility shift of the Axin protein detected by SDS-PAGE, suggesting that LRP6ICD promotes Axin phosphorylation (Figure 2.2G). However, in the presence of LRP6ICD, the total Axin signal is decreased even after λ phosphatase treatment, consistent with LRP6ICD mediating Axin degradation.

The ability of LRP6 to stabilize β-catenin is dependent on GSK3's phosphorylation of the serine residue on at least one of five Pro-Pro-Pro-Ser-Pro (PPPSP) motifs on LRP6 (Tamai et al., 2004; Zeng et al., 2005). If LRP6ICD accurately reconstitutes endogenous LRP6 signaling in extract, LRP6ICD's activity should be dependent on intact PPPSP motifs. An LRP6 construct in which all five PPPSP motifs

have been mutated to PPPAP (PPPAPX5) does not bind Axin or stabilize β-catenin in cultured cells (Tamai et al., 2004). This construct also fails to activate Wnt target genes in *Xenopus* ectodermal explants (Tamai et al., 2004). To test whether LRP6ICD signaling in egg extract requires intact PPPSP motifs, we expressed and purified LRP6ICD(PPPAPX5) protein from bacteria. In contrast to LRP6ICD, we find that LRP6ICD(PPPAPX5) does not inhibit β-catenin degradation or stimulate Axin degradation in egg extract (Figure 2.2F). We also find that LRP6ICD, but not LRP6ICD(PPPAPX5), is phosphorylated at PPPSP Ser1490 in egg extract as assayed by immunoblot with a previously characterized phospho-LRP6 (Ser1490) antibody (Tamai et al., 2004; Zeng et al., 2005) (Figure 2.8C). Requirement of these PPPSP motifs suggests LRP6ICD in extract functions in a manner that is similar to that of LRP6 in cultured cells and *Xenopus* embryos.

LRP6ICD Signals Independently of Dishevelled in *Xenopus* Egg Extract and Embryos.

Dishevelled (Dsh) is a cytoplasmic protein required for signaling downstream of Fz and upstream of the β-catenin destruction complex (Logan and Nusse, 2004). In cultured mammalian cells, overexpression of LRP6 that lacks its extracellular domain promotes Wnt signaling despite downregulation of Dsh by RNAi or overexpression of a dominant-negative form of Dsh (Li et al., 2002), suggesting the intracellular domain of LRP6 can signal independently of Dsh. More recently, it was shown that Dsh is required for LRP6 oligomerization and phosphorylation (Bilic et al., 2007), which are necessary for LRP6-mediated activation of Wnt/β-catenin signaling. Interestingly, LRP6 expressed

without its extracellular domain bypasses this requirement for Dsh and is constituitively oligomerized and phosphorylated (Bilic et al., 2007). These data suggest that LRP6ICD may mimic Dsh-activated LRP6 and circumvent the requirement for Dsh in Wnt/β-catenin signaling.

To test whether LRP6ICD signaling in *Xenopus* egg extract bypasses its requirement for Dsh, we immunodepleted endogenous Dsh from egg extract (Salic et al., 2000). Depletion of Dsh (Figure 2.3A) did not affect the ability of LRP6ICD to stabilize β-catenin or promote Axin degradation (Figure 2.3B). To determine if Dsh is required for LRP6ICD signaling *in vivo*, we tested whether Xdd1 (a dominant negative form of Dsh (Sokol, 1996)) prevents LRP6's activation of the Wnt/β-catenin pathway in *Xenopus* embryos. In mRNA co-injection experiments, Xdd1 inhibits Wnt8-induced secondary axis formation but has no effect on the ability of LRP6ICD to induce secondary axes (Figure 2.3C). Thus, our data in *Xenopus* egg extract and embryos demonstrate that LRP6ICD signals independently of Dsh and are consistent with a model in which LRP6ICD mimics Dsh-activated LRP6 in Wnt/β-catenin signaling (Bilic et al., 2007).

Phosphorylation and activation of LRP6 (upon Wnt signaling) are believed to be due to relief of a conformational constraint mediated by LRP6's extracellular domain, and this may explain why the intracellular domain of LRP6 mimics a constitutively phosphorylated and active form of the receptor (Bilic et al., 2007). Axin-bound GSK3 has been suggested to play a role in phosphorylation and activation of LRP6 (Zeng et al., 2008). Because phosphorylation of LRP6 is a prerequisite for its binding to Axin (Tamai et al., 2004), however, the initial phosphorylation of LRP6 may occur by a pool of GSK3 that is not bound to Axin. In egg extract where Axin has been immunodepleted, we find

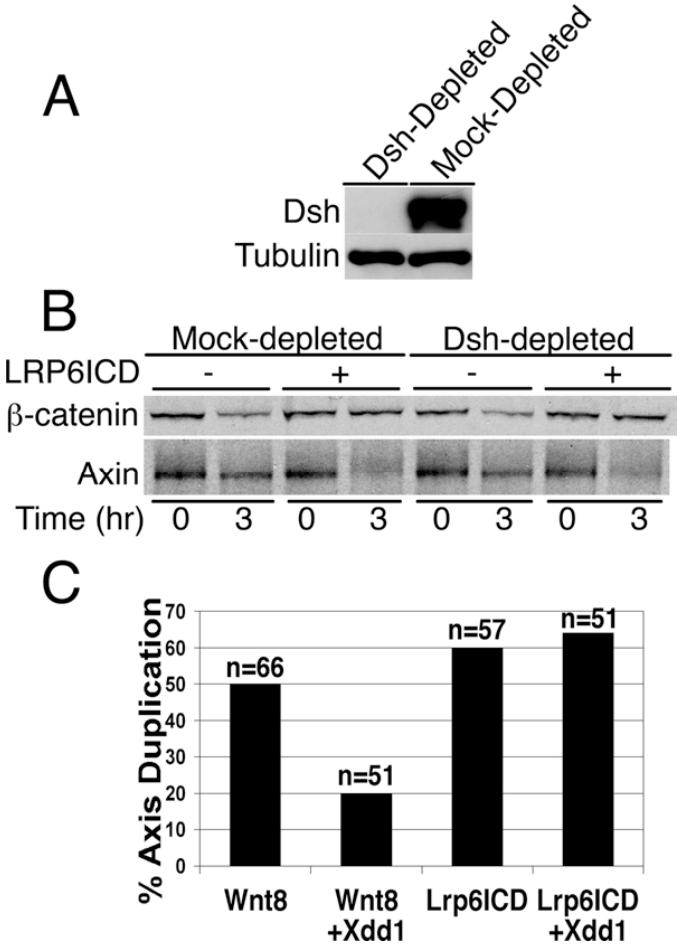


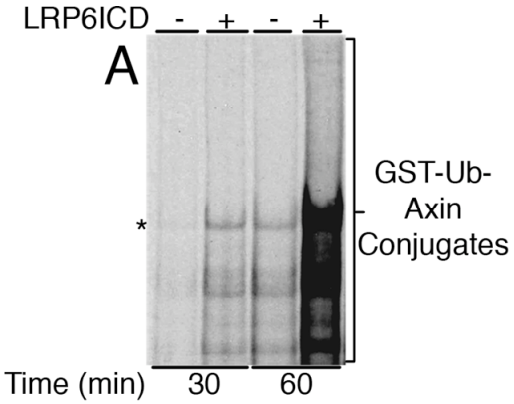
Figure 2.3: LRP6ICD Signals Independenly of Dsh in *Xenopus* Egg Extract and Embryos. (A) Immunoblot of Dsh-depleted and mock-depleted (Protein A beads) egg extract. (B) Dsh depletion does not affect the ability of LRP6ICD to promote β-catenin stabilization or Axin degradation in egg extract. (C) To compare Xdd1 mRNA-mediated suppression of Xwnt8 mRNA-induced and LRP6ICD mRNA-induced axis duplication, mRNAs were titrated to promote axis duplication in 50-60% of injected embryos. Dominant negative Dsh (Xdd1) (1 ng RNA) prevents axis duplication by Wnt8 (1pg RNA) but not by LRP6ICD (500 pg). For duplication assays, *Xenopus* embryos were injected in each ventral blastomere at the 4-cell stage. This experiment was performed by E.T.

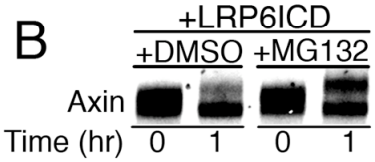
that LRP6ICD still becomes phosphorylated at PPPSP Ser 1490 as assayed by immunoblot, suggesting that initial LRP6 phosphorylation may occur independently of Axin.

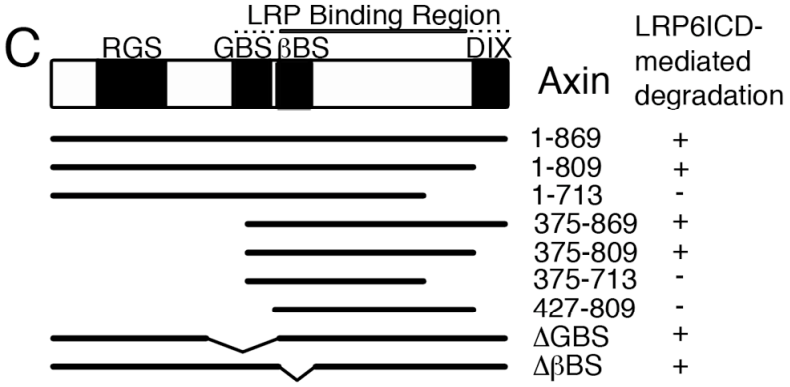
LRP6ICD-Mediated Axin Degradation Occurs Via the Ubiquitin/Proteasome Pathway and Is Distinct from GSK3-Regulated Axin Degradation.

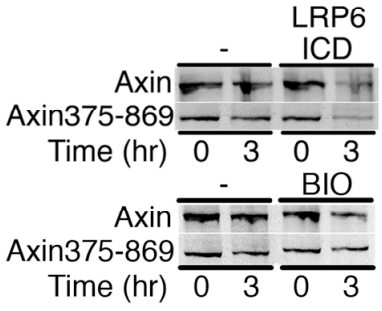
To identify the mechanism by which LRP6 promotes Axin degradation, we tested whether LRP6ICD induces Axin degradation via a ubiquitin-mediated, proteasome-dependent process. We find LRP6ICD promotes Axin ubiquitination in *Xenopus* egg extract (Figure 2.4A). Furthermore, we show that inhibition of the proteasome with MG132 prevents Axin degradation, leading to accumulation of a slower migrating form of Axin (Figure 2.4B). Thus, our data indicate that, consistent with results from intact *Xenopus* oocytes (Kofron et al., 2007), Axin degradation is proteasome-dependent in egg extract.

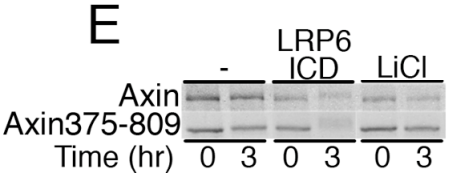
To uncover structural elements of Axin required for its LRP6-mediated degradation, we analyzed a panel of truncated Axin polypeptides. We identified a minimal Axin fragment (Axin375-809) that degrades in response to LRP6ICD (Figures 2.4C and 2.5). This minimal fragment includes the GSK3, β-catenin, and PP2A binding sites on Axin (Fagotto et al., 1999). However, deletion of the GSK3 or β-catenin binding domain from full-length Axin does not prevent its LRP6ICD-mediated turnover (Figure 2.4C); thus, binding of Axin to GSK3 or β-catenin may not be required for LRP6-mediated degradation of Axin. Interestingly, amino acids 375-427 appear to be required in the large N-terminal truncation mutants (compare Axin375-809 and Axin427-809) but


LRP6ICD - + - +
A
GST-Ub-
Axin
Conjugates
*
Time (min) 30 60


B
+LRP6ICD
+DMSO +MG132
Axin
Time (hr) 0 1 0 1


C
LRP Binding Region
RGS GBS βBS DIX
Axin
LRP6ICD-
mediated
degradation
1-869 +
1-809 +
1-713 -
375-869 +
375-809 +
375-713 -
427-809 -
ΔGBS +
ΔβBS +


D
LRP6
ICD
-
Axin
Axin375-869
Time (hr) 0 3 0 3
BIO
-
Axin
Axin375-869
Time (hr) 0 3 0 3


E
LRP6
ICD
-
LiCl
Axin
Axin375-809
Time (hr) 0 3 0 3 0 3

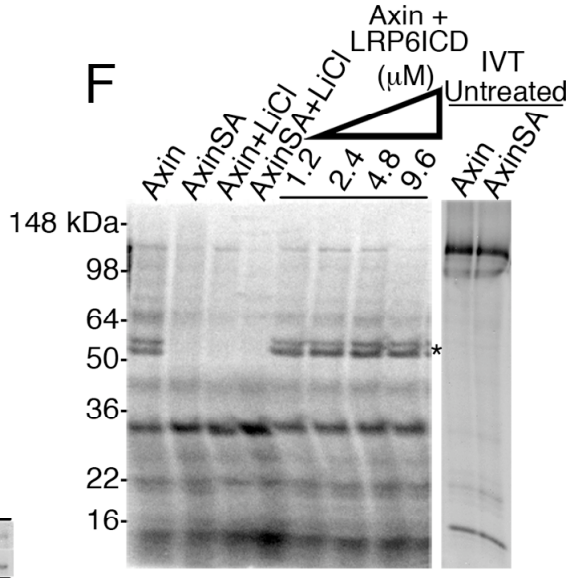

F
Axin +
LRP6ICD
(μM)
IVT
Untreated
Axin
AxinSA
Axin+LiCl
AxinSA+LiCl
1.2
2.4
4.8
9.6
Axin
AxinSA
148 kDa-
98-
64-
50-
36-
22-
16-
*

Figure 2.4: LRP6ICD Mediates Axin Degradation Independently of GSK3 Inhibition. (A) LRP6ICD stimulates addition of GST-ubiquitin to radiolabeled, IVT Axin in egg extract. GST-ubiquitin conjugates were pulled down with glutathione beads at indicated times and analyzed by SDS-PAGE and autoradiography. Asterisk indicates full-length Axin. This experiment was performed by K.K.J. (B) Addition of the proteasome inhibitor MG132 (1 mM) to egg extract inhibits LRP6ICD-mediated Axin degradation. This experiment was performed by Curtis Thorne. (C) Degradation of Axin mutants in egg extract in the presence of LRP6ICD. RGS, RGS domain; GBS, GSK3 Binding Site; βBS, β-catenin Binding Site; DIX, DIX domain. The indicated LRP5/6 binding region on Axin is based on previous Axin-LRP5 and Axin-Arr yeast two-hybrid studies (Mao et al., 2001b; Tolwinski et al., 2003); dotted lines represent large deletions of Axin that were not further mapped, and the borders of Axin-LRP5/6 interaction likely reside within the dotted lines. (D) LRP6ICD promotes degradation of Axin and Axin375-869, whereas the GSK3 inhibitor BIO (50 mg/ml, Calbiochem) promotes degradation of Axin but not Axin375-869. (E) LRP6ICD promotes degradation of Axin and Axin375-869, whereas the GSK3 inhibitor LiCl (50 mM) promotes degradation of Axin but not Axin375-869. (F) Inhibition of GSK3-mediated Axin phosphorylation (by LiCl (50 mM) or mutagenesis (AxinSA)), but not incubation with LRP6ICD, alters the trypsin proteolysis pattern of IVT Axin after incubation in egg extract for 30 min (note bands at level of asterisk). All experiments used equal concentrations of IVT Axin. An SDS-PAGE autoradiograph of IVT Axin and AxinSA prior to trypsin treatment is shown at right.

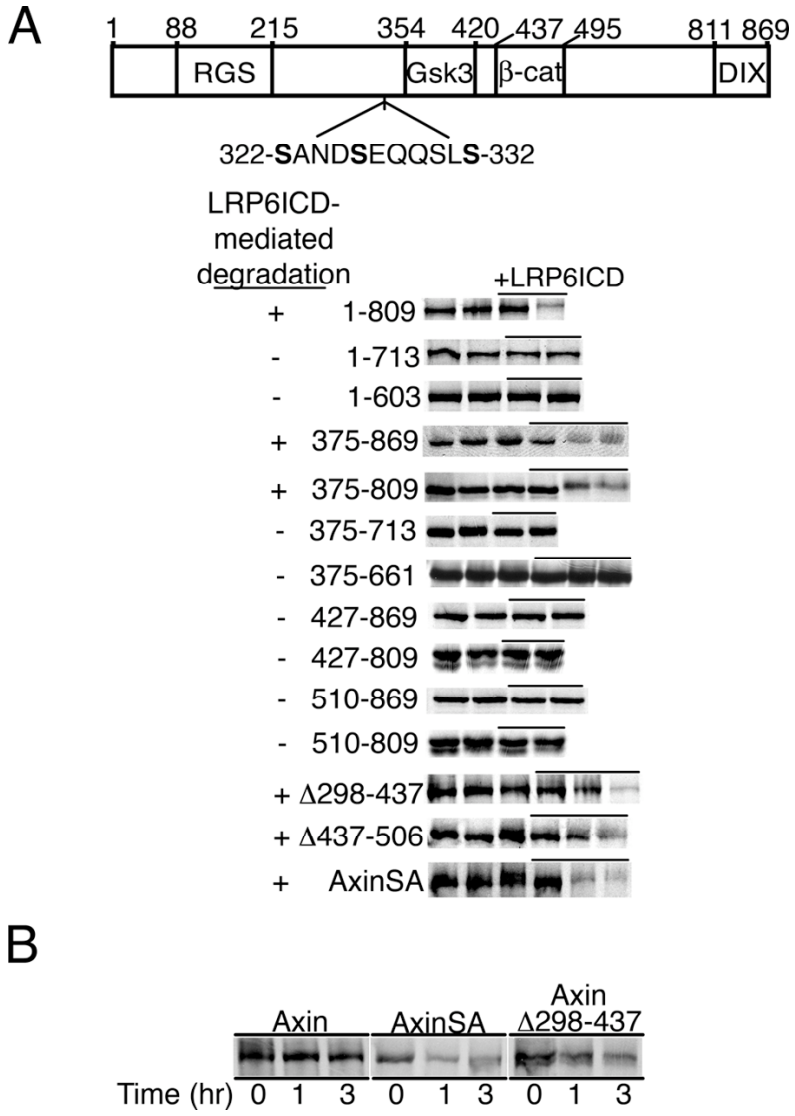


Figure 2.5: LRP6ICD Stimulates Degradation of Axin Truncation Mutants in Egg Extract. (A) IVT, radiolabeled Axin degradation assays in the presence or absence of LRP6ICD in *Xenopus* egg extract. Samples were analyzed at either 0 hr and 3 hr (two lanes) or 0 hr, 1 hr, and 3 hr (three lanes). In all experiments, wild-type Axin is used to confirm LRP6ICD activity (data not shown). Amino acids corresponding to mAxin's initiation methionine, RGS domain, GSK3 Binding Site, β-catenin Binding Site, and DIX domain are shown. Axin with internal deletions of the GSK3 and β-catenin binding sites are AxinΔ298-437 and AxinΔ437-506, respectively. Predicted GSK3 phosphorylation sites on Axin mutated in AxinSA are indicated with bold text. (B) To highlight effects of LRP6ICD on Axin stability, egg extracts with low basal Axin turnover were used for experiments in Figure 2.5A. In other batches of egg extract, Axin truncation mutants lacking putative GSK3 phosphorylation sites shown to regulate Axin stability have increased rates of basal degradation compared to wild-type Axin. In these extracts, LRP6ICD retains the ability to promote degradation of these Axin truncation mutants (data not shown).

not in the internally truncated AxinΔGBS; we believe this may result from abnormal folding of certain truncation mutants, redundancy within Axin regarding sequences required for LRP6ICD-mediated Axin degradation, and/or dimerization of certain Axin mutants with endogenous Axin (Luo et al., 2005). Notably, we find that the region of Axin identified to bind LRP5/6 by yeast two-hybrid assays (Mao et al., 2001b; Tolwinski et al., 2003), also appears to be required for its LRP6-mediated degradation (Figure 2.4C). These data are consistent with a model in which LRP6/Axin binding is required for LRP6-mediated Axin degradation.

Several models for Wnt pathway activation involve inhibition of GSK3, positing global inhibition of GSK3 within the cell or specific inhibition of GSK3 within the β-catenin destruction complex. Either mechanism would allow β-catenin levels to rise because its phosphorylation, which is necessary for its degradation, is blocked. Experiments suggest an inherent feed-forward mechanism whereby GSK3 inhibition also stimulates Axin degradation by preventing phosphorylation of Axin, which is normally required for its stability (Yamamoto et al., 1999). Thus, we tested whether LRP6ICD promotes Axin degradation by inhibiting GSK3-mediated phosphorylation of Axin.

If LRP6ICD promotes turnover of Axin by inhibiting its GSK3-mediated phosphorylation, Axin mutants that degrade in response to LRP6ICD should also be able to degrade in response to GSK3 inhibition. Alternatively, if LRP6ICD-mediated Axin turnover does not occur via GSK3 inhibition, certain Axin mutants may degrade in response to LRP6ICD but not in response to GSK3 inhibition. We find evidence in support of the latter model. Both LRP6ICD and the GSK3 inhibitor BIO (Figure 2.4D) promote turnover of full-length Axin; in contrast, Axin375-869 degrades in response to

LRP6ICD but not the GSK3 inhibitor BIO (Figure 2.4D). Another GSK3 inhibitor, lithium (50 mM), also promotes turnover of full-length Axin but not Axin375-869 (Figure 2.4E). Furthermore, Axin mutants lacking previously identified GSK3 phosphorylation and binding sites as well as an Axin mutant (AxinSA) in which predicted GSK3 phosphorylated serines are mutated to alanines (Yamamoto et al., 1999) degrade in response to LRP6ICD (Figures 2.4C and 2.5). Together, these data indicate that LRP6 is unlikely to promote Axin degradation via a mechanism that inhibits GSK3-mediated stabilization of Axin.

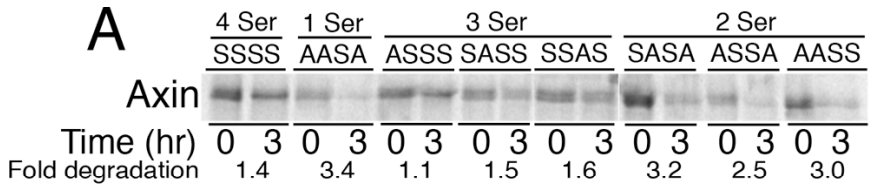
Distinct mechanisms underlying LRP6-mediated and GSK3 inhibition-mediated Axin degradation may induce different Axin conformations. Because changes in a protein's conformation may expose or conceal certain tryptic cleavage sites, a protein's tryptic proteolysis pattern is traditionally used to detect conformational changes (Liu et al., 2005; Moroney and McCarty, 1982; Stukenberg and Kirschner, 2001). Incubation of radiolabeled, IVT Axin in egg extract followed by partial trypsin proteolysis results in a characteristic Axin digestion pattern upon analysis by SDS-PAGE and autoradiography (Figure 2.4F). Trypsin digestion of Axin lacking GSK3 phosphorylation (either via mutation (AxinSA) or incubation with a GSK3 inhibitor (LiCl)) results in a proteolysis pattern distinct from wild-type Axin. Incubation of Axin with LRP6ICD, however, yields a digestion pattern that is indistinguishable from that of Axin alone. Because addition of LRP6ICD and inhibition of GSK3 phosphorylation have distinct effects on Axin conformation as assayed by trypsin digest, we propose that LRP6 signaling and GSK3 inhibition affect Axin, at least in part, through different mechanisms. These biochemical data are consistent with genetic evidence in *Drosophila* embryos that Arrow,

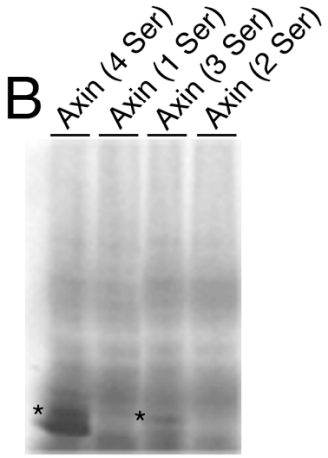
the *Drosophila* LRP5/6 ortholog, can promote Axin degradation in the absence of GSK3 activity (Tolwinski et al., 2003).

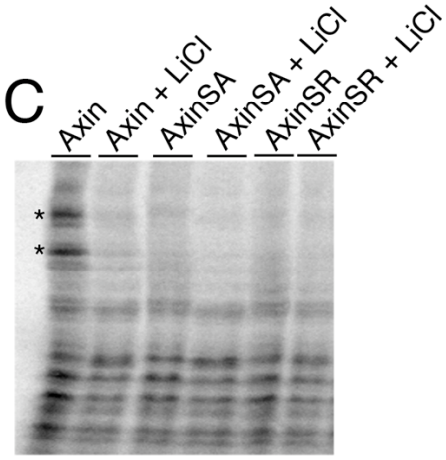
GSK3's Phosphorylation Sites on Axin Contribute Additively to Axin Stability.

To better understand how GSK3's phosphorylation of Axin regulates its stability, we made point mutations of serines in Axin's predicted GSK3 phosphorylation region (**S**AND**S**EQQ**S**L**S**) (Figure 2.6A). In previous work, mutation of this region to ***A***AND***A***EQQ***A***L**S** decreased the half-life of Axin transfected into cultured mammalian cells. Notably, the half-life of wild-type Axin could be decreased similarly by addition of a GSK3 inhibitor to these cells. As the serines in this region are GSK3 consensus sites, it was suggested that GSK3 phosphorylation of these sites regulates Axin stability. However, further investigation of these putative phosphorylation sites was not undertaken. We find that these serines contribute to Axin stability in an additive fashion. Wild-type Axin containing all four serines was most stable in egg extract, and degradation increased significantly as the number of serines mutated to alanine was increased (Figure 2.6A). Thus, we find that all of the **S**AND**S**EQQ**S**L**S** serines affect Axin stability, and that these serines contribute to Axin stability in an additive fashion. Such a finding is consistent with a mechanism whereby these phosphorylated serines contribute to binding of another protein that regulates Axin stability or contribute to Axin assuming a conformation with a decreased half-life.

Because we found that wild-type Axin incubated with a GSK3 inhibitor or AxinSA (***A***AND***A***EQQ***S***L**A**) has a distinct trypsin proteolysis pattern from wild-type Axin, we tested whether mutations of fewer serines also affected Axin conformation.


A
4 Ser
1 Ser
3 Ser
2 Ser
SSSS
AASA
ASSS
SASS
SSAS
SASA
ASSA
AASS
Axin
Time (hr) 0 3 0 3 0 3 0 3 0 3 0 3 0 3 0 3
Fold degradation 1.4 3.4 1.1 1.5 1.6 3.2 2.5 3.0


B
Axin (4 Ser)
Axin (1 Ser)
Axin (3 Ser)
Axin (2 Ser)
*
*


C
Axin
Axin + LiCl
AxinSA
AxinSA + LiCl
AxinSR
AxinSR + LiCl
*
*

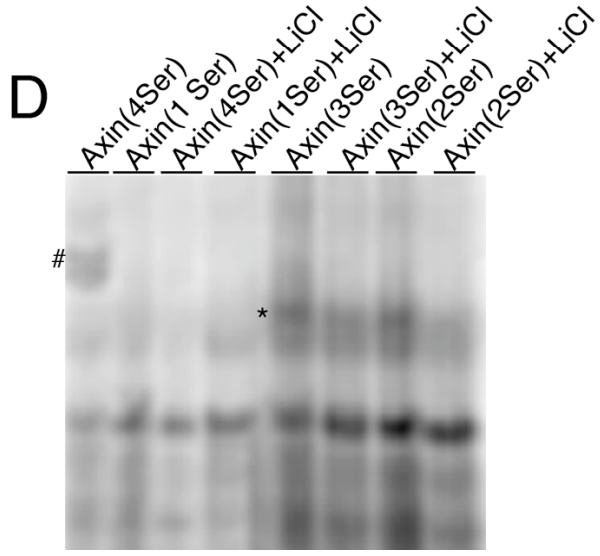

D
Axin(4Ser)
Axin(1 Ser)
Axin(4Ser)+LiCl
Axin(1Ser)+LiCl
Axin(3Ser)
Axin(3Ser)+LiCl
Axin(2Ser)
Axin(2Ser)+LiCl
#
*

Figure 2.6: GSK3's Phosphorylation Sites on Axin Contribute Additively to Axin Stability. (A) Basal degradation of Axin mutants with Ser-Ala substitutions in the GSK3 phosphorylation region ($\underline{S}^1$AND$\underline{S}^2$EQQ$\underline{S}^3$L$\underline{S}^4$) increases with the number of Ala mutations. For simplification, only serine residues (and not intervening residues) are noted in figure. Basal degradation of Axin with 4 Ser > 3 Ser > 2 Ser > 1 Ser. Fold degradation is (0 hr band)/(3 hr band) as quantified by ImageQuant analysis of digital PhosphorImager file. (B) Trypsin proteolysis pattern of Axin in egg extract is altered by mutations of GSK3 phosphorylation region. Axin(SSSS, 4 Ser) produces a trypsin proteolysis fragment that is less abundant in Axin(SSAS, 3 Ser) and absent in Axin(ASSA, 2 Ser) and Axin(AASA, 1 Ser). Note bands shown at right of asterisks. (C) As shown in Figure 2.4F, inhibition of GSK3-mediated Axin phosphorylation (by LiCl (50 mM)) or mutagenesis (AxinSA(AASA, 1 Ser)) alters the trypsin proteolysis pattern of IVT Axin after incubation in egg extract for 30 min. Notably, AxinSR(RRRS) has a proteolysis pattern similar to AxinSA(AASA). Note bands at levels of asterisks. (D) Ser-Ala substitutions in the GSK3 phosphorylation region of Axin affect LiCl-mediated alterations in their trypsin proteolysis patterns. Axin(SSSS, 4 Ser), Axin(SSAS, 3 Ser), and Axin(ASSA, 2 Ser) have LiCl-sensitive trypsin proteolysis pattern, while Axin(AASA, 1 Ser) does not. Note LiCl sensitive bands marked by * and #.

Given the effects of such mutations on Axin stability, we hypothesized that these serines would affect Axin's confirmation as assayed by trypsin digest in an additive fashion. Indeed, incubation of radiolabeled wild-type Axin in egg extract followed by trypsin proteolysis leads to a proteolysis pattern in which there is a prominent band that is decreased in intensity with mutation of one serine and absent with mutation of two or three serines (Figure 2.6B). We attempted to make an Axin mutant that would be constituitively stable and insensitive to GSK3 inhibition by replacing these serines with negatively charged, possibily phospho-mimetic aspartic acid residues; however, this mutant was most similar to the AxinSA mutant, suggesting that these substituted amino acids were not sufficiently similar to phosphorylated serines (Figure 2.6C). To further explore the role of these serines as GSK3 phosphorylation sites, we tested whether our serine to alanine substitution mutants were sensitive to GSK3 inhibition by assaying their trypsin proteolysis patterns. We find that the proteolysis patterns of mutants with four, three, and two serines retain sensitivity to LiCl-mediated GSK3 inhibition (Figure 2.6D). This result supports our hypothesis that these four serines contribute to Axin stability and conformation in an additive fashion. Although this mechanism for control of Axin stability appears to be independent of LRP6-mediated regulation of Axin stability and beyond the scope of this work, an understanding of the mechanism by which this region affects Axin stability will contribute significantly to our knowledge of the mechanism of Wnt/β-catenin signaling.

LRP6ICD-Mediated β-catenin Stabilization Does Not Require Axin Degradation.

Although we hypothesize that LRP6-mediated degradation of Axin, a required component of the β-catenin destruction complex, leads to β-catenin stabilization, we wanted to determine whether this is the only mechanism by which LRP6ICD stabilizes β-catenin. To explicitly test this model, we assessed whether LRP6ICD can stabilize β-catenin in egg extract in which endogenous Axin is replaced by a non-degradable Axin mutant, Axin1-713 (Figure 2.7D). Axin1-713, like full-length Axin, ventralizes *Xenopus* embryos (indicative of inhibition of Wnt/β-catenin signaling) (data not shown), stimulates β-catenin degradation in egg extract (Figure 2.7D), and binds LRP6ICD in egg extract (Figure 2.7A). Thus, Axin1-713 retains all measurable activities of full-length Axin except that it is not degraded in response to LRP6ICD (Figure 2.4C). Consistent with the requirement of Axin for destruction complex formation, immunodepletion of endogenous Axin from extract (Figure 2.7B) prevented β-catenin degradation (Salic et al., 2000) (Figure 2.7D). Addition of IVT Axin1-713 to Axin-depleted extract restored β-catenin degradation to an extent similar to that of addition of full-length Axin. We then tested whether LRP6ICD inhibits β-catenin degradation in Axin1-713-rescued extract. As shown in Figure 2.7D, LRP6ICD inhibits β-catenin degradation in extract where endogenous Axin is replaced by either full-length Axin or non-degradable Axin1-713. Thus, LRP6ICD can inhibit β-catenin degradation independently of Axin degradation in *Xenopus* egg extract.

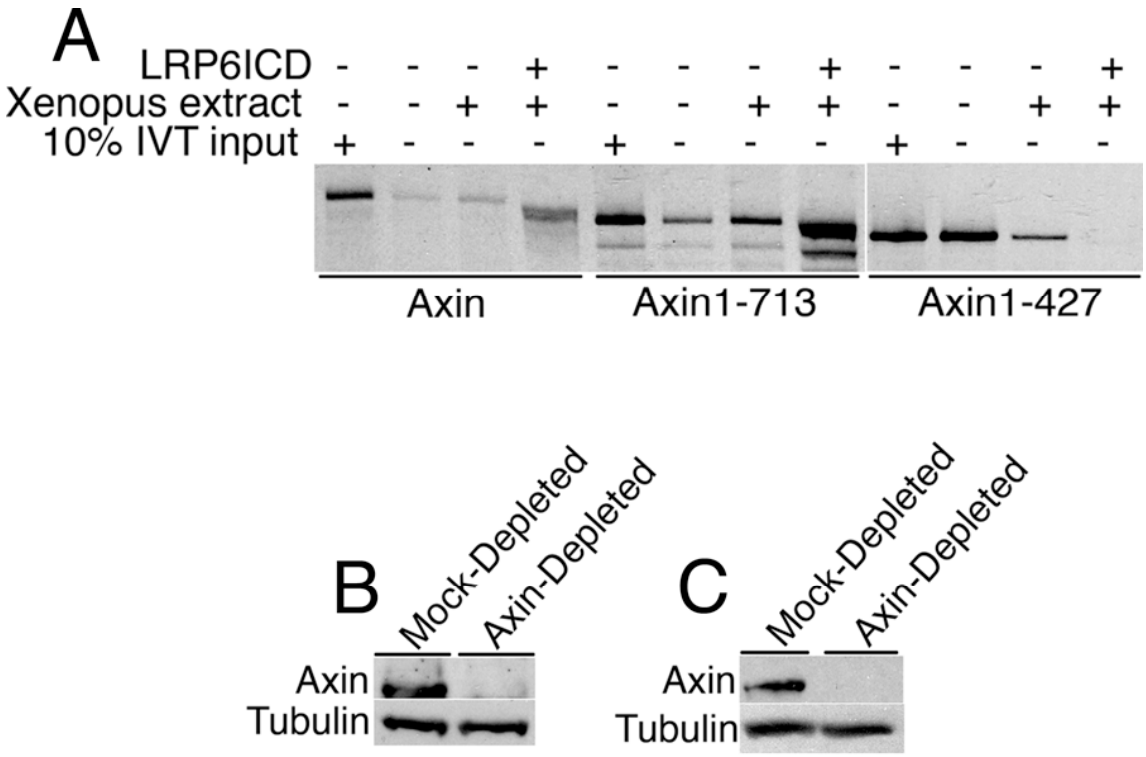
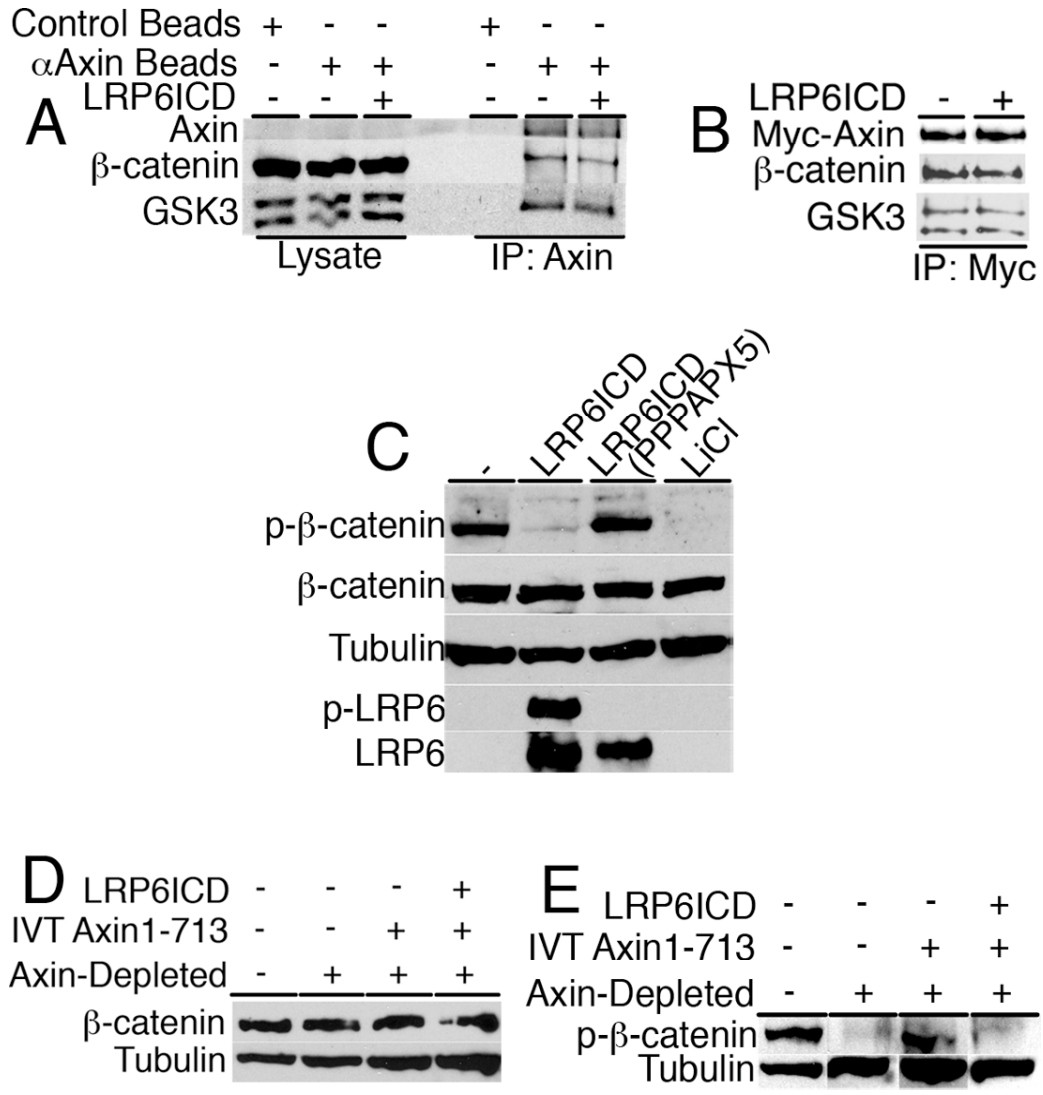

A
LRP6ICD
Xenopus extract
10% IVT input
Axin
Axin1-713
Axin1-427
B
Mock-Depleted
Axin-Depleted
Axin
Tubulin
C
Mock-Depleted
Axin-Depleted
Axin
Tubulin
D
Axin-Depleted Extracts
IVT Axin
IVT Axin1-713
LRP6ICD
LRP6ICD
β-catenin
Time (hr) 0 3 0 3 0 3 0 3 0 3 0 3

Figure 2.7. LRP6ICD Promotes β-catenin Stabilization in the Absence of Axin Degradation. (A) Axin and non-degradable Axin1-713, but not Axin1-427, bind LRP6ICD in egg extract. Radiolabeled Axin, Axin1-713, and Axin1-427 were incubated with 6XHis-tagged LRP6ICD and pulled down with nickel beads. Binding was performed in egg extract to increase the stringency of binding reaction conditions and because post-translational modification of LRP6 (e.g. phosphorylation) is important for Axin/LRP6 binding. The signal representing 10% of the total amount of IVT used in the binding experiments is indicated. LRP6ICD increases the amount of Axin and Axin1-713, but not Axin1-427, that is pulled down with nickel beads. IVT Axin and Axin1-713 pulled down with nickel beads in the presence of LRP6ICD also display a similar increase in mobility on SDS-PAGE. These data suggest Axin and Axin1-713 bind LRP6 in a similar manner. Binding of nickel beads to 6XHis-tagged LRP6ICD occupies the Ni^{++} sites on the nickel resin, potentially blocking its nonspecific binding to IVT Axin. This likely explains why there is less IVT Axin background binding in the lane with LRP6ICD compared to IVT Axin alone. (B) Western blot confirms immunodepletion of Axin from *Xenopus* Egg Extract. Axin immunoblot of mock (Protein A bead) and Axin-depleted egg extract from Figures 2.7D. Western blotting of tubulin is performed as loading control. (C) Western blot confirms immunodepletion of Axin from *Xenopus* egg extract. Axin immunoblot of mock (Protein A bead) and Axin-depleted egg extract from Figures 2.8D and 2.8E. Western blotting of tubulin is performed as loading control. (D) LRP6ICD inhibits β-catenin degradation in extract where endogenous Axin is replaced by non-degradable Axin1-713. Addition of IVT Axin or Axin1-713 restores the ability of Axin-depleted extract to degrade radiolabeled β-catenin. LRP6ICD inhibits both IVT Axin and Axin1-713-induced β-catenin-degradation. This experiment was performed by K.K.J. and C.S.C.

LRP6ICD Prevents GSK3-Mediated Phosphorylation of β-catenin.

We next sought to identify the mechanism by which LRP6 stabilizes β-catenin independently of Axin degradation. It has been proposed that LRP6 might inhibit β-catenin degradation by promoting dissociation of the β-catenin destruction complex (Nusse, 2005) (Figure 2.1). To test this model, we immunoprecipitated Axin from egg extract incubated in the presence or absence of LRP6ICD and immunoblotted for GSK3 or β-catenin. As shown in Figure 2.8A and B, LRP6ICD (at a concentration that fully inhibits β-catenin degradation in *Xenopus* egg extract) does not affect Axin's ability to bind GSK3 or β-catenin. Thus, our data suggest that LRP6 does not stabilize β-catenin by sequestering Axin from GSK3 or β-catenin.

Alternatively, LRP6 could stabilize β-catenin by directly preventing its phosphorylation within the destruction complex. CK1α phosphorylates β-catenin at Ser45 (P45) to prime it for GSK3's phosphorylation at Ser33/Ser37/Thr41 (P33/37/41), which is required for β-catenin polyubiquitination and degradation (Liu et al., 2002). Previous studies showed Wnt signaling inhibits GSK3-mediated β-catenin phosphorylation but does not inhibit CK1a-mediated β-catenin phosphorylation (Liu et al., 2002). We therefore tested whether LRP6ICD inhibits appearance of GSK3-phosphorylated β-catenin in egg extract. Significantly, LRP6ICD, like the GSK3 inhibitor lithium, inhibits GSK3-mediated phosphorylation of β-catenin (Figure 2.8C). If LRP6 stabilizes β-catenin through inhibition of β-catenin phosphorylation, LRP6ICD's requirement for intact PPPSP motifs to stabilize β-catenin should extend to LRP6ICD's inhibition of β-catenin phosphorylation. Indeed, LRP6ICD(PPPAPX5), which does not inhibit degradation of β-catenin (Figure 2.2F), does not inhibit GSK3's phosphorylation


A
Control Beads + - - + - -
αAxin Beads - + + - + +
LRP6ICD - - + - - +
Axin
β-catenin
GSK3
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B
LRP6ICD - +
Myc-Axin
β-catenin
GSK3
IP: Myc
C
-
LRP6ICD
LRP6ICD (PPPAPX5)
LiCl
p-β-catenin
β-catenin
Tubulin
p-LRP6
LRP6
D
LRP6ICD - - - +
IVT Axin1-713 - - + +
Axin-Depleted - + + +
β-catenin
Tubulin
E
LRP6ICD - - - +
IVT Axin1-713 - - + +
Axin-Depleted - + + +
p-β-catenin
Tubulin

Figure 2.8: LRP6ICD's Inhibition of GSK3-Mediated β-catenin Phosphorylation Stabilizes β-catenin in the Absence of Axin Degradation. (A) LRP6ICD does not affect the ability of Axin to bind GSK3 or β-catenin in egg extract. Endogenous Axin was immunoprecipitated from extract and immunoblotted for GSK3, β-catenin, and Axin. (B) LRP6ICD Does Not Inhibit Myc-Tagged Axin Binding to GSK3 or β-catenin in Egg Extract. IVT, Myc-Axin was incubated in egg extract in the presence or absence of LRP6ICD. Myc-Axin was then immunoprecipitated with Myc-conjugated beads and immunoblotted for Myc, β-catenin, or GSK3. IVT β-catenin was supplemented to extract to enhance its signal. (C) Incubation of LiCl (50 mM) or LRP6ICD (but not LRP6ICD(PPPAPX5)) in egg extract (30 min) inhibits phosphorylation of endogenous β-catenin at GSK3 target sites P33/37/41. Immunoblot of LRP6ICD from the same gel reveals LRP6ICD, but not LRP6ICD(PPPAPX5), is phosphorylated at the PPPSP Ser1490. All samples were blotted from a single gel. (D) Levels of endogenous β-catenin in egg extract are not affected by Axin depletion. Immunoblot for endogenous β-catenin demonstrates that manipulations shown in Figure 8E do not affect total β-catenin levels. This likely reflects the fact that the β-catenin involved in Wnt signal transduction represents only a small, cytoplasmic fraction of total β-catenin. The vast majority of β-catenin in cells and egg extracts belongs to a stable, membrane-bound pool of β-catenin that is not normally regulated by the destruction complex or Wnt signaling (Heasman et al., 1994; Peifer et al., 1994b). (E) LRP6ICD inhibits GSK3-mediated β-catenin phosphorylation in extract in which endogenous Axin is replaced by non-degradable Axin1-713. Axin depletion did not affect total β-catenin levels as assayed by immunoblot (Figure 2.8D). Depletion of endogenous Axin prevents β-catenin P33/37/41 phosphorylation. Addition of IVT Axin1-713 restores β-catenin phosphorylation in Axin-depleted extract. LRP6ICD inhibits IVT Axin1-713-induced β-catenin phosphorylation. Extracts were analyzed after 2 hr incubation. All samples were blotted from a single gel; intervening lanes were removed for clarity.

of β-catenin (Figure 2.8C). Notably, we find that LRP6's PPPSP serine Ser1490 is phosphorylated in extracts (Figure 2.8C). Thus, LRP6ICD inhibits phosphorylation of β-catenin likely through a mechanism that requires serine phosphorylated PPPSP motifs.

Phosphorylation of β-catenin by GSK3 requires its recruitment into the β-catenin destruction complex, which is mediated in part by Axin. Thus, it was possible that LRP6ICD-induced inhibition of GSK3's phosphorylation of β-catenin is a direct consequence of LRP6-mediated Axin degradation. To address this possibility, we tested whether LRP6ICD inhibits β-catenin P33/37/41 phosphorylation in egg extract in which Axin is replaced by non-degradable Axin1-713 (Figure 2.8D and E). Axin depletion (Figure 2.7C) from extract inhibited GSK3's phosphorylation of β-catenin, consistent with Axin's role as a required scaffold for this phosphorylation event. Addition of non-degradable IVT Axin1-713 to Axin-depleted extract restored β-catenin P33/37/41 phosphorylation. LRP6ICD blocked this Axin1-713-induced β-catenin phosphorylation (Figure 2.8E), demonstrating that LRP6ICD can inhibit phosphorylation of β-catenin by GSK3 independently of Axin degradation.

LRP6ICD in egg extract could specifically prevent β-catenin phosphorylation or act as a general GSK3 inhibitor (possibly by GSK3 sequestration (Mi et al., 2006)). If the former is correct, LRP6 should inhibit β-catenin phosphorylation without affecting phosphorylation of another GSK3 substrate (e.g. Tau) (Figure 2.9A). In egg extract supplemented with exogenous GSK3, recombinant Tau is phosphorylated at its well-characterized GSK3 target site Ser396 (P396) (Hong et al., 1997). In contrast to lithium, which robustly inhibits GSK3's phosphorylation of both β-catenin and Tau, LRP6ICD inhibits phosphorylation of β-catenin but not of Tau. Thus, our data indicate that levels

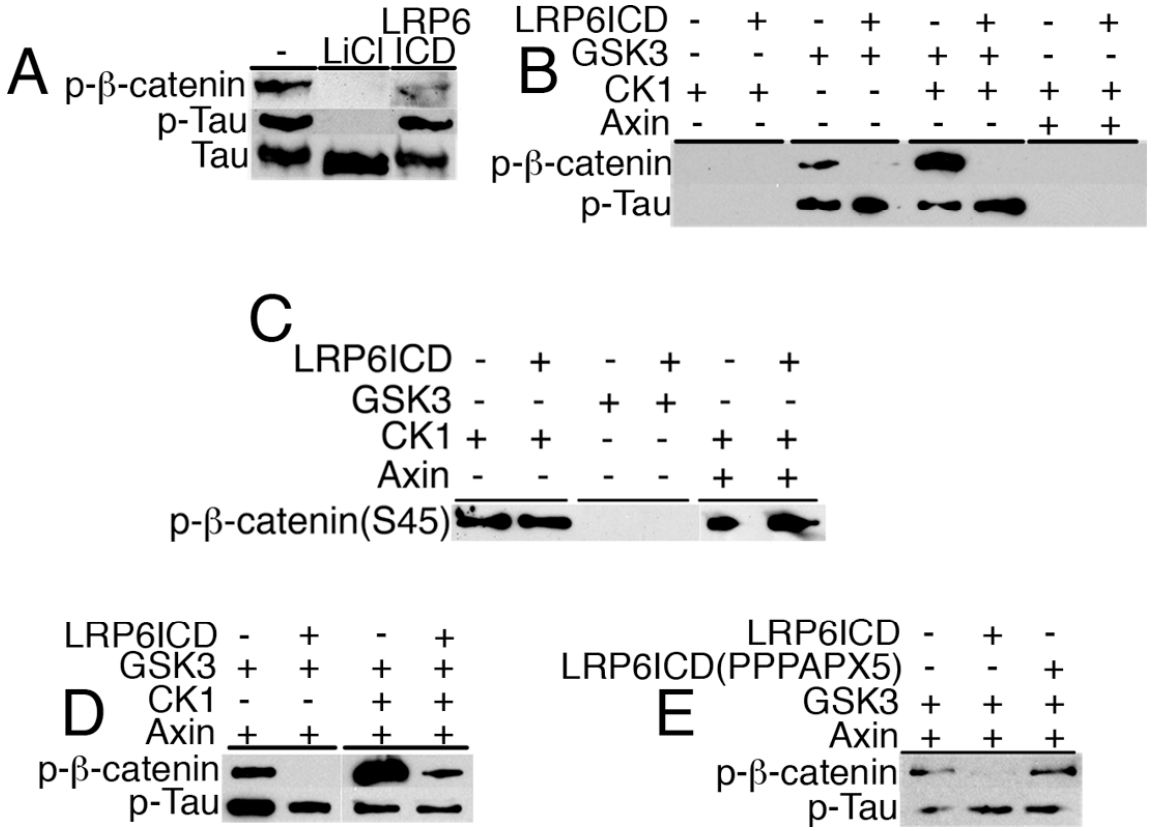


Figure 2.9: LRP6ICD Directly and Specifically Inhibits GSK3's Phosphorylation of β-catenin. (A) LiCl (50 mM) inhibits phosphorylation of β-catenin P33/37/41 and exogenous Tau P396 by GSK3, whereas LRP6ICD inhibits phosphorylation of β-catenin but not Tau by GSK3. Extract was supplemented with GSK3 to enhance detection of phosphorylated Tau as previously described (Yost et al., 1998). β-catenin and Tau from the same reaction sample were immunoblotted from a single gel; intervening lanes were removed for clarity. (B) and (D) In an *in vitro* kinase assay containing purified, recombinant Axin (0.1 μM), GSK3 (0.79 μM), CK1 (1.37 μM), Tau (0.34 μM) and β-catenin (0.22 μM), LRP6ICD (4.1 μM) inhibits phosphorylation of β-catenin P33/37/41 by GSK3 without inhibiting the phosphorylation of Tau P396 by GSK3. (C) LRP6ICD does not inhibit CK1 phosphorylation of β-catenin *in vitro*. In an *in vitro* kinase assay with recombinant, purified proteins, LRP6ICD does not inhibit phosphorylation of β-catenin P45 by CK1. For this figure, nitrocellulose membrane from Figure 2.9B was stripped and re-blotted with antibody to β-catenin P45. (E) In a kinase reaction in which recombinant Axin is absent, phosphorylation of β-catenin by GSK3 is inhibited by LRP6ICD but not by LRP6ICD(PPPAPX5). For (B), (C), (D), and (E), β-catenin and Tau were incubated in the same reaction and immunoblotted from a single gel.

of LRP6ICD that stabilize β-catenin in egg extract inhibit GSK3-mediated β-catenin phosphorylation without affecting global GSK3 activity. Our finding that LRP6ICD does not act by inhibiting total GSK3 activity is also supported by our data demonstrating that LRP6ICD and lithium have distinct effects on Axin's trypsin proteolysis pattern and that LRP6 promotes Axin degradation independently of GSK3 inhibition (Figures 2.4C, D, E, F, and 2.5). Although previous experiments suggested that LRP6 inhibits global GSK3 activity, the concentration of LRP6 intracellular domain in those experiments was not reported and may have been significantly greater than the concentration of LRP6ICD used in our experiments (Mi et al., 2006). Indeed, we detected inhibition of GSK3's phosphorylation of both β-catenin and Tau at higher concentrations of LRP6ICD than those required to inhibit β-catenin phosphorylation in our assays.

LRP6ICD Directly Inhibits GSK3-Mediated β-catenin Phosphorylation.

The simplest model for LRP6 signaling is that it directly inhibits β-catenin phosphorylation by GSK3. Alternatively, LRP6-mediated inhibition of β-catenin phosphorylation may require additional components. To determine if LRP6ICD is sufficient to inhibit GSK3-mediated β-catenin phosphorylation, we tested whether we could reconstitute LRP6 signaling with purified components.

In a kinase assay with purified, recombinant proteins, LRP6ICD inhibits GSK3-mediated phosphorylation of β-catenin at P33/37/41 without inhibiting CK1's phosphorylation of β-catenin at P45 (Figure 2.9B, C, and D). Importantly, the concentration of LRP6ICD tested does not inhibit GSK3's phosphorylation of Tau in the same reaction, demonstrating that inhibition of β-catenin phosphorylation by LRP6ICD is

not a result of general inhibition of GSK3 activity (Figure 2.9B and C). Thus, LRP6ICD preferentially inhibits GSK3's phosphorylation of β-catenin in a kinase assay with purified components.

The ability of GSK3 to phosphorylate β-catenin independently of Axin (albeit inefficiently) in our purified system allowed us to test whether LRP6ICD inhibits GSK3's phosphorylation of β-catenin directly or indirectly (via a conformational change of Axin upon its binding to LRP6ICD). Significantly, we find that Axin is not required for LRP6ICD's inhibition of β-catenin P33/37/41 phosphorylation (Figure 2.9B). In addition, CK1g's phosphorylation plays a role in LRP6 signaling *in vivo* (Davidson et al., 2005), but CK1 is not required for LRP6ICD activity in our kinase assay (Figure 2.9B and D). These results demonstrate that LRP6ICD can directly inhibit GSK3-mediated phosphorylation of β-catenin and that this inhibition does not require other components.

Next, we tested whether LRP6 requires intact PPPSP motifs to inhibit GSK3's phosphorylation of β-catenin in our purified system. Unlike LRP6ICD, LRP6ICD(PPPAPX5) does not inhibit GSK3's phosphorylation of β-catenin (Figure 2.9D), demonstrating that LRP6's PPPSP motifs are required for LRP6 to inhibit β-catenin phosphorylation *in vitro*. In a kinase assay with recombinant proteins, GSK3 phosphorylates LRP6 in a manner that requires intact PPPSP motifs (data not shown and (Mi et al., 2006)). Thus, we infer that phosphorylation of PPPSP serines by GSK3 is required for LRP6's ability to inhibit β-catenin phosphorylation in our purified, reconstituted system. Our purified system exhibits specific properties that are consistent with *in vivo* and egg extract data: 1) requirement for PPPSP serines (Tamai et al., 2004; Zeng et al., 2005), 2) specificity for inhibition of β-catenin and not Tau phosphorylation

(Figure 2.9A), and 3) inhibition of β-catenin phosphorylation by GSK3 but not CK1 (Liu et al., 2002). Thus, we believe these studies recapitulate distinct properties of LRP6-mediated signaling *in vivo*.

LRP6ICD Associates with β-catenin *In Vivo*.

Given that LRP6ICD is sufficient to inhibit GSK3-mediated β-catenin phosphorylation in a kinase assay with purified proteins, we hypothesized that LRP6ICD may directly interact with β-catenin to prevent its GSK3-mediated phosphorylation. To determine whether β-catenin and LRP6ICD can interact in cultured mammalian cells, we performed Bimolecular Fluorescence Complementation (BiFC) between β-catenin and LRP6ICD (Hu et al., 2002). In this assay, interacting proteins that are fused to N- and C-terminal halves, respectively, of yellow fluorescent protein (YFP) bring the two halves of YFP in close enough association to produce a functional, fluorescent YFP molecule. BiFC-mediated fluorescence requires a relatively stable protein-protein interaction in the range of several seconds and detects direct or very close interactions within protein complexes (Hu et al., 2002). Similar to fluorescence energy transfer (FRET), BiFC indicates a potential for physical interaction in a cell. As a positive control, cells transfected with N- and C-terminal halves of YFP fused to separate Glutathione S-Transferase (GST) proteins (which have been shown to oligomerize) produce cytoplasmic YFP fluorescence in approximately 50% of cells (Figure 2.10). In contrast, none of the cells transfected with N-and C-terminal halves of YFP fused to LRP6ICD and GST, respectively, or fused to GST and β-catenin, respectively, produce any detectable fluorescent signal (Figure 2.10). In addition, the individual fusion proteins, when

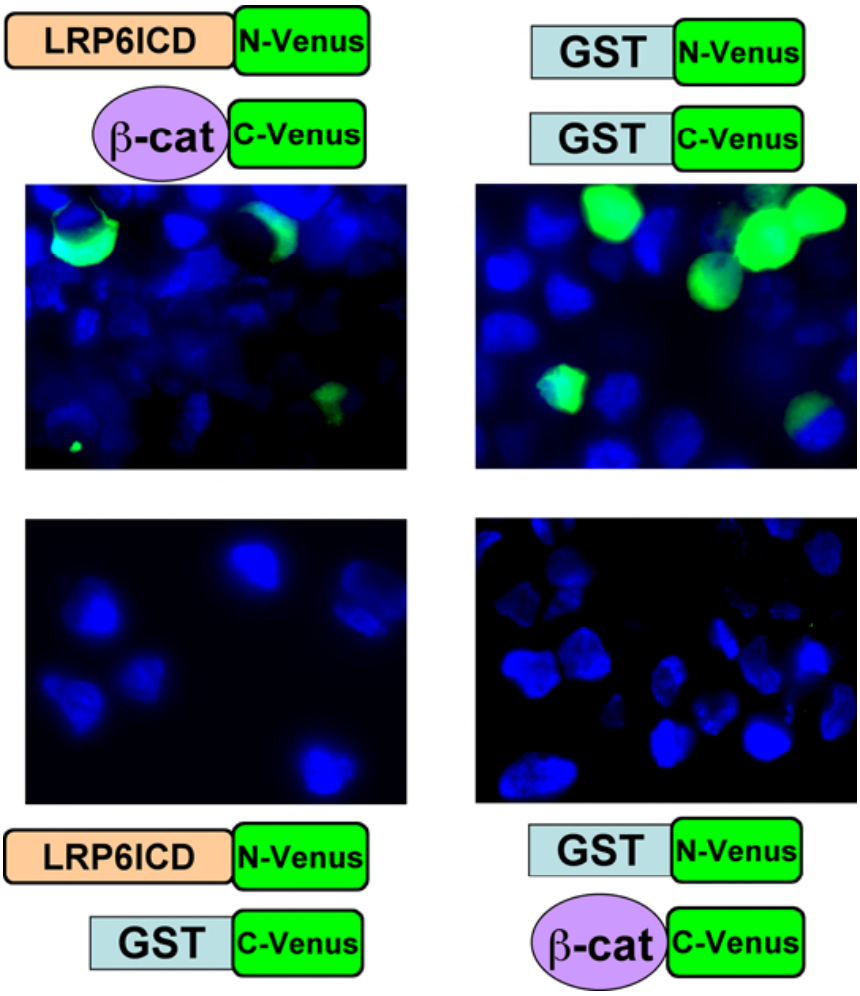


Figure 2.10: BiFC Analysis of LRP6ICD and β-catenin Interaction *In Vivo*. Fluorescence is detected in HEK293 cells transfected with plasmids encoding LRP6ICD-YN plus β-catenin-YC and GST-YN plus GST-YC (positive control). No fluorescence is detected in cells transfected with plasmids encoding LRP6ICD-YN plus GST-YC, β-catenin-YC plus GST-YN, or any of the individual fusion constructs. YN and YC represent the N-terminal (1-154) and C-terminal (155-238) halves of YFP, respectively. DAPI staining (blue) is used to visualize cell nuclei. This experiment was performed by K.K.J.

expressed in cells alone, do not produce fluorescence (data not shown). Importantly, cells transfected with N- and C-terminal halves of YFP fused to LRP6ICD and β-catenin, respectively, produce functional, fluorescent YFP in approximately 15% of cells (Figure 2.10). These results indicate that LRP6ICD and β-catenin form a stable interaction *in vivo* (likely within the Axin complex).

Discussion

We provide evidence that LRP6 can promote β-catenin stabilization independently of Axin degradation by inhibiting GSK3's phosphorylation of β-catenin. This mechanism is consistent with cultured cell experiments demonstrating Wnt-mediated stabilization of β-catenin in the absence of Axin degradation (Liu et al., 2005). Intriguingly, we find that LRP6 directly and specifically inhibits GSK3's phosphorylation of β-catenin *in vitro*, independently of Axin. It has been previously shown that addition of Wnt ligand to cultured mammalian cells rapidly induces recruitment of Axin to LRP5/6 (Mao et al., 2001b). We propose that this interaction between LRP5/6 and Axin serves to bring LRP5/6 in close proximity to β-catenin and GSK3, allowing for inhibition of β-catenin phosphorylation. Consistent with this hypothesis, we find that LRP6ICD and β-catenin can interact in cultured cells (Figure 2.10). We also find that LRP6 requires intact PPPSP motifs to directly inhibit GSK3's phosphorylation of β-catenin. Thus, we propose a working model (Figure 2.11). A Wnt signal induces GSK3's and CK1γ's phosphorylation of LRP5/6, which promotes the binding of Axin to LRP5/6 (Davidson et al., 2005; Zeng et al., 2005). Axin thereby brings β-catenin and GSK3 in close proximity to LRP5/6 where its phosphorylated PPPSP motifs are involved in

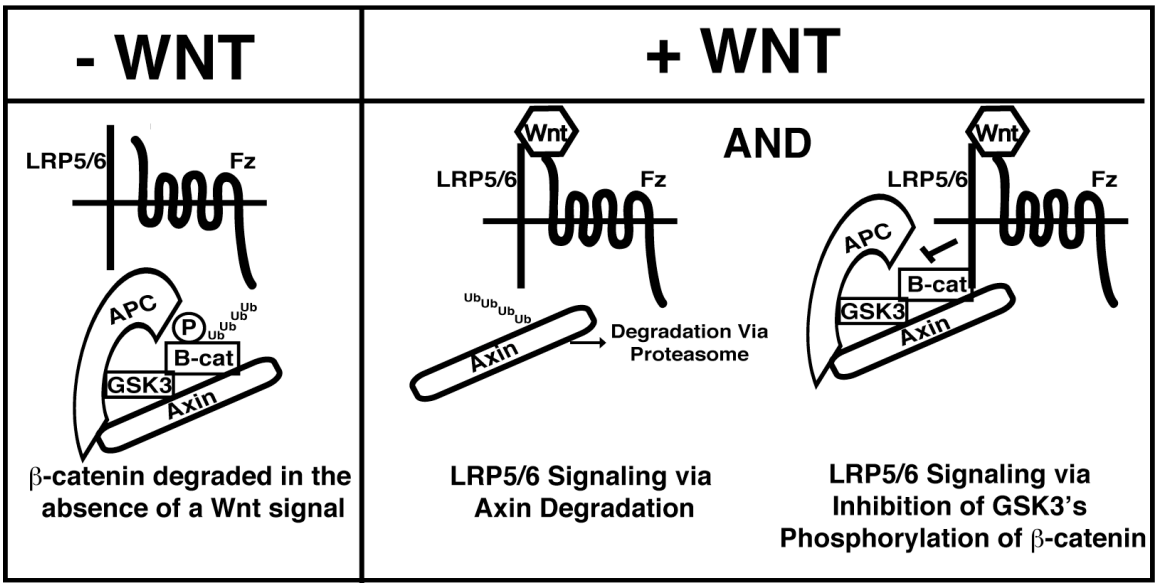


Figure 2.11. Our Model for the Mechanism by which LRP6 Stabilizes β-catenin. In the absence of a Wnt ligand, the destruction complex (Axin, APC, and GSK3) promotes phosphorylates of β-catenin, leading to its ubiquitin-mediated degradation. In the presence of a Wnt ligand, LRP6 promotes degradation of Axin, which may promote β-catenin stabilization by inhibiting destruction complex formation. Additionally, Wnt-activated LRP6 recruits and binds the destruction complex, allowing LRP6 to directly inhibit of GSK3's phosphorylation of β-catenin within the destruction complex.

mediating inhibition of GSK3's phosphorylation of β-catenin. Analysis of the molecular details of this interaction may help elucidate the mechanism by which LRP6 prevents β-catenin phosphorylation.

While the work described here was in press, Hendriksen *et al*. reported that dephosphorylated β-catenin accumulates at activated, phosphorylated LRP6 in response to canonical Wnt signaling in cultured mammalian cells (Hendriksen et al., 2008). This finding further supports our model in which Wnt-activated LRP6 directly inhibits GSK3's phosphorylation of β-catenin within the destruction complex at the plasma membrane (Figure 2.11). The results from Hendriksen *et al*. also argue against previously published models (Figure 2.1). In argument against LRP6 acting by binding Axin and sequestering it away from destruction complex components such as β-catenin, Hendrikson *et al*. find that Axin recruited to LRP6 in response to canonical signaling remains bound to β-catenin (Hendriksen et al., 2008). In argument against LRP6 acting solely by Axin degradation, Hendrikson *et al*. find that β-catenin phosphorylation and degradation are inhibited while β-catenin remains bound to Axin (Hendriksen et al., 2008). These findings by Hendrikson *et al*. also demonstrate that egg extracts and purified protein assays accurately recapitulate Wnt signaling mechanisms utilized in intact cells. Thus, we suggest that further use of the experimental systems described in our work will aid in elucidation of the precise mechanistic pathway by which LRP6 activates canonical Wnt signaling.

While some studies have shown that overexpression of the LRP5/6 intracellular domain alone is sufficient to promote canonical Wnt signaling (Mi et al., 2006; Mi and Johnson, 2005), other studies suggest that the LRP5 intracellular domain requires its

transmembrane domain or an N-terminal hydrophobic moiety (to promote membrane association) in order to activate the Wnt pathway (Mao et al., 2001b). In our experiments, LRP6ICD lacking such a motif is sufficient to induce ectopic Wnt/β-catenin signaling in *Xenopus* embryos and egg extract. Differences in the abilities of these LRP5/6 intracellular fragments to activate Wnt signaling may simply reflect differences in levels of expression and/or stability of the constructs rather than differences in localization.

Because Axin is the limiting factor in β-catenin destruction complex formation, we predict Axin degradation (though not required for all aspects of β-catenin stabilization) plays an important role in LRP5/6-mediated Wnt signal transduction (Lee et al., 2003). Thus, we suggest both LRP5/6-mediated inhibition of β-catenin phosphorylation and stimulation of Axin degradation contribute significantly to Wnt/β-catenin signaling (Figure 2.11). The existence of two mechanisms by which LRP5/6 mediates β-catenin stabilization may allow for more robust transduction of a Wnt signal. Furthermore, these two mechanisms are fundamentally different and could lead to distinct downstream responses. Regulation of the relative contributions of both mechanisms for stabilizing β-catenin would allow an organism to fine-tune sensitivity to Wnt signals for precise temporal and spatial control of tissue patterning. Moreover, it is likely that additional mechanisms not described here further contribute to the robustness and regulation of Wnt-mediated β-catenin stabilization (Liu et al., 2005; Yost et al., 1998).

Future Directions

What Are the Roles and Mechanisms of LRP6-Mediated Inhibition of GSK3's Phosphorylation of β-catenin?

Given our work in egg extract and *in vitro* kinase assays as well as the work of others showing that β-catenin's GSK3-mediated phosphorylation is inhibited on Axin at Wnt-activated LRP6 in cells (Hendriksen et al., 2008), the mechanistic basis of this phenomenon should be elucidated.

First, amino acids of LRP6ICD required for its inhibition of GSK3's phosphorylation of β-catenin should be identified. It has been shown that LRP6ICD acts in a modular fashion, such that five discrete PPPSP-containing regions contribute cooperatively to LRP6's activity. In support of this hypothesis, a single PPPSP-containing module retaining only 16 amino acids from LRP6 has been shown to promote canonical signaling in cultured cells and *Xenopus* embryos. Reconstitution of the activity of this 16 amino acid, single PPPSP-containing LRP6ICD fragment in egg extract and the *in vitro* kinase assay would significantly accelerate discovery of amino acids critical to LRP6ICD's inhibition of β-catenin phosphorylation. Thus, extensive mutation of conserved amino acids in this region can be undertaken to determine the molecular basis of this interaction. Study of these mutant fragments in the kinase assay with the purified mutant LRP6ICD fragment, GSK3, and β-catenin would provide a very specific, quantifiable readout of LRP6's inhibition of β-catenin phosphorylation. Study of the effects of these mutant fragments on β-catenin phosphorylation in egg extract may

support data from the *in vitro* kinase assay in a more biological context. Importantly, these LRP6ICD mutant fragments could be tested for their ability to bind Axin and promote Axin degradation in egg extract. Point mutants of single PPPSP-containing fragments of LRP6ICD that do not inhibit GSK3's phosphorylation of β-catenin in a kinase assay but do bind Axin and promote Axin degradation would be especially helpful in mechanistic studies because they would allow dissection of LRP6ICD's two major signaling activities: Axin degradation and inhibition of GSK3's phosphorylation of β-catenin. These LRP6ICD mutants that could promote Axin degradation without inhibiting GSK3's phosphorylation could be tested for their ability to stabilize β-catenin in egg extracts and promote Wnt-mediated transcription in *Xenopus* embryos and cultured cells. These experiments will be used to determine whether LRP6ICD's inhibition of GSK3-mediated β-catenin phosphorylation is required for Wnt signaling, which is an important, outstanding question in the study of Wnt signal transduction. Ideally, this question could be answered in a more biological, genetic context. Mutation of a key amino acid in a single PPPSP containing region could be applied to all of the remaining PPPSP modules to obtain a complete LRP6 that retains only the ability to promote Axin degradation without directly inhibiting β-catenin phosphorylation. Replacement of wild-type Arrow (or LRP5 and LRP6) with such a mutant Arrow (or LRP5 and LRP6) using homologous recombination in *Drosophila* (or mouse) could be used to determine whether LRP6's direct inhibition of β-catenin phosphorylation is necessary for canonical Wnt signal transduction in the context of development of a whole animal. Moreover, cell lines derived from such a mouse could be used to address more kinetic and mechanistic aspects of this question. Thus, mutation of LRP6ICD could

uncover its molecular basis for inhibition of β-catenin phosphorylation and its role in biology.

Second, the molecular basis for LRP6-mediated inhibition of GSK3's phosphorylation of β-catenin could be uncovered using mutants of β-catenin or GSK3. For example, LRP6 may directly bind β-catenin and affect its ability to be phosphorylated by GSK3. If this is the case, direct interaction between β-catenin and LRP6ICD could be assayed by immunoprecipitation experiments carried out with these recombinant proteins. If an interaction is detected, regions on β-catenin required for this interaction could be mapped and mutated to understand the molecular basis of this interaction. Identification of such a binding region may provide clues for how this binding event leads to inhibition of β-catenin's phosphorylation by GSK3. Alternatively, LRP6ICD may interact with GSK3 in a manner that affects its substrate specificity, interrupting its phosphorylation of β-catenin but not other substrates. Mutation of certain regions of GSK3 may be helpful in elucidating such a mechanism. As with LRP6ICD mutants, these mutations of β-catenin and GSK3 may affect LRP6ICD-mediated inhibition of GSK3 phosphorylation of β-catenin without inhibiting Axin degradation. Such mutants could be used to uncouple and study the role of these two mechanisms for LRP5/6 activity.

Third, structural studies of these proteins may directly reveal their mechanisms of action. As it has been suggested that a complex of LRP6ICD, Axin, β-catenin, and GSK3 is rather stable in cells, this complex may be reconstituted with recombinant proteins and used for crystallography studies to reveal the mechanism by which LRP6 directly inhibits GSK3's phosphorylation of β-catenin.

Lastly, identification of amino acids on LRP6, β-catenin, and GSK3 required for their functional interaction may have important medical applications. Such sites may be mutated in diseases affecting canonical Wnt signaling. In addition, a crystal structure of this functional complex could be used to initiate rational drug discovery to identify a small molecule inhibitor of LRP5/6-mediated inhibition of GSK3's phosphorylation of β-catenin.

What Are the Roles and Mechanisms of LRP6-Mediated Axin Degradation?

Our work has confirmed that LRP6 promotes degradation of Axin independently of GSK3's phosphorylation of Axin in a manner that likely requires LRP6/Axin binding. However, further work should be carried out to understand how LRP6 promotes Axin degradation. As we find that LRP6ICD promotes poly-ubiquitination of Axin, we can use the minimal Axin fragment that degrades in response to LRP6ICD in order to identify ubiquitinated lysines on Axin required for its degradation. As there are only about a dozen conserved lysines on this Axin fragment, each lysine can be mutated to determine which is necessary for Axin's LRP6ICD-mediated degradation in egg extracts. Notably, it may be necessary to mutate multiple lysines as there may be redundancy in this process. Alternatively, mass spectrometry of epitope-tagged Axin may be used to identify ubiquitinated lysines on Axin. Lysine mutants of Axin that prevent its LRP6ICD-mediated poly-ubiquitination should be tested for alterations in basal and GSK3 inhibition-mediated degradation. Preferably, a mutant that does not degrade in response to LRP6ICD but has a normal basal half-life and an unperturbed LiCl-mediated decrease in half-life can be identified. Replacement of wild-type Axin with such a

mutant of Axin in *Drosophila* (or Axin1 and Axin2 in mouse) by homologous recombination could be used to determine whether Axin degradation is required for canonical Wnt signaling in development of a whole organism. Cell lines from such a mouse could be used to answer mechanistic and kinetic aspects of this question. If canonical Wnt signaling is not inhibited or only partially inhibited by such mutations, LRP6's direct inhibition of GSK3's phosphorylation of β-catenin (or another mechanism) may be sufficient to promote Wnt signaling *in vivo*.

The mechanism and role of Axin degradation could also be explored by identification of an E3 ubiquitin ligase responsible for LRP6-mediated Axin poly-ubiquitination. Such an E3 could be identified by a mass spectrometry approach to identify Axin binding partners or by a functional loss-of-function screen to identify E3 ligases involved in Wnt signaling and Axin degradation. Mutation in a whole organism of such an E3 ligase for Axin can also be used to determine the role for Axin degradation in development. Of note, mutations that inhibit LRP6-mediated Axin degradation and mutations that inhibit LRP6-mediated inhibition of β-catenin phosphorylation can be combined to determine whether LRP6 may signal independently of both of these proposed mechanisms for LRP6-mediated Wnt signal transduction.

Identification of diseases with mutations of sites on Axin required for its LRP6-mediated degradation or mutations in Axin's E3 ligase may help uncover a molecular basis for certain human diseases. And, this work could be used to initiate discovery of drugs that could inhibit Wnt signaling by inhibiting degradation of Axin.

Thus, our work has suggested two mechanisms for LRP6-mediated activation of canonical Wnt signaling. Elucidation of the molecular basis for these mechanisms will

help determine the role they play in development and disease and may lead to improved diagnosis and treatment of diseases involving misregulation of canonical Wnt signaling.

BOOK 1: RECEPTER-MEDIATED ACTIVATION OF CANONICAL WNT SIGNALING

CHAPTER 3: THE ROLE OF HETEROTRIMERIC G PROTEINS IN CANONICAL WNT SIGNALING

Introduction

The Wnt co-receptors Frizzled (Fz) and low-density lipoprotein receptor-related receptor related proteins 5 or 6 (LRP5/6) bind Wnt ligands on the plasma membrane (reviewed in Logan and Nusse, 2004). Formation of an oligomer containing these proteins activates the Wnt/β-catenin, or canonical Wnt, signaling pathway. The mechanism by which Wnt- and LRP5/6-bound Fz transduces a signal is thought to occur through phosphorylation and activation of Dishevelled (Dsh) (Yanagawa et al., 1995; Zeng et al., 2008), which is required for phosphorylation and activation of the LRP5/6 intracellular domain (Bilic et al., 2007; Zeng et al., 2005). The phosphorylated LRP5/6 intracellular domain then promotes β-catenin stabilization by two mechanisms: direct inhibition of GSK3's phosphorylation of β-catenin and promotion of Axin degradation (Cselenyi et al., 2008; Mao et al., 2001b). In addition to this specific pathway, activation of Wnt co-receptors has other effects that promote Wnt/β-catenin signaling. Activated Dsh may bind GSK3 binding protein (GBP), which directly inhibits GSK3 activity within the β-catenin destruction complex (Yost et al., 1998). Activation of the receptor complex may also promote dissociation of the destruction complex (Liu et al., 2005). Wnt-dependent Ser9/Ser21 phosphorylation and inhibition of GSK3β/GSK3α may promote

Wnt/β-catenin signaling (Yokoyama et al., 2007b). In addition, it has been shown that Wnt-mediated activation of Rac1 plays a role in transporting β-catenin to the nucleus where it regulates transcription (Wu et al., 2008). Several other receptor-mediated mechanisms for Wnt/β-catenin signal transduction have been suggested. Thus, a great deal remains unknown regarding the complex mechanisms by which activated Wnt co-receptors stabilize β-catenin and lead to β-catenin-mediated transcriptional activity.

Fz is a seven-transmembrane, heptahelical protein with a predicted topology that is similar to heterotrimeric G protein coupled receptors (GPCRs) (Josefsson, 1999; Malbon, 2004). The human genome contains over 1,500 GPCRs that recognize a diverse set of ligands (Josefsson, 1999). Ligand-mediated activation of GPCRs promotes dissociation of the G protein heterotrimer into effector subunits, a Gα monomer and a Gβγ dimer (reviewed in Milligan and Kostenis, 2006; Smrcka, 2008). Specifically, ligand causes a conformational change of the GPCR which promotes dissociation of GDP from the Gα subunit to allow for binding of GTP and subsequent dissociation of the heterotrimer into a Gα monomer and a Gβγ dimer. Specific classes of Gα and Gβγ effectors promote highly diverse downstream signaling events. The Gα proteins may be classified into proteins of the Gαs (which stimulate cAMP production), Gαi (which inhibit cAMP production), Gαq/11 (which activate phospholipase C (PLC)), and Gα12/13 (which activate Rho family GTPases) families (Milligan and Kostenis, 2006). Importantly, many of these Gα proteins signal through multiple mechanisms that are distinct from those of their traditional family classifications. Of note, certain molecular processes modulated by effectors of Gα and Gβγ proteins have also been shown to

promote β-catenin stabilization, including cAMP production (Hino et al., 2005), PLC activation (Gao and Wang, 2007), and Rac1 activation (Wu et al., 2008).

There are several important implications for a role for G-proteins in Wnt/β-catenin signaling. The majority of drugs prescribed for human diseases target GPCRs (Jacoby et al., 2006). If Fz is a true GPCR, Fz may be an excellent target for drug discovery. For example, a drug that inhibits Fz's GPCR activity could be used to treat certain cancers caused by overactivation of Wnt/β-catenin signaling. Second, many important downstream responses in cells responding to a Wnt ligand occur within five minutes after incubation with Wnts. Dsh phosphorylation, recruitment of the destruction complex to LRP5/6, LRP5/6 phoshorylation, and inhibition of GSK3's phosphorylation of β-catenin all occur very rapidly in response to Wnts (Bryja et al., 2007; Zeng et al., 2008). GPCR-mediated dissociation of Gα and Gβγ and activation of their signaling targets can also occur extremely rapidly (Milligan and Kostenis, 2006), and Fz-induced heterotrimer dissociation could explain the mechanism behind immediate events that occur in Wnt signaling. Finally, elucidation of the effects of G proteins on β-catenin could suggest important crosstalk between events that activate GPCRs other than Fz and regulation of β-catenin activity in the cell. As a result, major questions in Wnt/β-catenin signaling are whether Fz is a *bona fide* GPCR and whether G proteins are involved in Wnt signaling.

Several lines of evidence support a model by which Fz affects Wnt signaling via GPCR activity. In *Drosophila*, Gαo (from the Gαi family) null cells in wing imaginal discs are deficient in transduction of a canonical Wnt signal (Katanaev et al., 2005). In this experimental system, overexpression of constituitively active GαoGTP, which

constituitively binds GTP, phenocopies Wnt signaling gain-of-function mutations (Katanaev et al., 2005). While these genetic data are promising, they do not address whether Gαo plays a direct, Fz-coupled role in Wnt signaling or whether Gαo couples to Wnt signaling indirectly through another GPCR.

Numerous experiments testing the role of G proteins in Wnt signaling have been performed in the murine F9 teratocarcinoma cell line. In this cell line, F9 cells differentiate into primitive endoderm in response to Wnt3a treatment. In F9 cells, Wnt3a-dependent differentiation is inhibited by pertussis toxin (PTX), which prevents heterotrimer dissociation via ADP ribosylation of Gα subunits of the Gαo and Gαi families (Liu et al., 1999b). Gαq and Gαo depletion also inhibit this process, suggesting that Wnt3a-mediated differentiation of F9 cells occurs through Gαo and Gαq (Liu et al., 1999b). To further study this process, a β2 Adrenergic Receptor-rat Fz1 chimera (β2AR-rFz1) was utilized that combined the extracellular and transmembrane regions of the β2AR and intracellular regions of Fz1 (Liu et al., 2001). Thus, a β2AR agonist could be added to cells in which the chimera was overexpressed to activate the intracellular regions of Fz1. While there are many caveats to the interpretations of experiments with such a chimeric protein, F9 cells overexpressing β2AR-rFz1 stabilized β-catenin and promoted β-catenin-dependent transcription in response to the β2AR agonist isoproterenol (Liu et al., 2001). As in endoderm differentiation, these β2AR-Rfz1-mediated events were PTX-sensitive and depended on Gαo and Gαq (Liu et al., 2001). Further work in F9 cells has led to a model in which Wnt-mediated activation of Fz activates Gαo and Gαq to promote PLC-dependent, casein kinase 2- (CK2) mediated Dsh phosphorylation, leading to β-catenin stabilization (Gao and Wang, 2006, 2007).

Nonetheless, the use of a single cell type and a chimeric β2AR-Rfz1 receptor in these studies significantly limits the interpretations that can be gleaned from these studies. Indeed, the majority of these data (except for a role for Gαo and Gαq) have not been replicated in other experimental systems.

A role for G proteins in canonical Wnt signal transduction has also been suggested in L929 and 3T3-L1 cells (Liu et al., 2005). In these cells, Wnt3a promotes a rapid dissociation of GSK3β from Axin1 and of GSK3α from Axin2 in a manner that is sensitive to PTX and dependent on Gαo and Gαq (Liu et al., 2005). This work further supports a role for Gαo and Gαq in canonical Wnt signaling by showing that rapid effects of recombinant Wnt3a (and not Wnt conditioned media which contains many other signaling ligands) on destruction complex integrity are G protein-dependent. Notably, the Axin-GSK3 dissociation event is correlated with β-catenin stabilization but not β-catenin-mediated transcription. Thus, an important caveat to these experiments is that these changes in destruction complex integrity and β-catenin stabilization may not be significant enough to affect Wnt-mediated transcription. It should also be noted that rapid Wnt-mediated changes in the composition of the destruction complex have not been reproduced in similar experiments (Bilic et al., 2007; Yamamoto et al., 2006).

In addition to the role of G proteins in canonical Wnt signaling, promising data supports a role for G proteins in non-canonical signaling. In zebrafish embryos and F9 cells, separate sets of Wnts and Fzs are involved in a pathway that proceeds through calcium flux and not β-catenin regulation (Ahumada et al., 2002; Liu et al., 1999c; Slusarski et al., 1997a). PTX and depletion of Gαt, Gαo, or Gβ2 inhibit Ca^{++}-mediated non-canonical signaling in these experimental systems (Ahumada et al., 2002; Liu et al.,

1999c). In *Drosophila*, overexpression of Gαo and GαoGTP promote planar cell polarity (PCP) (another non-canonical Wnt signaling pathway) phenotypes (Katanaev et al., 2005). Notably, Gαo activity but not GαoGTP activity requires Fz in this system, suggesting functional coupling between Fz and Gαo in PCP signaling. While these data suggest that both canonical and noncanonical Wnt signaling may proceed through G proteins, studies of G proteins and Wnt signaling should be interpreted even more cautiously and be controlled for crosstalk-mediated effects between canonical and non-canonical Wnt signaling pathways. Overall, experimental evidence from *Drosophila* and cultured mammalian cells supports the possibility of a role for G proteins (especially Gαo and Gαq) in canonical Wnt signaling; however, the above data does not address whether Fz activates Wnt/β-catenin signaling via GPCR activity.

Although Fz is a seven-transmembrane spanning receptor, significant divergence of Fz's primary structure from *bona fide* GPCRs leads to its special classification with the Hedgehog pathway protein Smoothened (Smo) (Josefsson, 1999). As it has been shown that many GPCRs have signaling properties that are independent of G proteins (Sun et al., 2007), it is possible that both Fz and Smo are evolutionarily derived from ancestral GPCRs but have subsequently lost the ability to promote heterotrimer dissociation. Similar to Fz, promising though preliminary studies addressing the role of G proteins in Hedgehog signaling have been performed (Riobo et al., 2006; Ruiz-Gomez et al., 2007). While work from several groups has suggested that Fz is a GPCR, definitive experiments have not been performed. Specifically, it has not been established that Fz promotes heterotrimer dissociation. Dissociation could be assayed traditionally via [γ-32 P]GTP hydrolysis experiments in membranes from Fz-expressing or Fz null *Drosophila* S2 cells

incubated with recombinant Wg protein (Cassel and Selinger, 1976). Alternatively, it could be tested whether Fz-Wnt affinity is modulated by incubation of cells with GTPγs (Guanosine 5'-(3-O-thio) triphosphate, a non-hydrolyzable form of GTP); such a shift in agonist binding in response to GTPγs is a characteristic common to all tested GPCRs (Maguire et al., 1976). Fluorescence resonance energy transfer (FRET) could also be performed with candidate Gα and Gβγ subunits to assay for recombinant Wnt-dependent heterotrimer dissociation (Janetopoulos et al., 2001). If indeed Fz promotes heterotrimer dissociation and the specific Gα and Gβγ proteins are identified, loss-of-function of these G proteins should lead to inhibition of Wnt/β-catenin signaling. Such an experiment must be controlled to eliminate Fz-independent roles of these G proteins in Wnt/β-catenin signaling. Specifically, it would be ideal to show that overexpression of constituitively active GαGTP displays a reduced reliance on Fz compared with overexpression of wild-type Gα (Katanaev et al., 2005). Thus, significant work remains in elucidating whether Fz acts as a GPCR in Wnt signaling.

It is important to note that G proteins may affect canonical Wnt signaling in a biologically and therapeutically significant way even if they do not couple to Fz's potential GPCR activity. Indeed, G proteins activated by other GPCRs may modulate β-catenin activity in important ways. For example, it has been suggested that Prostaglandin E2 stablizes β-catenin through Gαs signaling (Castellone et al., 2005). Also, gonadotropin-releasing hormone and parathyroid hormone are two hormones that bind their own GPCRs and modulate β-catenin levels (Gardner et al., 2007; Tobimatsu et al., 2006). It is likely that these G protein pathways impinge on Wnt signaling in ways that are relevant to development, physiology, and disease. In addition to evaluating Fz as a

GPCR, it will be important to uncover how other G protein signaling events regulate Wnt/β-catenin signaling (Figure 3.1).

To identify potential G proteins with a role in regulation of β-catenin stability, we added purified Gα and Gβγ subunits to *Xenopus* egg extract and assayed for modulation of β-catenin stability. We find that Gαo, Gαi, Gαq, and Gβγ may play roles in regulating β-catenin levels and address their mechanisms of action.

Methods

Xenopus Egg Extract and β-catenin Degradation Assays

Xenopus egg extract was prepared and degradation assays were performed as described (Salic et al., 2000). Dsh immunodepletions were performed and confirmed as described in (Salic et al., 2000). *Xenopus* egg extract was incubated with an equal volume of Protein A-Affiprep beads (BioRad) bound to Dsh polyclonal antibodies. Incubation was performed at 4°C for 2 hr with inversion every 10 min.

Heterotrimer Dissociation

Gαβγ heterotrimer was dissociated via incubation with AlF_4^- as previously described with modifications (Sternweis and Gilman, 1982). Undissociated Gαβγ was incubated with 25 mM NaF, 0.1 mM $AlCl_3$, and 10 mM $MgCl_2$ for 45 min on ice before addition to egg extract.

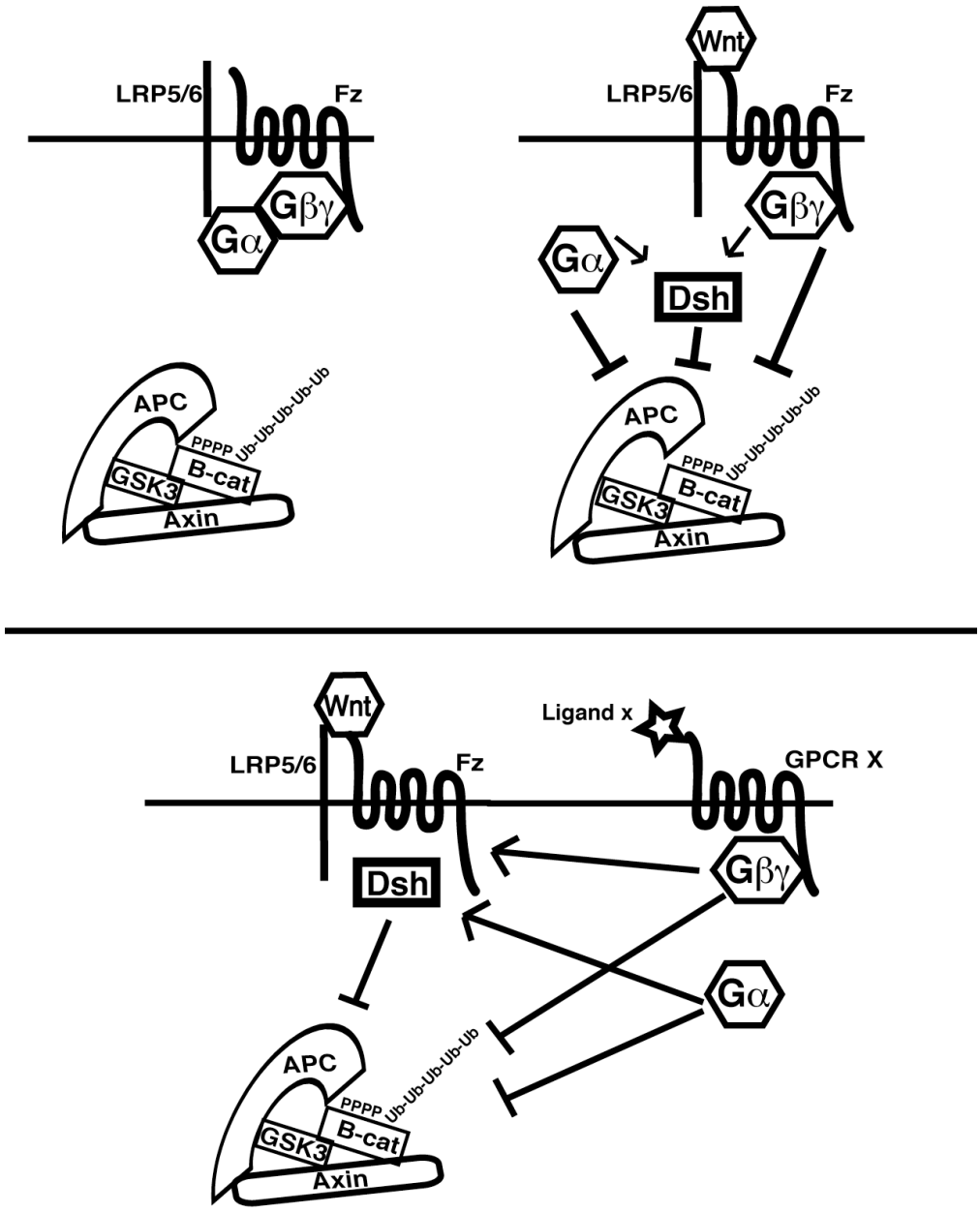


Figure 3.1: G Proteins May Directly or Indirectly Influence Wnt/β-catenin Signaling. (Top Panel) This schematic describes a mechanism whereby Fz acts as a Wnt-activated GPCR that promotes heterotrimer dissociation in a manner that leads to destruction complex inhibition. Gα or Gβγ may activate Dsh or directly inhibit destruction complex activity. (Bottom Panel) This schematic describes an indirect mechanism whereby activation of a GPCR other than Fz indirectly promotes Wnt-mediated destruction complex inhibition. Gα or Gβγ may directly inhibit the destruction complex or activate the Wnt receptor complex.

Immunoblotting

Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted. Bands were visualized using horseradish peroxidase-conjugated secondary antibodies and SuperSignal West Pico or Femto Chemiluminescent Substrate (Pierce). For reblotting, membranes were stripped by incubation in NaOH (0.4 M) for 15 min followed by 15 min in H_2O and reblocking.

β-catenin P33/37/41 antibody was purchased from Cell Signaling [1:1,000 kinase assay] [1:250 egg extract]. N-termal *Xenopus*-β-catenin antibody was a generous gift from Barry Gumbiner [1:3,000]. αTubulin was blotted with DM1α (Sigma) [1:5,000 dilution]. Axin antibody for immunoblot was purchased from R & D (Anti-human/mouse/rat Axin 1) [1:100]. Antibodies for Dsh and Axin immunopreipitation and immunoblotting were described previously (Salic et al., 2000) [1:100]. GSK3 was blotted with IH8 (Affinity Bioreagents) [1:500]. GSK3Ser9 was blotted anti-GSK3-Ser9 (Cell Signaling) [1:500]. Gβ was immunoprecipitated and blotted with S-13 (Santa Cruz) [1:300].

Immunoprecipitation

For IP, egg extract (50 μl) was incubated with or without G protein and IVT β-catenin (3 μl) for 45 min. Buffer A (700 μl) was added to extract with Protein A beads covalently conjugated to Axin, Dsh, or Gβγ antibody followed by 2 hr shaking at 4°C. Beads were washed with Buffer A (4 ml), eluted with sample buffer, and analyzed by SDS-PAGE and immunoblot.

Kinase Assay

Gαo (1 μM), Gαs (1 μM), GSK3 (0.79 μM) (NEB), and CK1 (1.37 μM) (NEB) were pre-incubated with 500 μM ATP and kinase buffer (20 mM HEPES (pH 7.5), 300 mM NaCl, 2 mM DTT, 1 mM EDTA, 10 mM $MgCl_2$, and 0.2% Tween 20) for 10 min at RT. His_6-β-catenin (0.22 μM) was then added and samples were removed for immunoblotting after 45 min at RT.

RNAi in S2R+ Cells

Fifty thousand cells were plated in wells of a 96-well plate in 50 μl serum-free media and left to adhere at RT for 1 hr. One microgram dsRNA per gene was then added to wells, and cells were incubated at RT for 30 min. dsDNA was prepared by *in vitro* transcription of linear dsDNA by PCR of genes from the *Drosophila* Gene Collection (DGC) with primers containing flanking T3 and T7 promoters. One hundred microliters serum-containing Schneider's media was added, and cells were incubated for 4 days at 27° C. After this period, 150 μl of serum- and Wg-containing media was added to cells for 1 day. After this period, media was removed and 75 μl Passive Lysis Buffer was added. Following 15 min of vigorous shaking, 20 μl was removed for cell titer and 40 μl for luciferase (SteadyGlo, Promega). Luciferase SuperTOPFlash signal was normalized to cell titer, and all experiments were performed in triplicate and averaged as displayed.

Results

Dissociation of the G Protein Heterotrimer Stabilizes β-catenin in *Xenopus* Egg Extract.

Previous work has shown that many molecular and kinetic aspects of cytoplasmic β-catenin regulation are recapitulated by radiolabeled, *in vitro* translated β-catenin in cytoplasmic *Xenopus* egg extract (Salic et al., 2000). To determine whether G proteins affect β-catenin stability in egg extract, we purified a G protein heterotrimer from porcine brain using previously described purification protocols (Milligan and Klee, 1985). The vast majority of Gα purified through this method has been shown to be Gαo (Gierschik et al., 1986), and we confirmed that the major Gα subunit in our purification was Gαo by immunoblot (Kristin Kalie Jernigan (KKJ), unpublished results). In addition, the remaining two major proteins from our purification were confirmed to be Gβ and Gγ by immunoblot (KKJ, unpublished results). Because this purified heterotrimer is present as an inactive, non-dissociated trimer that would not be predicted to activate Gα- and Gβγ-mediated signaling, we performed *in vitro* activation of Gαo and Gβγ via incubation with GDP-AlF_4^- to recapitulate GPCR-mediated heterotimer dissociation and activation (Sternweis and Gilman, 1982). Consistent with activation and trimer dissociation being required for G protein signaling, the inactive, non-dissociated G protein heterotrimer does not affect β-catenin stability in egg extract (Figure 3.2). However, the GDP-AlF_4^- dissociated Gα and/or Gβγ subunits significantly inhibit β-catenin degradation in egg extract (Figure 3.2). In this experiment, the inactive, non-dissociated trimer is an important control that suggests that the heterotrimer must be dissociated through an event

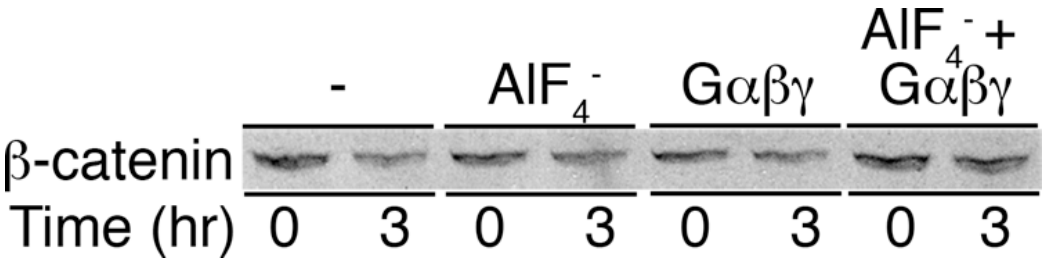


Figure 3.2: Dissociation of Gαβγ into Gαo and Gβγ Stabilizes β-catenin in Egg Extract. AlF_4^- treated Gαβγ (0.4 mg/ml), but not intact Gαβγ or AlF_4^- inhibits degradation of radiolabeled, IVT β-catenin in *Xenopus* egg extract.

such as GPCR activation to affect β-catenin. Thus, similar to the characterized Wnt pathway activators LRP6ICD (a constituitively active fragment of LRP6) and LiCl (a GSK3 inihibitor) (Cselenyi et al., 2008; Salic et al., 2000), dissociated Gαo and/or Gβγ stabilize β-catenin and suggest that either or both of these G proteins may play a role in canonical Wnt signaling. These data suggest two possible models: a direct "Fz as GPCR" model in which Wnt-activated Fz promotes trimer dissociation leading to β-catenin stabilization, or a "signaling crosstalk" model in which an unrelated, activated GPCR promotes trimer dissociation leading to β-catenin stabilization (Figure 3.1). Thus consistent with a role for GPCR-mediated G protein signaling in Wnt/β-catenin signaling, we find that dissociated Gαo and Gβγ but not intact Gαβγ stabilizes β-catenin in egg extract.

To determine whether Gαo or Gβγ purified from porcine brain affects β-catenin stability in egg extract, we tested whether recombinant Gαo or Gβγ stabilize β-catenin in *Xenopus* egg extract. Interestingly, each recombinant protein independently inhibited β-catenin degradation (Figure 3.3A and B), suggesting that Gαo and Gβγ may each play a role in regulating Wnt/β-catenin signaling. While Gαo has been suggested to play a positive role in canonical Wnt signal transduction, to our knowledge there have been no reports identifying a role for Gβγ in Wnt/β-catenin signaling.

Gαo, Gαi, Gαq, and Gβγ Stabilize β-catenin by Inhibiting its Phosphorylation by GSK3.

To further identify which Gα proteins may be involved in Wnt signaling, we tested whether other Gα subunits affect β-catenin degradation in *Xenopus* egg extract.

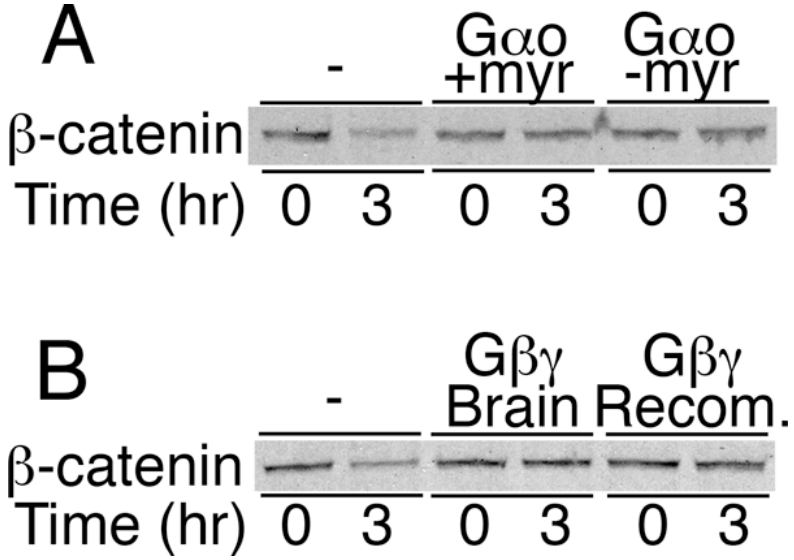


Figure 3.3: Gαo and Gβγ Each Stabilize β-catenin in Egg Extract. (A) Addition of bacterially expressed recombinant, myristoylated Gαo (5 μM) inhibits degradation of radiolabeled IVT β-catenin in egg extract. Non-myristoylated Gαo also stabilizes β-catenin and may be endogenously myristoylated in egg extract. (B) Porcine brain purified and dissociated Gβγ (7 μM) and SF9-expressed recombinant Gβγ both inhibit degradation of radiolabeled, IVT β-catenin in egg extract.

Gαo, Gαi1, Gαi2, Gαi3, Gαq, Gα11, Gα12, Gα13, and Gαt were expressed as recombinant proteins in bacteria or insect cells or were purified from tissue sources via conventional methods (KKJ, unpublished results). Their activities were confirmed by previously described methods. Addition of Gαo, Gαi1, Gαi2, and Gαq to *Xenopus* egg extract robustly stabilized β-catenin (KKJ, unpublished results), suggesting that these Gα proteins may play roles in Wnt/β-catenin signaling. Interestingly, overlapping experimental evidence from other groups also identified roles for Gαo and Gαq in canonical Wnt signaling. To our knowledge, Gαi has not been implicated in Wnt signaling; however, it should be noted that Gαo is in the same family (Gαi family) as Gαi1, 2, and 3 (Milligan and Kostenis, 2006). Our screen for Gα proteins involved in β-catenin regulation identified the two previously described Gα proteins suggested to be involved in Wnt signaling. We believe these findings validate our screen and confirm the utility of *Xenopus* egg extract in analyzing the role of G proteins in Wnt signaling. Overall, our studies confirm roles for Gαo and Gαq in canonical Wnt signaling and suggest novel roles for Gαi and Gβγ in regulation of β-catenin stability.

To identify how Gαo, Gαi, and Gαq stabilize β-catenin, we hypothesized that they could act either upstream or downstream of β-catenin's phosphorylation by GSK3 at Ser33/37/41 which is required for binding to the E3 ubiquitin ligase β–TRCP and subsequent poly-ubiquitination and degradation (Logan and Nusse, 2004). We find that Gαo activated by binding non-hydrolyzable GTPγs (GαoGTPγs) and Gβγ prevent GSK3's phosphorylation of β-catenin (Figure 3.4A and B). As has been previously described, LRP6ICD and the GSK3 inhibitor LiCl also inhibit β-catenin phosphorylation in this assay in *Xenopus* egg extract (Figure 3.4A) (Cselenyi et al., 2008). We next

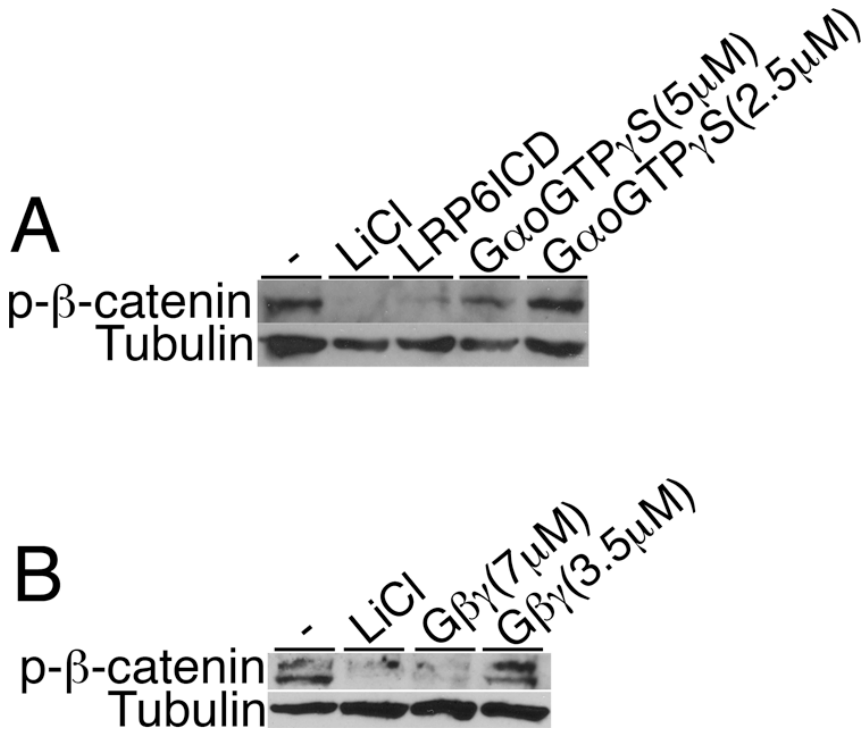


Figure 3.4: Gαo and Gβγ Each Inhibit GSK3's Phosphorylation of β-catenin in *Xenopus* Egg Extract. (A) Incubation of *Xenopus* egg extract with LiCl (50 mM), LRP6ICD (1.6 μM), or GαoGTPγS (5 μM, but not 2.5 μM) inhibits Ser33/37/41 phoshorylation of β-catenin. (B) Incubation of *Xenopus* egg extract with LiCl (50 mM) or porcine brain purified Gβγ (7 μM, but not 3.5 μM) inhibits Ser33/37/41 phoshorylation of β-catenin.

tested which G proteins inhibit GSK3's phosphorylation of β-catenin in *Xenopus* egg extract. Intriguingly, we find that all of the G proteins that stabilize β-catenin act at or upstream of the level of GSK3 phosphorylation (Figure 3.5). In addition, some of the proteins affect β-catenin phosphorylation within 30 minutes (Gαo and Gαi) while effects with others (Gαq) are not seen until after 2 hours of incubation in egg extract (data not shown). In addition to their shared membership in the Gαi family, the kinetic similarity of results with Gαo and Gαi may suggest that they work by a common mechanism that is distinct from that of Gαq. These data confirm our results from the β-catenin degradation assays and suggest a mechanism by which these proteins regulate β-catenin.

Gαo Directly Inhibits GSK3's Phosphorylation of β-catenin.

Most studies implicating a role for G proteins in Wnt/β-catenin signaling have focused on Gαo. Because there is most evidence in favor of a role for Gαo in Wnt signaling, we decided to focus on the mechanism by which Gαo inhibits β-catenin phosphorylation and degradation in biochemically amenable egg extract. Because Fz is required for Wnt-mediated phosphorylation and activation of Dsh (Gonzalez-Sancho et al., 2004), we hypothesized that Fz-dependent activation of Gαo may promote Dsh-mediated β-catenin stabilization. To test whether Gαo acts upstream of Dsh in β-catenin stabilization, we examined whether immunodepletion of endogenous Dsh prevents Gαo-mediated β-catenin stabilization (Salic et al., 2000). Surprisingly, depletion of endogenous Dsh does not prevent Gαo from stabilizing β-catenin (Figure 3.6A), suggesting that Fz-mediated activation of Dsh and Gαo may be mechanistically independent events.

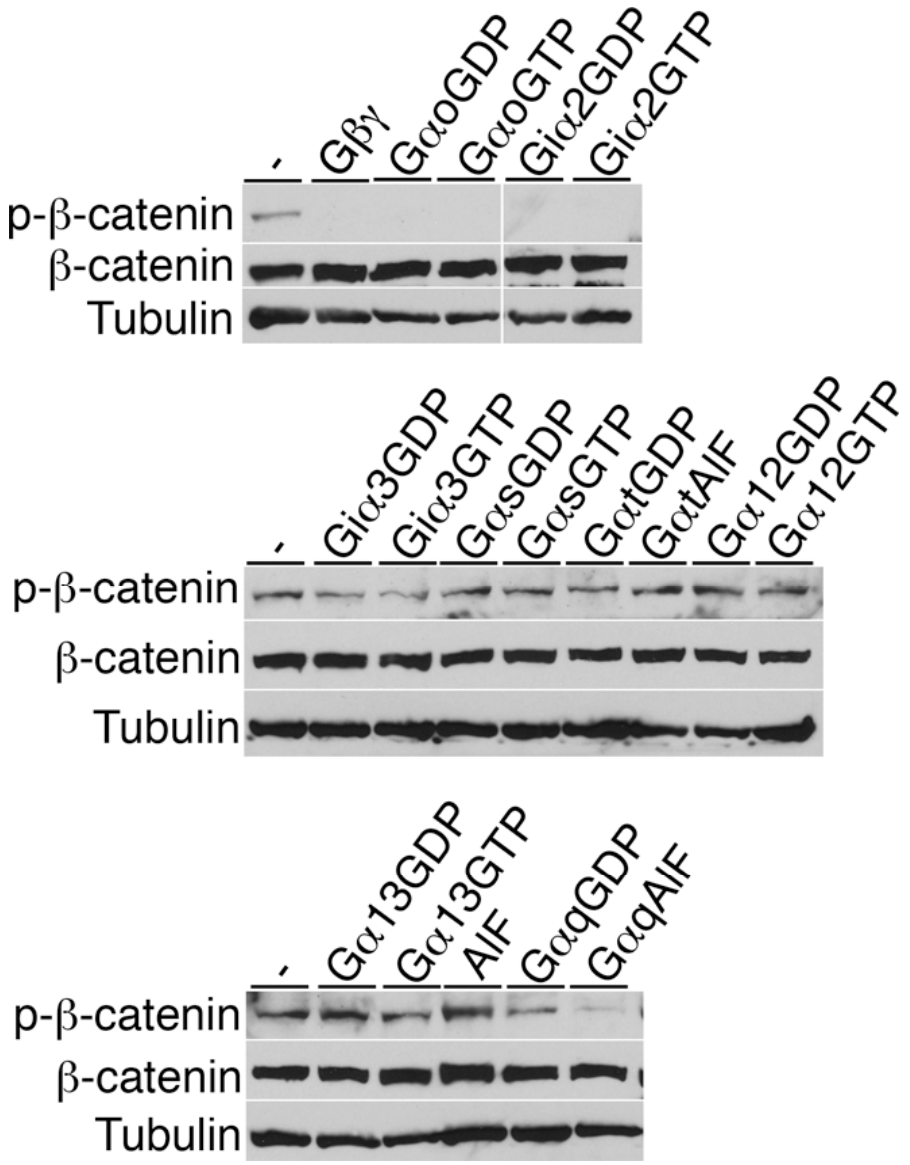


Figure 3.5: Gαo (5 μM), Gαi2 (7 μM), Gαi3 (5 μM), Gαq (0.5 μM), and Gβγ (7 μM) (but Not Gαs (5 μM), Gαt (5 μM), Gα12 (5 μM) and Gα13 (5 μM)) Inhibit GSK3's Phosphorylation of β-catenin in *Xenopus* Egg Extract. G proteins were incubated in extract for 2 hr, which were blotted for endogenous β-catenin. G proteins were obtained through methods described by KKJ, unpublished results. Because GDP forms of these proteins also stabilize β-catenin in egg extract, we predict that these proteins exchange GDP for GTP in egg extract.

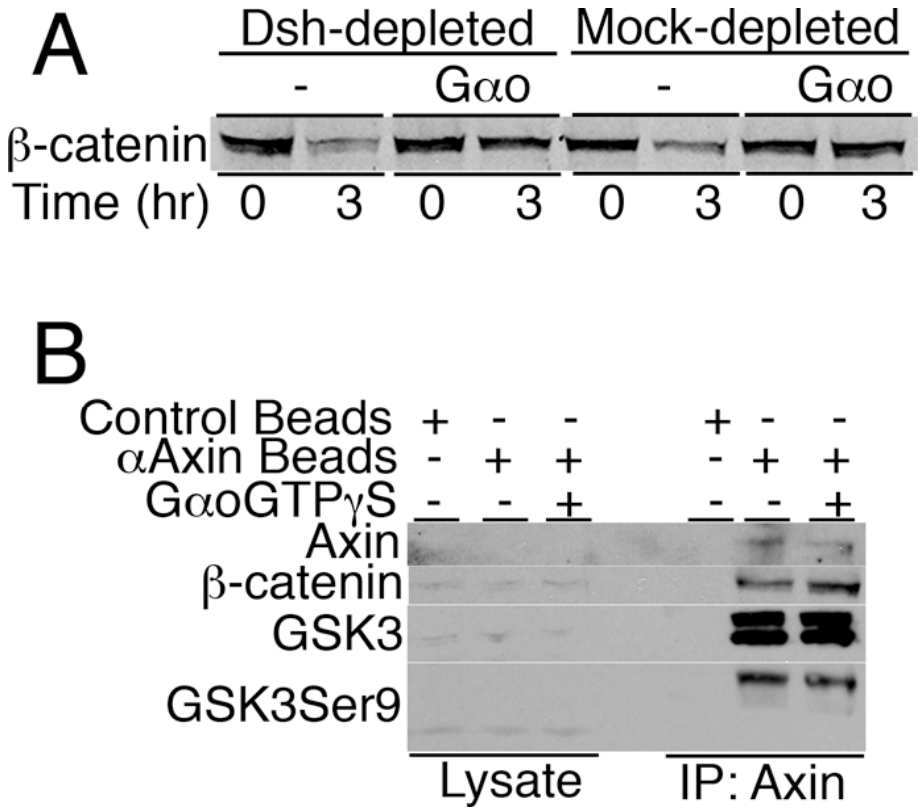


Figure 3.6: Gαo-Mediated β-catenin Stabilization Does Not Require Dsh or Involve Dissociation of the β-catenin Destruction Complex. (A) Gαo (5 μM) stabilizes β-catenin in mock- and Dsh-depleted egg extract. Depletion was confimed by immunoblot. (B) The amounts of Axin, β-catenin, GSK3, and GSK3Ser9 immunoprecipitated with endogenous Axin do not change when extract are incubated with Gαo (5 μM).

Previous work has suggested that Gαo stabilizes β-catenin by promoting dissociation of the destruction complex (Liu et al., 2005). Specifically, Gαo loss-of-function inhibits Wnt-mediated dissociation of Axin from GSK3. To test whether Gαo perturbs Axin's binding partners, we immunoprecipitated endogenous Axin from *Xenopus* egg extract in the presence or absence of a concentration of Gαo that stabilizes β-catenin. Importantly, we find that Gαo does not affect the amount of endogenous β-catenin, GSK3, or Axin that is immunoprecipitated by endogenous Axin (Figure 3.6B). Although we cannot rule out transient or subtle changes in destruction complex integrity, we are unable to detect Gαo-mediated dissociation of the β-catenin destruction complex.

Given that Gαo inhibits GSK3's phosphorylation of β-catenin independently of Dsh and destruction complex dissociation, we explored whether we could reconstitute Gαo's effects on β-catenin phosphorylation in a kinase assay using purified components. Interestingly, we find that in a kinase assay with recombinant β-catenin, GSK3, and Gαo; Gαo directly inhibits GSK3's phosphorylation of β-catenin (Figure 3.7). While this surprising result requires further analysis, it suggests that Gαo may bind and affect the conformation and activity of β-catenin or GSK3. As none of these functions have been previously attributed to Gαo, this experiment suggests a possible novel mechanism for Gαo signaling. Thus, we find that Gαo does not act upstream of Dsh or promote dissociation of the destruction complex; instead, Gαo may directly inhibit GSK3's phosphorylation of β-catenin.

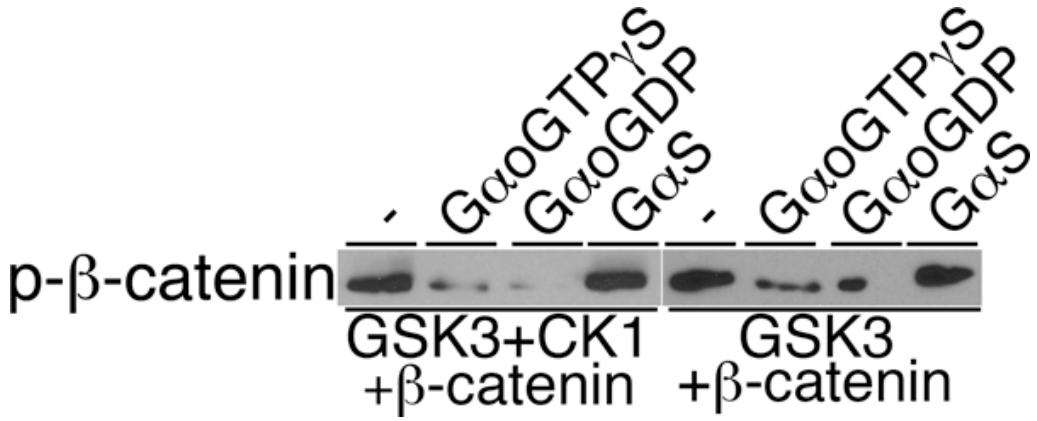
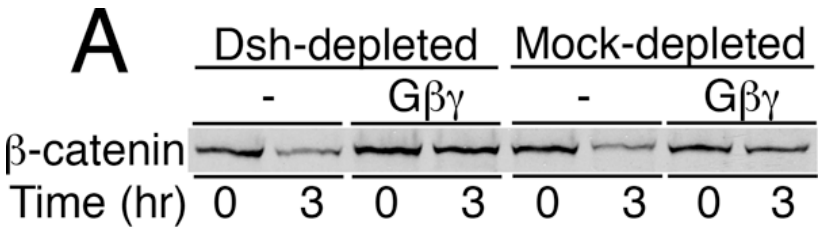


Figure 3.7: In a Kinase Assay with Purified, Recombinant Components, Gαo Directly Inhibits GSK3's Phosphorylation of β-catenin. Gαo, but not Gαs, inhibits phosphorylation of recombinant β-catenin in a kinase assay with GSK3 and CK1 or GSK3 alone.

Gβγ Inhibits Destruction Complex Activity.

Given the novelty and importance of a possible role for Gβγ in canonical Wnt signaling, we investigated our finding that Gβγ inhibits β-catenin phosphorylation and degradation. To analyze Gβγ activity in *Xenopus* egg extract, we utilized porcine brain-derived Gβγ that was dissociated and purified from Gαo. We confirmed that activity from this purification was attributable to Gβγ by expressing and purifying recombinant Gβγ. Both tissue-derived and recombinant Gβγ stabilize β-catenin in *Xenopus* egg extract (Figure 3.3B), suggesting that activity of brain-derived Gβγ truly results from Gβγ activity and not a contaminating protein from the purification. In the following experiments, only brain-derived Gβγ is utilized because this purification protocol allowed us to obtain larger quantities for analysis.

Because of the requirement of certain Fz residues for Dsh activation (Cong et al., 2004) and previous work suggesting binding between Gβγ and Dsh (Angers et al., 2006), we tested whether Gβγ activity requires Dsh in egg extract. As is the case for Gαo, Gβγ stabilizes β-catenin in *Xenopus* egg extract in which Dsh has been depleted (Figure 3.8A). This suggests that Gβγ likely acts independently of Dsh in Wnt/β-catenin signaling. We also tested whether Gβγ binds proteins of the destruction complex. Results from experiments in which endogenous or exogenous Gβ is immunoprecipitated from egg extract suggest that Gβγ may specifically bind Dsh as well as slowly migrating forms of GSK3 such as GSK3β(phospho-Ser9) (Figure 3.8B). Significant binding with β-catenin or LRP6ICD was not detected (Figure 3.8B). Although this experiment must be performed with antibody-free control beads to determine the extent of background binding between Gβγ and Dsh or GSK3, preliminary analysis suggests that endogenous



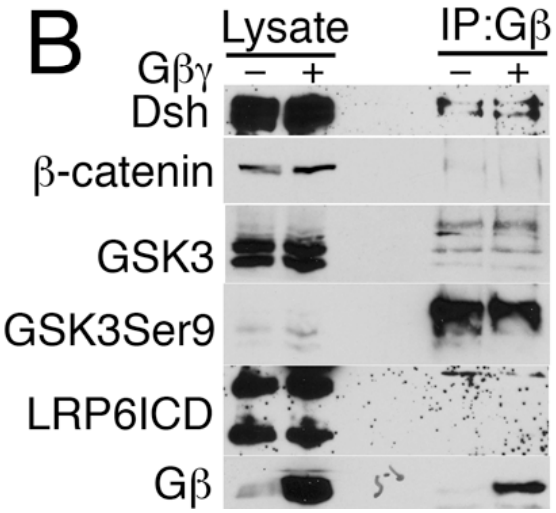


Figure 3.8: Gβγ Does Not Require Dsh to Stabilize β-catenin and May Bind Dsh and GSK3Ser9 in *Xenopus* Egg Extract. (A) Gβγ (7 μM) stabilizes β-catenin in mock- and Dsh-depleted egg extract. Depletion was confimed by immunoblot. (B) Endogenous or exogenous Gβγ (7μM) was immunoprecipitated from egg extract and blotted for Dsh, β-catenin, GSK3, LRP6ICD, and Gβγ. Importantly, the amount of Gβγ detected is increased in extract supplemented with exogenous β-catenin. An important caveat to conclusions drawn from this figure is that this experiment does not reveal the amount of protein immunoprecipitated by the Protein A agarose beads alone.

and exogenous Gβγ may bind Dsh and phosphorylated Ser9-inactivated GSK3. It is interesting to note that Gβγ may bind Dsh but not require Dsh for activity in egg extract. This discrepancy could result from a functional interaction that is required in intact cells but not in extract; alternatively, this binding event may be non-functional in Wnt/β-catenin signaling. The binding between Gβγ and phospho-Ser9 inactivated GSK3 may suggest that Gβγ may play a role in inactivating GSK3 within the destruction complex, leading to inhibition of β-catenin phosphorylation and degradation. Thus, further work should characterize potentially functional Gβγ interactions with proteins of the destruction complex.

As a result of promising physical interactions between the destruction complex and Gβγ, we tested whether Gβγ promotes dissociation of certain destruction complex components. In experiments in which endogenous Dsh or Axin is immunoprecipitated from egg extract, Gβγ does not appear to disrupt several known binding partners within the destruction complex (Figure 3.9). Thus, we do not detect Gβγ-mediated changes in physical associations within the destruction complex.

Because we have previously characterized effects of LRP6ICD in *Xenopus* egg extract, we thought it was possible that these proteins could synergize in the transduction of a Wnt/β-catenin signal (Cselenyi et al., 2008). Thus, we tested whether addition of Gβγ affected LRP6ICD signaling in extract. To our surprise, we found that Gβγ did not affect LRP6ICD's ability to stabilize β-catenin but strongly inhibited LRP6ICD's ability to promote degradation of Axin (Figure 3.10). The most straightforward interpretation of these results is that Gβγ inhibits phosphorylation and degradation of β-catenin within the destruction complex (independently of Axin degradation) in a manner that prevents

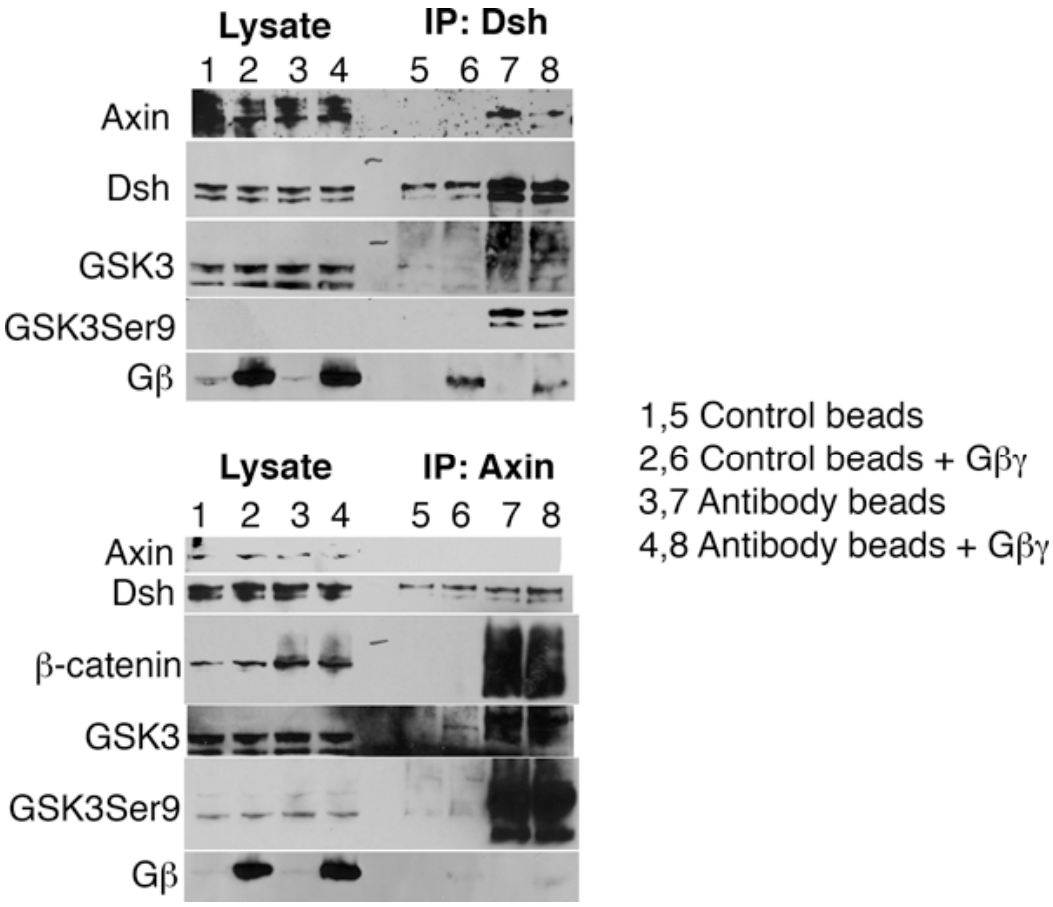


Figure 3.9: Gβγ Does Not Appear to Promote Dissociation of the β-catenin Destruction Complex in *Xenopus* Egg Extract. In egg extract in which endogenous Dsh is immunoprecipitated, addition of Gβγ (7 μM) does not change the amount of Dsh bound to GSK3. Although decreases in the amount of Dsh bound to Dsh or Axin appears to decrease, this result is not repeated in the converse experiment shown below. In egg extract in which endogenous Axin is immunoprecipitated, addition of Gβγ (7 μM) does not change the amount of Dsh bound to Dsh, β-catenin, or GSK3.

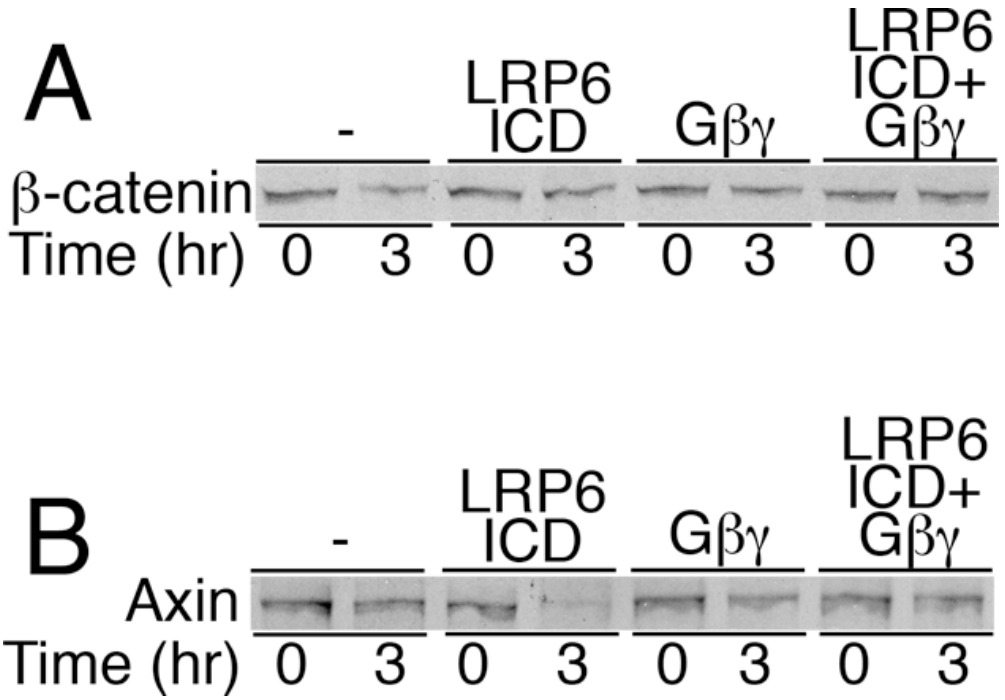


Figure 3.10: Gβγ Inhibits LRP6ICD-Mediated Axin Degradation in *Xenopus* Egg Extract. (A) In egg extract, incubation with LRP6ICD (1.6 μM), Gβγ (7 μM), or both LRP6ICD (1.6 μM) and Gβγ (7 μM) leads to stabilization of radiolabeled, IVT β-catenin. (B) In egg extract, incubation with LRP6ICD (1.6 μM) but not Gβγ or LRP6ICD (1.6 μM) and Gβγ (7 μM) leads to degradation of radiolabeled, IVT Axin.

LRP6ICD from binding Axin and promoting its degradation. This interpretation would be consistent with Gβγ binding GSK3 or Dsh within the destruction complex in a manner that precludes LRP6ICD binding to the destruction complex and promoting Axin degradation. Alternatively, Gβγ may activate known downstream effectors, such as PLC (Smrcka, 2008), in a manner that also precludes LRP6ICD's ability to bind the destruction complex and promote Axin degradation. Further examination of the ability of Gβγ to sequester the destruction complex from LRP6ICD may provide clues to its mechanism of action. Nonetheless, our results suggest Gβγ may promote Wnt signaling by binding and inactivating the destruction complex.

Discussion

This work establishes biochemically amenable *Xenopus* egg extract as a model system to study the effects of G protein signaling on β-catenin stability. We tested a panel of G proteins for modulation of β-catenin stability and identified Gαo, Gαi, Gαq, and Gβγ as potential regulators of Wnt/β-catenin signaling. While Gαo and Gαq have previously been implicated in Wnt/β-catenin signaling (Liu et al., 1999b), we also suggest possible roles for Gαi and Gβγ in this process. Further work must be undertaken to determine whether these proteins are required for Wnt signaling and whether they couple directly to GPCR activity of Fz or to other GPCRs. Answers to these questions along with mechanistic studies in egg extract and other systems will offer insight into regulation of β-catenin signaling.

Our preliminary mechanistic studies show that Gαo may play a role in directly inhibiting GSK3's phosphorylation of β-catenin. Further work should determine whether

Gαo directly binds these proteins or whether it signals through known Gαo signaling targets. Our studies with Gβγ suggest a novel role for this subunit in Wnt signaling. As with Gαo, the mechanism by which Gβγ inhibits β-catenin phosphorylation may be direct or indirect, through known Gβγ effectors. Surprisingly, neither Gαo nor Gβγ require Dsh for their effects on β-catenin stability. Perhaps, Gαi or Gαq directly couple to Fz to promote Dsh phosphorylation and activation. Alternatively, Fz-mediated activation of Dsh may not occur through GPCR-mediated G protein heterotrimer dissociation.

Future studies unraveling the roles of Gαo, Gαi, Gαq and Gβγ should attempt to determine whether Fz or other GPCRs endogenously activate these molecules to regulate Wnt/β-catenin signaling. These studies will lay the groundwork to determine whether Fz acts as a GPCR or whether G proteins are involved in crosstalk between other GPCR-mediated events and Wnt signaling. Such studies will be important in understanding the role of connections between Wnt signaling and G proteins in development, physiology, and disease. Indeed, identification of GPCR-mediated events that impinge on Wnt/β-catenin will provide a theoretical basis for the development of GPCR-modulating drugs with therapeutic potential for the treatment of cancers and other diseases caused by mis-regulation of Wnt/β-catenin signaling (Jacoby et al., 2006).

Future Directions

Is Gβγ required for Wnt Signaling?

Characterization of the role of Gβγ in Wnt signaling requires determination of whether Gβγ is required for Wnt/β-catenin signaling. Because there are 5 Gβ subunits

and 12 Gγ subunits in humans, we tested the requirement for Gβγ in Wnt signaling in *Drosophila* where there are only 3 Gβ subunits and 2 Gγ subunits. Thus, we performed small interfering (si) RNA-mediated RNAi for 2 of 3 of the Gβ subunits and both of the Gγ subunits in S2R+ TOPFlash reporter cells. These cells are stably transfected with a plasmid encoding a luciferase reporter driven by a promoter consisting of TCF/LEF binding sites, so β-catenin mediated transcription can be assayed by luciferase expression. Importantly, none of the single subunits or combinations thereof inhibited Wg-mediated TOPFlash transcriptional activity (Figure 3.11). However, we noted consistent increases in TOPFlash in cells where siRNA against Gβ76 was performed, suggesting a possible negative role for this endogenous protein in Wnt signaling (Figure 3.11). Nonetheless, experiments to determine whether this represents an off-target effect must be performed in order to validate that Gβ76 normally represses Wnt signaling. There are many important caveats to this experiment and the result that Gβγ knockdown does not inhibit Wnt signaling. First, we were unable to determine how much, if at all, these proteins were knocked-down as there are not commercially available antibodies that recognize these specific subunits. Second, knockdown of Gβγ has also been shown to inhibit all Gα-mediated signaling events, so even a positive result could not be attributed specifically to a role for Gβγ in Wnt signaling (Hwang et al., 2005). Thus, a different approach must be taken to determine whether Gβγ is necessary for Wnt signaling. The most common and rigorous approach to this question is to test whether overexpression of a Gβγ effector, the C-terminus of the β adrenergic receptor kinase (C-βARK), inhibits Wnt signaling by sequestering GPCR-activated, dissociated Gβγ (Koch et al., 1994). As

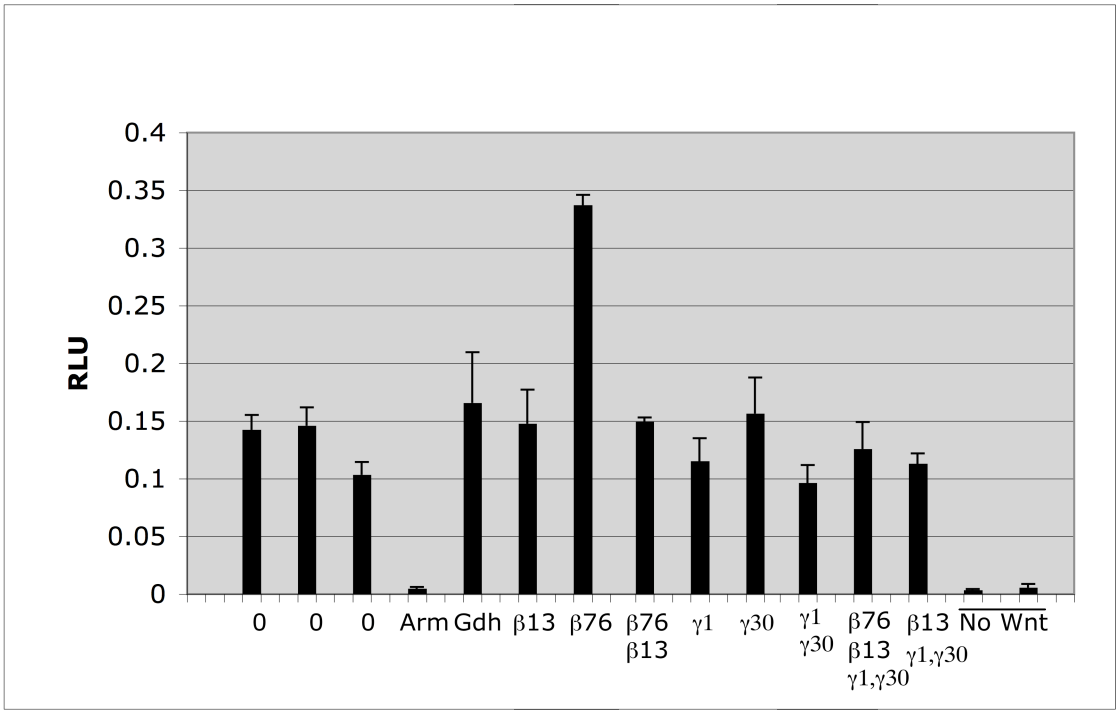


Figure 3.11: RNAi of Gβγ Subunits in *Drosophila* S2R+ TOPFlash Reporter Cells Does Not Inhibit Wg-Mediated TOPFlash Activation. 1 μg of each siRNA was added to S2R+ cells for 72 hr, Wg media was then added for 24 hr, and cells were harvested for TOPFlash and cell-titer assays. All experiments were performed in triplicate. Note that Arm siRNA inhibits the Wg-mediated luciferase signal (as expected) and Gβ76 siRNA increases the Wg-mediated luciferase signal. (RLU, relative light units; 0, no siRNA; Arm, Armadillo siRNA; Gdh, GAPDH siRNA)

preliminary results show promise, we will analyze the requirement of Gβγ in Wnt signaling using this approach.

Does Gβγ Directly Associate with Wnt Pathway Components?

To assess whether Gβγ stabilizes β-catenin by binding a protein involved in Wnt signaling, we will utilize multiple approaches to test candidate binding events. We will test whether Gβγ binds LRP6, Fz, Dsh, Axin, APC or β-catenin using co-immunoprecipition in egg extract or cultured cells. In cultured cells, we can determine whether Wnt3a treatment affects such binding events at specific time points (0, 5, 15, 30 60, and 120 minutes after Wnt treatement). When possible, we will perform these binding experiments using endogenous proteins to prevent overexpression-dependent binding artifacts. We will also test Gβγ's association with LRP6, Fz, Axin, and β-catenin using a Bimolecular Fluorescence Complementation assay (BiFC) approach (Hu et al., 2002). This method, also known as the split-YFP (Yellow Fluorescent Protein) approach, involves overexpressing two proteins with complementary halves of YFP in cultured cells. If the proteins interact, they bring the two halves of YFP close enough to make a functional, fluorescent YFP detectable by fluorescence microscopy. In addition to complementing results from co-immunoprecipitation experiments, BiFC has the added advantage of showing where in the cell the interaction is taking place. In parallel to the BiFC experiments, we will test whether Gβγ overexpression in cultured cells affects localization of whole YFP-tagged proteins such as LRP6, Axin, and β-catenin in order to assay for other Gβγ-mediated perturbations of Wnt components.

Does Gβγ Affect Wnt/β-catenin Signaling through Previously Described Gβγ Effectors?

If Gβγ does not directly interact with core components of Wnt signaling, it may modulate β-catenin levels via previously characterized effectors of Gβγ signaling such as PLC, adenylyl cyclase, phosphoinositide 3 kinase, and phospholipase D (Smrcka, 2008). To test this, we will utilize small molecule inhibitors of known Gβγ effectors to identify the pathway by which Gβγ modulates Wnt signaling. In egg extract, we tested whether incubation with small molecule inhibitors of Gβγ effectors inhibits the ability of purified Gβγ to stabilize β-catenin. As many of these inhibitors have not been characterized in egg extract, we can apply this approach to cell culture where incubation of such an inhibitor may inhibit Gβγ overexpression-mediated effects on the pathway. To further study such pathways, we can utilize molecular biological methods such as RNAi and overexpression of dominant negative proteins involved in the Gβγ-mediated signaling pathways.

Future Studies with Gα Subunits Potentially Involved in Wnt/β-catenin Signaling

The future directions mentioned above are also pertinent to Gα-mediated modulation of Wnt/β-catenin signaling. We can test whether Gαi is required for Wnt3a-mediated signaling in cell culture by applying small hairpin (sh) RNA to Gαi family members. Unlike Gβγ RNAi, Gαi RNAi should only affect Gαi mediated processes (Hwang et al., 2005). To determine whether Gαo, Gαi, and Gαq directly interact with known Wnt pathway components, co-immunoprecipitation in cell culture and egg extract as well as BiFC experiments may provide important mechanistic leads. In addition, in

cell culture and egg extract we can inhibit known Gαo-, Gαi-, and Gαq-mediated signaling processes with small molecules, RNAi, and dominant negative proteins. These Gα-dependent signaling pathways may be required for their effects on Wnt/β-catenin signaling, providing clues to their mechanisms of action.

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BOOK 2: N-GLYCOLSYLATION IN *XENOPUS* EMBRYOGENESIS

CHAPTER 4: INTRODUCTION TO GLYCOSYLATION IN DEVELOPMENT

Glycosylation in Metazoan Development.

The addition of carbohydrate moieties to proteins regulates their maturation, activity, localization, and/or stability (reviewed in Varki and Chrispeels, 1999). Several major types of glycosylation have been documented (Varki and Chrispeels, 1999). In N-glycosylation, a specific lipid-linked oligosaccharide, referred to as the LLO, consisting of 3 glucose molecules (Glc_3), 9 mannose molecules (Man_9), and 2 N-acetylglucosamine molecules ($GlcNAc_2$) attached by a diphosphate linkage to a dolichol lipid is transferred onto a protein. The LLO is transferred onto certain asparagine (Asn, N) residues within the consensus sequence Asn-X-Thr/Ser on proteins that are trafficked through the endoplasmic reticulum (ER). The protein's LLO is then modified as the protein matures through the ER and Golgi apparatus. Because N-glycosylation occurs in the ER, N-glycosylation targets proteins involved the cell's secretory pathway such as secreted or membrane proteins. In one class of O-glycosylation, a single N-acetylgalactosamine (GalNAc) is transferred onto a serine or threonine (Ser or Thr, O) residue on a protein in the ER or Golgi. Complex carbohydrate oligomers are often added to the initial single GalNAc, affecting activity, localization, and stability of proteins of the secretory pathway in a manner similar to that of N-glycosylation. Proteoglycans are heavily O-glycosylated proteins that are a major component of the extracellular matrix. In the Golgi, long, anionic O-linked carbohydrate chains, called glycosaminoglycans (GAGs), are attached

to these proteins before they are secreted outside of the cell. Their anionic character plays a role in regulating protein interactions, trafficking, and stability outside of the cell. A second major class of O-glycosylation involves addition of a single GlcNAc onto Ser or Thr sites and occurs on proteins in the cytoplasm and nucleus, not proteins of the secretory pathway. In this class of O-glycosylation, the single GlcNAc is not modified into a carbohydrate oligomer. Instead, this single carbohydrate moiety is a transient protein modification that plays a role similar to phosphorylation or ubiquitination. Some idea of the importance of glycosylation in biology can be gleaned from the fact that at least 1% of proteins encoded in the human genome are involved in glycosylation or modification of glycosylated proteins (Ohtsubo and Marth, 2006).

Given the importance and ubiquity of glycosylation in the maturation and activity of membrane-associated and secreted proteins, it is not surprising that protein glycosylation plays important roles in cell-cell communication during metazoan development. It is reasonable to predict that complete inhibition of glycosylation would prevent many processes required for animal development. However, many developmental programs proceed normally when animals are challenged with partial loss-of-function defects in glycosylation, and various though specific development defects have been reported (Table 4.1) (Haltiwanger and Lowe, 2004). Interestingly, the diversity of developmental defects caused by perturbation of proteins involved in glycosylation suggests that this is an intricately regulated biological process. Multiple studies have analyzed developmental phenotypes resulting from loss-of-function of certain proteins involved in glycosylation and traced them to specific defects in proteins involved in cell signaling (Haltiwanger and Lowe, 2004). For example, in mice and

Enzyme	Phenotype associated with enzyme deficiency
GlcNAc-1-phosphotransferase	Embryonic lethality (E4.5)
GlcNAcT-I	Embryonic lethality (E9.5) with defects in vascularization, neural tube formation, and situs inversus of the heart
α-mannosidase-II	Dyserythropoiesis and SLE-like autoimmune disease
α-mannosidase-IIx	Spermatogenic failure, male sterility
GlcNAcT-II	Frequent postnatal lethality with defects in multiple physiologic systems
	Survivors phenocopy human CDG-IIa disease
GlcNAcT-III	Neurological deficit and tumor inhibition reported upon gene truncation
	No defect yet reported upon complete gene deletion
GlcNAc-IVa	Viable, under further study
GlcNAcT-V	Immune dysfunction with hyperactive T cells and intestinal hyperplasia
Polypeptide GalNAcT-1	Viable/under further study
Polypeptide GalNAcT-8	Viable/under further study
Core 2 GlcNAcT-I	Myeloid leukocytosis and inflammation deficit
Polypeptide-O-fucosyltransferase	Not reported
Lunatic fringe β1-3GlcNAcT	Defective somite formation, axial skeleton deformities, perinatal death
Radical fringe β1-3GlcNAcT	Viable, no reported phenotype
Manic fringe β1-3GlcNAcT	Not reported
Braniac 1 β1-3GlNAcT	Defective implantation with early embryonic lethality
Myd gene (putative glycosyltransferase POMGNT1)	Neuro-muscular disease similar to human muscular dystrophy
Ceramide glucosyltransferase	Embryonic lethality at gastrulation (E6.5 to E7.5)
Ceramide galactosyltransferase	Myelin abnormalities, paralysis, and postnatal death; arrested spermatogenesis
β1-4GalNAcT (GM2/GD2 synthase)	Myelin and axonal degeneration, male sterility with defective testosterone transport
EXT1 α4GlcNAc & β4GlcA transferase	Embryonic lethal, gastrulation defect
N-deactylase/N-sulfotransferase-1 (NDST-1)	Defective pulmonary surfactant formation, postnatal respiratory distress and lethality
N-deactylase/N-sulfotransferase-1 (NDST-2)	Defects in mast cell heparin synthesis and granule formation
Heparan sulfate 2-sulfotransferase (HS-2OST)	Renal agenesis with neonatal lethality
GPI synthesis: X-linked *Pig-A*	Lethality if mutation is germline or prevalent in somatic tissue
Hyaluronan synthase-2 (Has-2)	Embryonic lethality (E9.5); defects in cardiac morphogenesis and mesenchyme formation
β1-4 GalT-1	Multiple defects including epithelial and endocrine abnormalities, frequent postnatal lethality; CDG-IId defect in humans
ST6Gal-I	Immunodeficiency with attenuated B cell function
ST3Gal-I	Cytotoxic T cell apoptosis, enhanced CD8ab-MHC class I binding
ST3Gal-II	Viable/under further study
ST3Gal-III	Viable/under further study
ST3Gal-IV	Inflammation response deficit, vWF and platelet deficiencies
ST8Sia-I (GD3 synthase)	Viable alone. Lethal audiogenic seizures in collaboration with GM2/GD2 synthase deficiency
ST8Sia-IV (PST)	Abnormal neuronal development and LTP deficit
ST8Sia-II (STX)	Viable, under further study
FUT1	Viable, under further study
FUT2	Viable, under further study
FUT4	Partial neutrophil adhesion deficit, subtle selectin ligand defects
FUT7	Defective selectin ligands, neutrophil adhesion, and lymphoid homing
FUT8	Not yet reported
FUT9	Not yet reported
GST-3 (LSST, HEC-GlcNAc6ST)	Moderate defect in lymphocyte homing and HEV-borne L-selectin ligands
β1-3 GalT	Cataracts, model for hyperacute acute xenograft rejection
Galgt2 β1-4GalNAcT	Viable, under further study
GDP-4-keto,6-deoxymannose epimerase/reductase (FX)	Strain-dependent embryonic lethality; block in GDP-fucose synthesis conditionally defective fucosylation in all tissues
UDP-GlcNAc 2-epimerase	Embryonic lethality; bock to CMP-sialic acid synthesis with sialic acid deficiency
O-GlcNAc transferase: X-linked *Ogt*	Lethality unless mutation is paternally acquired in females

Table 4.1: Phenotypes Associated with Mutations in Mice that Perturb Glycosylation Are Shown in This Table Adapted from (Haltiwanger and Lowe, 2004).

Drosophila, loss-of-function of O-fucose: β1,3-N-acetylglucosaminyl-transferase leads to defects in Notch signaling-dependent developmental programs by inhibiting O-fucosylation of Notch proteins (Moloney et al., 2000). In mice, a defect in an enzyme required for synthesis of all GAGs on proteoglycans, UDP-glucose dehydrogenase, leads to a severe loss of all extracellular matrix GAGs but specifically impinges on FGF (and not BMP or Wnt) signaling (Garcia-Garcia and Anderson, 2003). Thus, global and ubiquitous regulators of glycosylation have been shown to disrupt specific signaling events in development.

Because many specific developmental and molecular perturbations have been reported to occur as a result of defects in proteins involved in glycosylation, these proteins should not be considered "housekeeping" proteins as they may be dynamically and intricately regulated modulators of development. For example, a cell could fine-tune its response to an extracellular signal through up- or down-regulation of a certain gene involved in glycosylation that specifically affects the activity of a protein or proteins involved in that signaling pathway. Similarly, evolution of specific cell-biological processes could occur through genetic changes that affect cis-regulatory elements of a gene involved in glycosylation, which would affect its expression and lead to cell-signaling changes in certain tissues of an organism. Defects in several modulators of glycosylation have very specific biological effects, which suggests that regulation of these proteins may play important roles in physiology and evolution.

While there have been many loss-of-function experiments supporting a role for dynamic regulation of enzymes involved in glycosylation, less work has focused on overexpression of these enzymes. To further evaluate the candidacy of these genes as

important regulatory nodes in physiology and evolution, it will be important to determine whether increases in the expression of such genes also have specific and significant biological consequences. In support of this hypothsis, a number of pathologic mutations in humans create ectopic glycosylation sites that may lead to disease (Vogt et al., 2005). Work exploring the dynamics of expression of genes involved in glycosylation may also provide some answers to these questions.

Glycosylation Defects Cause Human Developmental Disorders.

Consistent with animal studies demonstrating that many defects in glycosylation have specific phenotypes that are not embryonically lethal, mutations in an increasing number of genes involved in glycosylation have been reported to cause congenital disorders in humans (Figure 4.1) (Freeze, 2006). Several human disorders affecting O-glycosylation have been identified. Defects in heparan sulfate proteoglycan biosynthesis have been shown to cause hereditary multiple exostoses (Simmons et al., 1999). And, defects in O-glycosylation of FGF-23 have been linked with familial tumoral calcinosis (Kato et al., 2006). In Ehlers-Danlos syndrome, patients present with delayed speech and motor development, failure to thrive, hypotonia, and distinctive connective tissue defects (Lawrence, 2005). This syndrome is likely caused by defects in xylose-based GAG synthesis (Quentin et al., 1990). Disorders affecting O-glycosylation are summarized in Table 4.2 (Freeze, 2006). Disorders affecting N-glycosylation are classified as Congenital Disorders of Glycosylation (CDGs) (reviewed in Eklund and Freeze, 2006). Type 1 CDGs affect synthesis or transfer of the LLO onto Asn residue of N-glycosylated proteins, whereas Type 2 CDGs affect processing of the LLO on N-glycosylated proteins.

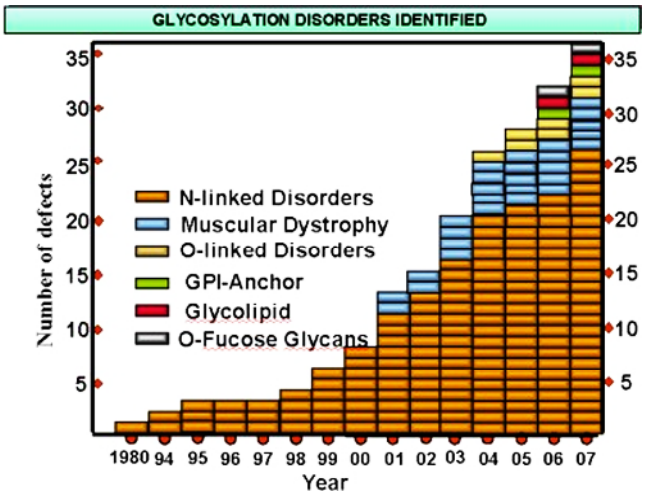

Figure 4.1: The Number of Identified Human Disorders Affecting Glycosylation Has Greatly Increased During the Last 30 Years. Figure from (http://www.burnham.org/default.asp?contentID=143).

Disorder	Gene	Enzyme	OMIM	Key Features
Defects in O-glycosylation pathways				
Walker–Warburg syndrome	*POMT1/ POMT2*	*O*-mannosyltransferase 1	236670	Type II lissencephaly, cerebellar malformations, ventriculomegaly, anterior chamber malformations, severe delay; death in infancy
Fukuyama muscular dystrophy	*FCMD*	Fukutin, a putative glycosyltransferase	253800	Cortical dysgenesis, myopia, weakness and hypotonia; 40% have seizures
Congenital muscular dystrophy type 1C (MDC1C)	*FKRP*	Fukutin-related protein, a putative glycosyltransferase	606612	Hypotonia, impaired motor development, respiratory muscle weakness
Congenital muscular dystrophy type 1D (MDC1D)	*LARGE*	Putative glycosyltransferase	608840	Muscular dystrophy with profound mental retardation
Hereditary inclusion-body myopathy-II (IBM2)	*GNE*	UDP-GlcNAc epimerase/kinase	600737	Adult onset with progressive distal and proximal muscle weakness; spares quadriceps
Ehlers–Danlos syndrome	*B4GALT7*	β1,4-Galactosyltransferase 7	130070	Progeroid Ehlers-Danlos syndrome; macrocephaly, joint hyperextensibility
Hereditary multiple exostosis	*EXT1/ EXT2*	Glucuronyltransferase/GlcNAc transferase	133700	Multiple exostoses (diaphyseal, juxtaepiphyseal)
Chondrodysplasias	*DTDST/ SLC26A2*	Sulphate anion transporter	222600 600972 256050	Diastrophic dysplasia: airway collapse, early death in severe cases, adults reported. Achondrogenesis Ib: usually stillborn or early death of respiratory failure. Atelosteogenesis II: pulmonary hypoplasia, fatal in infants
Spondylo-epimetaphyseal dysplasia	*ATPSK2*	3′-phosphoadenosine-5′-phosphosulphate synthase	603005	Abnormal skeletal development and linear growth
Macular corneal dystrophy types I and II	*CHST6*	Keratan sulphate 6-0-sulphotransferase	217800	Corneal clouding and erosions, painful photophobia
Familial tumoral calcinosis	*GALNT3*	GalNAc transferase	211900	Massive calcium deposits in skin and tissue
Tn syndrome	*COSMC*	Chaperone of β1,3GalT	230430	Anaemia, leukopaenia, thrombocytopaenia (somatic mutation)
Defects in glycolipid synthesis				
Paroxysmal nocturnal haemoglobinuria	*PIGA*	PI-GlcNAcT	311770	Complement-mediated haemolysis (somatic mutation)
Amish infantile epilepsy	*SIAT9*	Sia2,3Galβ1,4Glc-Cer synthase	609056	Tonic-clonic seizures, arrested development, neurological decline

CDG, congenital disorder of glycosylation; Cer, ceramide; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Sia, sialic acid.

Table 4.2: Human Diseases Resulting from Defects in O-Glycosylation Are Shown in This Table from (Freeze, 2006).

CDG patients often present with mental and psychomotor retardation and dysmorphic features. Coagulopathies and gastrointestinal problems are slightly less common features of CDGs. In this group of disorders, partial loss-of-function, autosomal recessive mutations in global regulators of N-glycosylation lead to specific syndromes with shared and distinct features. A summary of disorders in N-linked glycosylation is shown in Table 4.3 (Freeze, 2006), and a diagram displaying their underlying metabolic defects in N-glycosylation is shown in Figure 4.2 (Freeze, 2006). The specific developmental and molecular processes perturbed in CDGs and other genetic disorders where glycosylation is disrupted remain important medical and biological questions. Indeed, elucidation of the molecular mechanisms by which loss-of-function of certain regulators of glycosylation leads to the symptoms of these disorders may offer insight into targets for therapeutic intervention.

Analyses of defects in glycosylation in model organisms suggest that many proteins involved in glycosylation could conceivably be evolutionarily modulated to fine-tune specific aspects of biology. In support of this concept, it has been suggested that CDGs may be the result of evolutionary pressure to globally downregulate N-glycosylation. For example, a heterozygous loss-of-function point mutation R141H in phosphomannomutase (PMM2) (the enzyme mutated in CDG Type 1a) is found in 1/70 Northern Europeans, while the homozygous mutation is thought to be lethal (Schollen et al., 2000). Given such a high prevalence for this mutation, there may be a selective advantage to a partial loss-of-N-glycosylation via PMM2 R141H heterozygosity (Schollen et al., 2000). It has been suggested, though not tested, that this heterozygous mutation is protective against viral infections by limiting viral coat production, a process

Disorder	Gene	Enzyme	OMIM	Key Features
CDG-Ia	*PMM2*	Phosphomannomutase II	212065	Mental retardation, hypotonia, esotropia, lipodystrophy, cerebellar hypoplasia, stroke-like episodes, seizures
CDG-Ib	*MPI*	Phosphomannose isomerase	602579	Hepatic fibrosis, protein-losing enteropathy, coagulopathy, hypoglycaemia
CDG-Ic	*ALG6*	Glucosyltransferase I Dol-P-Glc: Man_9-$GlcNAc_2$-P-P-Dol glucosyltransferase	603147	Moderate mental retardation, hypotonia, esotropia, epilepsy
CDG-Id	*ALG3*	Dol-P-Man:Man_5-$GlcNAc_2$-P-P-Dol mannosyltransferase	601110	Profound psychomotor delay, optic atrophy, acquired microcephaly, iris colobomas, hypsarrhythmia
CDG-Ie	*DPM1*	Dol-P-Man synthase I GDP-Man: Dol-P-mannosyltransferase	603503	Severe mental retardation, epilepsy, hypotonia, mild dysmorphism, coagulopathy
CDG-If	*MPDU1*	Man-P-Dol utilization 1/Lec35	608799	Short stature, icthyosis, psychomotor retardation, pigmentary retinopathy
CDG-Ig	*ALG12*	Dol-P-Man:Man_7-$GlcNAc_2$P-P-Dol mannosyltransferase	607143	Hypotonia, facial dysmorphism, psychomotor retardation, acquired microcephaly, frequent infections
CDG-Ih	*ALG8*	Glucosyltransferase II Dol-P-Glc: Glc_1-Man_9-$GlcNAc_2$-P-P-Dol glucosyltransferase	608104	Hepatomegaly, protein-losing enteropathy, renal failure, hypoalbuminaemia, oedema, ascites
CDG-Ii	*ALG2*	Mannosyltransferase II GDP-Man: Man_1-$GlcNAc_2$-P-P-Dol mannosyltransferase	607906	Normal at birth; mental retardation, hypomyelination, intractable seizures, iris colobomas, hepatomegaly, coagulopathy
CDG-Ij	*DPAGT1*	UDP-GlcNAc: Dol-P-GlcNAc-P transferase	608093	Severe mental retardation, hypotonia, seizures, microcephaly, exotropia
CDG-Ik	ALG1	Mannosyltransferase I GDP-Man: $GlcNAc_2$-P-P-Dol mannosyltransferase	608540	Severe psychomotor retardation, hypotonia, acquired microcephaly, intractable seizures, fever, coagulopathy, nephrotic syndrome, early death
CDG-Il	*ALG9*	Mannosyltransferase Dol-P-Man: Man_6- and Man_5-$GlcNAc_2$-P-P-Dol mannosyltransferase	608776	Severe microcephaly, hypotonia, seizures, hepatomegaly
CDG-IIa	*MGAT2*	GlcNAc transferase 2	212066	Mental retardation, dysmorphism, stereotypies, seizures
CDG-IIb	*GLS1*	Glucosidase I	606056	Dysmorphism, hypotonia, seizures, hepatomegaly, hepatic fibrosis; death at 2.5 months
CDG-IIc	*SLC35C1/ FUCT1*	GDP-fucose transporter	266265	Recurrent infections, persistent neutrophilia, mental retardation, microcephaly, hypotonia; normal transferrin
CDG-IId	*B4GALT1*	β1,4 galactosyltransferase	607091	Hypotonia (myopathy), spontaneous haemorrhage, Dandy–Walker malformation
CDG-IIe	*COG7*	Conserved oligomeric Golgi complex subunit 7	608779	Fatal in early infancy; dysmorphism, hypotonia, intractable seizures, hepatomegaly, progressive jaundice, recurrent infections, cardiac failure
CDG-IIf	*SLC35A1*	CMP-sialic acid transporter	605634	Thrombocytopaenia, no neurological symptoms; normal transferrin, abnormal platelet glycoproteins
CDG-II/COG1	COG1	Conserved oligomeric Golgi complex subunit 1	606973	Hypotonia, growth retardation, progressive microcephaly, hepatosplenomegaly, mild mental retardation
Mucolipidosis II and III	*GNPTA*	UDP-GlcNAc: lysosomal enzyme, GlcNAc-P transferase	252500	Coarsening features, organomegaly, joint stiffness, dysostosis, median neuropathy at the wrist; MLIII is less severe than MLII, which presents in infancy
Congenital dyserythropoietic anaemia (CDA II)	Unknown	Unknown	224100	Anaemia, jaundice, splenomegaly, gall bladder disease

CDG, congenital disorder of glycosylation; Dol, dolichol; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Man, mannose.

Table 4.3: Human Diseases Resulting from Defects in N-Glycosylation Are Shown in This Table from (Freeze, 2006).

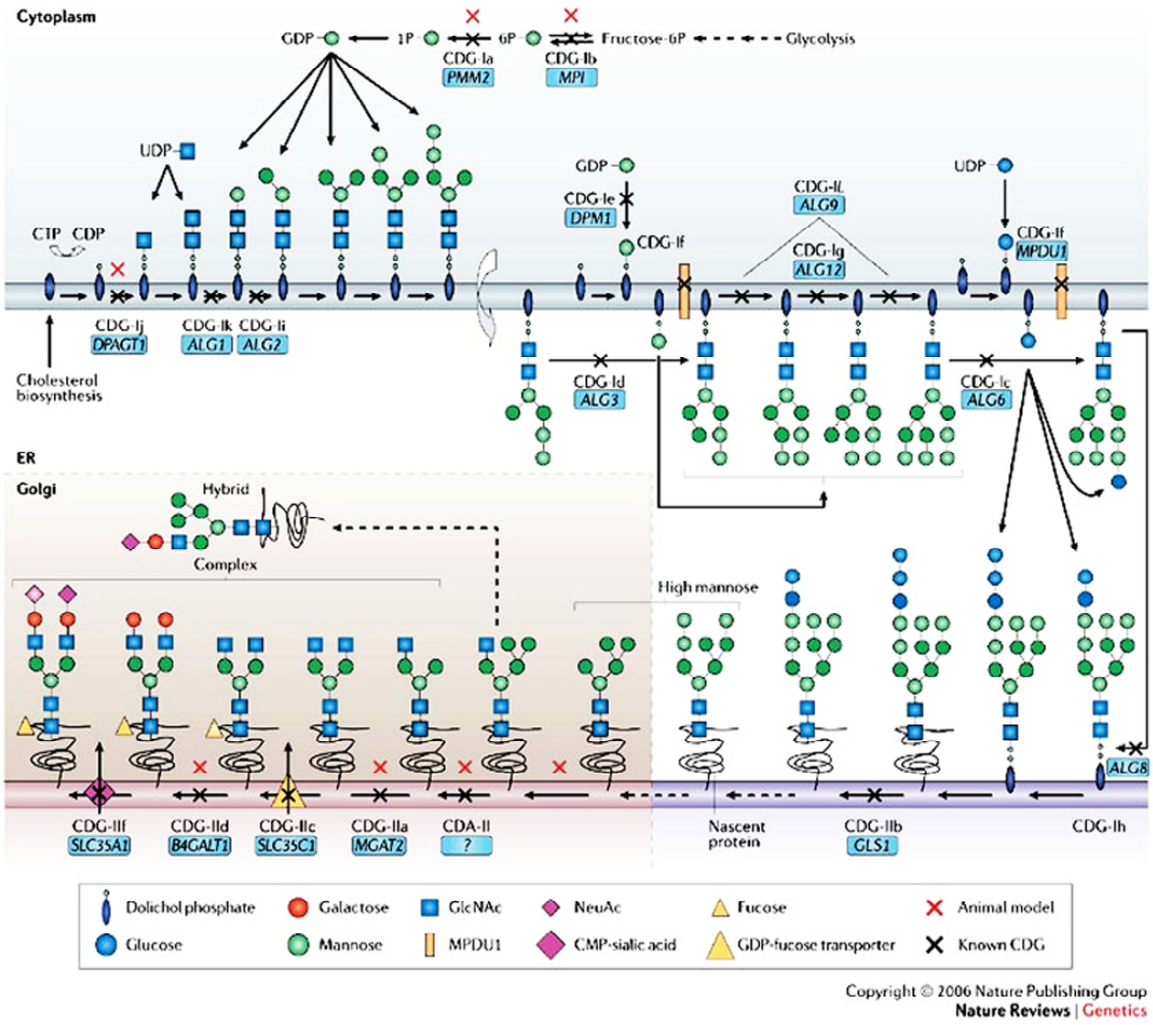

Figure 4.2: A Diagram Detailing the Molecular and Metabolic Defects Associated with CDGs is Shown from (Freeze, 2006).

that requires host N-glycosylation (Freeze and Westphal, 2001). Although the possibility of a selective advantage for decreased N-glycosylation is attractive, there is currently no direct evidence that addresses this hypothesis.

While a mechanistic molecular link has not been established between mutations found in CDGs and their signs and symptoms, clinical and experimental evidence provide some initial clues. Parameters for CDG diagnosis include signs and symptoms, detection of hypoglycosylated serum Transferrin, specific enzymatic assays, and DNA sequencing (Eklund and Freeze, 2006). As hypo-N-glycosylation of Transferrin can be readily detected in most CDG patients, it is possible that hypo-N-glycosylation of other specific proteins may directly cause CDG signs and symptoms. As CDGs cause many diverse symptoms, the molecular etiology of these symptoms may or may not be identical. In support of the former possibility, certain mutations in fibroblast growth factor (FGF) signaling lead to diseases with a similar array of signs and symptoms as CDGs. For example, microcephaly, dysmorphic palate, 4,5 digit syndactyly, and strabismus are common to several CDGs as well as genetic disorders affecting FGF signaling (Chen and Deng, 2005; Toydemir et al., 2006). However, it is also conceivable that the same glycosylation defect could affect different signaling events in different tissues. Thus, it will be important to determine whether CDGs cause disease through one or multiple signaling pathways.

Consistent with N-glycosylation playing important roles in protein folding in the ER, CDG Type 1a,b, and c patient-derived dermal fibroblasts display an activated Unfolded Protein Response (UPR) (Lecca et al., 2005; Shang et al., 2002). The UPR promotes cellular adaptation to ER stress via expression of ER chaperones, enhanced

production of mature LLOs, global inhibition of translation, and/or apoptosis (reviewed in Schroder, 2008). The precise UPR-induced program has been shown to depend on the level of ER stress: expression of ER chaperones and enhancement of mature LLOs occurs at a lower level of UPR activation, while apoptosis is the most drastic response that occurs when there is a higher level of ER stress and UPR activation. In CDG Type 1a,b, and c dermal fibroblasts, enhanced production of mature LLOs is detected, but other aspects of UPR are not activated (Shang et al., 2002). Thus, UPR-mediated adaptive responses to ER stress have been noted in CDG patient cells. It is not known whether CDGs cause more drastic UPR responses in other tissues during different developmental periods. This is an important question because developmental defects that result from CDGs could be caused by UPR activation in certain tissues (not by hypo-N-glycosylation of certain proteins *per se*). Although several loss-of-function studies of enzymes involved in glycosylation in model organisms have demonstrated UPR activation, it is not clear whether the developmental phenotype results directly from hypoglycosylation or indirectly through upregulation of the UPR (Haecker et al., 2008; Yuan et al., 2007). Thus, it will be important to determine whether chronic UPR activation contributes to the developmental disorders and phenotypes resulting from perturbations in glycosylation.

Finally, the relationship between defects in N- and O-linked glycosylation requires further experimental inquiry. A number of patients have presented with defects in both N- and O-linked glycosylation (Jaeken and Matthijs, 2007). In some cases, it is not known whether these patients have defects in an enzyme that affects both N- and O-linked glycosylation or whether perturbation of one process affects the other via UPR-mediated responses or other uncharacterized mechanisms. Interestingly, certain

symptoms of Type 1 CDGs can be alleviated by subcutaneously delivered heparin therapy (Liem et al., 2008). It was unexpected that a defect in N-glycosylation could be treated with an O-glycosylated GAG. Perhaps, the primary N-glycosylation defect could perturb O-glycosylation; alternatively, the primary N-glycosylation defect could be suppressed by heparin-mediated alteration of the extracellular matrix. In support of the former possibility, several aspects of the UPR would be expected to affect expression and maturation of secreted O-glycosylated proteins. A defect in N-glycosylation could activate the UPR, which could inhibit O-glycosylated proteoglycan secretion, a defect that could be suppressed by exogenous heparin therapy. Alternatively, hypo-N-glycosylation of certain proteins may be rescued *in trans* by alteration of the extracellular matrix with exogenous heparin. Thus, the relationship between O- and N-linked glycosylation defects should be further studied to better understand the mechanism by which CDGs cause their symptoms.

Many rudimentary questions remain in understanding the mechanism by which CDG mutations cause their respective diseases. It will be important to determine whether CDGs impact a single or numerous of cell signaling pathways. The extent of the contribution of the UPR to CDG symptoms should also be explored. And, crosstalk between O- and N-linked glycosylation defects in producing CDG symptoms requires study. In addition to furthering our understanding of these diseases and their effects on human development, answers to these questions will help determine whether drugs that activate or inhibit cell signaling pathways, drugs that inhibit UPR, or GAGs or small molecules that affect O-linked glycans will have therapeutic potential in treatment of CDGs.

Of the 18 identified CDGs, therapy has only been effective in two subtypes (Freeze, 2006). Patients with CDG Type1b have mutations in phosphomannose isomerase (PM1), which converts fructose-6-phosphate to mannose-6-phosphate. The patients' coagulopathy, protein-losing enteropathy, and liver fibrosis can be drastically improved when their diets are supplemented with mannose (Niehues et al., 1998). Patients with CDG Type2c have mutations in the GDP-fucose transporter and exhibit immunological defects and neurological deficits, which can be improved when their diets are supplemented with fucose (Marquardt et al., 1999). Given that these CDGs are metabolic enzymes, supplementing the product or substrate of the defective enzyme seems a reasonable and cost effective therapeutic goal. Because these disorders are often quite rare, however, this therapeutic strategy has not been attempted for many CDGs. Moreover, there may be issues concerning effective delivery, solubility, and stability of these metabolic intermediates that must be overcome. As a result, there is a need for patient cell lines and whole organism models to test candidate therapies for these diseases.

Currently untreated CDGs may have fairly straightforward but untested therapies. To begin to address this medical problem, development of whole organism animal models of these diseases should be initiated. Such models could then be used to test candidate therapies. Certain CDG genes such as PMI have been knocked-out in mice, but the null mutation causes embryonic lethality and is, thus, a poor model for the human disease (DeRossi et al., 2006). Instead of creating null mutations, endogenous CDG genes in the mouse could be replaced by genes containing mutations orthologous to those found in CDG patients. Alternatively, partial loss-of-function of CDG genes could be

analyzed more quickly in other vertebrate organisms such as *Xenopus* or zebrafish by partial knockdown with Morpholino oligonucleotides. In addition to utility in developing therapeutics, animal models for CDGs will be an invaluable resource for establishing the link between loss of function of CDG genes and the molecular mechanisms by which they promote their disease phenotypes. In any of the above organisms, defects in tissue development could be identified and traced to their molecular roots. In addition, the contribution of UPR and crosstalk between N- and O-linked glycosylation to disease phenotype could be explicitly tested. Thus, development of model organisms to study CDGs will be beneficial to patients suffering from these disorders and will offer researchers an opportunity to directly identify the molecular mechanisms by which CDG mutations cause their diseases. Overexpression studies in these organisms as well as study of CDG gene expression patterns will also shed light on the question of whether these genes are dynamically regulated in biological processes. If so, it should be productive to study whether dysregulation of these genes plays a role in the pathogenesis of other diseases such as cancer or neurological disease. Consistent with this hypothesis, inhibition of N-glycosylation with tunicamycin shows greater toxicity in certain cancer cells than in non-transformed cells, implying therapeutic potential for regulation of N-glycosylation in cancer (Contessa et al., 2008).

Basic biological studies as well as clinical studies of patients with congenital disorders that perturb glycosylation suggest that enzymes that regulate glycosylation could potentially be dynamically regulated in development, physiology, disease, and evolution. Studies that directly address these hypotheses will be of general value to understanding biology as well as curing human disease.

BOOK 2: N-GLYCOSYLATION IN *XENOPUS* EMBRYOGENESIS

CHAPTER 5: NAGK AND DPAGT1 REGULATE ANTEROPOSTERIOR PATTERNING IN *XENOPUS* EMBRYOGENESIS

Introduction

More than 1% of the human genome encodes proteins that are involved in glycosylation (Ohtsubo and Marth, 2006). The extent to which proteins involved in glycosylation are dynamically regulated in physiology, development, disease, and evolution is not known. Two major types of glycosylation have been identified: N-(Asn)-linked and O-(Ser or Thr)-linked glycosylation (reviewed in Varki and Chrispeels, 1999). In N-glycosylation, a specific 14-residue, lipid-linked precursor oligosaccharide (LLO) is transferred to certain Asp residues on proteins trafficked through the endoplasmic reticulum (ER). This LLO is then trimmed and modified in the ER and Golgi apparatus. N-glycosylation occurs primarily on proteins that are secreted outside the cell or that span the plasma membrane. N-glycosylation regulates the proper maturation and folding of these proteins as well as their localization, activity, and stability.

Although complete inhibition of N-glycosylation is likely incompatable with any proper functioning of biological processes that occur on the plasma membrane or in the extracellular matrix, partial loss-of-function of proteins regulating N-glycosylation leads to a group of human diseases called Congenital Disorders of Glycosylation (CDGs) (reviewed in Freeze, 2006; Haltiwanger and Lowe, 2004). CDGs are caused by autosomal, partial loss-of-function, recessive mutations in a several global regulators of

glycosylation. Most CDGs are diagnosed in part via detection of hypo-N-glycosylated serum Transferrin (Eklund and Freeze, 2006). While symptoms of CDGs differ depending on the particular mutated gene, many CDGs have similar symptoms. Patients with CDGs often present with psychomotor retardation, characteristic dysmorphic features, coagulopathies, and gastrointestinal problems. The biological and molecular defects by which mutations in CDGs cause their symptoms are not known. To better understand the how global perturbation in N-glycosylation causes specific aspects of CDG disease, it will be important to develop a whole organism model system whereby effects of partial loss of N-glycosylation on vertebrate development can be studied in detail.

In addition to a better understanding of CDG pathogenesis, a model organism for CDGs could be used to test candidate therapeutics for these rare diseases. Therapies for only two types of CDG have been identified. However, dietary mannose supplementation for CDG Type 1b (Niehues et al., 1998) and dietary fucose supplementation for CDG Type 2c (Marquardt et al., 1999) are cost-effective therapies that work by supplementing the product or substrate of the defective enzyme. Development of therapies for CDGs could be significantly accelerated given animal models for these diseases.

Here, we suggest use of the *Xenopus laevis* embryo as a model organism to study pathogenesis and treatment of CDGs. In a screen for kinases that regulate *Xenopus* embryogenesis, we identified N-acetylglucosamine kinase (NAGK) as a regulator of anteroposterior pattering. The developmental defect was phenocopied by perturbation of expression of dolichol-phosphate N-acetylglucosamine phosphotransferase (DPAGT1), a

rate-limiting enzyme required specifically for N-glycosylation (Lehrman, 1991; Lehrman et al., 1988) and a CDG gene mutated in CDG Type 1j (Wu et al., 2003). Analysis of loss-of-function phenotypes from these embryos suggests that they have defects in FGF signaling. In addition, overexpression of these genes and study of their expression patterns through development suggest that NAGK and DPAGT1 are dynamically regulated modulators of vertebrate development.

Methods

DNA Constructs, mRNA synthesis, and MOs

cDNAs encoding 232 human kinases from the Harvard Institute of Proteomics (HIP) FLEXGene human kinase cDNA collection (pDNR-dual complete set) (www.hip.harvard.edu/research/kinases/index.htm) were amplified by PCR with primers designed to facilitate *in vitro* transcription followed by translation in *Xenopus* embryos or egg extracts. A PCR-amplified fragment of the pCS2 Poly(A) sequence was first obtained (5' oligo sequence: GACCATTCGTTTGGCGCGCGGGCCTGAGATCC-AGACATGATAAGATAC; 3' oligo sequence: GAATTAAAAAACCTCCCACACC-TCCCCCTGAACCTG). A second PCR-amplified fragment of the human kinase with a 3' oligonucleotide designed to overlap with the 5' oligonucleotide of the CS2 Poly(A) fragment primer was also obtained (5' oligo sequence: GGCCCGCGCGCCAAACGA-ATGGTC and 3' oligo sequence: CCAAGCCTTCTAATACGACTCACTATAGGG-AGACAGTGAGCGAGGAAGCGGCCGC). Both fragments were then "sewn" together in a third PCR reaction to constitute a single fragment with a 5' human kinase and a 3'

poly(A) tail. For experiments performed subsequent to the screen, kinases were cloned into pCS2. Kinase-dead NAGK T128M was created by inducing a T-M mutation in human NAGK by a PCR-based approach. DNA encoding full-length human DPAGT1 was obtained from OpenBiosystems (#7207845). Recombinant human NAGK protein was made by bacterial expression of pMAL-NAGK with 4 hr IPTG induction at 37^0C and purified on amylose resin. Capped RNA was synthesized using mMessage mMachine (Ambion). MOs designed against NAGK's 5' UTR (CTCCCCCATATACAGCA-GCCATCGC) and DPAGT1's 5' UTR and start sequence (CCGGCATGTTTGCCAATAGTTTACG) were obtained from Gene Tools, LLC.

Xenopus Egg Extract Degradation Assay

Xenopus egg extract was prepared and degradation assays were performed as in (Salic et al., 2000).

In Situ Analysis

In situ analysis was performed as described (Harland, 1991). Probes against Brachyury (Smith et al., 1991), Chordin (Sasai et al., 1994), Wnt8 (Christian et al., 1991), Twist (Hopwood et al., 1989), N-Cam (Kintner and Melton, 1987), Otx30 (Pannese et al., 1995), and Collagen 2 (Su et al., 1991) were made and used as previously described. Probe against NAGK was constructed against a 1.4 kb fragment of *Xenopus laevis* NAGK (#5515556, OpenBiosystems, pCMV-SPORT6) using Sp6 for the antisense strand.

FGF Animal Cap Assay

Embryos were soaked 0.1X MMR (Marc's Modified Ringers) with or without tunicamycin (2 ug/ml or 4 ug/ml) or glucosamine (100 mM) starting at the 8-cell stage. At stage 8 (before the onset of gastrulation), ectodermal caps were explanted from tunicamycin, glucosmaine, or control treated embryos and cultured in 0.75X MMR supplemented with recombinant xFGF8 (100 ng/ml). After 1 hr incubation, animal caps were transferred to an Eppendorf tube, media was removed, and caps were homogenized in SDS-PAGE sample buffer and flash frozen. Lysates were thawed and analyzed by SDS-PAGE and immunoblot for phospho-ERK (Cell Signaling) and total ERK (Cell Signaling).

Results

NAGK Overexpression Posteriorizes *Xenopus* Embryos.

In an overexpression screen to identify novel kinases that regulate vertebrate development, 29 pools each consisting of 8 mRNAs encoding different human kinases were injected into four-cell stage *Xenopus laevis* embryos. Embryos were then analyzed for developmental defects. mRNAs from pools that perturbed development were then injected individually to identify the overexpressed kinase mRNA affecting development. In Figure 5.1, we show embryological phenotypes associated with overexpression of two single kinases without characterized roles in early vertebrate development, B-lymphocyte tyrosine kinase (BLK) and dual-specificity tyrosine-phosphorylation regulated kinase 2 (DYRK2). BLK and DYRK2, along with hematopoetic cell kinase (HCK) and cyclin

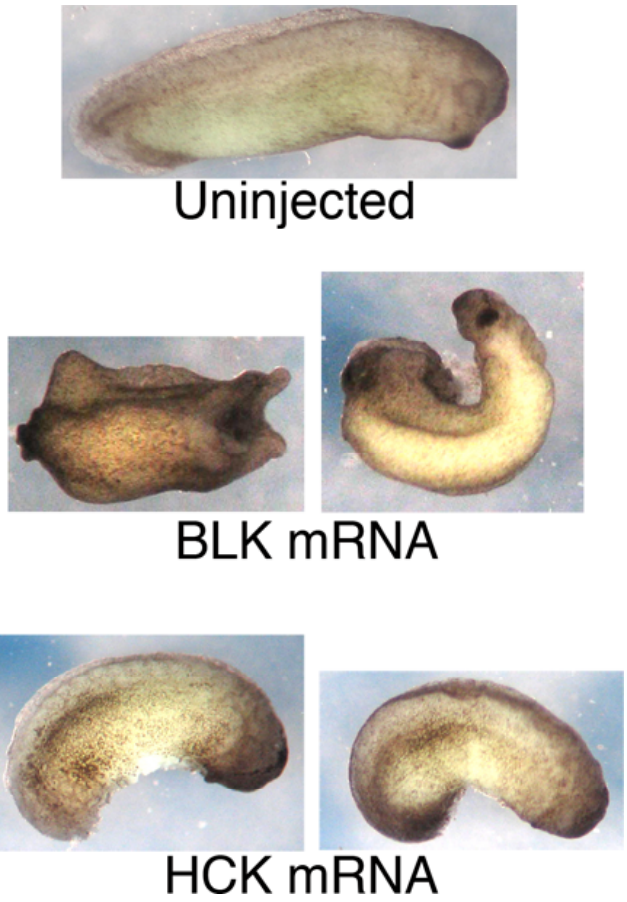

Figure 5.1: Overexpression of BLK and HCK Ventralize or Posteriorize *Xenopus* Embryos. Injection into each dorsal blastomere of 2- and 4-cell embryos of mRNA ecoding either human BLK (100 pg) or HCK (170 pg) inhibits development of anterior trunk and head tissues in *Xenopus* embryos. Screen performed by E.T. and C.S.C.

dependent kinase 2 (CDK2) (data not shown), prevented formation of head and trunk tissues in *Xenopus* embryos. Interestingly, both BLK and HCK belong to the c-Src family of kinases (Kefalas et al., 1995).

In parallel to the developmental screen, a screen utilizing the same mRNA pools was performed in cytoplasmic *Xenopus laevis* egg extract to identify kinase regulators of β-catenin stability (Salic et al., 2000). In canonical Wnt signaling, β-catenin stability is a major regulatory node by which the pathway is activated or inhibited (Logan and Nusse, 2004). Single exogenous mRNAs were translated in *Xenopus* egg extracts to assay for effects on β-catenin stability. Overexpression of several single kinases had effects on β-catenin levels that may relate to their perturbations of *Xenopus* development. For example, BLK and DYRK2 overexpression both appeared to stabilize β-catenin in this assay (data not shown). Further characterization must be performed to determine whether these candidates modulate *Xenopus* development through regulation of β-catenin.

Overexpression of NAGK did not affect β-catenin levels but had one of the strongest and most penetrant phenotypes identified from the developmental screen. Overexpression of mRNA encoding NAGK and injection of recombinant human NAGK protein both robustly inhibited formation of anterior trunk and head structures, a phenotype described as posteriorized (Figure 5.2).

NAGK Loss-of-Function Anteriorizes *Xenopus* Embryos.

As overexpression of NAGK inhibited formation of anterior structures, we hypothesized that NAGK plays an endogenous role in antagonizing or limiting

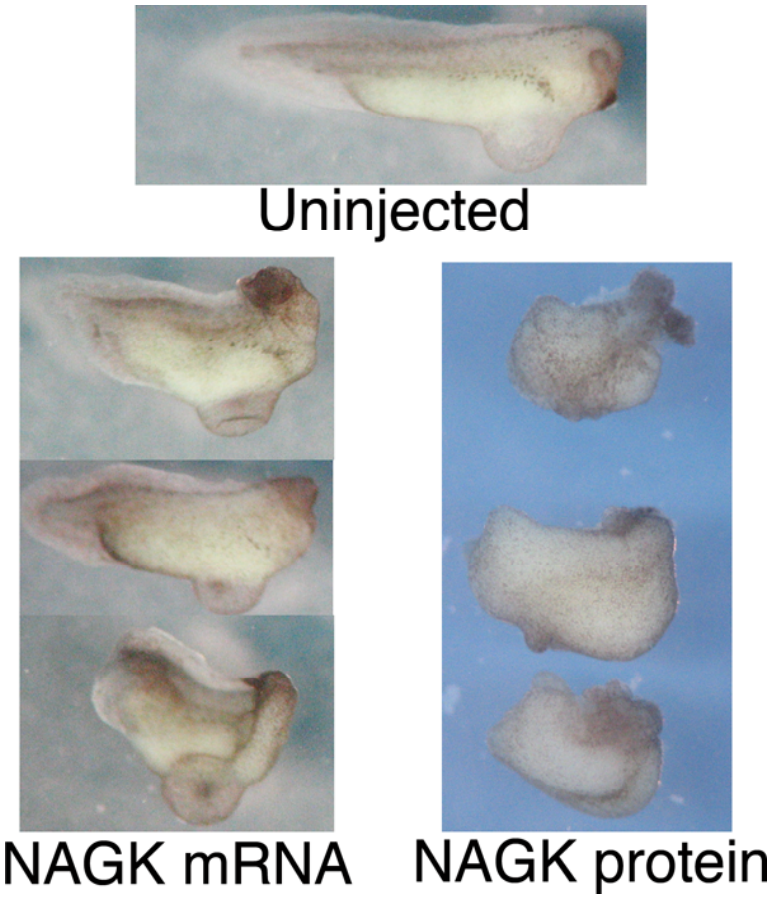

Figure 5.2: Overexpression of NAGK Posteriorizes *Xenopus* Embryos. Injection into each dorsal blastomere of 4-cell embryos of mRNA encoding human NAGK (1 ng) or recombinant protein encoding MBP-tagged human NAGK (20 pg) prevents formation of anterior structures in *Xenopus* embryos assayed at Stage 35.

specification of anterior structures. If this were the case, inhibition of NAGK would be expected to promote expansion of anterior structures at the expense of truncated posterior structures; such a phenotype is commonly referred to as "anteriorized". We performed NAGK loss-of-function through three experimental perturbations. First, we designed Morpholino oligonucleotides (MOs) to inhibit translation of endogenous NAGK. Dorsal injection of NAGK MO anteriorized *Xenopus* embryos (Figure 5.3B). Second, we hypothesized that overexpression of a kinase-dead mutant of NAGK (NAGK T128M) would compete with endogenous NAGK for substrate binding and prevent phosphorylation of the substrate. An orthologous T228M mutation in the ATP binding site of the similar sugar kinase, glucokinase, has been shown to potently abolish kinase activity (Mahalingam et al., 1999), and this ATP binding region is highly conserved between glucokinase and NAGK (Berger et al., 2002). In support of this "dominant negative" strategy, dorsal overexpression of NAGK T128M anteriorized embryos, phenocopying MO-mediated NAGK loss-of-function (Figure 5.3C). Third, we injected a small molecule competitive inhibitor of NAGK that mimics its substrate (3-O-methyl N-acetylglucosamine) (Miwa et al., 1994; Zeitler et al., 1992) into dorsal blastomeres, which also promotes anteriorization of embryos (Figure 5.3D). Thus, NAGK overexpression posteriorizes embryos, and NAGK loss-of-function (via three distinct experimental perturbations) anteriorizes embryos. These experiments demonstrate that endogenous NAGK is required to limit anterior structure formation in *Xenopus* embryos.

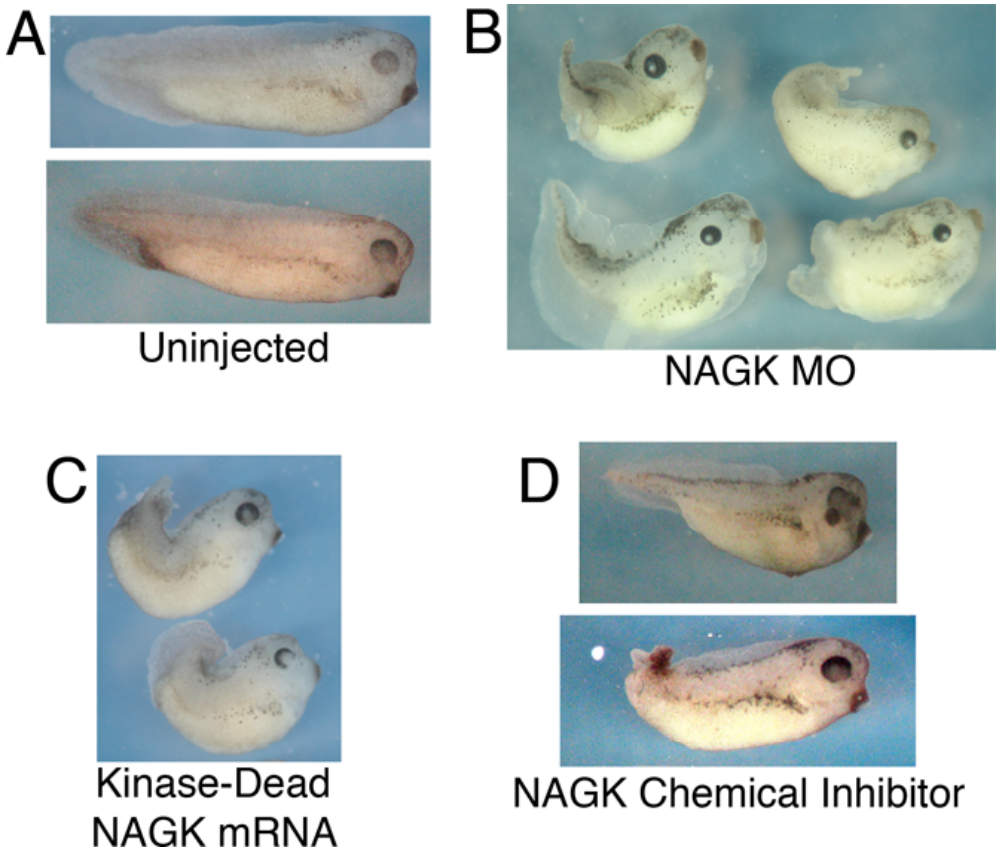

Figure 5.3: Endogenous NAGK Is Required in *Xenopus* Development to Limit Specification of Anterior Tissues. (A) Uninjected control embryos for experiments shown in B,C, and D. (B) Injection into each dorsal blastomere of a 4-cell embryo of NAGK MO (1.3 pmol) leads to increased specification of anterior structures at the expense of posterior structures (anteriorization). (C) Injection into each dorsal blastomere of a 4-cell stage embryo of mRNA encoding NAGK T128M anteriorizes embryos. (D) Injection into each dorsal blastomere of a 4-cell embryo of 3-O-methyl N-acetylglucosamine (4 uM) anteriorizes embryos though to a lesser extent than NAGK MO or NAGK T128M. Note ectopic cement gland (another indicator of anteriorization) in upper panel.

Perturbations of NAGK and DPAGT1 Lead to Similar Developmental Defects.

NAGK is the first enzyme in the salvage pathway that converts free, cytoplasmic N-acetylglucosamine (GlcNAc) generated from degradative cellular pathways into UDP-GlcNAc, which is then transferred onto oligosaccharides that are incorporated into glycosylated proteins or glycosaminoglycans (Hinderlich et al., 2000). Given structural studies performed with NAGK, the active site of the kinase is not predicted to have kinase activity towards protein substrates (Berger et al., 2002). Of note, we did not discover any phenotypes when we overexpressed two other enzymes from this pathway (GlcNAc-6-P mutase and UDP-GlcNAc pyrophosphorylase) (data not shown), possibly suggesting that NAGK is a rate-limiting step in this process. We were initially surprised that an enzyme that appeared to play a global role in promoting glycosylation has such a strong and specific effect on tissue specification during development.

To test whether NAGK acts on anteroposterior patterning via alteration of glycosylation and to determine whether NAGK disrupts development through effects on N- or O-linked glycosylation, we investigated the effect of perturbation of a rate-limiting enzyme with a specific role in N-linked glycosylation, DPAGT1. DPAGT1 transfers the first sugar (GlcNAc) onto ER lipid-linked dolichol to initiate construction of the LLO that is transferred onto the Asn residue of N-glycosylated proteins in the ER (Lehrman et al., 1988). Embryos in which DPAGT1 was overexpressed were posteriorized to an extent similar to those injected with NAGK (Figure 5.4A). Moreover, DPAGT1 MO and tunicamycin, a potent, well-characterized DPAGT1 inhibitor (Lehrman et al., 1988), both strongly anteriorized embryos (Figure 5.4B and 5.4C). Thus, developmental phenotypes produced by alterations in NAGK phenocopy those caused by alterations in the rate-

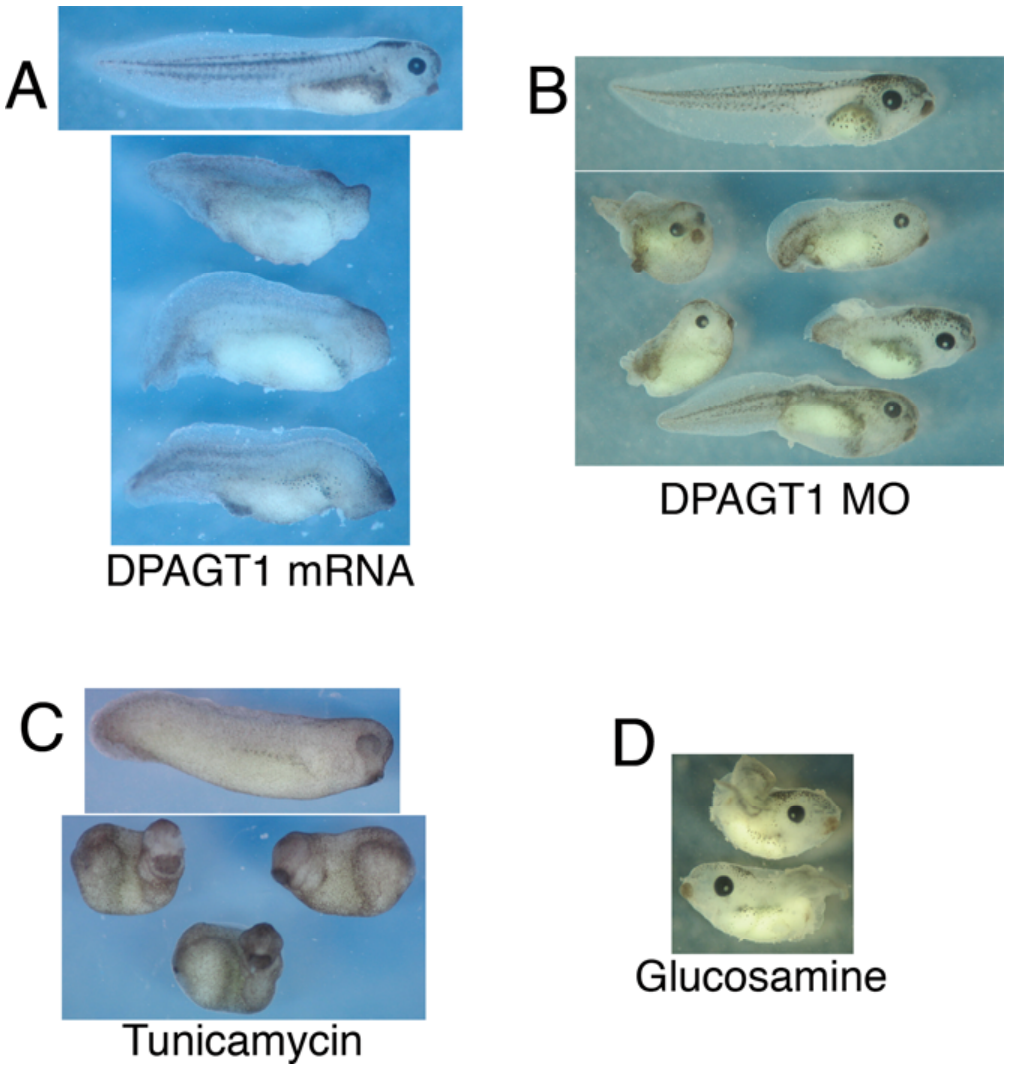

Figure 5.4: Gain- and Loss-of-Function of a Key Rate-Limiting Enzyme Specific to N-Linked Glycosylation Phenocopy NAGK Disruption in *Xenopus* Embryos. (A) Injection into each dorsal blastomere of a 4-cell embryo of mRNA encoding DPAGT1 (0.4 ng) posteriorizes embryos. An uninjected embryo is shown above. (B) Injection into each dorsal blastomere of a 4-cell embryo of DPAGT1 MO (1.3 pmol) anteriorizes embryos. An uninjected embryo is shown above. (C) Soaking *Xenopus* embryos from the 8-cell stage until Stage 16 in .1X MMR with 2 ug/ml tunicamycin anteriorizes *Xenopus* embryos. A control embryo is shown above. (D) Soaking *Xenopus* embryos from the 8-cell stage until Stage 16 in .1X MMR with 100 mM glucosmaine anteriorizes *Xenopus* embryos.

limiting step of N-glycosylation. Previous work in cultured cells demonstrated that incubation with glucosamine inhibits both N- and O-linked glycosylation (Little et al., 2008); and, soaking *Xenopus* embryos with glucosamine promotes anteriorization, similar to what is seen with NAGK and DPAGT1 loss-of-function (Figure 5.4D). These data support a model in which NAGK regulates anteroposterior patterning of *Xenopus* embryos by its effects on N-glycosylation.

In addition to its well-characterized role as a rate-limiting enzyme in N-glycosylation, DPAGT1 is mutated in CDG Type 1j (Wu et al., 2003). Like many patients who suffer from severe CDGs, the patient identified with defective DPAGT1 presented with mental retardation, microcephaly, seizures, and muscular hypotonia (Eklund and Freeze, 2006). Because the specific developmental and molecular perturbations that link deficits in N-glycosylation and CDG disease symptoms have not been identified, the DPAGT1 (and NAGK) loss-of-function phenotypes that we have identified in *Xenopus* embryos may provide a model system to study the molecular pathogenesis of CDGs.

Specific Tissues Are Misspecified in Embryos with N-glycosylation Defects.

To determine which tissues are improperly specified in embryos in which NAGK or DPAGT1 are overexpressed and inhibited, we utilized *in situ* analysis of well-characterized developmental mRNA markers. In a preliminary experiment performed towards this end, we analyzed markers for early mesoderm (Brachyury) (Smith et al., 1991), Spemann's organizer (Chordin) (Sasai et al., 1994), ventral mesoderm (Wnt8) (Christian et al., 1991), neural crest (Twist) (Hopwood et al., 1989), neural tissue (neural

cell adhesion molecule (N-Cam)) (Kintner and Melton, 1987), anterior neurectoderm (*Xenopus* orthodenticle 30 (Otx30)) (Pannese et al., 1995), and axial/somitic mesoderm (Collagen 2) (Su et al., 1991). We compared wild-type albino embryos with embryos treated with tunicamycin. While differences between expression of certain markers such as Twist, Otx30, N-Cam, and Collagen-2 were noted, expression of these mRNAs is so dynamic that there is significant variation even between identically treated embryos (Figure 5.5-5.7). Although tunicamycin treatment is experimentally simple, tunicamycin-treated embryos exhibit certain off-target phenotypes that differ from NAGK or DPAGT1 loss-of-function phenotypes (Figure 5.4C). To avoid these problems, future experiments will be performed in which only one side of the embryo will be injected with NAGK MO or GPT MO and the contralateral side will be injected with lacZ mRNA for β-galactosidase lineage tracing to mark the contralateral side and serve as an injection control (Hassler et al., 2007). As a result, wild-type and perturbed tissue can be more easily compared, and MO-mediated perturbation will decrease off-target effects seen in tunicamycin-treated embryos.

FGF Signaling Is Impaired in Embryos with N-Glycosylation Defects.

The phenotype that results from NAGK or DPAGT1 loss-of-function most closely resembles the phenotype of embryos in which FGF signaling has been impaired (Delaune et al., 2005; Park et al., 2002; Tsang et al., 2002). In embryos in which FGF signaling, NAGK, or DPAGT1 have been inhibited, a characteristic expansion of anterior structures at the expense of trunk and tail tissue, which become ventrally curved, is noted (Figure 5.8). This precise phenotype does not occur in embryos in which other signaling

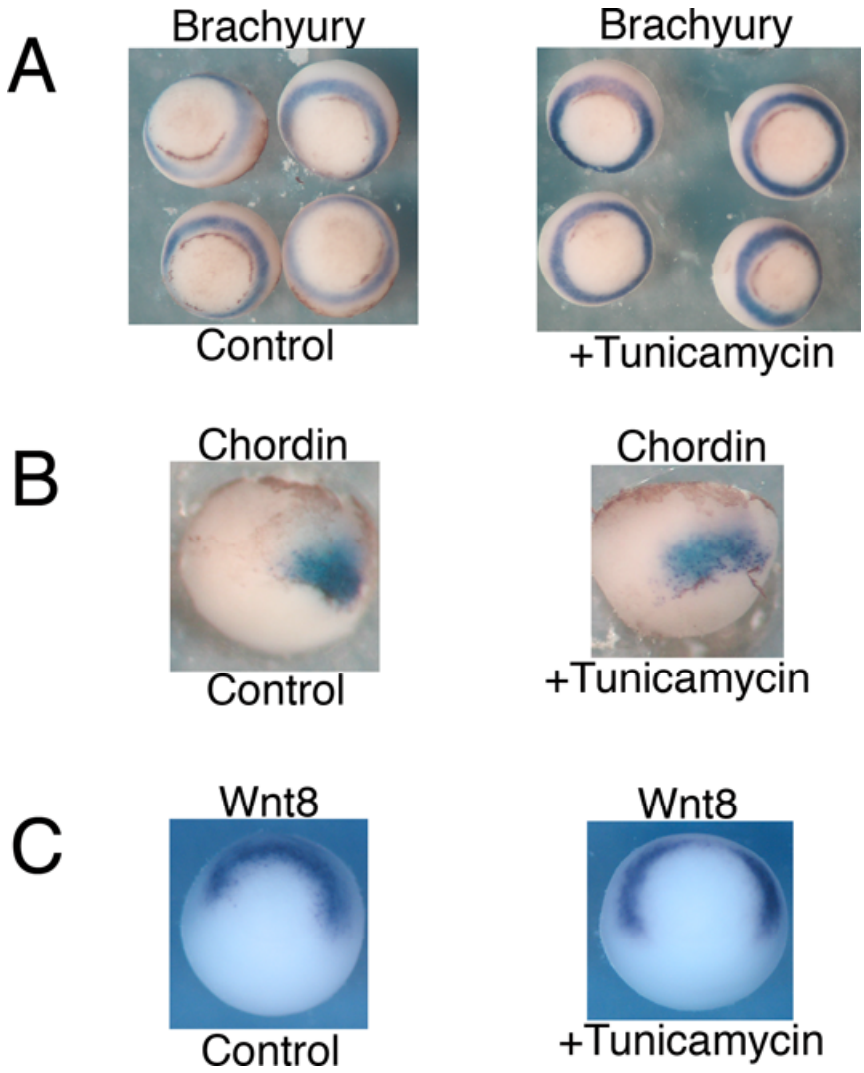

Figure 5.5: ***In Situ*** **Analysis of Early Embryonic Markers for Mesoderm, Organizer, and Ventral Mesoderm Suggest These Tissues Are Not Perturbed in Tunicamycin-Treated Embryos.** (A) Analysis of Brachyury RNA expression in Stage 10.5 embryos suggests tunicamycin does not disrupt mesoderm early formation. (B) Analysis of Chordin RNA expression in Stage 11 embryos suggests tunicamycin does not disrupt formation of Spemann's organizer. (C) Analysis of Wnt8 RNA expression in Stage 10.5 embryos suggests tunicamycin does not disrupt ventral mesoderm formation.

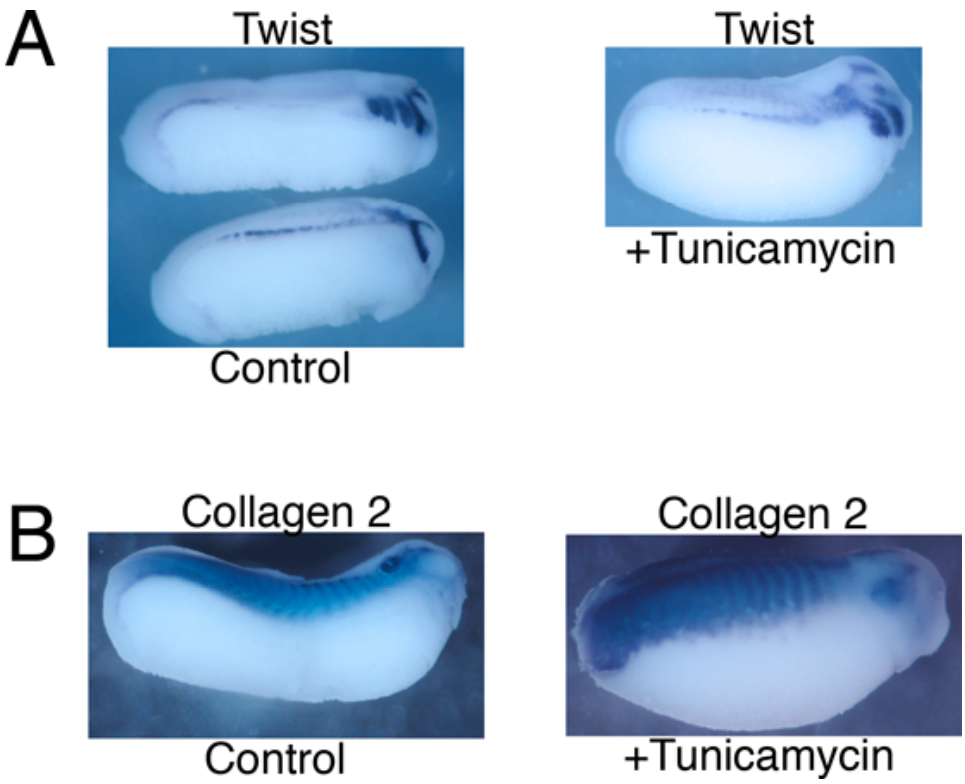

Figure 5.6: ***In Situ*** **Analysis of Early Embryonic Markers for Neural Crest and Somites Suggests These Tissues May Be Misspecified in Tunicamycin-Treated Embryos.** (A) Analysis of Twist RNA expression in Stage 26 embryos suggests tunicamycin may promote anterior displacement of branchial arch derivatives and decreased Twist expression around optic placodes. Thus, development of neural crest development may be disrupted in these embryos. (B) Analysis of Collagen 2 RNA expression in Stage 26 embryos suggests tunicamycin promotes increased posterior staining of tissues expressing Collagen 2, a marker of somitic mesoderm. This alteration in expression pattern suggests a convergence and extension defect (Ela Knapik, personal communication).

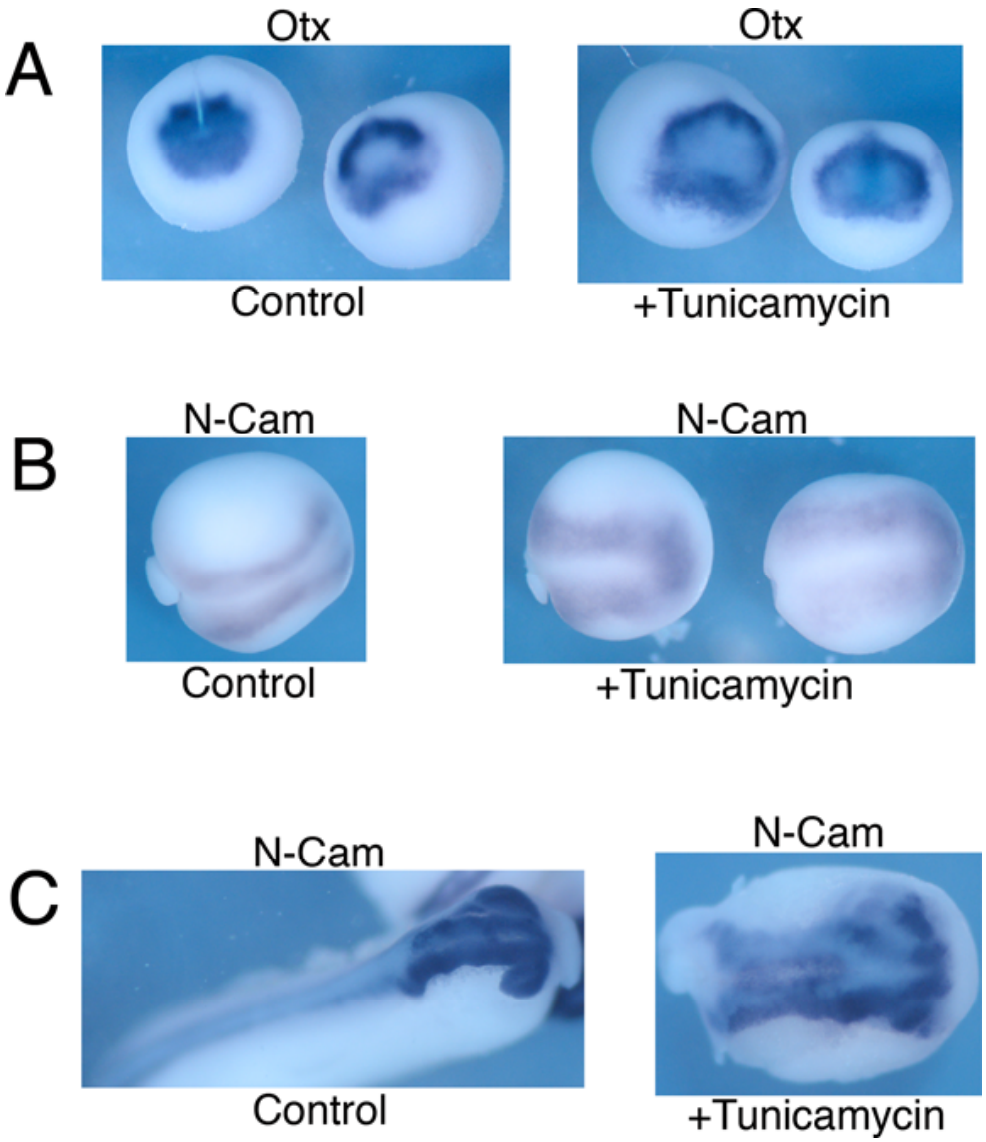

Figure 5.7: ***In Situ*** **Analysis of Early Embryonic Markers for Anterior Neurectoderm and Pan-Neural Tissue Suggests These Tissues May Be Misspecified in Tunicamycin-Treated Embryos.** (A) Analysis of Otx30 RNA expression in Stage 15 embryos suggests tunicamycin may promote expansion of anterior neurectoderm, consistent with the anteriorized phenotypes of these embryos. (B) Analysis of N-Cam RNA expression in Stage 15 embryos suggests tunicamycin may promote expansion of neural tissue in neurula stage embryos. Note expansion of N-Cam expression in the neural folds. (C) Analysis of N-Cam mRNA expression in Stage 26 embryos suggests tunicamycin may also affect neural tissue later in development. Note disparate N-Cam staining around optic placodes and neural tube. The alterations in N-Cam expression patterns suggest convergence and extension defects (Ela Knapik, personal communication).

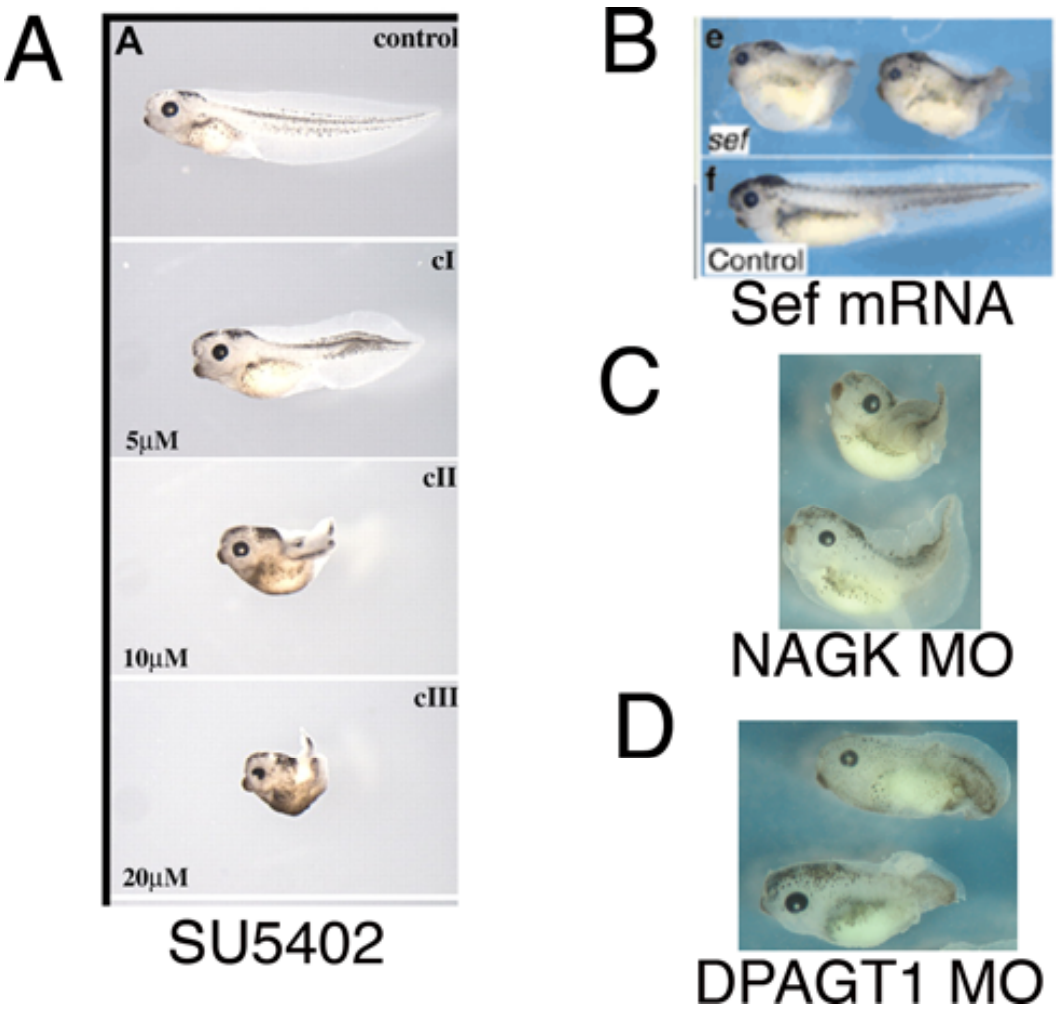

Figure 5.8: NAGK and DPAGT1 Loss-of-Function Phenocopy Loss-of-Function of FGF Signaling in *Xenopus* Embryogenesis. (A) Panel from (Delaune et al., 2005). Embryos soaked in increasing concentrations of the FGF receptor inhibitor SU5402 display increasingly shortened A-P axes, anteriorization, and dorsal curvature of the tail. (B) Panel from (Tsang et al., 2002). Dorsal mRNA injection of a negative regulator of FGF signaling, Sef, also promotes a shortened A-P axis anteriorization, and dorsal curvature of the tail. (C) and (D) Like embryos shown in (A) and (B) where FGF signaling is inhibited, loss-of-function of NAGK (1.3 pmol MO) and DPAGT1 (1.3 pmol MO) causes a shortened A-P axis, anteriorization, and dorsal curvature of the tail.

pathways such as Wnt, bone morphogenetic protein (BMP), or Hedgehog are perturbed. As result, we directly tested whether FGF signaling is inhibited in embryos in which N-glycosylation has been inhibited using a previously described method (Wang et al., 2004). Embryos were soaked in tunicamycin-containing medium starting at the 8-cell stage. At stage 8 (before the onset of gastrulation), ectodermal caps were explanted from tunicamycin or control embryos and cultured in media supplemented with recombinant xFGF8. Importantly, FGF signaling as assayed by immunoblot for phospho-ERK was compromised in tunicamycin-treated embryos (Figure 5.9). Although this experiment should also be carried out with NAGK MO or DPAGT1 MO (to control for off-target effects of tunicamycin) and with rescue by overexpression of NAGK or DPAGT1 (again to determine specificity), the data from this experiment suggest that loss-of-function of N-glycosylation directly inhibits FGF signaling in *Xenopus* embryos.

NAGK Has a Dynamic Developmental Expression Pattern.

Although metabolic genes such as NAGK and DPAGT1 are sometimes considered "housekeeping" genes that are not dynamically controlled to regulate important biological processes during development, our data suggests that this is not the case for NAGK and DPAGT1. Of note, both genes have contrasting partial gain- and loss-of-function phenotypes. This implies that levels of the activity of these proteins must be tightly regulated to allow for proper development. As a result, we hypothesized that NAGK and DPAGT1 would display dynamic patterns of expression. Indeed, *in situ* analysis of NAGK expression demonstrates that this gene is dynamically regulated (Figure 5.10). In blastula stages, NAGK is preferentially expressed in the cells of the

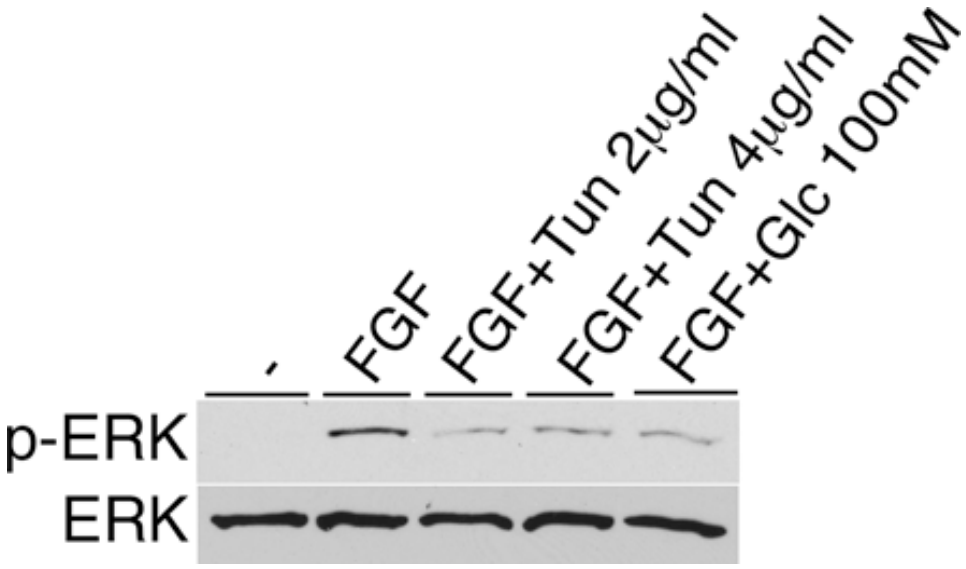

Figure 5.9: Tunicamycin Directly Inhibits FGF Signaling in Animal Caps. Embryos were soaked .1X MMR with or without tunicamycin (2 ug/ml or 4 ug/ml) or glucosamine (100 mM) starting at the 8-cell stage. At stage 8 (before the onset of gastrulation), ectodermal caps were explanted from tunicamycin, glucosmaine, or control treated embryos and cultured in .75X MMR supplemented with recombinant xFGF8 (100 ng/ml). After 1hr incubation, FGF signaling was as assayed by immunoblot for phospho-ERK and total ERK.

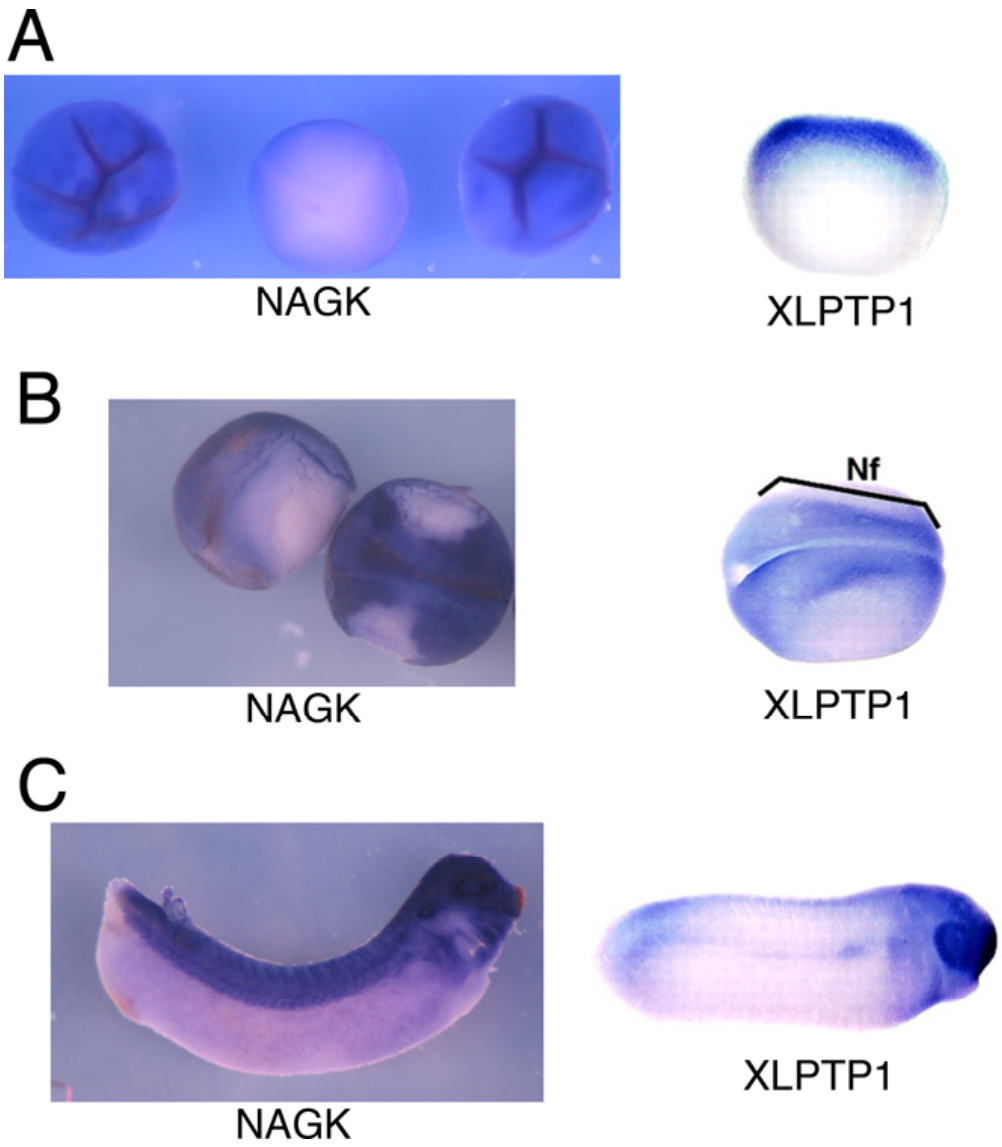

Figure 5.10: Endogenous NAGK Is Expressed in Tissues Undergoing FGF Signaling. XLPTP1 is a protein involved in FGF signaling with an expression pattern that is similar to that of several FGF receptors. The expression pattern of XLPTP1 shown here has been taken from figures from (Park et al., 2002). (A) Like XLPTP1 (Park et al., 2002) (side view, animal side up), NAGK is expression is enhanced at the animal hemisphere of early blastula stage embryos (left and right embryos: top view of animal hemisphere; center embryo: bottom view of vegetal hemisphere). (B) Like XLPTP1 (Park et al., 2002), NAGK expression is enhanced at the neural folds (NF) of neurula stage embryos. (C) Like XLPTP1 (Park et al., 2002), NAGK in the tailbud stages is enhanced at optic placodes, otic placodes, throughout head tissues, and in the somites.

animal pole. In neurula stages, NAGK seems to be preferentially expressed at the neural folds. And in tailbud stages, NAGK is strongly expressed in the somitic and head regions. Although we have not obtained data regarding the spatiotemporal regulation of DPAGT1 in *Xenopus* embrygenesis, studies suggest DPAGT1 is dynamically expressed in the hamster (Mota et al., 1994).

Given the data that suggests that inhibition of N-glycosylation directly inhibits FGF signaling, we considered the possibility that NAGK could be more highly expressed in tissues that undergo FGF signaling. Intriguingly, the expression pattern of NAGK strongly resembles the expression patterns of certain FGF receptors as well as intracellular modulators of FGF signaling (Figure 5.10) (Golub et al., 2000; Park et al., 2002). These data suggest that certain global regulators of N-glycosylation may need to be upregulated in tissues undergoing FGF signaling. Our finding suggests a further possibility: certain global regulators of N-glycosylation may be direct transcriptional targets of FGF signaling. Nonetheless, overexpression data and expression pattern analysis suggest that expression levels of NAGK and DPAGT1 must be dynamically adjusted to certain levels that allow for proper regulation of developmental events.

Discussion

N-glycosylation Regulates FGF Signaling in *Xenopus* Development.

We have characterized the sugar kinase NAGK as a novel regulator of anteroposterior patterning in *Xenopus* embryogenesis. Given the similarity of phenotypes derived from perturbation of NAGK and DPAGT1 (Figure 5.2, 5.3, and 5.4), we suggest

that NAGK affects development through its role in N-glycosylation. Although we were surprised that perturbations of global regulators of N-glycosylation had such specific developmental phenotypes, defects in such proteins (e.g. DPAGT1 (Wu et al., 2003)) cause CDGs with very specific symptoms in humans. Because the phenotypes of embryos with loss-of-function in NAGK or DPAGT1 resemble the phenotype of embryos in which FGF signaling is inhibited (Figure 5.5), we tested whether inhibition of DPAGT1 directly inhibits FGF signaling and found this to be the case (Figure 5.9). Moreover, examination of NAGK's expression pattern reveals that the gene displays dynamic temporal-spatial regulation through development and is most highly expressed in tissues undergoing FGF signaling (Figure 5.10). Thus, we suggest that NAGK is a dynamically regulated modulator of FGF signaling in vertebrate development.

It is not known how global perturbation of N-glycosylation inhibits FGF signaling. Partial inhibition of N-glycosylation may lead to hypoglycosylation of an FGF signaling component and thus reduce its proper maturation, trafficking, activity, or stability. Direct analysis of FGF ligands, receptors, or other N-glycosylated components of FGF signaling may reveal defects in such a protein that could suggest a molecular basis by which perturbation of N-glycosylation inhibits FGF-directed developmental processes. It will also be interesting to speculate why a certain component of FGF signaling is more sensitive to changes in N-glycosylation than other N-glycosylated proteins required for development. Nonetheless, identification of the FGF-associated protein inhibited by partial loss-of-N-glycosylation will help elucidate the connection between defects in N-glycosylation and FGF signaling. While we only noted defects in FGF signaling, it is possible that partial loss-of-N-glycosylation impacts other

developmental events in *Xenopus*. To test this, it will be important to determine whether activation of FGF signaling downstream of the glycosylation-induced defect can suppress all or only some of the developmental defects induced by partial loss-of-N-glycosylation.

Unexpectedly, we find that overexpression of NAGK and DPAGT1 also cause a defect in anteroposterior patterning (Figure 5.2 and 5.4). While loss-of-function anteriorizes embryos, gain-of-function posteriorizes embryos. These data suggest that the amount of N-glycosylation must be titrated within a certain range to allow for proper development. This hypothesis is supported by the dynamic temporal-spatial pattern of NAGK's mRNA expression pattern. Overall, these data suggest that certain regulators of N-glycosylation are not merely "housekeeping" genes but are dynamically regulated modulators of development. Future work testing whether overexpression of these genes actually promotes enhanced FGF signaling may suggest that precise NAGK and/or DPAGT1 levels directly determine the extent of FGF signaling in cells.

Elucidation of NAGK and DPAGT1 as developmental regulators implies that these genes and possibly other global regulators of glycosylation may be modulated to control certain physiological, pathological, and evolutionary events. As a result, we suggest that activation or inhibition of expression of these genes may have direct and specific effects on a variety of biology phenomena that deserve detailed experimental investigation. Such studies may reveal how alterations in the level of N-glycosylation play important and specific roles in physiology, pathology, and evolution. For example, mutations that upregulate expression of NAGK or DPAGT1 may be oncogenic by promoting FGF-mediated carcinogenesis. In evolution, mutations in the cis-regulatory

elements of NAGK or DPAGT1 may alter the amount of FGF-mediated signal transduction in a certain tissue to alter its morphology and function.

Xenopus May Be Used as a Model Organism to Study Pathogenesis and Treatment of CDGs.

The mechanism by which loss-of-function mutations in global regulators of N-glycosylation cause symptoms of CDGs has not been identified (Freeze, 2006). To address this problem, we suggest that partial loss-of-function of DPAGT1 in *Xenopus* embryos may constitute a model system to study CDGs. Specifically, our work implies a hypothesis for the molecular basis of CDG pathogenesis: mutations in CDG genes perturb human development by specifically impinging upon proper FGF signaling. Consistent with this hypothesis, certain mutations in FGF signaling in humans cause developmental disorders with symptoms identical to those caused by CDGs. For example, microcephaly, dysmorphic palate, 4,5 digit syndactyly, and strabismus are common to several CDGs as well as genetic disorders affecting FGF signaling (Chen and Deng, 2005; Freeze, 2006; Toydemir et al., 2006). While much work remains to test the link between CDGs and defective FGF signaling in humans, our use of *Xenopus* as a model system to study CDGs has generated a hypothesis regarding the molecular pathogenesis of this group of human disorders.

Xenopus may also be used as a model system to understand other important aspects of CDGs. It has been shown that cells derived from CDG patients display an activated UPR (Lecca et al., 2005; Shang et al., 2002). Inhibition of N-glycosylation may cause hypo-N-glycosylated proteins to fold incorrectly. As a result, protein trafficking

through the ER is defective and the UPR is activated. It is thus possible, though untested, that chronic activation of UPR may indirectly promote CDG pathogenesis. To test whether UPR plays a role in the developmental phenotypes seen in embryos with loss-of-function in NAGK or DPAGT1, one can examine whether downregulation of certain UPR-mediated events suppresses the primary developmental defect. Alternatively, one can test whether small molecule activators of the UPR that do not directly inhibit N-glycosylation cause similar developmental defects to those seen in cells where NAGK or DPAGT1 are perturbed.

In addition to serving as a model system to understand the pathogenesis of CDGs, *Xenopus* could also be used to test candidate therapeutics for specific CDGs. As CDGs are caused by defects in metabolic enzymes, supplementation of the product or substrate of an enzyme mutated in a particular CDG has successfully alleviated disease symptoms of two types of CDGs (Marquardt et al., 1999; Niehues et al., 1998). Such a therapeutic strategy could be tested in *Xenopus* for treating currently intractable CDGs. First, knockdown of the *Xenopus* ortholog of the particular CDG gene could be used to model the disease. Then, it could be tested whether supplementation with a certain carbohydrate metabolite via soaking or direct injection could suppress the phenotype. A proof-of-principle experiment could first be undertaken to validate this approach. CDG patients with mutations in PMI are treated with mannose therapy (Niehues et al., 1998). Can PMI-mediated developmental defects in *Xenopus* be similarly suppressed with mannose supplementation? Other therapeutic strategies may also be tested in *Xenopus*, such as activation of FGF signaling. Thus, we suggest the use of *Xenopus* to develop treatments

for CDGs. As a final note, the similarity of phenotypes caused by perturbation of NAGK and the CDG gene DPAGT1 suggests that NAGK could itself be a candidate CDG gene.

Future Directions

The experiments described in this chapter are part of a work in progress. Here, I describe further experiments that must be performed to support the stated results.

To confirm MO-mediated knockdown of NAGK and DPAGT1 translation, it is necessary to immunoblot for these proteins in control embryos and MO-injected embryos. As these proteins are highly conserved in human and mouse to which multiple antibodies have been raised, I predict that commercially available antibodies will suffice to confirm MO-knockdown of these genes.

To test whether NAGK and DPAGT1 MO-dependent phenotypes specifically result from decreases in translation of these proteins, we must rescue MO-mediated knockdown of NAGK and DPAGT1 with mRNA overexpression of NAGK and DPAGT1, respectively. This experiment is absolutely required to interpret the current MO data. Given that our overexpression experiments employ human NAGK and DPAGT1, these overexpressed mRNA's themselves will avoid MO-mediated translation inhibition. However, as we note phenotypes for both gain- and loss-of-function with these genes, we will likely need to carefully titrate the amount of mRNA to obtain rescue.

In addition to mRNA-mediated rescue of NAGK MO, rescue of NAGK MO with the NAGK's metabolic product GlcNAc-6-P would be an even more specific rescue than that performed with mRNA. This rescue would confirm that NAGK's role in these

phenotypes concerns its conversion of GlcNAc to GlcNAc-6-P and not some uncharacterized role for NAGK outside of glycosylation and GlcNAc phosphorylation.

I have assumed that NAGK and DPAGT1 affect the same developmental and molecular processes based on their similar embryonic phenotypes. To be able to state this with greater confidence, we must assay for functional interaction between these genes. For example, does NAGK overexpression increase the severity of DPAGT1 mRNA-mediated developmental defects? Does NAGK-MO increase the severity of DPAGT1 MO-mediated developmental defects? In addition, can NAGK overexpression rescue DPAGT1 MO-mediated defects, and can DPAGT1 overexpression rescue NAGK-MO-mediated defects? While the former two experiments seem more likely to be successful as the perturbations need only converge on a single process, positive results with the latter two experiments would have interesting and unexpected implications for cross-regulation of N-glycosylation that could inform CDG therapy development.

As stated in the text, the developmental survey for tissues perturbed in embryos with gain- and loss-of-function in NAGK or DPAGT1 should be performed in a different manner than shown in Figure 5.6, 5.7, and 5.8. The left or right side of the embryo should be injected with NAGK (or DPAGT1) MO or mRNA, and the contralateral side should be injected with a lineage tracer to mark control injection. It will be easier to interpret differences in highly dynamic and specific expression patterns if a control side of a single embryo can be compared with an experimentally perturbed side (Hassler et al., 2007).

The animal cap assay directly assaying FGF signaling should be repeated with NAGK MO or DPAGT1 MO instead of the less specific drug tunicamycin. In addition,

mRNA rescue of these MO effects in this assay would better support the conclusion that knockdown of these genes inhibits FGF signaling. To increase the sensitivity of the assay, animal caps could be incubated with FGF ligand for periods significantly shorter than 1 hr (e.g. 5 or 10 minutes) (Wang et al., 2004).

Lastly, *in situ* analysis of temporal-spatial mRNA expression of NAGK and GPT1 in the embryo must be repeated to obtain embryos with less background staining. The use of albino embryos or bleached pigmented embryos may aid this endeavor because many of the stained tissues are located in highly pigmented areas of the embryo.

While the previous suggested experiments are required for reasonable interpretation of the current data, the following future directions are aimed to further probe interesting aspects of this project.

If loss-of-function of NAGK and DPAGT1 perturb development through FGF signaling, experimental activation of FGF signaling via injection of mRNA encoding FGF ligand or receptor may suppress these defects in N-glycosylation. If complete rescue of the phenotype is achieved, it is likely that NAGK and DPAGT1 completely act through FGF signaling. If only certain aspects of the developmental phenotypes are rescued, it is possible that NAGK and DPAGT1 also affect other biological processes that could be further explored.

Although the phenotypes of embryos in which NAGK or DPAGT1 are perturbed most closely resemble defects in FGF signaling, certain aspects of the phenotypes could also be explained by alterations in Wnt or BMP signaling. The extensive survey of tissue specification via *in situ* hybridization for developmental markers may shed light on whether disruptions of tissue resembles perturbations of pathways other than FGF

signaling. In addition, the animal cap assay where FGF signaling was directly assayed could also be performed for Wnt and BMP signaling. To directly assay for disruption of Wnt signaling in embryos with perturbed N-glycosylation, embryos could be injected with mRNA encoding Wnt8, and after 1 hr whole blastulas could be assayed for decreases in phosphorylated β-catenin. To directly assay for disruption of BMP signaling, embryos could be injected with BMP ligand and after 1 h whole embryos could be assayed for phospho-Smad.

Immunoblotting for endogenous or exogenous, tagged N-glycosylated proteins involved in FGF signaling could reveal mobility shifts induced by mRNA or MO-mediated perturbation of NAGK or DPAGT1. Such shifts may result from hypo- or hyper-glycosylation of these proteins, which could be further tested by treatment of lysates with N-glycosidases to remove N-glycans from these proteins. These experiments could identify improperly glycosylated proteins involved in FGF signaling. In addition, mutation of existing N-glycosylation sites (or creation of new ones) on these proteins could directly test whether improper glycosylation of these proteins causes the developmental phenotypes observed in embryos in which NAGK or DPAGT1 are altered. In this manner, the molecular target by which perturbation of NAGK or DPAGT1 disrupt FGF signaling can be elucidated.

As expression of NAGK appears to be highest in tissues where FGF signaling occurs, it is possible that NAGK could be a transcriptional target of FGF signaling in the embryo. This could comprise a positive feedback loop to regulate FGF signaling. Determination of such a mechanism would support the role for NAGK as an important, dynamic regulator of FGF signaling in development. One could easily test whether gain-

or loss-of-function of FGF signaling (by conventional mRNA or MO injection) enhances or decreases expression of NAGK (as assayed by RT-PCR or *in situ* hybridization for NAGK).

Because I have suggested the use of *Xenopus* as a model organism to study CDGs and their therapies, it would be fairly straightforward to test this hypothesis with a proof-of-principle experiment. I propose that CDG Type 1b or CDG Type 2c and their successful treatments could be recapitulated in *Xenopus* embryos. CDG Type 1b is caused by a mutation in PMI and treated with mannose supplementation (Niehues et al., 1998). CDG Type 2c is caused by a mutation in FUCT1 and treated with fucose supplementation (Marquardt et al., 1999). I predict that MOs directed against PMI and FUCT1 would cause developmental defects in *Xenopus* similar to those seen with NAGK or DPAGT1 MO and that the defects could be suppressed by injection or soaking with mannose or fucose, respectively. Such experiments would greatly strengthen the case for using *Xenopus* as a model for CDGs.

It is predicted that CDGs are vastly undiagnosed, and mutations in genes causing CDGs continue to be identified. Thus, it is possible that mutations in NAGK, like mutations in DPAGT1, may cause CDGs in humans. Databases with partially mapped congenital disorders may be used to explore such a possibility (Taniguchi et al., 2006).

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