ROLE OF LRP6 IN THE WNT/β-CATENIN PATHWAY AND ITS REGULATION BY HETEROTRIMERIC G PROTEINS

By

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To my family

and

my husband, Jimmy

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A, alanine

AC, adenylyl cyclase

APC, adenomatous polyposis coli

β2-AR, β2- adrenergic receptor

cAMP, 3'-5'-cyclic adenosine monophosphate

CK1, casein kinase 1

CT, Curtis Thorne

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

Dsh, dishevelled

DTT, Dithiothreitol

EDTA, ethylenediamine tetraacetic acid

EGTA, ethylene glycol tetraacetic acid

Fz, Frizzled

GAP, GTPase activating protein

GDP, guanosine diphosphate

GEF, Guanine nucleotide exchange factor

GPCR, G protein coupled receptor

GSK3, glycogen synthase kinase 3

GTP, guanosine triphosphate

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

LRP5/6, low-density lipoprotein receptor-related protein

P, proline

PCR, polymerase chain reaction

PMSF, phenylmethanesulphonyfluoride

PTX, Pertussis toxin

RGS, Regulator of G protein signaling

RNA, Ribonucleic acid

S, serine

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

TRIS, tris(hydroxymethyl)aminomethane

Ub, ubiquitin

BOOK 1: INTRACELLULAR MECHANISM OF WNT/β-CATENIN SIGNAL TRANSDUCTION

CHAPTER 1: INTRODUCTION TO CELL SIGNALING

Introduction

The Wnt/β-catenin signaling pathway is highly conserved throughout metazoan and is required for the coordination of diverse developmental programs, stem cell maintenance, cell growth and cell proliferation. Multiple disease states have been attributed to the misregulation of Wnt/β-catenin signaling; most notably colorectal cancer in which upwards of 80% of tumors contain a mutation that renders the pathway constitutively active. Normally, the pathway is activated when the Wnt ligand binds to the Frizzled and low-density lipoprotein receptor related protein 6 (LRP6) receptors resulting in inhibition of phosphorylation and degradation of the co-transcriptional activator β-catenin. Elevated levels of β-catenin translocate to the nucleus and activate target gene expression. Within Chapter 2 and 3, I describe our findings on the coordination and regulation of the early intracellular signaling events that result in β-catenin stabilization. Chapter 1 provides a framework for the following Chapters including an introduction to cellular signaling, G protein signaling, and Wnt/β-catenin signaling.

How a cell communicates

Cells are remarkable communicators. Communication is the means by which cells understand their environment, maintain relationships with their surroundings, and in turn

provide cues to other cells and tissues. Just as humans have created a multitude of methods of communication, cells too have evolved intricate means of communicating. It is by these intricate means of communication that a single fertilized cell develops into an embryo with specific tissues. Specialized tissue develops further into organ systems, and these organ systems maintain homeostasis. On the simplest level, cells communicate by creating a signal that is sent to another cell. After recognition of the signal, the cell processes the signal and a response is produced. The mechanism by which a cell recognizes, processes, and responds to a signal is termed signal transduction.

Signal transduction governs and coordinates developmental processes in all metazoans. We understand this in great part from the work of Hans Spemann in the early 1900's. At this time, the events that transpired during embryonic development eluded many. To this end, in his Nobel Lecture in 1935, Hans Spemann stated

"How does that harmonious interlocking of separate processes come about which makes up the complete process of development? Do they go on side by side independently of each other (by "self-differentiation", Roux), but from the very beginning so in equilibrium that they form the highly complicated end product of the complete organism, or is their influence on each other one of mutual stimulation, advancement or limitation?"

Hans Spemann, along with Hilde Mangold, observed that transplantation of the dorsal lip of the blastopore from an amphibian embryo entering gastrulation onto the opposite side of another embryo resulted in formation of two body axes in the graphed embryo. One of the two axes was formed from the endogenous dorsal lip of the embryo while the other was formed from the grafted dorsal lip tissue. Tissue that would normally have formed the ventral side of the animal, instead, developed into a complete dorsal axis when the dorsal lip was graphed on it. The dorsal lip was then coined the organizer since it was able to redirect the fate of the tissue on which it was grafted (Spemann and Mangold,

2001). This landmark discovery provided crucial evidence that cells signal to one another to induce changes and coordinate developmental processes. For this work Spemann was awarded the Nobel Prize in Medicine or Physiology in 1935.

To date 18 signaling pathways have been identified. In general, all signaling pathways utilize a similar mechanism to transduce a signal. A ligand is released into the extracellular environment and bound by a cell surface receptor. This receptor-ligand interaction results in receptor activation and subsequent transduction of the signal into the cell. Intracellular signal transduction is accomplished by second messengers, or cellular switches, that pass the signal from one messenger to another until the final message is received resulting in a physiological response such as movement, transcriptional activation, cell death, etc.

Of the 18 signaling pathways identified, 5 pathways have been attributed to coordinating the events of metazoan development (Gerhart, 1999). While the conserved core processes (including mitosis, histone/DNA chromatin complexes, membrane dynamics, etc) of simple eukaryotes were established at least 2 billion years ago, the coordination of mutli-cellularity that has evolved since that time. Much of this coordination is due to the evolution of signaling pathways that allow cell-cell communication, which was not required for the survival of the simple single-celled eukaryote. The evolution of body plan is attributed to the Cambrain period (543-505) in which all existing members of each phyla were present. It has been observed that species from diverse phyla, such as flatworms and mice, are composed to the same tissue types during development, although the organization of these tissues differs. Essentially 5

signaling pathways (including the Wnt/β-catenin signaling pathway), which are also conserved through these diverse phyla, regulate this organization (Gerhart, 1999).

To a certain extent, it is unfortunate that mechanisms of signal transduction are coined pathways. A pathway suggests that there is only one direct way by which a signal can be sent from a source to produce a directed outcome of a cell. It also suggests that each pathway functions independently of the others. However, evidence suggests that crosstalk exists between pathways, such that activation of a receptor of one pathway can affect the outcome of another signaling pathway. Additionally, second messengers of one signaling mechanism can be used to transduce the signal of another signaling system. Mechanisms of signal transduction may exist more as a signaling network than a signaling pathway (van Amerongen and Nusse, 2009).

Historical Perspective: G protein Signaling

The first glimpse of a mechanism for cell signaling came in 1957 with the discovery of cyclic AMP (cAMP) and adenylyl cyclase (AC) by Rall and Sutherland. For this work Sutherland was awarded a Nobel Prize in Medicine or Physiology in 1971 (Berthet et al., 1957a) (Sutherland and Rall, 1958). They had observed that the kinase that phosphorylates glycogen could be activated by challenge with the hormones epinephrine and glucagon (Robinson and Sutherland, 1971) as well as treatment with fluoride. This same response was identified in canine liver homogenates (Berthet et al., 1957b), which produced a heat soluble factor produced by the enzyme adenylyl cyclase (AC) that was later crystallized and identified as cAMP (Berthet et al., 1957a; Sutherland and Rall, 1958). At this time, it was thought that the receptor, which received the

hormone signal, also produced AC activity. Evidence from biochemical and genetic studies later indicated that the receptor and AC were two individual proteins. These studies utilized a murine lymphoma cell line sensitive to high levels of cAMP. After selection of a population of cells that were resistant to the high cAMP levels, cells were identified to have wild type ligand binding capabilities but no AC activity (Insel et al., 1976). In addition, turkey erythrocytes lacking AC activity while containing functional receptors were fused with erythroleukemic cells containing a functional AC that lack functional receptors. The resulting fused cells were able to respond to the agonist and produce cAMP, indicating that the activities of the receptor and AC are independent entities (Orly and Schramm, 1976).

In addition to the receptor (β-adrenergic receptor) and enzyme (AC), evidence suggested that a third member of this pathway was involved in the hormone stimulation of cAMP production. Glucagon (the receptor's ligand) and fluoride stimulation of cAMP production was not competitive, and addition of fluoride could not stimulate cAMP production beyond glucagon. This suggested that fluoride acted on a different site than glucagon (Birnbaumer, 1991). Also, hormonal stimulation of AC was dependent on the presence of GTP, GDP, or GMP-PCP, and each was equally effective at stimulating AC activity (Rodbell et al., 1971). In the absence of a hormone, Gpp(NH)p, a non-hydrolyzable analog of GTP, was found to maximally stimulate AC activity. This indicated that AC system involved a GTPase, which was active in the presence of GTP.

Cassel and Selinger demonstrated that GTP was hydrolyzed in turkey erthrocyte membranes in the presence of catecholamines, and that this activity was dependent on magnesium and blocked by addition of an antagonist (Cassel and Selinger, 1976). They

proposed a model whereby AC is bound by GDP in its basal state and activation of AC results in replacement of GTP for GDP. Challenge with hormones resulted in hydrolysis of the bound GTP to GDP by AC, placing AC back into its basal state (Cassel and Selinger, 1976). At this time, it was also found that cholera toxin activated AC activity but inhibited GTPase activity stimulated by catecholamines (Cassel and Selinger, 1977; Sharp and Hynie, 1971).

Additional genetic and biochemical evidence from other groups challenged the Cassel and Selinger model and suggested that another protein could be attributed with the guanine nucleotide dependence. A mutant S49 lymphoma cell line, termed cyc-, was identified to be resistant to β-adrenergic receptor agonists, fluoride, Gpp(NH)p, and cholera toxin, but not addition of cAMP. This indicated that AC is functional in these mutant cells and is a separate entity from the guanine nucleotide regulated protein (Bourne et al., 1975). It was the work of a number of independent groups that together, through the use of alternative methods, identified a protein that is distinct from the receptor and AC that comprised the guanine nucleotide dependent signal transduction activity. Protein eluded from a GTPγS sepharose resin from lysate treated with fluoride and detergent could add guanine nucleotide regulation back to its flow-through (Pfeuffer, 1977). Also, sucrose density gradient centrifugation revealed a 42kDa protein that co-migrated with AC activity. Another group solubilized and combined membranes from the cyc- cell line (lacking detectable AC activity while expressing wild type receptors) and mouse L cells (containing wild type AC while lacking receptors) and obtained AC activity after treatment with agonist, fluoride and guanine nucleotide (Ross and Gilman, 1977b). After killing the AC activity in the L cell extract, and addition to the cyc-

membranes, they were able to recapitulate guanine nucleotide, fluoride, and hormone-stimulated activity (Ross and Gilman, 1977a) (Ross et al., 1978). The culmination of these studies provided strong evidence that another player, separate from the receptor and AC, served as a transducer between the receptor and AC.

Purification of the AC regulator subunit (Gs) was accomplished by analyzing GTP and fluoride-stimulated AC activity in the cyc- membranes (Northup et al., 1980; Sternweis et al., 1981). Gs were found to be composed of three polypeptides, later named Gα, β, and γ. It was also determined that while these polypeptides were absent in the cyc- membranes, but present in wild type S49 wild type membranes, consistent with previous experiments in cyc- membranes (Johnson et al., 1978). Al Gilman and Martin Rodbell were awarded the Nobel Prize in Medicine of Physiology in 1994 for their discovery of heterotrimeric G proteins and G protein signaling.

Heterotrimeric G protein Signaling

In a simplified model of heterotrimeric G protein signaling, a ligand binds to a G protein coupled receptor (GPCR) resulting in a conformational change such that the GPCR Gα protein has decreased affinity for its bound GDP (Figure 1.1). Because the intracellular concentration of GTP is higher than GDP, Gα quickly becomes bound to GTP. The Gα subunit is in an inactivate state when bound to GDP and active when bound to GTP. The activated Gα dissociates from the Gβγ dimer, and each of the dissociated subunits can interact with effector proteins. The length of effector activation is dictated by the intrinsic rate of GTP hydrolysis, as well as by Gα's interactions with GTPase Activating Proteins (GAP). Once GTP is hydrolyzed to GDP, the Gα subunit is

returns to its inactive state, binds Gβγ, associates with its receptor, and is ready for another round of G protein activation.

GPCRs represent one of the largest families of proteins in the human genome. The distinguishing feature of this family of proteins is the presence of seven α-helical transmembrane domains. 27% of all Food and Drug Administration (FDA)-approved drugs target this protein family (Williams and Hill, 2009), indicating that GPCRs are of interest to the pharmaceutical industry. Although present in lower eukaryotes (i.e. yeast and slime molds), GPCRs evolved 530 million years ago along with the appearance of multicellular organisms (Brown, 2007). The International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification has classified human GPCRs in 4 main categories based on sequence comparison; Class one is typified by rhodopsin, Class two is typified by the secretin receptor, Class three is typified by the glutamate receptor, and Class four is typified by the frizzled and smoothened receptors (Foord et al., 2005). Together, there are approximately 360 known functional GPCRs in the human genome.

Although originally identified to regulate cAMP levels, heterotrimeric G proteins have been found to mediate a number of signal transduction pathways. Currently there are 16 genes for Gα subunits in mammals (Table 1.1). Based on sequence similarity, Gα subunits have been separated into four families; G_s, G_i, G_q, and G_{12}. The types of proteins that Gα subunits interact with and activate, or effecter proteins, are as diverse as enzymes and ion channels (Table 1.1) (Cabrera-Vera et al., 2003). Also, the rate of nucleotide exchange (the amount of time it takes for a Gα subunit to release GDP and bind GTP), which is the rate-limiting step in G protein activation, varies greatly between

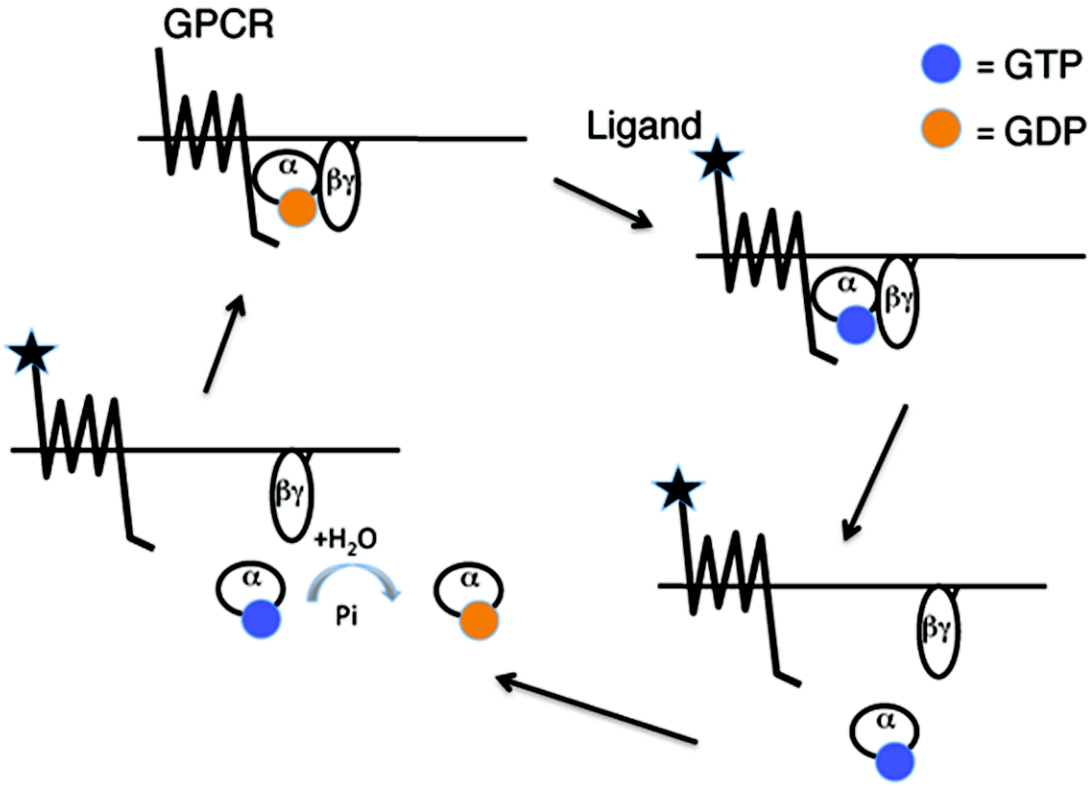


Figure 1.1 Cycle of heterotrimeric G protein activation. Starting in the top left hand corner, in the resting state, of off state, the heterotrimer is in association with a GPCR at the plasma membrane and the Gα subunit is bound to GDP. Ligand binding to the G protein coupled receptor (GPCR) results in a conformational change such that the GPCR induces its associated Gα protein to exchange its bound GDP for GTP rending the Gα subunit active. The activated Gα dissociates from the Gβγ dimer and each of the dissociated subunits can interact with effector proteins. The length of this dissociation is dictated by the intrinsic rate of GTP hydrolysis as well as the by Gα's interactions with GTPase Activating Proteins (GAPs). Once GTP is hydrolyzed to GDP, the Gα subunit is placed back into its inactive state, binds Gβγ, and associates with its receptor ready for another round of G protein activation.

Table 1.1 Gα families and effectors

Family	Subtype	Effector
Gs	Gαs(s)	Adenylyl Cyclase
	Gαs(l)	
	Gαolf	
Gi	Gαi1	Adenylyl Cyclase Ca^{2+} and K^{+} channels
	Gαi2	
	Gαi3	
	GαoA	
	GαoB	
	Gαz	
	Gαt1	Phosphodiesterase
	Gαt2	
	Gαgust	
Gq	Gαq	Phospholipase C β
	Gα11	
	Gα14	
	Gα15	
	Gα16	
G12	Gα12	Rho family GTPases
	Gα13	

(T.M. Caberera-Vera, Endocrine Reviews, 24 (6): 765-781)

individual Gα subunits. For example, the dissociation rate of Gαi2 is 0.07 min^{-1} at 30°C while Gαq has an extremely low affinity for GTP in the absence of an activated receptor (Hepler et al., 1993; Linder et al., 1990).

Originally thought to function as a regulator of Gα protein activity, Gβγ subunits are also a diverse class of proteins that can interact with and activate numerous effectors (Table 1.2). Five Gβ units and twelve Gγ subunits have been identified in both human and mouse genomes. Gβ and γ are always found in complex, however the variability in the composition of this dimer has not been correlated to signaling potential, except for $G\beta_1\gamma_1$ which has only been shown to associate with Gαt in the visual system (Smrcka, 2008). Similar to Gα, Gβγ interacts with and activates a number of proteins, including dynamin, ion channels, and phosphoinositide 3 kinase γ (Smrcka, 2008).

Both Gα and βγ are post-translationally modified with lipid modifications promoting their membrane association. All Gα subunits (except Gαt) are palmitolyated and G_i family are also myristoylated. Gγ subunits are isoprenylated, and this isoprenylation is required for Gβγ membrane localization. These lipid modifications are not only required for proper localization of Gα and Gβγ, but also for their interaction with effectors and receptors (Taussig et al., 1993; Yasuda et al., 1996; Cabrera-Vera et al., 2003) .

Crystal structures of inactive (GDP-bound) and active (GTP-bound) Gα, Gβγ, and the heterotrimeric Gαβγ have provided invaluable insight into the mechanism of heterotrimeric G protein activation (Coleman et al., 1994; Lambright et al., 1994; Lambright et al., 1996; Noel et al., 1993; Sondek et al., 1994; Wall et al., 1995). These

Table 1.2 Gβγ effector proteins

Physiological Gβγ Effectors	K^+ channel
	GPCR Kinase 2 and 3
	Phospholipase C β1, β2, β3
	Adenylyl cyclase
	N type Ca^{2+} channels
	P/Q type Ca^{2+} channels
	Phosphoinositide 2 kinase γ
	SNAP-25
	P-Rex1 Rac GEF
Proteins regulated by Gβγ	p21 activated kinase
	Raf-1
	Dynamin
	Ras GRF (a Ras GEF)
	Dbl (a Rho GEF)
	Bruton's Tyrosine Kinase
	Tubulin/microtubules
	Histone deacetylase 5
	P114 RhoGEF
	RGS4
	RGS3
	ATP sensitive K^+ channel
	Phospholipase D1
	IP_3 receptor 1
	T type Ca^{2+} channel
	Rac/cdc42 GEF

Physiological Effector: Gβγ binding protein for which a clear physiological role for Gβγ-effector intereaction has been established
Gβγ regulated protein: Gβγ binding protein whose activity is regulated by Gβγ but no physiological role established for the intereaction
(A.V. Smrcka, Cell Mol Life Sci. 2008 Jul;65(14):2191-214)

structures reveal that Gα contains a GTPase domain and a helical domain. It is the helical domain that is the most divergent between Gα subunit families indicating that this region determines its effector and receptor interactions. Three flexible regions within Gα, as well as regions in the N and C terminal regions of Gα, appear to be responsible for the conformation change in Gα after receptor activation. In addition, this conformational change allows for nucleotide exchange (Marin et al., 2001). Gβγ interacts with Gα though a hydrophobic pocket present on Gα. Gβ is composed of a β-propeller with seven WD-40 repeats. The Gγ subunit tightly interacts with Gβ's coiled-coil domain such that Gβ and γ can only be separated under denaturing conditions (Smrcka, 2008).

In addition to the GPCR, heterotrimeric G proteins, and the G protein's effector, other players in the G protein signaling pathway further regulate signaling. One such protein is a Regulator of G-protein Signaling (RGS) domain containing protein. The RGS domain is a conserved 130 amino acid sequence that was identified in different genes at the same time by different groups (De Vries et al., 1995; Druey et al., 1996; Koelle and Horvitz, 1996). These RGS domain containing proteins bind Gα-GTP and accelerate its intrinsic GTPase activity by stabilizing the transition state for GTP hydrolysis. For this reason, RGS proteins are called GAPs, or GTPase Activating Proteins (De Vries et al., 2000). The GAP activity of RGS proteins provides significant means of negatively regulating G proteins signaling (Berman et al., 1996). RGS proteins can also inhibit Gα activity by binding and physically preventing Gα interaction with effectors (Hepler et al., 1997), as well as diminish Gβγ signaling by accelerating heterotrimer reformation (De Vries et al., 2000). A RGS domain is typically composed

of 9-α helices (Tesmer et al., 1997). Within the human genome, there are 37 genes containing at least one RGS domain. These genes are further classified into 8 subfamilies (Soundararajan et al., 2008). Some RGS proteins often contain other structural protein domains, such as PDZ (PSD95/DlGα/zo-1) and DEP domains (Dishevelled/EGL-10/Pleckstrin), which have been shown to be important for regulating G protein signaling (Ballon et al., 2006; Tsunoda et al., 1997).

Arrestins are another class of proteins that regulate heterotrimeric G protein signaling. This class of proteins is classified as adaptor proteins that modulate desensitization and trafficking of GPCRs. Within the arrestin family there are four members, only two of which are expressed outside the visual system. Arrestins function either by inhibiting GPCR interaction with G proteins or by inducing clathrin-mediated endocytosis of the GPCR. (Buchanan and DuBois, 2006).

Targeting heterotrimeric G proteins signaling pathways as avenues for treatment of disease and illness has been a lucrative endeavor. Successful treatments for conditions as diverse as high blood pressure and depression have targeted various G protein coupled signaling pathways (Donati and Rasenick, 2003; Lindsey et al., 2009). Because G proteins signal to coordinate cell movement, differentiation, and gene expression, G protein signaling pathways have been proposed to be promising targets for novel chemotherapies and cancer treatments.

Historical Perspective: Wnt/β-catenin signaling

The history of Wnt signaling is a beautiful culmination of discoveries from many disciplines of biological investigation that spans over 30 years. The history of Wnt/

β-catenin signaling highlights this signaling pathways role in both development and disease.

In 1975, a *Drosophila* mutant lacking wings and halteres, named *wingless,* was reported (Sharma and Chopra, 1976). Based on the mutant phenotype, the *wingless* locus was hypothesized to be involved in development. This hypothesis was confirmed a few years later when Nusslein-Volhard and Wieschaus identified *wingless* as a segmentation coordination gene in a *Drosophila* mutagenesis screen for genes required for segmentation (Nusslein-Volhard and Wieschaus, 1980). For identifying genes required for segmentation, including *wingless*, Nusslein-Volhard and Wieschaus were awarded the Nobel Prize in Physiology or Medicine in 1995.

Two years later, Nusse and Varmus identified the mammalian homolog of wingless while investigating for novel oncogenes by analyzing mouse mammary tumor virus (MMTV) insertion sites that resulted in tumorigenesis (Nusse and Varmus, 1982; Rijsewijk et al., 1982). Injection of mouse *int-1* RNA into *Xenopus leavis* embryos resulted in dual axis formation, indicating that much like *Drosophila int-1*, or *wg*, mouse *int-1* was not only an oncogene, but also involved in patterning vertebrates (McMahon and Moon, 1989). This further substantiated current thought that cellular oncogenes function normally to coordinate development in metazoan organisms (Bishop, 1983). To provide clarity to this new field of study, a mnemonic for *wingless* and *Int-1* gave birth to the term Wnt (Nusse et al., 1991).

Mutagenesis screens for genes that regulate patterning in the *Drosophila* embryo identified multiple genes that were later found to be part of a Wnt signaling pathway. These genes, named after the phenotype shown in their segmentation or polarity defect,

include *armadillo* (the *Drosophila* ortholog of β-catenin) (Wieschaus and Riggleman, 1987), *dishevelled* (Dsh) (Perrimon and Mahowald, 1987), *shaggy* (the *Drosophila* ortholog of glycogen synthase kinase 3, GSK3)(Ripoll et al., 1988), *and frizzled* (Fz) (Schubiger and Palka, 1986). Further genetic analysis placed these genes in the Wnt pathway in the following order; *wg* signals through *fz* to inhibit *shaggy*, and *dishevelled* and *armadillo* are downstream of *wg* (Bhanot et al., 1996; Noordermeer et al., 1994; Siegfried et al., 1992). Further genetic and biochemical analysis provided additional insight into the mechanism of signaling.

Negative regulators of the pathway were identified first by their effect in vertebrates. In 1949, the *Fused* mutant mouse was identified and described by axis duplication in embryos. Injection of RNAs for this gene in *Xenopus* embryos resulted in a loss of body axis indicating that this gene was a negative regulator of Wnt signaling (Zeng et al., 1997). To avoid confusion with the unrelated *Drosophila* gene *fused*, this gene was named Axin, for *ax*is *in*hibition. Another negative regulator of the pathway, Adenomatous Polyposis Coli (APC), gained attention early due to its linkage to colon cancer (i.e. familial adenomatous polyposis). β-catenin was identified in APC immunoprecipitates (Rubinfeld et al., 1993), and APC was found to regulate the stability of β-catenin such that expression of APC results in reduced β-catenin levels in SW480 cell line, known to have high levels of β-catenin and a mutant APC (Munemitsu et al., 1995).

Additional Wnt signaling components were identified in the 1990's and early 2000's by a combination of methods. One example of this is the LRP5/6, low density lipoprotein related receptor 5/6. LRP6 was identified by *Drosophila* genetics (Wehrli et

al., 2000), mouse genetics (Pinson et al., 2000), and *Xenopus* injection studies (Tamai et al., 2000).

The Wnt ligand can signal independently of β-catenin to activate two addition pathways; the Planar Cell Polarity (PCP) pathway that signals through small GTPases including Rac and Rho, and the Wnt/Ca2+ pathway which signals through Jun kinase (JNK). These pathways utilize a number of proteins that function in the Wnt/β-catenin pathway, however, it is not understood the extent of cross talk within the Wnt signaling pathways. These "noncanonical" Wnt pathways will not be discussed at this time.

Current Models of Wnt/β-catenin Signaling

The key to canonical Wnt signaling is regulating cytosolic and nuclear levels of the cotranscriptional activator β-catenin. When the Wnt pathway is off or inactive, cytosolic β-catenin is maintained at a low level by the action of the β-catenin destruction complex. When the pathway is "on", the destruction complex is inhibited, the cytosolic level of β-catenin increase, and β-catenin can enter the nucleus to activate a Wnt transcriptional program (Figure 1.2).

The β-catenin destruction complex is responsible for maintaining low levels of cytosolic β-catenin in the cell. The destruction complex includes the scaffold proteins Axin and APC, and the kinases GSK3 and Casein Kinase I α (CK1α). Through specific binding domains, Axin interacts with GSK3, CK1α, and β-catenin to coordinate the series of phosphorylation events required to mark β-catenin for degradation. CK1α phosphorylates β-catenin at serine 45 to prime β-catenin for phosphorylation at serines 33 and 37, and threonine 41 by GSK3 (Amit et al., 2002; Liu et al., 2002). These sites of

phosphorylation provide a recognition site for its E3 ubiquitin (Ub) ligase (β-TRCP), resulting in its subsequent ubiquitination and degradation by the proteasome (Hart et al., 1999; Latres et al., 1999). Additionally, GSK3 and CK1α phosphorylate APC and Axin, resulting in their increased association with β-catenin (Jho et al., 1999; Rubinfeld et al., 2001; Xing et al., 2004), further enhancing β-catenin degradation (Lee et al., 2003). Consistent with their roles within the destruction complex, both Axin and APC are tumor suppressors.

The relationship between APC and Axin is somewhat paradoxical. Axin levels have been calculated to be exceedingly lower then other Wnt pathway components in *Xenopus* oocytes, suggesting that modulation of Axin stability could be a point of pathway regulation (Lee et al., 2003). In *Drosophila*, APC plays a positive role in Wingless signaling by promoting degradation of Axin. Additionally, the domain of APC that is required for APC-mediated Axin degradation is not the same domain that interacts with β-catenin (Takacs et al., 2008). Conversely, Axin over-expression can facilitate ubiquitin-mediated degradation of APC (Choi et al., 2004).

Wnt ligands, for which the pathway was named, activate the Wnt signaling pathway. This is a conserved family of 19 ligands present in all metazoans. They are cysteine rich proteins that are ~350-400 amino acids in size. Wnt ligands are glycosylated, (which is required for secretion) and lipidated, contributing to the hydrophobicity and poor solubility of the Wnt ligand (Komekado et al., 2007).

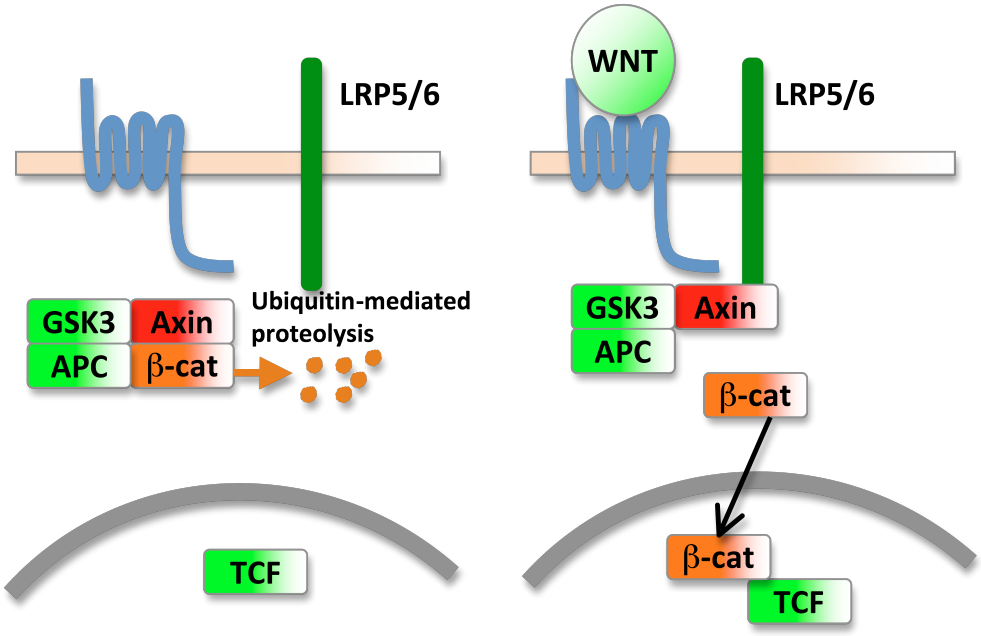


Figure 1.2: Schematic of Canonical Wnt Signaling. In the absence of a Wnt ligand (left side of figure), β-catenin concentrations are kept low in the cytoplasm through the action of the destruction complex, which consists of two scaffold proteins, Axin and the adenomatous polyposis coli (APC) protein, as well as a kinase, glycogen synthase kinase 3 (GSK3). The GSK3 in destruction complex phosphorylates β-catenin resulting in its recognition by an E3 ubiquitin ligase and its subsequent polyubiquitination and proteasome-mediated degradation. In the presence of a Wnt ligand (right side of figure), the ligand binds co-receptors Fz and LRP5/6, resulting in inhibition of destruction complex. Since the destruction complex is inhibited, β-catenin is not degraded and it accumulates in the cytoplasm. β-catenin can then translocate to the nucleus and interact with transcription factors of the T cell factor/lymphoid enhancer factor (TCF/LEF) family that activates a transcriptional program. Figure adapted from (Tolwinski and Wieschaus, 2004).

To activate the Wnt pathway, the Wnt ligand binds to the Frizzled and LRP5/6 co-receptors. The Frizzled receptor contains seven α-helical transmembrane domains and LRP5/6 contains a single transmembrane domain. Both of these receptors are required to transduce a Wnt signal intracellularly. In mammals, there are 10 Frizzled genes, and only one LRP5 and one LRP6. When comparing LRP5 and 6, LRP6 is required for embryogenesis (Pinson et al., 2000) while LRP5 is required for bone homeostasis (Kato et al., 2002). However, both genes must have some redundant roles in development since mice homozygous null for LRP5 and 6 die during gastrulation, earlier than homozygous LRP6 mutant alone. LRP6 also exhibits stronger signaling activity in both *Xenopus* (Tamai et al., 2000) and mammalian cells (Holmen et al., 2002). Based on this difference in activity, I will only refer to LRP6.

A key event in Wnt signaling is activation by phosphorylation of the intracellular domain of LRP6. The intracellular domain of LRP6 (as well as LRP5 and the *Drosophila* ortholog *arrow*) contains 5 repeated PPPSPxS motifs (P, proline; S, serine or threonine; x, variable residue). This intracellular domain alone is able to activate Wnt signaling (Mao et al., 2001a; Mao et al., 2001b) and a mutant lacking this domain is unable to transduce a Wnt signal and acts in a dominant negative fashion to inhibit Wnt and Fz signaling (Tamai et al., 2000). These PPPSPxS motifs in the intracellular domain of LRP6 are thought to be dually phosphorylated by the same kinases that phosphorylate β-catenin; GSK3 and CK1 (Davidson et al., 2005; Zeng et al., 2005). In contract to the β-catenin, GSK3 is the priming kinase for CK1 and it appears that the GSK3 phosphorylation of LRP6 is Wnt induced (Pan et al., 2008). In support of this, the GSK3α/β null ES cells lack most, if not all, Wnt induced LRP6 phosphorylation (Zeng et

al., 2008). The phosphorylated PPPSPxS motifs provide a binding domain for Axin such that Axin is recruited to the membrane after Wnt stimulation (Davidson et al., 2005; Tamai et al., 2004; Zeng et al., 2005). Additionally, GSK3 bound to Axin mediates further LRP6 phosphorylation (Zeng et al., 2008).

Frizzled function is required for LRP6 phosphorylation and activation (Zeng et al., 2008). Frizzled signaling has been linked to Dishevelled (Dsh). Frizzled and Dishevelled were identified to interact through the PDZ domain of Dsh and the c-terminal domain of Fz (Wong et al., 2003). Dsh has been shown to be recruited to the membrane after Wnt stimulation (Umbhauer et al., 2000).

Dishevelled is a 500-600 amino acid scaffold protein composed of DIX, PDZ, and DEP domains as well as a SH3 protein-binding motif (Wallingford and Habas, 2005). Mammals have three Dishevelled genes; (Dvl1, Dvl2, Dvl3) while model organisms, such as *Drosophila* and *Xenopus*, only have one Dishevelled gene (Dsh). For the sake of simplicity, I will refer to Dishevelled as Dsh, unless a study sited a specific mammalian Dishevelled, or Dvl.

Fz-Dsh interaction and Dsh function are required for LRP6 activation and phosphorylation (Bilic et al., 2007; Zeng et al., 2008). Dsh and Axin have been shown to interact (Itoh et al., 2000; Kishida et al., 1999). One model for LRP6 activation proposes that Fz-Dsh interaction recruits Axin-GSK3 to the membrane, and that GSK3 on Axin phosphorylates and activates LRP6 (Zeng et al., 2008). One weakness in this model is that the intracellular domain of LRP6 must be phosphorylated by GSK3 for Axin to bind. Axin, however, is recruited to the membrane after LRP6 phosphorylation. One source of GSK3 is Axin bound GSK3. This model suggests a feed-forward loop that amplifies the

GSK3 phosphorylation on LRP6. In support of this model, loss of one PPPSPxS domain results in loss of LRP6 signal and loss of two or more PPPSPxS domain results in a substantial loss of signaling capacity. This indicates that there is a requirement for full phosphorylation in order to transmit a signal through the activated LRP6 receptor (MacDonald et al., 2008; Wolf et al., 2008). A model that supports this stepwise activation of LRP6 by phosphorylation is an "initiation and amplification" model. The "initiation" step requires both Frizzled and Arrow (*Drosophila* LRP5/6 ortholog). In support of this model, a Frizzled-Arrow fusion is unable to fully rescue an *arrow*$^{-}$ mutant, indicating that Arrow/LRP6 serves a second downstream function to amplify signaling (Baig-Lewis et al., 2007). In addition, a chimeric Arrow protein containing the Torso dimerization domain potentiated Wingless (Wg) signaling, but is unable to initiate a Wg signal on its own. This suggests that Arrow dimerization, which is believed to take place after signal initiation, is not enough to initiate signaling. However, coexpression of the Fz/Arr chimera and the chimeric Arr resulted in a synergistic activation of signaling to the level of Wg expression itself. This implies that both initiation and amplification are required for robust Wg signaling (Baig-Lewis et al., 2007). Another model for LRP6 activation requires Fz-LRP6 clustering driven by Dvl, Axin, and GSK3 to form a "signalosome" (Bilic et al., 2007). In this model, Wnt stimulates Dvl mediated coaggregation of LRP6 with other Wnt signaling components (including Fz8, Axin, and GSK3) forming a "signalosome" which triggers CK1γ phosphorylation of LRP6. It has been proposed that Dvl (the mammalian ortholog of *Xenopus* Dsh) and Axin form large complexes with additional Wnt components that allow for weak yet dynamic interaction (Schwarz-Romond et al., 2007). In support of this theory, the DIX domain of Dvl is

required for Wnt induced receptor-complex formation (Bilic et al., 2007; Schwarz-Romond et al., 2007). Taken together, Wnt signaling stimulates dynamic protein interactions that culminate on LRP6 activation.

There are two schools of thought surrounding Wnt mediated inhibition of β-catenin phosphorylation; one is LRP6 dependent, while the other is LRP6 independent. Constitutively active LRP6 can inhibit β-catenin degradation, assumably independent of Fz and Dsh (He et al., 2004). Recent evidence suggests that phosphorylated LRP6 can directly inhibit β-catenin phosphorylation, resulting in β-catenin stabilization (Cselenyi et al., 2008; Piao et al., 2008; Wu et al., 2009). It has also been reported that β-catenin at the membrane is dephosphorylated in response to Wnt (Hendriksen et al., 2008). In support of a LRP6 independent β-catenin phosphorylation mechanism, over-expression of Dsh in *Drosophila* or addition of recombinant Dsh into *Xenopus* egg extract results in β-catenin stabilization in the assumed absence of LRP6 (Salic et al., 2000; Wehrli et al., 2000). Another mechanism for inhibition of β-catenin degradation involves dissociation of destruction complex or stimulation of Axin degradation by recruiting Axin away from the destruction complex to LRP6 or to Fz-Dsh at the membrane (Cliffe et al., 2003; Tamai et al., 2004). Also, Gαo signaling through Fz, as well as Protein Phosphatase 2 (PP2) dephosphorylation, has been proposed to lead to the dissociation of GSK3-Axin and disruption of the destruction complex (Liu et al., 2005; Luo et al., 2007). It has also been reported that Axin is degraded in response to signaling in *Xenopus* egg extract and *Drosophila* (Lee et al., 2003; Mao et al., 2001b; Tolwinski et al., 2003), although LRP6 can still inhibit β-catenin independent of Axin degradation (Cselenyi et al., 2008). However, the timing of Axin degradation lags behind β-catenin stabilization, suggesting

that destabilization of Axin is not a primary means of stabilizing β-catenin, but instead may serve to augment the length of time that the pathway is activated (Liu et al., 2005; Willert et al., 1999; Yamamoto et al., 1999).

In addition to signaling through the Frizzled and LRP6 receptors, studies in *Drosophila*, *C. elegans* and mice have identified atypical receptor tyrosine kinases (RTK) of the Ryk or Ror families function as Wnt receptors as well (Forrester et al., 2004; Green et al., 2007; Lu et al., 2004). In Wnt/β-catenin signaling, Ryk binds Wnt-3a and Fz to form a tertiary complex. The intracellular domain of Ryk interacts with Dsh, which is required for TCF activation in response to Wnt. Signaling through Ryk is vital for Wnt3a induction of neurite outgrowth of basal root ganglia (Lu et al., 2004). Roles for RTK and Ror have also been implicated in Wnt/JNK signaling (Minami et al., 2009; Oishi et al., 2003).

Heterotrimeric G proteins in Wnt/β-catenin Signaling

Ever since the Frizzled receptor was cloned and identified to contain seven α-helical transmembrane domains (Vinson et al., 1989), it has been thought that Fz may signal through heterotrimeric G proteins. A number of heterotrimeric G protein subunits as well as classic G protein signaling cascade members have been implicated in Wnt signaling.

Pertussis Toxin (PTX), an exotoxin produced by *Bordetella pertussis*, inhibits G_i family G proteins by ADP-Ribosylating the Gβ subunit thereby inhibiting its interaction with effectors and GPCRs. Wnt8 treatment of mouse F9 teratocarcinoma cells expressing Rat Fz-1 results in primitive endoderm (PE) formation. (Liu et al., 1999).

PTX treatment of F9 cells inhibits Wnt8 stimulated primitive endoderm formation suggesting that Wnt8 signals through G_i family of Gα subunits. Additionally, PTX treatment inhibited signaling through a β2-AR/Fz1 chimeric receptor (containing the extracellular and transmembrane domains of the β2-adrenergic receptor fused with the intracellular domains of the Frizzled receptor) (Gao and Wang, 2007; Malbon et al., 2001) inhibited Wnt3a stimulated β-catenin stabilization (Liu et al., 2005). PTX also suppressed effects of Fz over-expression in *Drosophila* ommatidia (Katanaev et al., 2005). Depletion by RNAi of Gαo from F9 cells also inhibited Wnt8 stimulated primitive endoderm formation (a read-out of Wnt/β-catenin pathway activation). Depletion of Gαq has the same effect as depletion of Gαo (Liu et al., 1999). Also, stimulation of a β2-AR/Fz1 chimeric receptor with β2-AR agonists requires Gαo (Liu et al., 2001), Gαo mediates Fz signaling in *Drosophila* (Katanaev et al., 2005), Gαo disrupts the GSK3-Axin2 interaction thereby stabilizing β-catenin (Liu et al., 2005), and Gαo binds and recruits Axin to the plasma membrane (Egger-Adam and Katanaev, 2009). Gαq is also required for signaling through β2-AR/Fz1 chimeric receptor, and is thought to inhibit the destruction complex (Liu et al., 2001; Liu et al., 2005), possibly through direct inhibition of GSK3 (Najafi, 2009). Knockdown of Gαq also abolishes Wnt3a stimulated inositol pentaphosphate (IP_5) production, whereas overexpression of a constitutively active Gαq stimulates IP_5 production (Gao and Wang, 2007). In addition to Gαo and Gαq, Gαs and Gα12/13 have been implicated in Wnt/β-catenin signaling. Signaling downstream of Prostaglandin E2 (PGE2) stimulates Gαs to bind the RGS domain of Axin, resulting in the inhibition and dissociation of GSK3 and subsequent β-catenin stabilization (Castellone et al., 2005). Binding of Gα12 and Gα13 to E-cadherin

cytoplasmic tails results in the release of membrane bound β-catenin and subsequent increase in the cytoplasmic concentration of β-catenin (Meigs et al., 2001). Additionally, Gα12 has been shown to interact with the RGS domain of Axin (Stemmle et al., 2006).

Positive and negative roles for Gβγ have been recently implicated for signaling in the Wnt/β-catenin pathway. Gβγ was first identified to interact with Dishevelled through a TAP-tag screening approach for novel Dishevelled binding partners (Angers et al., 2006). $G\beta_2\gamma_2$ has been proposed to provide a feedback mechanism by binding Dsh and signaling through phospholipase C (PLC) resulting in Dsh degradation through the lysosomal pathway (Jung et al., 2009). In support of this, injection of $G\beta_2\gamma_2$ RNA into *Xenopus laevis* embryo inhibited axis duplication induced by Xwnt8 or Dsh (Jung et al., 2009). Using *Drosophila* genetics, knock down of *Gβ13Fγ1* in *Drosophila* wings discs resulted in the loss of expression of short-range Wg target genes and produced a *wg* phenotype (Egger-Adam and Katanaev, 2009). The association between Dsh and Gβ was also confirmed in this study, concluding that Gβγ interacts with and recruits Dsh to the plasma membrane. However, over-expression of *Gβ13Fγ1* in *Drosophila* wing discs resulted in down-regulation of Wg signaling, much like the *Xenopus laevis* injection experiments. This indicates that Gβγ may play both a positive and negative role in the pathway; initial recruitment of Dsh to the membrane by Gβγ can serve to activate signaling and sustained or persistent membrane localization results in Dsh degradation (Egger-Adam and Katanaev, 2009).

Axin and other RGS domain containing proteins have been implicated in Wnt signaling. As previously mentioned, Axin has been proposed to interact with Gα12, Gαs, and Gαo through its RGS domain (Castellone et al., 2005; Egger-Adam and

Katanaev, 2009; Stemmle et al., 2006). Although both Gα12 and Gαs were shown to preferentially interact with Axin in an active state (GTP bound or treated with aluminum fluoride), Axin does not enhance the GTPase activity of the Gα subunits. This suggests that Axin does not act as a GAP (GTPase Activating Protein), but rather serves as a scaffold to bring signaling proteins in close association like the Gα12/13 RGS-domain containing RhoGEFS (Castellone et al., 2005; Stemmle et al., 2006; Tanabe et al., 2004). Similar to the phenotype observed after injection of a Wnt negative regulator, injection of *Xenopus* embryos with RNA for rat *RGS4* (Gi and Gq GAP) and human *RGS2* (Gq specific GAP) resulted in a ventralized phenotype (Wu et al., 2000). *rRGS4* was also able to inhibit *Xwnt-8* induction of axis duplication in *Xenopus* embryos, suggesting that RGS proteins modulate signaling through Wnts by attenuating G protein signaling (Wu et al., 2000). RGS19 was identified in a screen for RGS domain containing proteins to inhibit β-catenin signaling. Expression of constitutively active Gαo rescued Wnt/β-catenin signaling inhibition by RGS19 suggesting that RGS19 regulates Wnt/β-catenin signaling by inactivating Gαo (Feigin and Malbon, 2007).

Although Frizzled has not been identified as a bona-fide G protein coupled receptor, there are multiple lines of evidence that strongly imply that Frizzled signals through heterotrimeric G proteins (Malbon, 2004). (1) Frizzled is heptahelical, containing 7 α-helical transmembrane domains like all other G protein coupled receptors (Vinson et al., 1989). (2) Like other GPCRs, the N-terminus of Frizzled is on the extracellular side of the cell, while the C-terminal domain faces the intracellular side. The N-terminal domain is multiply N-glycosylated, and the C terminal sides (and internal loops) contain multiple predicted sites of phosphorylation, like other GPCRs.

(3) Signaling through Frizzled (or the β2-AR/Fz chimera) is PTX sensitive (Gao and Wang, 2007; Katanaev et al., 2005; Liu et al., 1999; Liu et al., 2005; Malbon et al., 2001). (4) Signaling through Frizzled can be induced by GTP analogs. (Liu et al., 2005). (5) There is a decrease in Fz signaling when expression of G proteins is knocked down (Gao and Wang, 2007; Katanaev et al., 2005; Liu et al., 2001; Liu et al., 1999), and (6) Fz signaling is activated when G proteins expression or activity is enhanced (Katanaev et al., 2005) (Gao and Wang, 2007). (7) There is an increase in Fz affinity for Wnts when the concentration of GTP is low (Liu et al., 2001). Significantly, Wnt stimulated Fz signaling has not been shown to increase Gα nucleotide exchange and Frizzled has not been shown to have GEF activity.

Although it has not been definitely determined that Fz signals through G proteins, multiple known GPCRs have been shown to signal to activate β-catenin signaling (Yi et al., 2008) (Malbon, 2005). Prostanoid receptors, EP2 and EP4, are GPCRs that have been shown to signal to stabilize β-catenin and activate β-catenin transcriptional activation after activation by PGE2 (Castellone et al., 2005; Fujino and Regan, 2001; Fujino et al., 2002). Signaling through PGE2 requires signaling by Gβγ and Gαs. When activated by PGE2, Gβγ signals through Phosphoinositol 3-kinase (PI3K) to Akt, which phosphorylates and inhibits GSK3. At the same time, Gαs signals to release GSK3 from Axin resulting in destruction complex destabilization and subsequent β-catenin stabilization (Castellone et al., 2005). Also, PGE2 signals through cAMP/PKA to directly regulate β-catenin signaling in hematopoietic stem cells (HSC) and the hematopoietic niche during embryogenesis (Goessling et al., 2009). Another GPCR, the lipid metabolite lysophosphatidic acid (LPA) receptor, stimulates cell proliferation in

colon cancer cells through stabilization of β-catenin. This mechanism for β-catenin stabilization is through Gq signaling to PKC, which phosphorylates and inhibits GSK3, resulting in β-catenin stabilization (Yang et al., 2005). Signaling by the platelet-derived growth factor (PDGF) through its GPCR activates the p68 RNA helicase to stimulate β-catenin nuclear translocation. It is believed to do this by blocking GSK3 phosphorylation of β-catenin and displacing β-catenin from Axin (Yang et al., 2006; Yang et al., 2007). Although it is not known if this signaling is transduced through heterotrimeric G proteins, activation of the parathyroid hormone receptor (PTH1R) GPCR by the parathyroid hormone (PTH) in osteoblasts requires activation of the Wnt co-receptor, LRP6, through phosphorylation by PKA. PTH mediated β-catenin stabilization and subsequent transcriptional activation has been attributed to the stimulation of bone anabolism (Wan et al., 2008). Also, treatment of cells with the Gonadotropin-Releasing Hormone (GnRH), which signals though a GPCR, inhibits β-catenin degradation through inhibition of GSK3 in mouse gonadotrope cells (Gardner et al., 2007). Although it is possible that Frizzled itself does not signal through heterotrimeric G proteins, other GPCRs activated by their ligands are able to utilize the intracellular Wnt/β-catenin signaling components to stabilize β-catenin.

β-arrestins, adaptor proteins that modulate desensitization and trafficking of GPCRs, have also been implicated to play a role in Wnt/β-catenin signaling. Mouse embryonic fibroblasts (MEFs) genetically depleted of β-arrestin 1 and/or 2 are unable to responded to Wnt3a activation of β-catenin signaling (Bryja et al., 2007). Also injection of *Xenopus laevis* with β-arrestin morpholinos blocked axis duplication when injected

with *Xwnt8*, suggesting that β-arrestins are required for Wnt/β-catenin signaling (Bryja et al., 2007).

Although there is a body of evidence that indicates that Wnt/β-catenin signaling is a G protein coupled signaling pathway, it is still not widely accepted that heterotrimeric G proteins moderate Wnt signaling.

BOOK 1: INTRACELLULAR MECHANISM OF WNT/β-CATENIN SIGNAL TRANSDUCTION

CHAPTER 2: Gβγ PROMOTES LRP6 MEDIATED β-CATENIN/TCF SIGNALING BY STIMULATING MEMBRANE ASSOCIATION AND ACTIVATION OF GSK3

Introduction

Heterotrimeric G proteins, which consist of a Gα subunit and an associated Gβγ dimer, mediate a multitude of physiological responses from a host of diverse ligands. Activated G protein-coupled receptors (GPCRs) catalyze exchange of GDP for GTP on Gα, resulting in subunit dissociation and G protein activation. The signal is terminated upon hydrolysis of GTP by Gα the intrinsic GTPase activity of the Gα subunit and its reassociation with Gβγ. Both Gα and Gβγ activate many downstream effectors.

The Wnt/β-catenin pathway is conserved throughout metazoa and required for coordination of diverse developmental programs, stem cell maintenance, and cell growth and proliferation. In the absence of a Wnt ligand, cytoplasmic β-catenin levels are kept low by the destruction complex, which is composed of Adenomatous Polyposis Coli (APC), Axin, and Glycogen Synthase Kinase 3 (GSK3). GSK3 phosphorylates β-catenin, thereby marking it for ubiquitination and proteasome-mediated degradation. Binding of Wnt to Frizzled and Low-Density Lipoprotein Receptor-Related protein 6 (LRP6) co-receptors results in inhibition of β-catenin destruction and a subsequent rise in cytoplasmic β-catenin levels. β-catenin enters the nucleus to initiate a transcriptional program with T cell factor/lymphoid enhancer factor (TCF/LEF) family members

(MacDonald et al., 2009). The initial discovery that the Wnt receptor, Frizzled, contains seven predicted transmembrane domains, a topology characteristic of GPCRs, suggested that Frizzled signals through heterotrimeric G proteins (MacDonald et al., 2008).

Activation of LRP6 is dependent on phosphorylation of conserved motifs within its intracellular domain (Davidson et al., 2005) (Zeng et al., 2005). Previous studies have suggested that a pool of Axin-bound GSK3 that translocates to the membrane via Dishevelled (Dsh) is responsible for initial phosphorylation of LRP6 at the plasma membrane (Zeng et al., 2008; Zeng et al., 2005). Phosphorylated LRP6 provides additional docking sites for cytoplasmic Axin-GSK3 complexes to promote further phosphorylation of LRP6. Binding of this complex to phospho-LRP6 results in inhibition of GSK3 with a subsequent decrease in β-catenin phosphorylation (Cselenyi et al., 2008; Piao et al., 2008; Wu et al., 2009). The mechanism for initiation of the early phosphorylation events of LRP6 is not known. Our evidence suggests that Gβγ recruits and activates GSK3 to phosphorylate LRP6, placing Gβγ in a pivotal role in initiation of LRP6 activation.

Methods

Constructs and purified proteins

Human $G\beta_1$ was subcloned into pCS2-HA vector. $hG\beta_1$ alanine mutations were constructed using QuikChange site mutagenesis kit (Stratagene) following manufacturer's protocol. Sequence encoding amino acids 493-689 of mouse adrenergic receptor kinase β1 was subcloned into pCS2 vector. Gγ2CAAX pCS2 was generated by

PCR-based site-directed mutagenesis. Oligonucleotide primer sequences are available upon request. All other constructs used have been described previously. Gαs and myristoylated GαoA, GαoB, Gαi2, and Gαi3 were expressed and purified from bacteria, and $G\beta_1\gamma_2$ and Gαq were purified from Sf9 cells as previously described (Hepler et al., 1994; Kreutz et al., 2006; Lee et al., 1994; Linder et al., 1990; Ueda et al., 1994). Heterotrimeric G proteins were purified from porcine brain (Wampler's Sausage Factory, TN) and Gαt from bovine rod outer segments using previously described protocols (Sternweis and Robishaw, 1984) (Papermaster and Dreyer, 1974). Gβγ was concentrated to 1 mg/mL in 50 mM HEPES pH 8.0, 1 mM EDTA, 1 mM DTT, 10 mM MgSO4, and 40 mM β-octyl glucoside. GSK3 and CKI proteins were purchased from New England Biolabs.

G protein activation

To activate GαoA, GαoB, Gαi2, Gαi3, and Gαs, proteins were diluted 1:20 (v/v) with 50mM HEPES pH 8, 1mM EDTA, 1mM DTT, 10mM $MgSO_4$, and 10μM GTPγS and incubated at 30°C for 30 min. Proteins were concentrated to their original volumes using Centricon-10 (Millipore). To activate Gα12, protein was diluted 1:1 (v/v) with 80mM HEPES pH 8, 1mM EDTA, 1mM DTT, 22mM $MgSO_4$, 0.05% Lubrol, and 20μM GTPγS and incubated at 30°C for 90mins. Gα13 was activated by diluting 1:1 (v/v) with 80mM HEPES pH 8, 1mM EDTA, 1 mM DTT, 3mM $MgSO_4$, 0.05% Lubrol, and 20μM GTPγS and incubated at 30°C for 90mins. Excess GTPγS was removed using Zeba Desalt Spin Column (Pierce). To activate purified brain heterotrimer, Gαt, and Gαq, proteins were incubated with 10mM NaF, 30μM $AlCl_3$, and 10mM $MgCl_2$ for 45mins on ice.

[^{35}S]β-Catenin degradation and phospho-β-catenin assays

Xenopus egg extract preparation and [^{35}S]β-catenin degradation assays were performed as previously described (Salic et al., 2000). Samples were removed at 0 and 3hr for SDS-PAGE and autoradiography. To detect β-catenin that has been phosphorylated by GSK3 in extract, anti-β-catenin-phospho-Ser33/Ser37/Thre41 antibody (Cell Signaling) was used for immunoblotting. Anti-α-tubulin (DM1α, Sigma) was control.

TOPflash assay

HEK-293 STF cells were cultured in DMEM media supplemented with 100units/mL penicillin, 100unit/mL streptomycin, 10% FBS. Lipofectamine 2000 transfection reagent (Invitrogen) was used for all transfections. Cells were lysed 48hrs post-transfection with 1X Passive Lysis Buffer (Promega) and luciferase activity measured with Steady Glo (Promega). Luciferase activities were normalized to cell number using Cell Titre Glo (Promega).

Co-immunoprecipitation studies

For co-immunoprecipitation from cells, cells were washed with cold PBS and then lysed on ice for 30min using non-denaturing lysis buffer (NDLB): 50mM Tris pH 7.4, 300mM NaCl, 5 mM EDTA, 1% (w/v) Triton X-100, and protease inhibitors (1mg/ml leupeptin, pepstatin, and chymostatin). Lysates were diluted to 1mg/ml with NDLB and incubated with antibody beads for 3hrs with rotation at 4°C. Beads were then washed 3X with NDLB and 1X with PBS. Proteins were eluted from beads with sample buffer and

processed for SDS-PAGE followed by immunoblotting. To prepare antibody beads, mouse anti-HA(12CA5), rat anti-HA(Roche), or rabbit anti-Gβ(1:300, Sigma) antibodies were cross-linked to Protein G or Protein A magnetic beads(New England Biolabs) following manufacturer's protocols. The following antibodies were used for immunoblotting: rabbit anti-Gβ(Sigma), mouse anti-HA(12CA5), mouse anti-Myc (9E10), and rabbit anti-VSVG(Bethyl Laboratories). Anti-α-tubulin (DM1α, Sigma) was used as loading control. For co-immunoprecipitation from *Xenopus* egg extract, extract was diluted 10X with PBS, incubated with anti-Gβ beads, and beads processed as above. Eluted samples were immunoblotted using anti-Gβ and anti-GSK3(BD Transduction).

Membrane protein isolation and detection of LRP6

For membrane preparations, cells were incubated in media containing 0.6mM sodium vanadate in DMEM (without serum) for 3hrs. Cells were then lysed and membrane-associated proteins isolated using ProteoExtract Native Membrane Protein Extraction kit(Calbiochem). To detect endogenous LRP6, endogenous phospho-LRP6, and transfected VSVG-LRP6, the following antibodies were used: rabbit anti-LRP6(Cell Signaling), rabbit anti-phospho-LRP6(Ser1490) (Cell Signaling), and rabbit anti-VSVG(Bethyl Laboratories), respectively.

***In vitro* protein binding assay**

For *in vitro* binding of $G\beta_1\gamma_2$ and GSK3, recombinant $G\beta_1\gamma_2$ was incubated with MBP or MBP-GSK3 protein in binding buffer (10mM HEPES pH 7.4, 300mM NaCl, 5mM $MgCl_2$, 1mM EGTA, 0.1% Triton X-100, and 0.5mg/mL BSA) at 4˚C for 45mins.

Protein G magnetic beads(New England Biolabs) cross-linked to anti-MBP antibody(Sigma) were added and the reaction incubated at 4˚C for 45mins. Beads were washed 4X with binding buffer, 2X times with PBS, and samples were eluted and analyzed by immunoblotting.

***In vitro* kinase assay**

Tau (200nM; r-Peptide, Tau-441) or LRP6ICD (100nM) were pre-incubated with 200nM or 400nM GSK3 (New England Biolabs), respectively, 200μM ATP, 20μCi [γ-^{32P}]ATP, GSK3 kinase buffer (20mM HEPES pH 7.5, 300mM NaCl, 2 mM DTT, 1mM EDTA, 10mM $MgCl_2$, 0.2% Tween-20) and increasing concentrations of recombinant $G\beta_1\gamma_2$, Go, or BSA in a total reaction volume of 20μl. Samples were incubated at room temperature and aliquots removed at 30mins (Tau) or 15mins (LRP6ICD) for analysis by SDS-PAGE and autoradiography.

Molecular modeling

The software program MacPyMol was used to generate a model of the $G\beta_1\gamma_2$ dimer(Protein Databank entry 1xhm) (Davis et al., 2005).

Statistical analysis

All luciferase assays were repeated atleast three times in triplicate. Values are presented as mean± standard deviation. We compared X by Analysis of Variance (ANOVA) to test the global hypothesis of equal mean intensities for all treatment conditions. The global

hypothesis of all ANOVA analyses were rejected ($p < 0.05$). All possible pairwise contrasts were evaluated using Tukey's HSD procedure.

Results

Biochemical screen identifies G proteins that regulate β-catenin turnover.

The *Xenopus* egg extract system has been used to recapitulate numerous complex GTP-dependent phenomena such as microtubule dynamics, translation, DNA replication, nuclear envelope reformation, and mitotic spindle assembly (Blow et al., 1987; Gard and Kirschner, 1987; Kornbluth et al., 1994). It contains cytosol and other cellular components including plasma membrane, organelles, amino acids, and nucleotides at or near physiological levels (Ma et al., 1998). Furthermore, this system has been shown to recapitulate the regulation and degradation of exogenous, [^{35}S]β-catenin via components of the Wnt pathway (Salic et al., 2000)(Figure 2.1)**.** To assess whether *Xenopus* egg extract could be used to test the role of G proteins in β-catenin/TCF signaling, we used purified G protein heterotrimers (primarily Go) from brain tissue for reconstitution (Sternweis and Robishaw, 1984). We detected modest inhibition of [^{35}S]β-catenin degradation upon addition of heterotrimer, with greater inhibition upon G protein activation by AlF_4^- (Figure 2.2A). Partial activity of the intact heterotrimer may have resulted from nucleotide exchange (GDP for GTP) of Go within these crude egg extract, which have been documented to contain high concentrations of GTP (Blow et al., 1987; Ma et al., 1998).

To identify G proteins involved in β-catenin/TCF signaling, we tested the four major classes of Gα subunits for their capacity to inhibit degradation of [^{35}S]β-catenin in

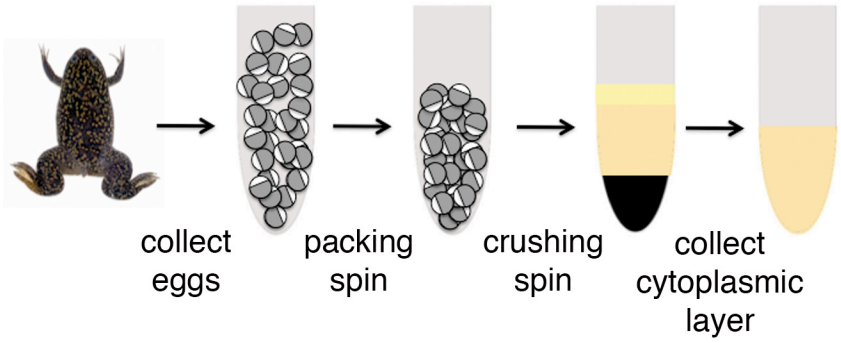
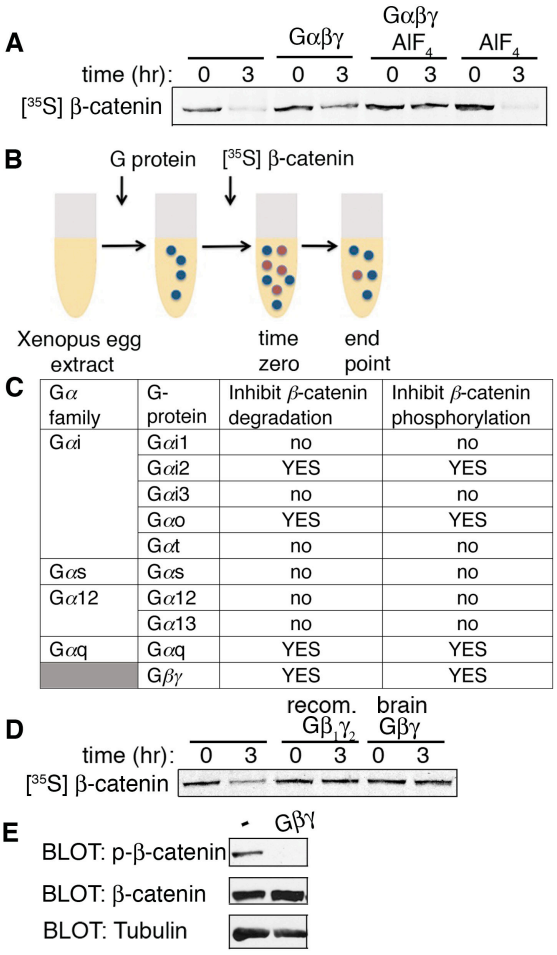


Figure 2.1: A schematic showing the preparation of *Xenopus* egg extract used for β-catenin degradation and phosphorylation assays. Unfertilized *Xenopus laevis* eggs were collected from females stimulated to ovulate by injection with human gonadotropin, de-jellied, and extracts prepared with minor modifications. Briefly, de-jellied eggs were spun in a microcentrifuge at low speed (2,000 rpm for 2 min at 4˚ C) to remove excess buffer. Eggs were then crushed by centrifugation at high speed (15,000 rpm for 2 min at 4˚ C), releasing their contents into the centrifuge tube. After the crushing spin, three layers of lysate could be readily observed: a dark pellet enriched in nuclei and pigments, a cytosolic layer, and a layer containing egg yolk and lipids. The cytosolic layer was removed and subjected to subsequent re-centrifugation (2-3X) at maximum speed. The final extract (~50-100 mg/ml) was used for all subsequent biochemical reconstitutions.



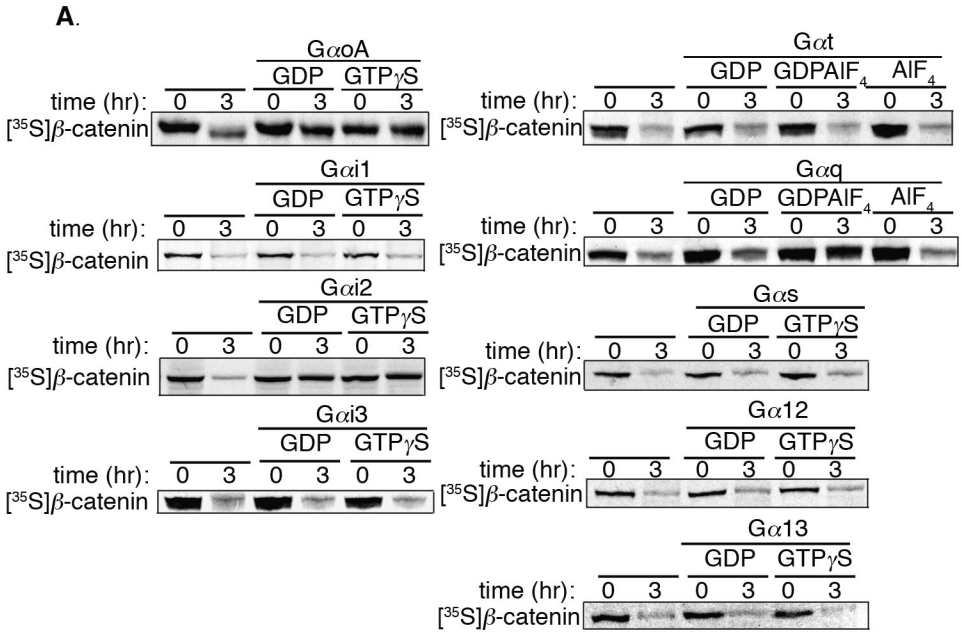
C

Gα family	G-protein	Inhibit β-catenin degradation	Inhibit β-catenin phosphorylation
Gαi	Gαi1	no	no
	Gαi2	YES	YES
	Gαi3	no	no
	Gαo	YES	YES
	Gαt	no	no
Gαs	Gαs	no	no
Gα12	Gα12	no	no
	Gα13	no	no
Gαq	Gαq	YES	YES
	Gβγ	YES	YES

Figure 2.2: G proteins inhibit β-catenin degradation in *Xenopus* egg extract. (A) Addition of purified brain G protein (5 μM) inhibits degradation of radiolabeled in vitro translated (IVT) [^{35}S]β-catenin in *Xenopus* egg extract and is potentiated by AlF_4^-. (B) Schematic of assay used to assess the effects of G protein subunits on IVT [^{35}S]β-catenin degradation in *Xenopus* egg extract. (C) Table summarizing the activities of the major classes of G proteins subunits on IVT [^{35}S]β-catenin turnover and phosphorylation of endogenous β-catenin by GSK3 in *Xenopus* egg extract. (D) Both recombinant $G\beta_1\gamma_2$ (5μM) and brain Gβγ (5μM) inhibit β-catenin degradation. (E) Addition of brain Gβγ (5μM) to *Xenopus* egg extract inhibits phosphorylation of endogenous β-catenin at GSK3 target sites 33/37/41. Extract was analyzed after 2 hr incubation. Tubulin is loading control. Figures are representative of experiments performed three times.

Xenopus egg extract (Figure 2.2B,C). The specificity of each G protein to inhibit [^{35}S]β-catenin turnover was further assessed by immunoblotting for endogenous β-catenin phosphorylation by GSK3 (Figure 2.2C; Figure 2.3). Both inactive (GDP-bound) and active forms were tested. For activation, proteins were bound to a GTPγS analog or treated with GDP-AlF_4^- (if nucleotide exchange is extremely slow in the absence of receptor as in the case of Gαt and Gαq). We found that, in addition to the previously implicated Gαo and Gαq, Gαi2 inhibited exogenous β-catenin degradation and phosphorylation of endogenous β-catenin by GSK3 (Gao and Wang, 2007; Liu et al., 2001; Katanaev et al., 2005; Liu et al., 2005; Najafi, 2009). β-catenin was stabilized in extracts by the GDP-bound forms of Gαo and Gαi2 but not Gαq. This is likely due to dramatic differences in affinity for GTP and the rates of nucleotide exchange between Gα subunits (GDP dissociation rate of Gαo = 0.19 min^{-1} at 30^o C; GDP dissociation rate of Gαi2 = 0.07 min^{-1} at 30^o C; Gαq has very low affinity for GTP in the absence of receptor) (Hepler et al., 1993; Linder et al., 1990). Interestingly, Gβγ protein purified from brain tissue and recombinant $G\beta_1\gamma_2$ inhibited β-catenin phosphorylation and degradation (Figure 2.2D,E; Figure 2.4). Additionally, Gβγ synergized with Gαo to inhibit β-catenin degradation, suggesting that Gαo and Gβγ act cooperatively (Figure 2.5A).

We maintain that concentrations of Gα and Gβγ used in our assays are likely to be physiologically relevant. The effective concentrations of Gα and Gβγ in our biochemical assays are in the low micromolar range, well below the predicted concentrations of heterotrimeric G proteins at the plasma membrane (~1 mM) (Taussig et al., 1993). Furthermore, because membrane localization of G proteins is not homogeneous, local concentrations of G proteins within the plasma membrane may actually exceed 1 mM


A.
GαoA
GDP GTPγS
time (hr): 0 3 0 3 0 3
[35S]β-catenin
Gαt
GDP GDPAlF4 AlF4
Gαi1
Gαq
Gαi2
Gαs
Gαi3
Gα12
Gα13

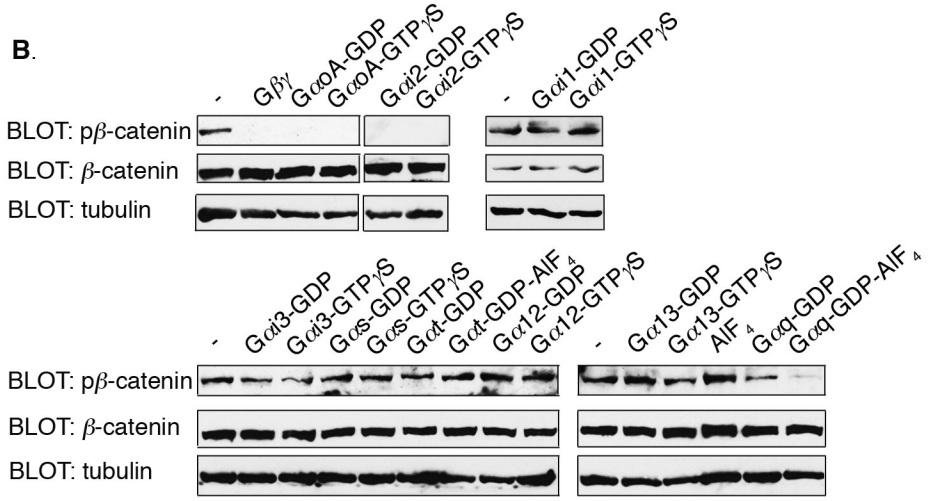

B.
- Gβγ GαoA-GDP GαoA-GTPγS Gαi2-GDP Gαi2-GTPγS
- Gαi1-GDP Gαi1-GTPγS
BLOT: pβ-catenin
BLOT: β-catenin
BLOT: tubulin
- Gαi3-GDP Gαi3-GTPγS Gαs-GDP Gαs-GTPγS Gαt-GDP Gαt-GDP-AlF4 Gα12-GDP Gα12-GTPγS
- Gα13-GDP Gα13-GTPγS AlF4 Gαq-GDP Gαq-GDP-AlF4

Figure 2.3: Screen for heterotrimeric G protein subunits that inhibit β-catenin degradation and phosphorylation in *Xenopus* egg extract. (A) Degradation of in vitro-translated [^{35}S]β-catenin degradation in extract is inhibited upon addition of GαoA (1.0 μM), Gαi2 (5.0 μM), or Gαq (0.5 μM). (B) Similarly, GαoA (1.0 μM), Gαi2 (5.0 μM), and Gαq (0.5 μM) each inhibit GSK3 phosphorylation of endogenous β-catenin in extracts as detected by a β-catenin phospho-33/37/41 antibody. All Gα preparations were estimated to be ~50-80% active based on nucleotide binding and/or fractional protection from trypsin proteolysis. Tubulin is loading control. Experiments were repeated at least four times with representative results shown.

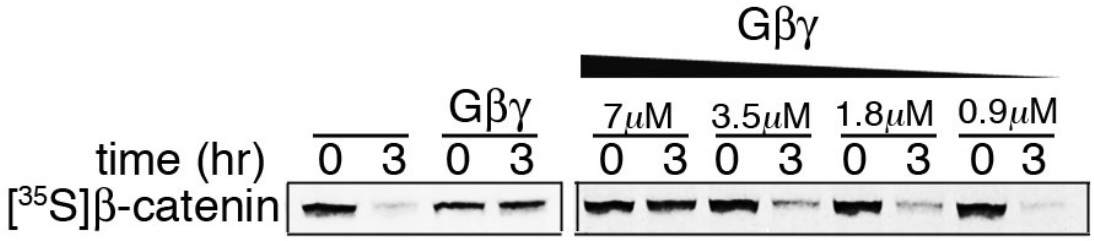


Figure 2.4: $G\beta_1\gamma_2$ inhibits [^{35}S]β-catenin degradation in a dose-dependent manner. Addition of increasing concentrations of $G\beta_1\gamma_2$ resulted in increased inhibition of β-catenin turnover in extracts. This experiment was repeated three times with representative results shown.

(Taussig et al., 1993). Finally, there is a wide range of concentrations at which G protein subunits interact with specific effectors**.** For example, the interaction between Gαi and adenylylcyclase occurs at high nanomolar concentrations, whereas interaction between Gαi and K+ channels occurs at the picomolar concentrations (Breitwieser and Szabo, 1988; Taussig et al., 1993).

Gβγ binds and recruit GSK3 to the membrane.

We hypothesize that the mechanism by which Gβγ inhibits β-catenin degradation in extract involves its sequestration of GSK3 into membranes, thereby preventing phosphorylation of cytoplasmic β-catenin. Isoprenylation of Gγ targets Gβγ subunits to the plasma membrane. Thus, Gβγ added to *Xenopus* egg extract (which contains membranous organelles) is likely incorporated into membranes. Furthermore, Gβγ has been previously shown to interact with three kinases, all of which are translocated to the membrane after binding Gβγ (Smrcka, 2008). In support of our hypothesis that Gβγ sequesters GSK3 into membranes, addition of GSK3 (but not Casein Kinase I) to extract reversed the inhibitory effects of Gβγ on β-catenin degradation (Figure 2.5B). Confirming an interaction between GSK3 and Gβγ, we next asked if Gβγ and GSK3 could form a complex in *Xenopus* egg extract. In agreement with our hypothesis, we show that endogenous GSK3 co-immunoprecipitated with endogenous Gβγ from extract (Figure 2.5C). When co-expressed in cultured mammalian cells, GSK3 similarly co-immunoprecipitated with $G\beta_1\gamma_2$ (Figure 2.5D). In contrast, we were unable to demonstrate co-immunoprecipitation of GSK3 and Gαo when both were co-expressed in cultured mammalian cells (data not shown). Finally, we observed interaction between

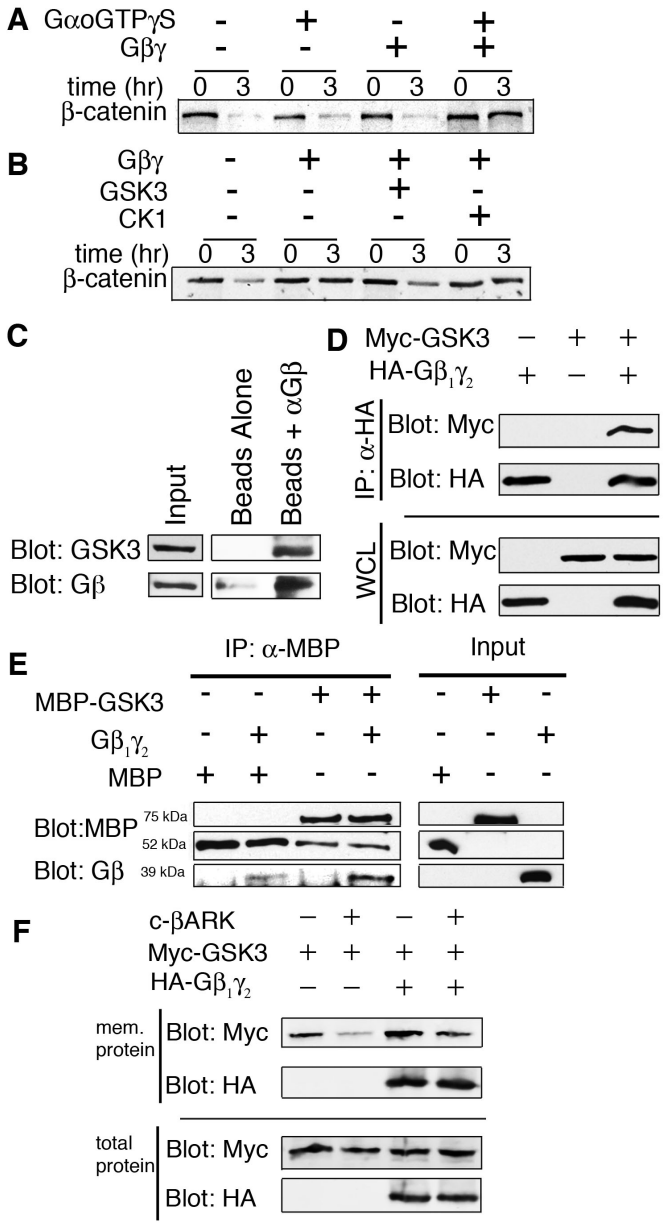

A
GαoGTPγS - + - +
Gβγ - - + +
time (hr) 0 3 0 3 0 3 0 3
β-catenin
B
Gβγ - + + +
GSK3 - - + -
CK1 - - - +
time (hr) 0 3 0 3 0 3 0 3
β-catenin
C
Input
Beads Alone
Beads + αGβ
Blot: GSK3
Blot: Gβ
D
Myc-GSK3 – + +
HA-Gβ1γ2 + – +
IP: α-HA
Blot: Myc
Blot: HA
WCL
Blot: Myc
Blot: HA
E
IP: α-MBP
Input
MBP-GSK3 - - + + - + -
Gβ1γ2 - + - + - - +
MBP + + - - + - -
Blot:MBP
75 kDa
52 kDa
Blot: Gβ
39 kDa
F
c-βARK – + – +
Myc-GSK3 + + + +
HA-Gβ1γ2 – – + +
mem. protein
Blot: Myc
Blot: HA
total protein
Blot: Myc
Blot: HA

Figure 2.5: G$\beta_1\gamma_2$ binds and promotes GSK3 membrane localization. (A) $G\beta_1\gamma_2$ synergizes with GαoA to inhibit β-catenin degradation. [^{35}S]β-catenin was added to *Xenopus* egg extract and incubated with buffer, $G\beta_1\gamma_2$ (0.5μM), GαoA-GTPγS (0.25μM), or $G\beta_1\gamma_2$ (0.5μM) plus GαoA-GTPγS (0.25μM). (B) The ability of $G\beta_1\gamma_2$ to inhibit β-catenin degradation in extract was reversed by addition of GSK3. [^{35}S]β-catenin was added to extract and incubated with $G\beta_1\gamma_2$(5μM), $G\beta_1\gamma_2$ plus GSK3(10units/μl), or $G\beta_1\gamma_2$ plus CKI (100units/μl). (C) GSK3 co-immunoprecipitates with Gβ from *Xenopus* egg extract. Anti-Gβ antibody beads were incubated with extract, washed, eluted with sample buffer, and immunoblotted for Gβ and GSK3. (D) GSK3 co-immunoprecipitates with $G\beta_1\gamma_2$ from cultured cells. HEK-293 cells were transfected with Myc-GSK3 and HA-$G\beta_1\gamma_2$, lysed, HA-$G\beta_1\gamma_2$ immunoprecipitated, and Myc-GSK3 detected by immunoblotting. (E) GSK3 binds $G\beta_1\gamma_2$ in vitro. MBP-GSK3 (0.66μM) or MBP (0.66μM) was incubated with $G\beta_1\gamma_2$ (0.6μM) and immunoprecipitated with anti-MBP antibody. Samples were washed, eluted, and immunoblotted for Gβ and MBP. (F) $G\beta_1\gamma_2$ enhances association of GSK3 with membrane fractions, and this effect is reversed by c-βARK. HEK-293 cells were transfected with c-βARK, Myc-GSK3, and HA-$G\beta_1\gamma_2$. Cell membranes were isolated and immunoblotted for Myc and HA. Data shown are representative of experiments performed at least three times.

purified Gβγ and GSK3 (Figure 2.5E). These results suggest that GSK3 and Gβγ directly interact.

To test whether Gβγ binds GSK3 to promote membrane localization, we expressed Myc-GSK3 with or without HA-$G\beta_1\gamma_2$ in HeLa cells and isolated membrane-associated proteins. An increased amount of GSK3 was membrane-associated when $G\beta_1\gamma_2$ was co-expressed (Figure 2.5F). Because cellular knockdown of Gβγ has been shown in previous studies to be complicated by subsequent loss of Gα signaling (Hwang et al., 2005; Krumins and Gilman, 2006). To circumvent this problem, we used the C-terminal domain of β-adrenergic receptor kinase (c-βARK), which binds and inhibits free Gβγ (Koch et al., 1994). In support of our model, we found that inhibition of both HA-$G\beta_1\gamma_2$ and endogenous Gβγ by c-βARK reduced membrane localization of Myc-GSK3 (Figure 2.5F).

Gβγ is required for LRP6-stimulated β-catenin/TCF signaling.

By promoting membrane localization of GSK3, $G\beta_1\gamma_2$ could enhance the activity of GSK3 towards its membrane substrates such as LRP6. HA-$G\beta_1\gamma_2$ expression resulted in a 2 to 5-fold enhancement of LRP6-stimulated TOPflash activity (a measure of β-catenin/TCF transcriptional activation), which was blocked by c-βARK (Figure 2.6A, Figure 2.7A,B). Consistent with the importance of $G\beta_1\gamma_2$ membrane localization for its synergy with LRP6, overexpression of a $G\gamma_2$ CAAX mutant lacking the isoprenylation modification (required for Gγ membrane localization) dramatically reduced enhancement of LRP6-stimulated TOPflash activity (Figure 2.7C). Finally, c-*β*ARK decreased

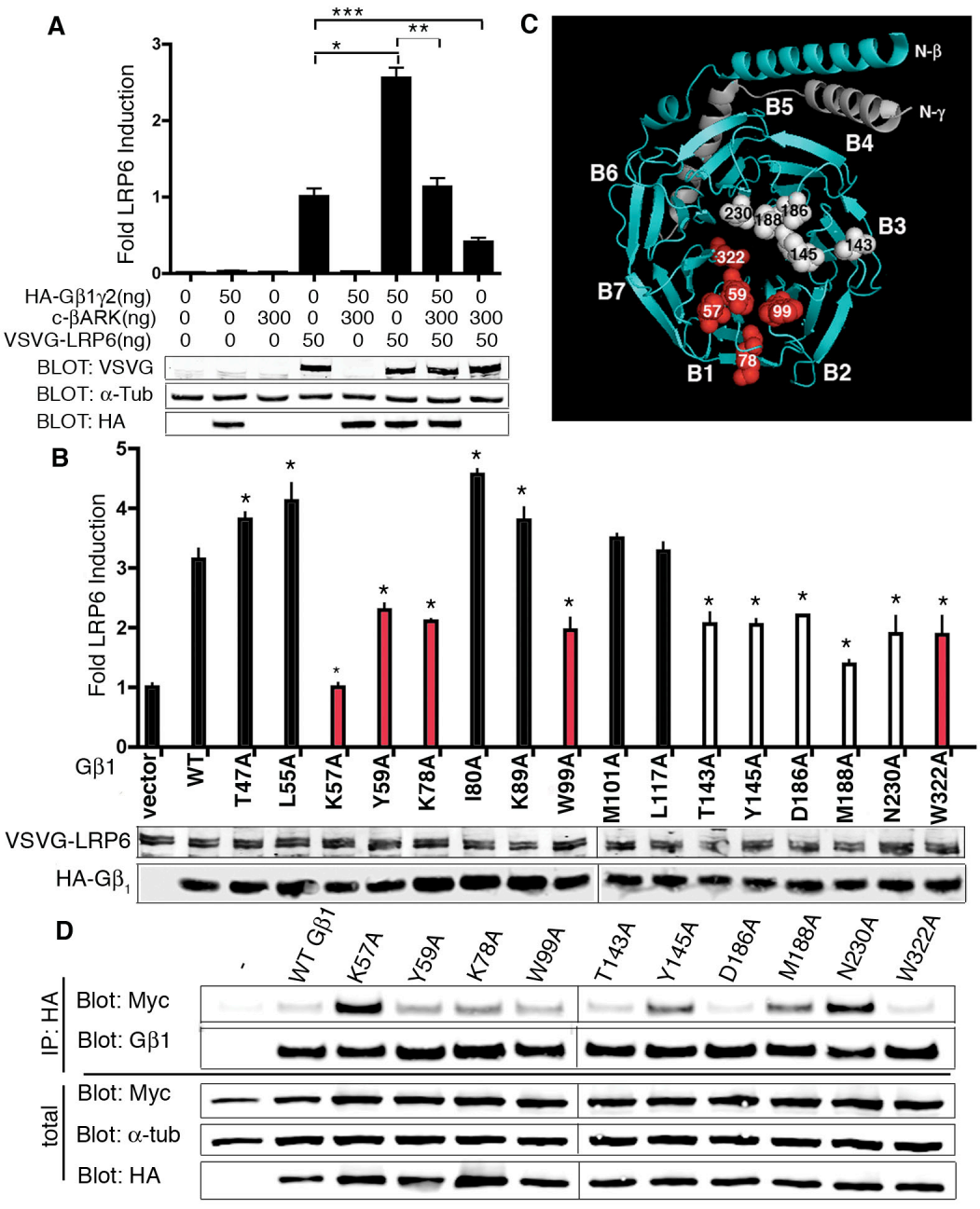

A
Fold LRP6 Induction
HA-Gβ1γ2(ng)
c-βARK(ng)
VSVG-LRP6(ng)
BLOT: VSVG
BLOT: α-Tub
BLOT: HA
C
N-β
N-γ
B5
B4
B6
B3
B7
B1
B2
230
188
186
143
145
322
59
57
99
78
B
Fold LRP6 Induction
Gβ1
vector
WT
T47A
L55A
K57A
Y59A
K78A
I80A
K89A
W99A
M101A
L117A
T143A
Y145A
D186A
M188A
N230A
W322A
VSVG-LRP6
HA-Gβ1
D
WT Gβ1
K57A
Y59A
K78A
W99A
T143A
Y145A
D186A
M188A
N230A
W322A
IP: HA
Blot: Myc
Blot: Gβ1
total
Blot: Myc
Blot: α-tub
Blot: HA

Figure 2.6: LRP6 activation of β-catenin/TCF signaling is regulated by $G\beta_1\gamma_2$. (A) $G\beta_1\gamma_2$ enhances LRP6 stimulation of TOPflash, and c-βARK inhibits the effects of $G\beta_1\gamma_2$ on LRP6 activity. HEK-293 STF cells were transfected with various combinations of HA-$G\beta_1\gamma_2$, VSVG-LRP6, and c-βARK. Lysates were assayed by TOPflash and immunoblotting. *P=$8.48\text{x}10^{-13}$,**P=$3.35\text{x}10^{-12}$,***P=$1.09\text{x}10^{-6}$ (ANOVA family-wise) (B) Scanning alanine mutagenesis of $G\beta_1$ identifies amino acids that mediate LRP6 activation. VSVG-LRP6 (40ng) was transfected alone or with the indicated $G\beta_1$ mutant (50ng) plus $G\gamma_2$ (50ng) into HEK-293 STF cells. Lysates were assayed by TOPflash and immunoblotting (anti-HA for $G\beta_1$ and anti-VSVG for LRP6). Red and white bars represent mutants that map to two clusters on the surface of $G\beta_1$. For Topflash assays, experiments were performed in triplicate, and luciferase activity is fold-induction compared to LRP6 (mean ± s.d.). *P<0.005 (ANOVA family-wise). (C) The crystal coordinates of $G\beta_1\gamma_2$. $G\beta_1$ is shown in teal and $G\gamma_2$ in gray. The seven β-propellers of Gβ are labeled B1-B7. Two clusters of amino acids required for $G\beta_1$-enhanced LRP6 activity are in red and white. (D) Co-immunoprecipitation of GSK3 with $G\beta_1$ point mutants. HEK-293 cells were transfected with Myc-GSK3, HA-$G\beta_1$ point mutants, and $G\gamma_2$. Cells were lysed, HA-$G\beta_1$ immunoprecipitated, and Myc-GSK3 detected by immunoblotting. Each experiment was repeated three times with representative results shown.

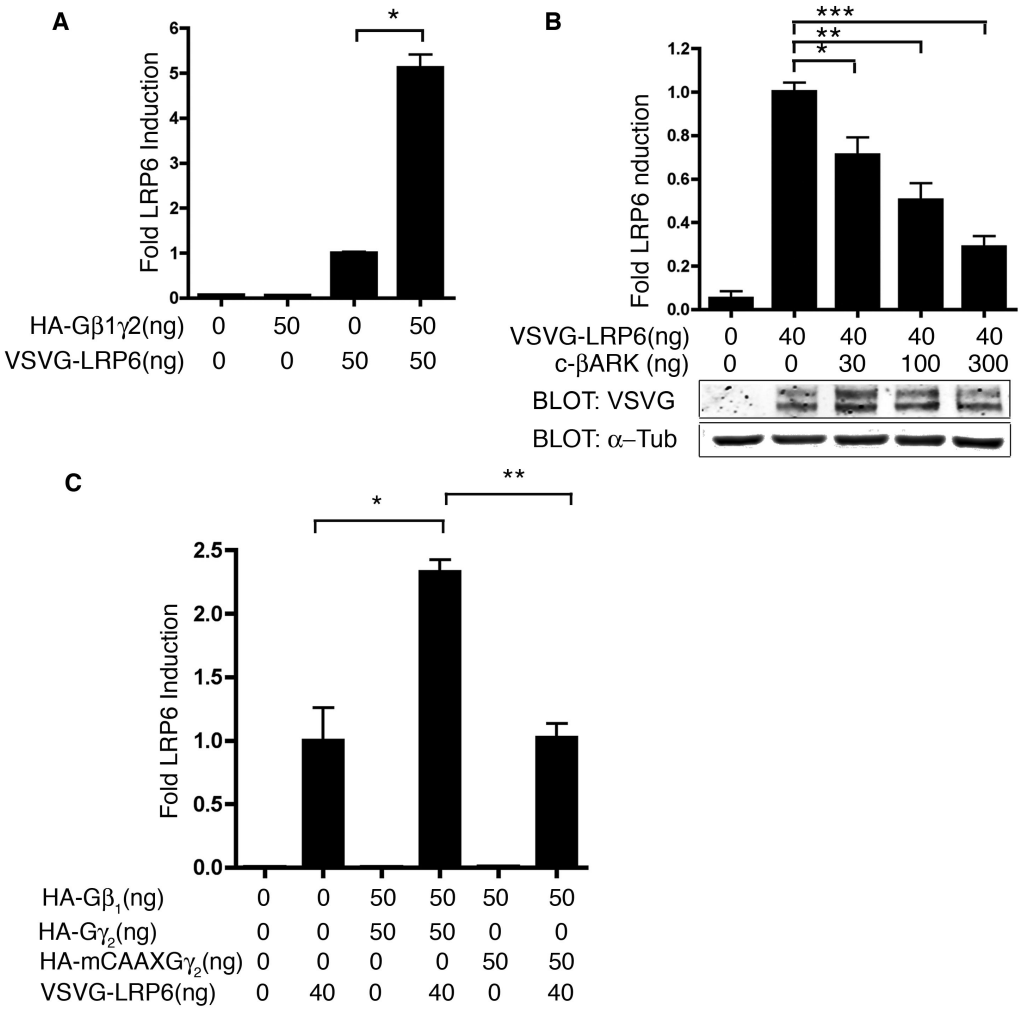


Figure 2.7: $G\beta_1\gamma_2$ enhancement of LRP6 stimulation of TOPflash is inhibited by inhibition of $G\beta\gamma$ and loss of $G\gamma_2$ membrane localization. (A) $G\beta_1\gamma_2$ enhanced LRP6 stimulation of TOPflash. HEK-293 STF cells (stably transfected with TOPflash, a luciferase-based β-catenin/TCF reporter) were transfected with HA-$G\beta_1\gamma_2$ and/or VSVG-LRP6 and lysates assayed for luciferase activity. *P=0.0001 (ANOVA family-wise). (B) VSVG-LRP6 activation was inhibited by c-βARK. HEK-293 STF cells were transfected with VSVG-LRP6 plus increasing concentrations of c-βARK. Lysates were assayed by TOPflash and immunoblotting. *P=0.001, **P=$9.94\text{x}10^{-6}$, ***P=$3.41\text{x}10^{-7}$ (ANOVA family-wise). (C) Membrane localization of $G\beta_1\gamma_2$ is required for its capability to enhance LRP6 activity. In contrast to wild-type $G\gamma_2$, expression of a $G\gamma_2$ CAAX mutant (lacks signal for isoprenylation) did not enhance LRP6 activity as assessed by TOPflash. The amount of plasmid transfected is indicated. Luciferase activity is fold-induction compared to LRP6 (mean ± s.d.). Experiments were performed in triplicate.
*P= $4.12\text{x}10^{-7}$, **P=$6.81\text{x}10^{-7}$ (ANOVA family-wise).

TOPflash activation by LRP6 alone (Figure 2.6A, Figure 2.7B), suggesting that endogenous Gβγ contributed to LRP6 signaling. c-βARK did not appear to exert its inhibitory effect by acting on other components of the β–catenin pathway, because we were unable to detect interaction of c-βARK with Axin, GSK3, or Dsh by co-immunoprecipitation. Raising the possibility that c-βARK does not exert its effects on Wnt signaling through its interactions with known components of the pathway (data not shown).

We did not observe any effects of HA-$G\beta_1\gamma_2$ on Wnt3a-stimulated activity in cultured cells (Figure 2.8A). This may suggest that, similar to CK1γ, $G\beta_1\gamma_2$ becomes limiting when LRP6 is present in excess (Davidson et al., 2005). c-βARK expression also had no effect on Wnt3a-stimulated activity (Figure 2.8B). One possibility is that Gβγ may signal through a distinct mechanism upstream of LRP6 (e.g. via non-Wnt3a-mediated pathways). Expression of c-βARK inhibited Dsh-stimulated activation (Figure 2.9A), indicating a requirement of Dsh for Gβγ. There was no detectable effect of HA-$G\beta_1\gamma_2$ or c-βARK when the pathway was stimulated by LiCl, a GSK3 inhibitor, confirming that Gβγ acts upstream of the β-catenin destruction complex (Figure 2.8C,D). These data suggest that Gβγ functions upstream of the destruction complex, at the level of LRP6 and Dsh.

Identification of critical $G\beta_1$ residues that mediate LRP6 activity and GSK3 binding.

Previous studies identified critical residues on $G\beta_1$ that mediate its interaction with specific effectors (Ford et al., 1998; Scott et al., 2001). To determine whether these

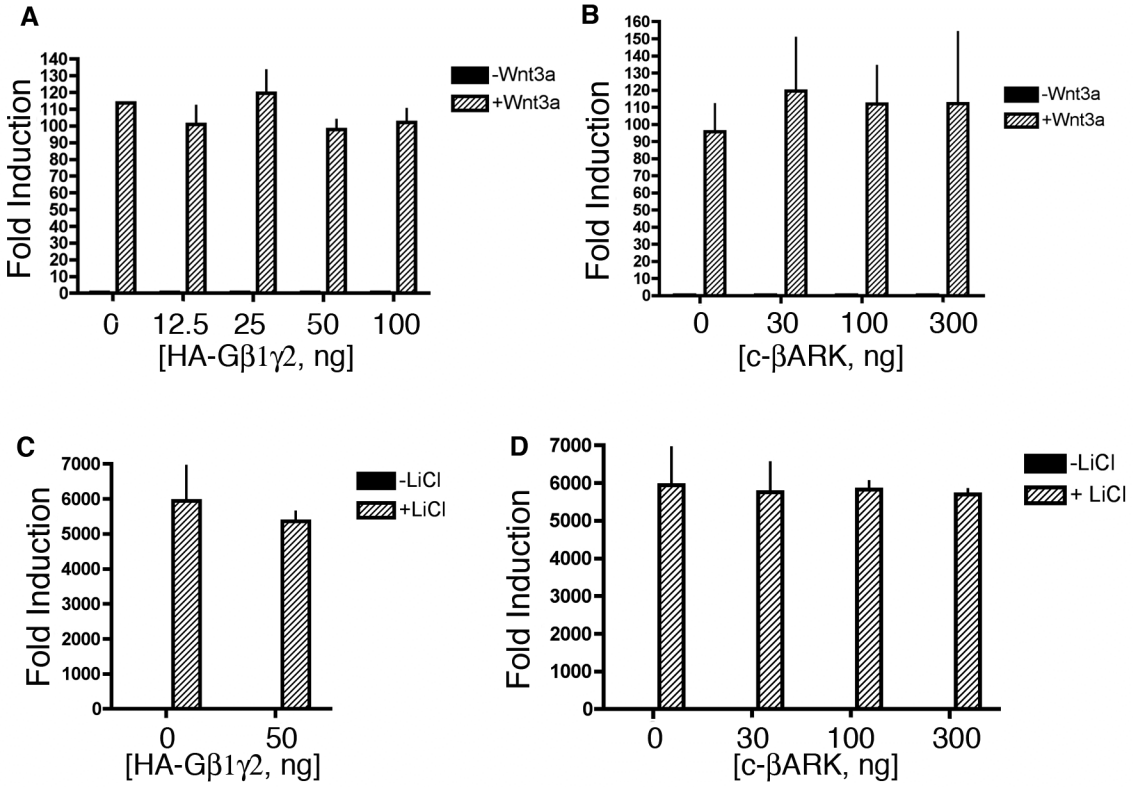


Figure 2.8: $G\beta_1\gamma_2$ does not enhance activation by Wnt3a or LiCl treatment, and activation by Wnt3a or LiCl is not blocked by c-βARK. HEK-293 STF cells were transfected with HA-$G\beta_1$ plus $G\gamma_2$ (A,C) or c-βARK (B,D) and treated overnight with Wnt3a-conditioned media (A,B) or 30 mM LiCl (C,D). Transfected cells were then lysed and processed for TOPflash. The amount of plasmid used for transfection is indicated. Luciferase activity is fold-induction compared to control (mean ± s.d.). Experiments were performed in triplicate. Statistical significant differences were not observed.

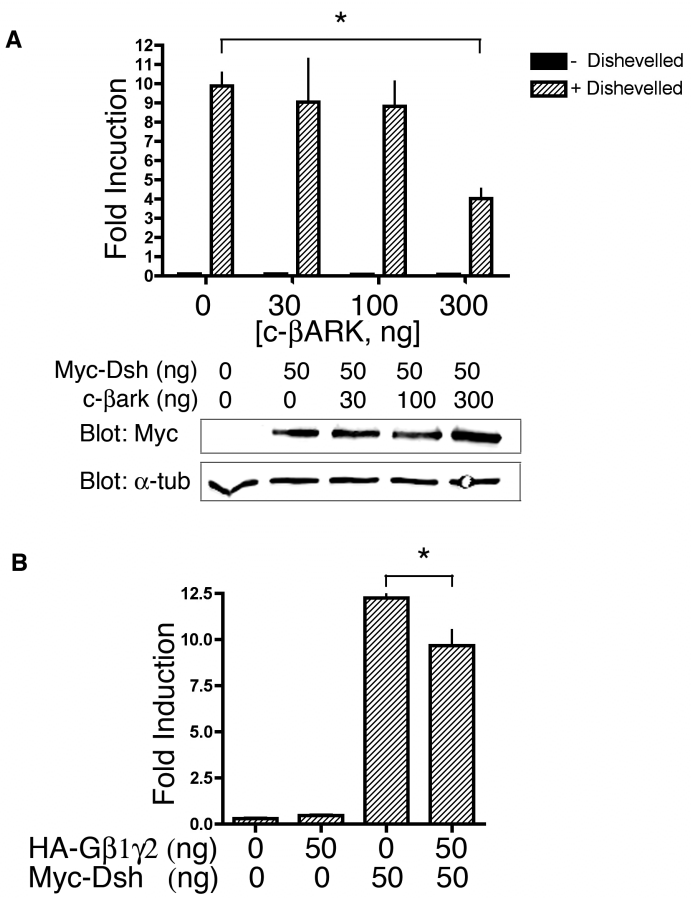


Figure 2.9: Dsh-mediated activation is inhibited by c-βARK. (A) HEK-293 STF cells were transfected with Myc-Dsh plus increasing concentrations of c-βARK. Transfected cells were then lysed and processed for TOPflash or immunoblotting. *P=3.85x10^{-5} (ANOVA family-wise)**.** (B) HEK-293 STF cells were transfected with HA-Gβ_1 and Gγ_2; Myc-Dsh; or HA-Gβ_1, Gγ_2 and Myc-Dsh. *P=8.94x10^{-5} (ANOVA family-wise). Lysates were assayed for TOPflash activity. The amount of each plasmid used for transfection is indicated. Luciferase activity is fold-induction compared to control (mean ± s.d.). Experiments were performed in triplicate.

sites also mediate GSK3 binding and/or LRP6 activation, we generated a series of single alanine mutants of $G\beta_1$. We found that a subset of these Gβ1 mutants exhibited a decreased capacity to activate LRP6 (Figure 2.6B). Gβ is a WD40-repeat protein that forms a seven-bladed β-propeller structure (Smrcka, 2008). Based on previous X-ray crystal structures of Gβ, residues altered in this subset of mutants are clustered within two regions of $G\beta_1$ (predominantly within blades 1, 3, and 4; Figure 2.6C). For the subset of $G\beta_1$ mutants with impaired LRP6 activation, we next performed co-immunoprecipitation experiments to test the capacity of each mutant to bind GSK3. Surprisingly, we found that several of these $G\beta_1$ mutants bound GSK3 more avidly than wild-type $G\beta_1$ (Figure 2.6D). These results support a model in which dynamic interaction between Gβγ and GSK3 is required to promote LRP6 activation.

Gβγ enhances GSK3 kinase activity towards LRP6.

To further understand how $G\beta_1\gamma_2$ activates LRP6 signaling, we tested whether $G\beta_1\gamma_2$ enhances phosphorylation of LRP6 by GSK3. We transfected cells with $G\beta_1\gamma_2$ and GSK3, isolated membrane-associated proteins, and immunoblotted with an antibody that recognizes GSK3-phosphorylated sites on LRP6. When GSK3 was co-expressed with $G\beta_1\gamma_2$, there was an increase in phosphorylated LRP6 above that observed when GSK3 was expressed alone (Figure 2.10A). LRP6 was also present in $G\beta_1\gamma_2$ immunoprecipitates, suggesting that they form a complex (Figure 2.11B). Consistent with a recent study, we also detected Axin in $G\beta_1\gamma_2$ co-immunoprecipitates (Figure 2.11C) (Jung et al., 2009)**.** Because c-βARK inhibits Dsh-stimulated activation, we tested whether $G\beta_1\gamma_2$ binds Dsh. Consistent with previous reports, we found that Dsh co-

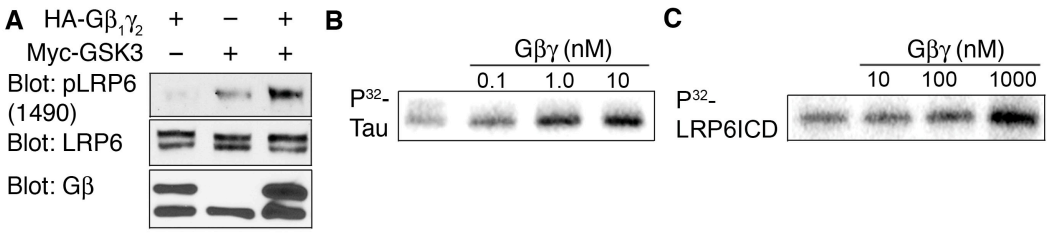


Figure 2.10: $G\beta_1\gamma_2$ enhances phosphorylation of LRP6 by GSK3. (A) $G\beta_1\gamma_2$ increases phosphorylation of endogenous LRP6 when co-transfected with GSK3. HEK-293 cells were transfected with Myc-GSK3 with or without HA-$G\beta_1\gamma_2$ and lysates immunoblotted with anti-phospho-LRP6 (Ser1490). (B,C) $G\beta_1\gamma_2$ enhances GSK3 phosphorylation of Tau (B) and LRP6ICD (C) *in vitro*. Each experiment was repeated three times with representative results shown.

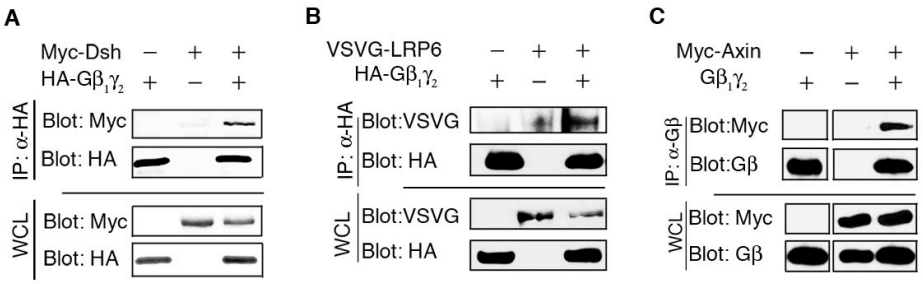


Figure 2.11: Dsh, Axin, and LRP6 form complexes with $G\beta_1\gamma_2$. (A) Dsh co-immunoprecipitates with $G\beta_1\gamma_2$ from cultured cells. HEK-293 cells were transfected with HA-$G\beta_1$ (1 μg), $G\gamma_2$ (1 μg), and Myc-Dsh (1 μg). Cells were lysed, HA-$G\beta_1$ immunoprecipitated, and Myc-Dsh detected by immunoblotting for the Myc tag. (B) LRP6 co-immunoprecipitates with $G\beta_1\gamma_2$ from cultured cells. Membrane proteins were isolated from HEK-293 cells transfected with HA-$G\beta_1$ (1 μg), $G\gamma_2$ (1 μg), and VSVG-LRP6 (1 μg). HA-$G\beta_1$ was immunoprecipitated and VSVG-LRP6 detected by immunoblotting for the VSVG tag. (C) Axin co-immunoprecipitates with $G\beta_1\gamma_2$ from cultured cells. HEK-293 cells were transfected with HA-$G\beta_1$ (1 μg), $G\gamma_2$ (1 μg), and Myc-Axin (1 μg). Cells were lysed, $G\beta_1$ immunoprecipitated, and Myc-Axin detected by immunoblotting for the Myc tag. Experiments were repeated at least three times with representative results shown.

immunoprecipitates with $G\beta_1\gamma_2$ (Figure 2.11A) (Angers et al., 2006; Jung et al., 2009; Egger-Adam and Katanaev, 2009). In summary, Gβγ appears to act in a complex with Dsh, Axin, and GSK3 to promote phosphorylation of LRP6.

To determine if Gβγ directly affects GSK3 activity, we performed *in vitro* kinase assays. Gβγ enhanced GSK3 phosphorylation of Tau, a known GSK3 target, whereas undissociated heterotrimeric Go and BSA did not enhance GSK3 activity towards Tau (Figure 2.10B, Figure 2.12). Gβγ also enhanced GSK3 phosphorylation of the intracellular domain of LRP6 (LRP6ICD) on the PPPSP (Pro-Pro-Pro-Ser-Pro) motif, which has been shown to provide a docking site for Axin and is required for LRP6 activity (Figure 2.10C, Figure 2.13). Interestingly, Gβγ stimulates GSK3 phosphorylation of LRP6ICD to a much lesser degree than for Tau (Figure 2.10C). This may reflect differences in the affinity of GSK3 for Tau versus LRP6ICD and a previously reported feedback mechanism by which phosphorylated LRP6 inhibits GSK3 activity (Cselenyi et al., 2008; Piao et al., 2008; Wu et al., 2009). Alternatively, LRP6 could simply compete with Gβγ for binding to GSK3. This latter model is consistent with our observation that $G\beta_1$ mutants with increased affinity for GSK3 are unable to activate β-catenin/TCF signaling.

Discussion

GSK3 is the fourth identified Gβγ kinase effector (in addition to G protein coupled-receptor kinase 2, Bruton's tyrosine kinase, and Phosphoinositide 3 kinase γ) (Smrcka, 2008). In each case, the mechanism of action of Gβγ appears to involve membrane translocation and, in the case of PI3Kγ, BTK, and GSK3, stimulation of kinase

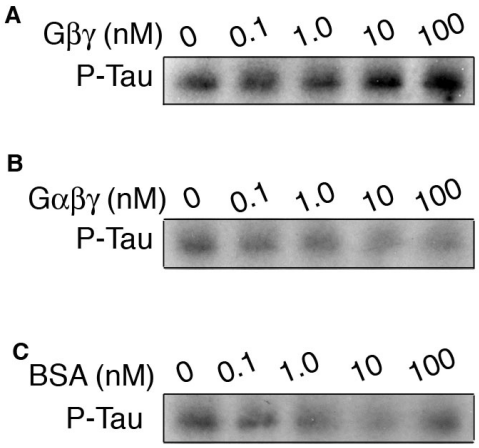


Figure 2.12: Free G$\beta\gamma$ is required for enhanced GSK3 activity towards Tau and LRP6ICD. Phosphorylation of Tau by GSK3 is enhanced by free Gβγ (A), but not by G protein heterotrimer purified from brain (B) or BSA (C). Experiments were repeated three times with representative results shown.

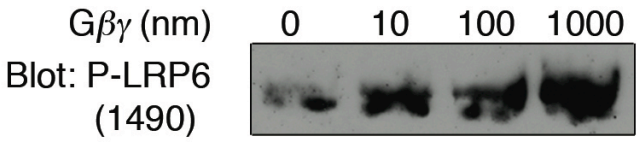


Figure 2.13: **Phosphorylation of LRP6ICD by Gβγ-stimulated GSK3 occurs at the PPPSP Ser-1490 site as detected by immunoblotting**. This experiment was repeated three times with representative results shown.

activity. It has recently been shown that regulation of GSK3 by Gβγ plays a critical role in controlling the stability of Snail, a GSK3 substrate, during zebrafish gastrulation (unpublished data, Speirs et al.). Thus, Gβγ may control diverse signaling pathways through regulation of GSK3.

$G\beta_2\gamma_2$ has been reported to negatively regulate Wnt-1 signaling by a feedback mechanism that stimulates the degradation of Dsh (Jung et al., 2009). Our results, however, are consistent with a recent study in *Drosophila* indicating that Gβγ acts to positively promote β-catenin/TCF signaling (Egger-Adam and Katanaev, 2009). The simplest model to explain the activation of β-catenin/TCF signaling by Gβγ would invoke a receptor-mediated mechanism. In contrast to its effects on Dsh and LRP6, however, c-βARK did not inhibit Wnt3a activation of β-catenin/TCF signaling. One possibility is that receptor-mediated activation of the heterotrimer and subsequent association of Gβγ and GSK3 is a rapid and tightly coupled process. Indeed, there is evidence that certain GPCRs and their G proteins remain tightly associated upon activation by ligand (Gales et al., 2005). Thus, overexpression of c-βARK, which binds free Gβγ, may not be sufficient to disrupt Frizzled receptor-associated Gβγ. In support of a role for Gβγ in Wnt signaling, it has recently been shown that *Drosophila* Gβγ is required for proper activation of the Wnt pathway (Egger-Adam and Katanaev, 2009). Alternatively, it is possible that signaling through $G\beta_1\gamma_2$ may be independent of Wnt/Frizzled. To date, Frizzled has not been demonstrated to be a bona fide GPCR; there is evidence, however, that traditional GPCRs can activate β-catenin/TCF signaling. Parathyroid receptor I has been reported to activate LRP6 to signal β-catenin/TCF-mediated transcription, and the Gonadotropin releasing hormone receptor has been shown

to inhibit GSK3 activity and upregulation of β-catenin/TCF target genes (Gardner et al., 2007; Wan et al., 2008). In the future, it will be important to distinguish whether Gβγ mediates β-catenin signaling via classic GPCRs or via Frizzled.

We propose a model in which both Gα and Gβγ regulate β-catenin/TCF signaling (Figure 2.14). Our biochemical screen using purified recombinant proteins and *Xenopus* egg extract demonstrates a role for Gαo and Gαq, as well as Gαi2, in the inhibition of β-catenin degradation. Additionally, our biochemical studies support a positive role for Gβγ in β-catenin/TCF signaling. Artificially tethering GSK3 to the plasma membrane is sufficient to stimulate Wnt signaling in cultured mammalian cells and axis duplication in *Xenopus* laevis embryos; thus, the β-catenin/TCF pathway appears to be very sensitive to GSK3 membrane localization (Zeng et al., 2005). Our data support a model in which Gβγ acts in concert with Dsh to recruit and activate GSK3 (either free or possibly in association with Axin) at the plasma membrane; membrane-associated GSK3 phosphorylates LRP6, which binds to the β-catenin degradation complex to inhibit β-catenin turnover.

Future Directions

Dissection of the nature of GSK3 and Gβγ interaction

Our studies indicate that the interaction between Gβγ and GSK3 is direct. Our mutagenesis analysis shows that the sites of interaction of GSK3 on Gβγ maps to the same interaction sites of known Gβγ effectors. We do not know how Gβγ enhances the kinase activity of GSK3. Mutagenesis of GSK3 may reveal specific sites of interaction on

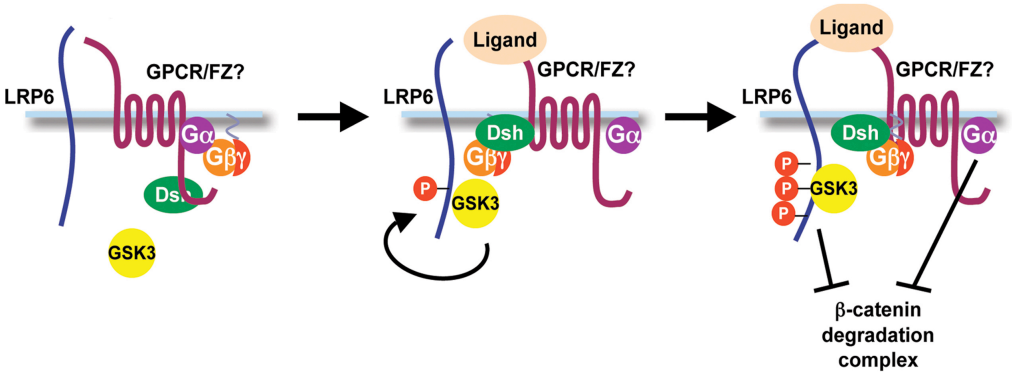


Figure 2.14: Model for regulation of β-catenin/TCF signaling by Gα and Gβγ.

GSK3 required for its interaction with Gβγ. It would also be interesting and beneficial to understand at a structural level the nature of Gβγ's activation of GSK3 kinase activity. Obtaining the crystal structure of Gβγ and GSK3 may reveal this information. Both Gβγ and GSK3 have been crystallized and their structure has been solved (Dajani et al., 2001; Wall et al., 1995). Additionally, we can take advantage of the 57A Gβ mutant, which has increased affinity for GSK3. It is possible that Axin, LRP6 and or Dsh may be required to form a stable complex between Gβγ and GSK3 and these proteins may be required to assemble a protein complex that can be crystallized.

Role of Gβγ within current LRP6 signaling models

There are 2 current models for LRP6 signaling: the (1) initiation and amplification model (Baig-Lewis et al., 2007) and the (2) signalosome model (Bilic et al., 2007). In the initiation and amplification model, Frizzled and LRP6 are required to receive the Wnt signal, and activation (and dimerization) of LRP6 is required for amplification of signaling. Neither step alone can successfully transduce a Wnt signal such that both are required for robust signaling (Baig-Lewis et al., 2007). In the signalosome model, Wnt stimulates Dishevelled and Axin polymerization and coaggregation of LRP6 with other Wnt signaling components. This results in LRP6 phosphorylation (by CK1γ) and receptor activation (Bilic et al., 2007). If Gβγ signals downstream of Wnt, it is possible that Gβγ fits in either of these models of LRP6 signaling.

In the initiation and amplification model, Gβγ may play roles both the initiation and amplification steps of the model. Signaling through Frizzled could result in the dissociation of Gα and Gβγ, allowing Gβγ to interact with and activate GSK3 at the

membrane resulting in the initial phosphorylation of LRP6 to initiate signaling. This initial phosphorylation of LRP6 by GSK3 at LRP6's PPPSP motifs could provide a docking site for Axin. Because Gβγ can interact with Axin and Dishevelled, Gβγ may aid in the robust phosphorylation of LRP6 by the GSK3 on Axin and promote signal amplification (Angers et al., 2006; Jung et al., 2009; Egger-Adam and Katanaev, 2009).

As previously mentioned, Gβ can interact with multiple Wnt pathway components, including LRP6, GSK3, Axin, and Dishevelled (Angers et al., 2006; Jung et al., 2009; Egger-Adam and Katanaev, 2009). In the signalosome model, all of these proteins are likely components of the signalosome. Since Gβ can interact with each of these proteins, it is possible that Gβγ could also be part of the signalosome. Gβγ may stabilize the signalosome as well as promote LRP6 phosphorylation and activation.

Role of Gβγ signaling downstream of known GPCR

Prostaglandin Receptor Signaling

Prostaglandin signaling is required for homeostasis and is involved in multiple physiological processes including digestion, reproduction, and immunity (Wang and Dubois, 2006). Prostglandin E2 (PGE2) also plays a strong role in the promotion of cancer progression and is involved in tumor cell proliferation, invasion, angiogenesis, and immunosuppression in numerous tumor types, including colorectal, hepatocellular, and breast cancer (Wang and Dubois, 2006). PGE2 is synthesized from arachidonic acid by cyclooxygenase (COX)-1 and -2 [also known as prostaglandin endoperoxide synthases (Ptgs)] producing PGC2 and PGH2. PGH2 is then converted to PGE2 by Prostaglandin

E synthase (Ptges). PGE2 can signal by binding its G protein coupled receptor, E-prostanoid (EP)1-4 (Regan, 2003; Wu, 2006) .

Recent work by the Solnica Krezel lab has found that PGE2 signaling is required for cell movement during gastrulation in the developing zebrafish embryo (Speirs, unpublished). There was an increase in E-cadherin message and protein in embryos injected with a morpholino targeting *ptges*, which knocked down production of PGE2. This indicates that PGE2 signaling is linked to E-cadherin production (Speirs, unpublished). Snail is a co-transcriptional repressor that binds the E-box of the E-cadherin gene promoter to inhibit its expression (Barrallo-Gimeno and Nieto, 2005). Snail proteins levels can be regulated post-transcriptionally by phosphorylation (by GSK3) and its subsequent ubiquitination by β-TRCP and proteasomal degradation. Speirs *et. al.* found that levels of Snail were much lower in ptges morphants (lacking PGE2) compared to zebrafish embryos injected with nonsense morpholino suggesting that PGE2 regulates E-cadherin levels by regulating Snail protein stability. The PGE2 receptor is a GPCR and signaling by PGE2 acts in part through heterotrimeric G proteins. Overexpression of Gβγ subunits in the pgtes morphants blocks degradation of Snail indicating that Gβγ signals downstream of receptor activation to regulate Snail protein stability. In collaboration with the Drs. Speirs and Solnic Krezel, we have shown that in cell culture experiments Gβγ can interact with zebrafish GSK3, and that this interaction is enhanced in the presence of PGE2 (Speirs, unpublished). This suggests that the interaction of Gβγ and GSK3 downstream of PGE2 stimulation stabilizes Snail protein and inhibits E-cadherin expression.

There are many connections between Wnt and PGE2 signaling. Axin2, a Wnt transcriptional target, has been shown to act as a nucleocytoplasmic chaperon for GSK3 by binding GSK3 in the nucleus and exporting it into the cytoplasm, thereby stabilization Snail in the nucleus (Yook et al., 2006). In this way, Wnt/β-catenin transcriptional activation can stabilized Snail and reduce E-cadherin protein expression. Snail has also been shown to activate β-catenin target gene expression by providing a positive feedback loop for Wnt dependent transcription; Snail can activate β-catenin target gene expression, inducing Axin2, which in turn helps to stabilize Snail by removing GSK3 form the nucleus (Stemmer et al., 2008).

The involvement of Axin2 in Snail stabilization provides interesting insight into the mechanism of Gβγ inhibition of GSK3 phosphorylation on Snail. Since we know that Gβγ normally localizes to the membrane and we have found that Gβγ interacts with Axin it would be interesting to determine whether Axin2 plays a role in PGE2 signaling through Gβγ. Our studies indicate that Gβγ can enhance the kinase activity of GSK3, suggesting the possibility that additional GSK3 substrates are phosphorylated to regulate Snail stability. One such GSK3 substrate is LRP6 and it is possible that LRP6 plays a role in PGE2 signaling.

It is also possible that the Gβ mutants that bind GSK3 with a higher affinity would be better at stabilizing Snail in zebrafish embryos. In addition, it would be interesting to see is c-βark can reverse the Snail rescue by Gβγ in the *ptges* morphants. Because PGE2 signaling enhances the association of Gβγ and GSK3 in culture cells, GSK3 may localize to the plasma membrane after PGE2 signaling in embryos.

Parathyroid Receptor Signaling

The parathyroid hormone (PTH) is a hormone that targets bone, kidney, and intestine, and acts to regulate calcium metabolism (Jilka, 2007; Potts and Gardella, 2007). PTH signals by binding the Parathyroid hormone 1 receptor (PTH1R) which activates Gαs, Gαq, and Gβγ resulting in the production of 3',5'-cyclic adenosine-5'-monophosphate (cAMP), and subsequent activation of protein kinase A (PKA) (Abou-Samra et al., 1991; Juppner et al., 1991). Although it is thought that the PKA signals to mediate the PTH response, the exact molecular mechanism is not known.

Wnt signaling is also essential for skeletal biology and recently a link between bone mass and gain of loss of function of LRP5 and the Wnt antagonist sclerostin was identified (Baron et al., 2006; Balemans and Van Hul, 2007; Glass and Karsenty, 2007). Additionally, PTH activates β-catenin signaling in osteoblasts by signaling through the Wnt co-receptor LRP6 (Wan et al., 2008). As with treatment with Wnt3a, PTH treatment of cells expressing the PTH1R receptor stimulated TOPflash activity and stabilized cytoplasmic β-catenin. PTH also stimulated PTH-PTH1R-LRP6 complex formation, LRP6 phosphorylation, and membrane recruitment of Axin. Furthermore chemical inhibition of PKA with H89 inhibited PTH stimulated TOPflash, but not stimulation by Wnt3a, suggesting that Wnt3a and PTH may signal through similar but not identical downstream signaling events (Wan et al., 2008).

In addition to the endocrine factor PTH, PTH1R signals through the paracrine/autocrine factor PTHrR (Parathyroid hormone related protein) (Kronenberg et al., 1996). Although PTH1R signals through functionally distinct ligands, there is only one isoform of the receptor. This one isoform of PTH1R has been shown to signal

through multiple subclasses of Gα subunits including Gαs, Gαq/11, Gαi/o, and Gα12/13, and has been linked to multiple signaling pathways (Abou-Samra et al., 1991; Bringhurst et al., 1993; Cheung et al., 2005; Offermanns et al., 1996). This indicates that PTH1R can promiscuously interact with multiple heterotrimeric G proteins. One explanation for this receptor's promiscuity is that the receptor interacts with Gβγ directly such that Gβγ links the receptor to the Gα subunits. Structure function analysis identified specific sites within the c-terminal tail of PTH1R that is required for high affinity interaction with Gβγ and for Gα signaling (Mahon et al., 2006). Structural analysis (by X-ray crystallography) of Gβγ bound to the C-terminal tail of PTH1R indicates that the regions of predicted membrane-facing region of Gβ is oriented to the membrane spanning receptor, and the region of interaction is highly conserved for isoforms 1-4 of Gβ (Johnston et al., 2008). This suggests that the interaction of PTH1R with Gβγ may explain PTH1R's promiscuity as well as provide a mechanism for receptor mediated Gα activation (Johnston et al., 2008).

Since PTH1R binds Gβγ and can complex with and activate LRP6, it would be interesting to know if PTH1R signals through Gβγ interaction with GSK3. If this is the case, one would expect that PTH treatment might enhance the interaction of Gβγ and GSK3. Overexpression of Gβγ along with PTH treatment might also enhance TOPflash activation and c-βark expression may inhibit signaling. It is possible that over-expression of Gβγ could enhance PTH stimulated phosphorylation of LRP6 and expression of the Gβγ inhibitor c-βark could decrease PTH stimulated LRP6 phosphorylation. This would provide further evidence that Gβγ-GSK3 interaction can signal downstream of numerous GPCRs.

Wnt signaling components as bona fide G protein signaling proteins

Axin

The scaffold protein Axin contains a RGS (regulator of G protein signaling) domain, a conserved domain that acts as a GTPase activating protein (GAP) by binding a Gα subunit unit when bound to GTP, stabilizing the GTP hydrolysis transition state, and enhancing the rate of GTPase hydrolysis. This decreases the amount of time that the Gα subunit is bound to GTP and active. The RGS domains may not have GAP activity and instead limit Gα-GTP activity by binding the Gα and inhibiting it from interacting with effectors. Mutagenesis analysis revealed that Axin's RGS domain is also the site of its interaction is APC, the other scaffold protein required for destruction complex formation and function (Behrens et al., 1998; Hart et al., 1998; Kishida et al., 1998). Structural analysis found that APC localizes to the opposite face of the RGS domain from the G protein binding site. This suggests that Axin may be able to interact with APC and Gα proteins at the same time and that Axin's interaction with APC does not inhibit it from interacting with Gα as well. Axin's RGS domain appears to lack GTPase activity, although it interacts with various Gα subunits including Gαo, Gαs, and Gα12 (Castellone et al., 2005; Egger-Adam and Katanaev, 2009; Stemmle et al., 2006). There are no reports, however, that demonstrate that Axin's RGS domain has GAP activity. Unpublished preliminary data from Dr. Elliott Ross' lab (UT Southwestern) suggests that Axin contains a low level of GTPase activity. Mutational analysis of Axin's RGS domain followed by GTPase assays may provide insight into whether or not Axin's RGS domain contains GAP activity.

Because Gαo and Axin interact through Axin's RGS domain, we could also express and purify Gαo and Axin (or a fragment of Axin containing its RGS domain) in a complex, and perform structural analysis to directly identify the nature of this interaction.

Frizzled

Although there is a body of evidence that suggests that Frizzled is a GPCR, it has not been directly demonstrated that Frizzled signals through heterotrimeric G proteins. A preliminary manuscript from Nature Proceedings reports that Frizzled containing bacterial membranes interacts with Gαo in a pertussis toxin sensitive manner, and can activate Gαo nucleotide exchange after treatment with Wnts (Katanaev and Buestorf1, 2009). This Wnt stimulated activation of Gαo nucleotide exchange is also sensitive to inhibition by secreted Fz-related proteins (sFRP). Although this report has not been peer reviewed, this is the first reported attempt to demonstrate that Fz is a bona fide GPCR.

There are a number of issues with this report. First of all, the biochemical assays were conducted with partially purified components. The Frizzled receptor was expressed in *E.coli* cells then membranes were isolated. The Frizzled proteins was never isolated from the bacterial membrane, so there could have been contribution by the *E.coli* protein in the membrane protein prep. Also, they saw a bell shaped response in their guanine nucleotide binding assay such that at low and high concentrations of Wnt ligand they saw only low levels of nucleotide exchange. They attributed this to inhibitor action of carrier proteins within the Wnt preparation. The use of purified Wnt3a ligand should be able to resolve this issue of carrier proteins.

Altogether, the data reported was rather weak, however it does provide a foundation for others to more definitely illustrate a role for Frizzled as a functional GPCR.

BOOK 1: INTRACELLULAR MECHANISM OF WNT/β-CATENIN SIGNAL TRANSDUCTION

CHAPTER 3: LRP6 TRANSDUCES A CANONICAL WNT SIGANL INDEPENDENTLY OF AXIN DEGRADATION BY INHIBITING GSK3'S PHOSPHORYLATION OF β-CATENIN

The work described in this chapter has been published (Cselenyi CS, Jernigan KK et al., 2008)

Introduction

The best-characterized form of Wnt signaling is the Wnt/β-catenin, or canonical Wnt, pathway (Logan and Nusse, 2004). During Wnt/β-catenin signaling, a Wnt ligand binds transmembrane coreceptors Frizzled (Fz) and low-density lipoprotein receptor-related proteins 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 1α (CK1 α) (Logan and Nusse, 2004). CK1 α 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 coreceptor 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 is 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, reduction of Axin levels represents a potentially robust mechanism for β-catenin stabilization (Lee et al., 2003). As a result, LRP5/6-mediated Axin degradation is 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 ≈2 h before substantial changes in Axin levels are detected (Willert et al., 1999; Liu et al., 2005). 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. 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., 1994). Here, we address whether LRP6 can stabilize β-catenin independently of Axin degradation. We reconstituted LRP6 signaling in biochemically tractable *Xenopus* egg extract, which has been used to accurately reconstitute cytoplasmic aspects of Wnt signal transduction (Salic et al., 2000; Lee et al., 2001; Major et al., 2007). 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 Ax in and subcloned in pCS2. AxinΔ298–437 and AxinΔ437–506 have been previously described (Logan and Nusse, 2004). AxinSA harbors Ser-Ala mutations at the following predicted GSK3 phosphorylation sites: SANDSEQQSLS. We refer to mouse Ax in (GenBank Accession: XM914907) amino acid (aa) 126 as ‘‘start methionine’’ (CSLMQSP). Mouse and human LRP6ICD (aa 1397–1614) (GenBank Accessions: NM008514 and NM002336, respectively) were subcloned into pET11-D or pCS2 using a PCR-based approach. For Lrp6ICD mRNA (SI Figure 3.3C 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) , LRP6I CD with a 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 OD600 of 0.3 at 37°C and induced with isopropyl-D-thiogalactoside (IPTG) (0.3 μg/ml) for 9 h. 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 (1μg/ml) for 4 h and purified on glutathione resin.

mRNA Synthesis and RT-PCR.

Capped RNA for embryo injection was synthesized from linearized plasmid DNA templates by using mMessage mMachine (Ambion). Animal caps were cut from stage 9 embryos and cultured in 75% Marc's modified Ringers (MMR) [0.1 M NaCl, 2 mM KCl, 1 mM MgSO4, 2 mM CaCl2, 5 mM Hepes (pH 7.4)] until stage 11. RT-PCR for *siamois*, *Xnr3*, and ODC were performed by using primers and conditions previously described (www. hhmi.ucla.edu/derobertis/index.html).

Tau Phosphorylation.

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

***Xenopus* Egg Extract Degradation Assay and Depletion**.

Xenopus egg extract was prepared and degradation assays were per for med as described (Logan and Nusse, 2004). Extract was incubated with LRP6ICD at a concentration of 1.6 μM unless other wise noted. In Axin-depletion experiments, IVT proteins were made in wheat germ lysate (Promega). Dsh and Ax in immunodepletions were performed and confirmed as described (Logan and Nusse, 2004), 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 h with inversion every 10 min. As shown in Figure 3.6D, Axin antibody was covalently conjugated to Protein A magnetic beads (NEB) for depletion.

Phosphatase Treament.

Samples (0.8 μl) from a *Xenopus* egg extract degradation assay were added to λ–phosphatase buffer (12.5 μl) and 400 units of 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 h 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 h 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.

mRNA Synthesis and RT-PCR.

Capped RNA for embryo injection was synthesized from linearized plasmid DNA templates by using Immunoprecipitation (IP). For IP, egg extract (50 μl) was incubated with or without LRP6ICD and IVT β-catenin (3 μl) for 2 h. Buffer A (700 μl) was added

to extract with Protein A beads covalently conjugated to myc or Axin antibody followed by 2 h shaking at 4°C. Beads were washed with Buffer A (4 ml), eluted with sample buffer, and analyzed by SDS/PAGE and immuno-blot.

Kinase Assay.

LRP6ICD or LRP6ICD(PPPAPX5) (4.1 μM), MBP-Axin (0.1 μM) , GSK3 (0.79 μM) (NEB), and CK1 (1.37μM) (NEB) were preincubated with 500 μM ATP and kinase buffer [20 mM Hepes (pH 7.5), 300 mM NaCl, 2 mM DTT, 1 mM EDTA, 10 mM MgCl2, and 0.2% Tween 20] for 10 min at RT. His6-β-catenin (0.22 μM) 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 by using horseradish peroxidase-conjugated secondary antibodies and SuperSignal West Pico or Fempto Chemiluminescent Substrate (Pierce). For reblotting, membranes were stripped by incubation in (0.4 M) for 15 min followed by 15 min in H2O and reblocking. α-Tubulin was blotted with DM1α (Sigma) (1:5,000 dilution). β-catenin P33/37/41 antibody was purchased from Cell Signaling Technology (1:1,000 kinase assay; 1:250 egg extract). β-catenin P45 antibody was purchased from Cell Signaling Technology (1:500). N-terminal *Xenopus* β-catenin antibody was a generous gift from Barry Gumbiner (1:3,000). Antibodies for Axin immunodepletion and immuno-precipitation were described previously. Axin antibody

for immunoblot was purchased from R & D (anti-human/mouse/rat Axin 1) (1:100). Antibodies to Dsh were described previously (1:100). GSK3 was blotted with IH8 (Affinity Bioreagents) (1:500). His6 tag was blotted with the MCA1396 antibody (Serotec). Phospho-LRP6 (Ser-1490) was purchased from Cell Signaling Technology (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 Technology (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% FBS and antibiotics. Cells were grown at 37°C in 5% CO2 for 24 h and then incubated at 30°C for 20 h. 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 MgCl2, 2 mM EGTA] supplemented with 11.66% wt/vol sucrose. Slides were mounted with VectorShield containing DAPI stain and imaged by using a Nikon Eclipse 80i fluorescence microscope with a Nikon 60A 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 coreceptor. 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, 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 without its transmembrane domain (LRP6ICD; Figure 3.1A,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 explants (Figure 3.1C,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

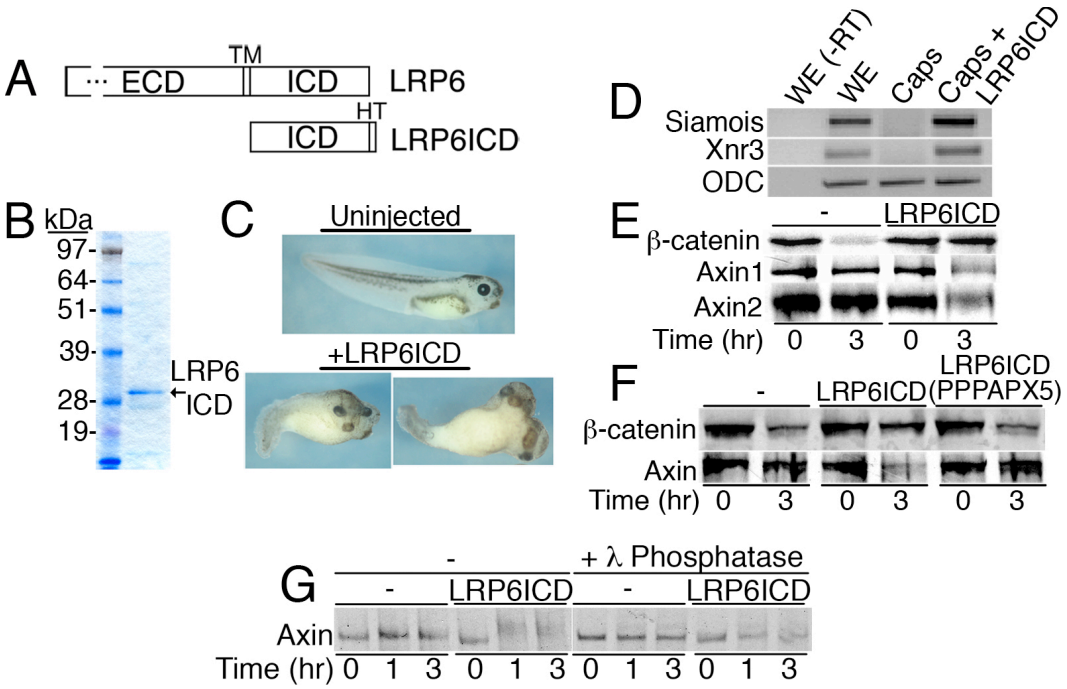


Figure 3.1: Recombinant LRP6ICD activates Wnt signaling *in vivo* and in *Xenopus* egg extract. (A) LRP6ICD spans the intracellular domain of mouse LRP6 (aa1397-1614) and does not include its transmembrane domain. ECD, extracellular domain; ICD, intracellular domain; TM, transmembrane domain; HT, 6Xhistidine tag. (B) Coomassie-stained gel of recombinant LRP6ICD (1 μg) purified from bacteria. (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). (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). (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. (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.

protein prevents degradation of radiolabeled, *in vitro*-translated (IVT) β-catenin in *Xenopus* egg extract (Figure 3.1E). Consistent with a proposed mechanism for LRP6 signaling, we demonstrate that LRP6ICD also stimulates degradation of IVT Axin and Axin2 (Figure 3.1E). We also tested whether LRP6ICD induces phosphorylation of Axin. We find that λ-phosphatase reverses the LRP6ICD-mediated upward mobility shift of the Axin protein detected by SDS/PAGE, suggesting that LRP6ICD promotes Axin phosphorylation (Figure 3.1G). 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 depends 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 depend 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, LRP6ICD(PPPAPX5) does not inhibit β-catenin degradation or stimulate Axin degradation in egg extract (Figure 3.1F), even when added at a concentration 2-fold higher than that used for LRP6ICD (data not shown). We also find that LRP6ICD, but not LRP6ICD (PPPAPX5), is phosphorylated at PPPSP Ser-1490 in egg extract (Tamai et al., 2004; Zeng et al., 2005) (Figure 3.6C). 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 Disheveled in *Xenopus* egg extract and embryos.

Disheveled (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 down-regulation of Dsh by RNAi or overexpression of a dominant-negative form of Dsh (Li et al., 2002), suggesting that 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 constitutively 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 3.2A) did not affect the ability of LRP6ICD to stabilize β-catenin or promote Axin degradation (Figure 3.2B). To determine whether 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 coinjection experiments, Xdd1 inhibits Wnt8-induced

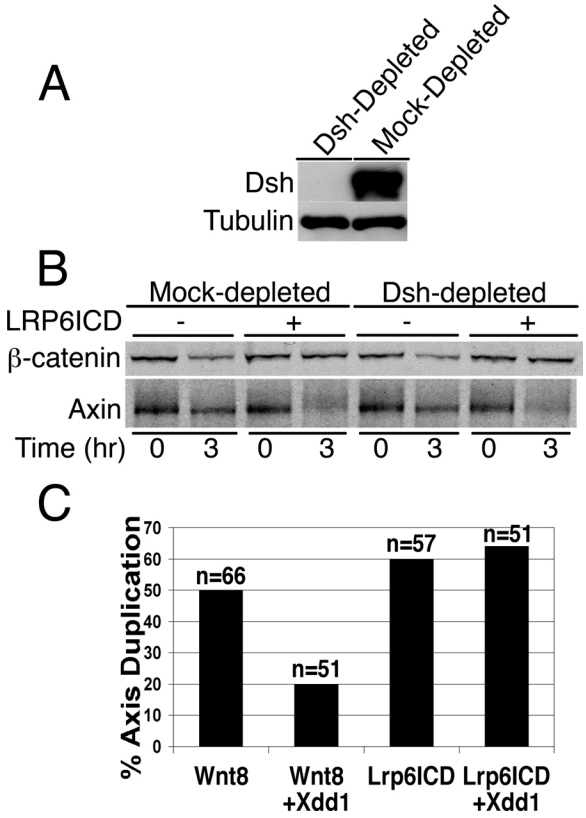


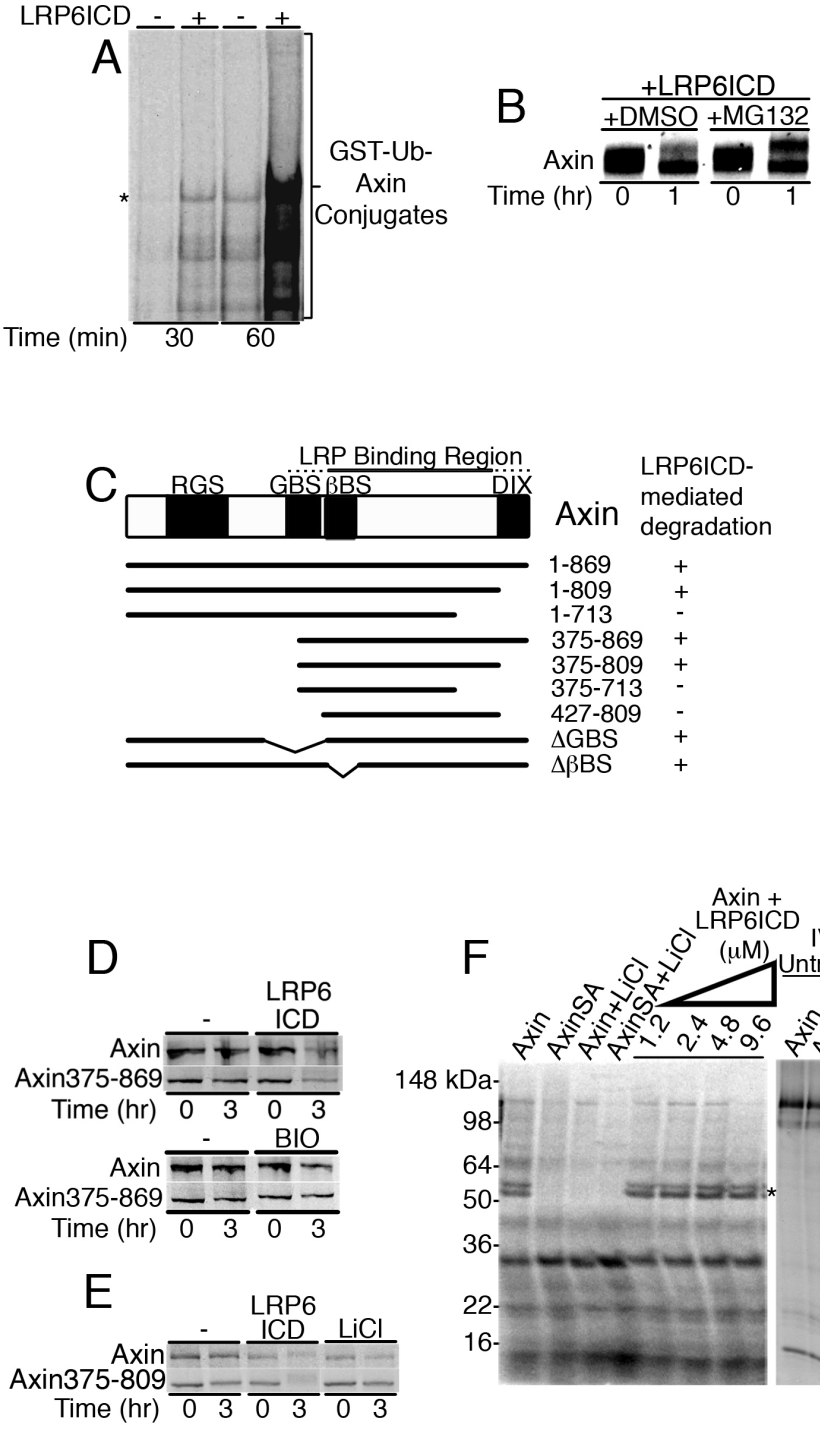
Figure 3.2: LRP6ICD signals independently of Dsh in *Xenopus* egg extract and embryos. (A) Immunoblot of Dsh-depleted and mock-depleted (Protein A beads) egg extract. (B) Dsh depletion does not affect the ability of LRP6ICD to promote β-catenin stabilization or Axin degradation in egg extract. (C) To 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.

secondary axis formation but has no effect on the ability of LRP6ICD to induce secondary axes (Figure 3.2C). 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).

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 that LRP6ICD still becomes phosphorylated at PPPSP Ser 1490 as assayed by immunoblot (data not shown), 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 an ubiquitin-mediated, proteasome-dependent process. We find that LRP6ICD promotes Axin ubiquitination in *Xenopus* egg extract (Figure 3.3A). Furthermore, we show that inhibition of the proteasome with MG132 prevents Axin degradation, leading to accumulation of a more slowly migrating form of Axin (Figure 3.3B). Thus, our data indicate that, consistent with results from intact *Xenopus* oocytes (Kofron et al., 2007), Axin degradation is proteasome dependent in egg extract.


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
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Axin
Axin375-869
Time (hr) 0 3 0 3
BIO
E
LRP6
ICD
LiCl
Axin375-809
F
Axin +
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(μM)
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Untreated
Axin
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Axin+LiCl
AxinSA+LiCl
1.2
2.4
4.8
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148 kDa-
98-
64-
50-
36-
22-
16-

Figure 3.3: 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. (B) Addition of the proteasome inhibitor MG132 (1 mM) to egg extract inhibits LRP6ICD-mediated Axin degradation. (C) Degradation of Axin mutants in egg extract in the presence of LRP6ICD. RGS, RGS domain; GBS, GSK3 Binding Site; bBS, 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.

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 (Figure 3.3C, Figure 3.4). This minimal fragment includes the GSK3, β-catenin, and PP2A binding sites on Axin (Fagotto et al., 1996). However, deletion of the GSK3 or β-catenin binding domain from full-length Axin does not prevent its LRP6ICD-mediated turnover (Figure 3.3C); 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 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 as binding LRP5/6 by yeast two-hybrid assays (Mao et al., 2001a; Tolwinski et al., 2003) also appears to be required for its LRP6-mediated degradation (Figure 3.3C). 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 3.3D) promote turnover of full-length Axin; in contrast, Axin375–869 degrades in response to LRP6ICD but not the GSK3 inhibitor BIO (Figure 3.3D). Another GSK3 inhibitor, lithium (50 mM), also promotes turnover of full-length Axin but not Axin375–869 (data not shown). 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 (Figure 3.3C, Figure 3.4). 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

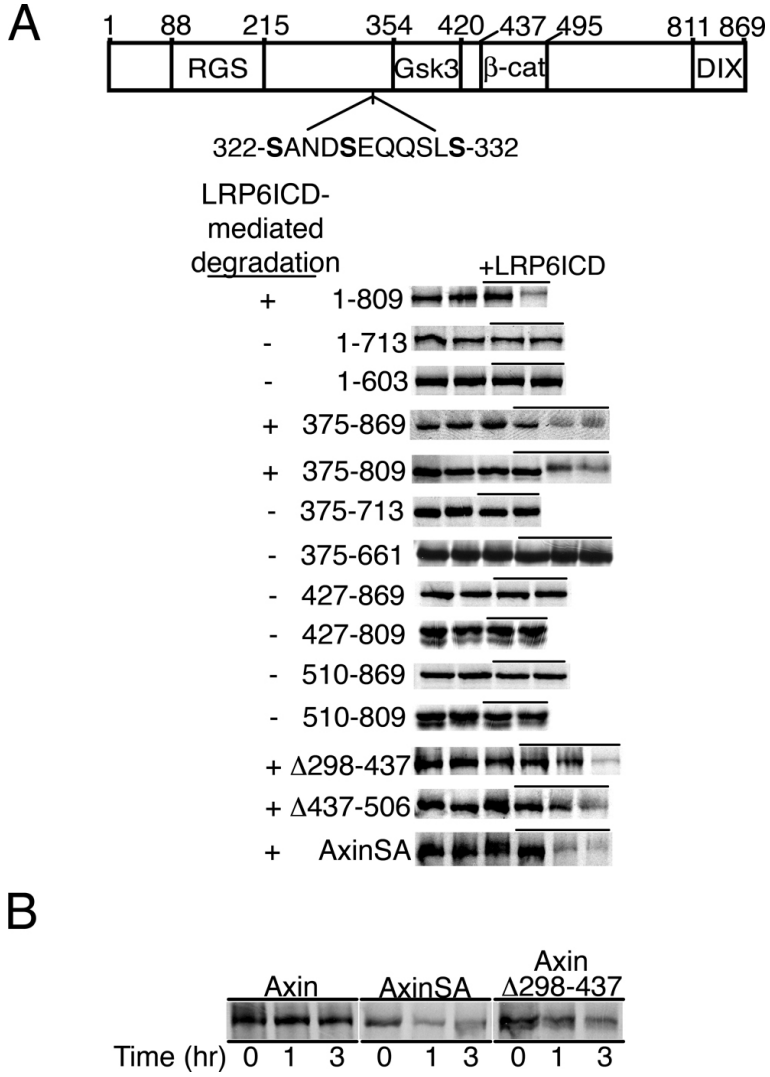


Figure 3.4: LRP6ICD stimulates degradation of Axin truncation mutants in egg extract. (A) IVT, radiolabeled Axin degradation assays in the presence or absence of LRP6ICD in Xenopus egg extract. Samples were analyzed at either 0 hr and 3 hr (two lanes) or 0 hr, 1 hr, and 3 hr (three lanes). In all experiments, 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 AxinD298-437 and AxinD437-506, 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).

characteristic Axin digestion pattern upon analysis by SDS/PAGE and autoradiography (Figure 3.3F). 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 that of 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 through different mechanisms. These data are consistent with 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).

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 test this model, we assessed whether LRP6ICD can stabilize β-catenin in *Xenopus* egg extract in which endogenous Axin is replaced by a non-degradable Axin mutant, Axin1-713 (Figure 3.5D). Axin1-713, like full-length Axin, ventralizes *Xenopus* embryos (indicative of inhibition of Wnt/β-catenin signaling) (data not shown), stimulates β-catenin degradation and binds LRP6ICD in *Xenopus* egg extract (Figure 3.5A,D). Thus, Axin1-713 retains all measurable activities of full-length Axin except that it is not degraded in response to LRP6ICD (Figure 3.3C). Consistent with the requirement of Axin for destruction complex formation, immunodepletion of endogenous

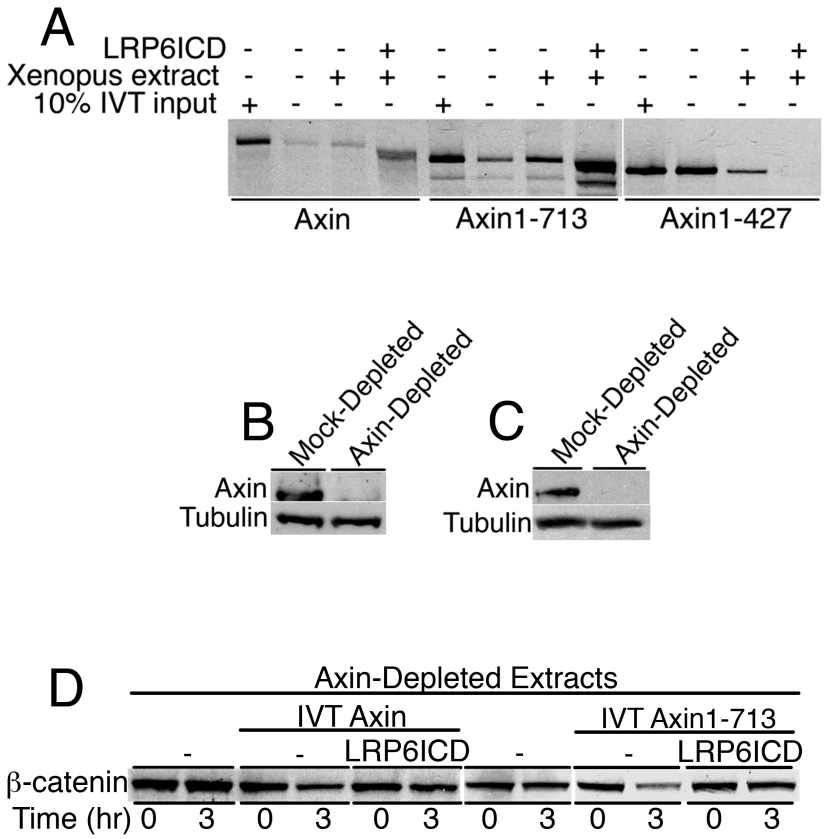

A
LRP6ICD
Xenopus extract
10% IVT input
Axin
Axin1-713
Axin1-427
B
Mock-Depleted
Axin-Depleted
Axin
Tubulin
C
Mock-Depleted
Axin-Depleted
Axin
Tubulin
D
Axin-Depleted Extracts
IVT Axin
IVT Axin1-713
LRP6ICD
β-catenin
Time (hr) 0 3 0 3 0 3 0 3 0 3 0 3

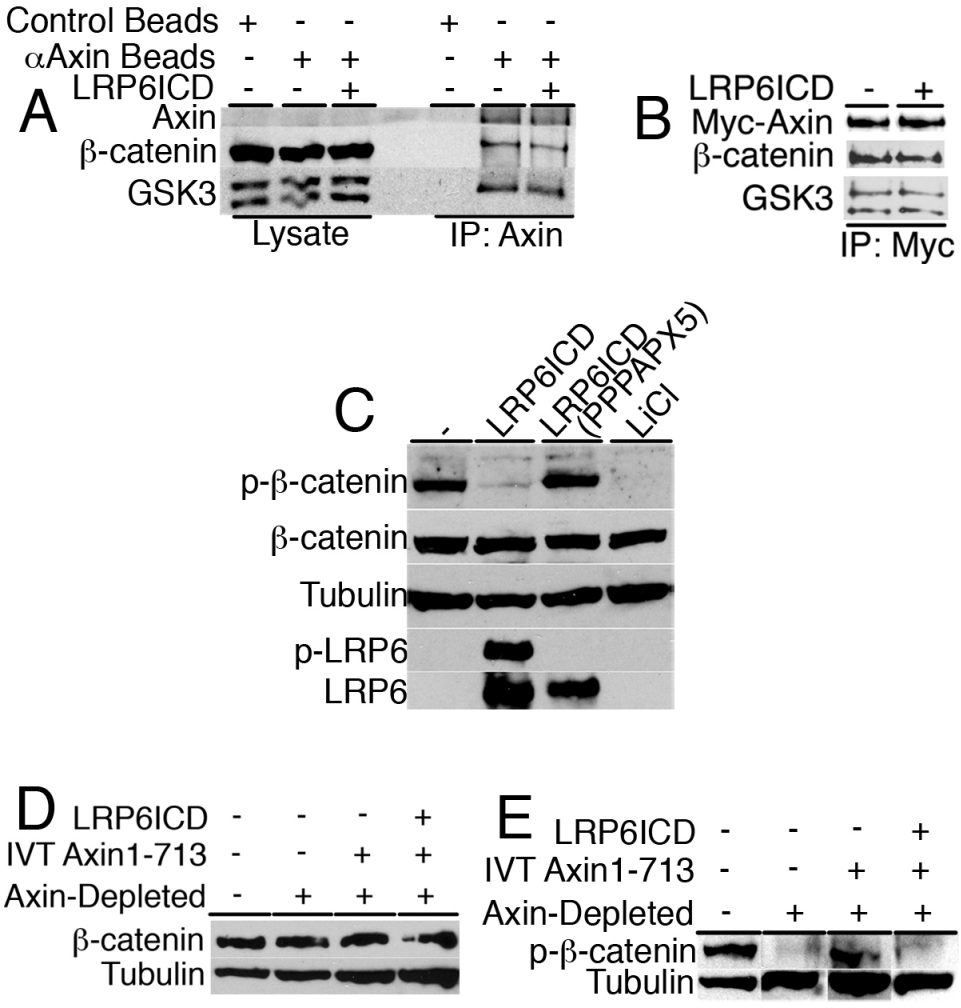
Figure 3.5: LRP6ICD promotes β-catenin stabilization in the absence of Axin degradation. (A) Axin and non-degradable Axin1-713, but not Axin1-427, bind LRP6ICD in egg extract. Radiolabeled Axin, Axin1-713, and Axin1-427 were incubated with 6XHis-tagged LRP6ICD and pulled down with nickel beads. Binding 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.

Axin from extract (Figure 3.5B) prevented β-catenin degradation (Salic et al., 2000) (Figure 3.5D). 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 3.5D, 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.

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). To test this model, we immunoprecipitated Axin from *Xenopus* egg extract incubated in the presence or absence of LRP6ICD and immunoblotted for GSK3 or β-catenin. As shown in Figure 3.6A and B, LRP6ICD (at a concentration that 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 sequester 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 Ser-45 (P45) to prime it for GSK3's phosphorylation at Ser-33/Ser-37/Thr-41 (P33/37/41),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Control Beads + - - + - -
αAxin Beads - + + - + +
LRP6ICD - - + - - +
Axin
β-catenin
GSK3
Lysate
IP: Axin
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 3.6: 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., 1994). (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. 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

which is required for β-catenin polyubiquitination and degradation (Liu et al., 2002). Previous studies showed that Wnt signaling inhibits GSK3-mediated β-catenin phosphorylation but does not inhibit CK1α-mediated β-catenin phosphorylation (Liu et al., 2002). We therefore tested whether LRP6ICD inhibits the appearance of GSK3-phosphorylated β-catenin in *Xenopus* egg extract. Significantly, LRP6ICD, like the GSK3 inhibitor lithium, inhibits GSK3-mediated phosphorylation of β-catenin (Figure 3.6C). 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 3.1F), does not inhibit GSK3's phosphorylation of β-catenin (Figure 3.6C). Notably, we find that LRP6's PPPSP serine Ser-1490 is phosphorylated in extracts (Figure 3.6C). 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 was a direct consequence of LRP6-mediated Axin degradation. To address this possibility, we tested whether LRP6ICD inhibits β-catenin P33/37/41 phosphorylation in *Xenopus* egg extract in which Axin is replaced by non-degradable Axin1-713 (Figure 3.6D,E) Axin depletion (Figure 3.5B) 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 3.6D), demonstrating that LRP6ICD can inhibit phosphorylation of β-catenin by GSK3 independently of Axin degradation.

LRP6ICD in *Xenopus* 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 3.7A). In *Xenopus* egg extract supplemented with exogenous GSK3, recombinant Tau is phosphorylated at its well characterized GSK3 target site Ser-396 (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 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 (Figure 3.3 C,D,E). 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.

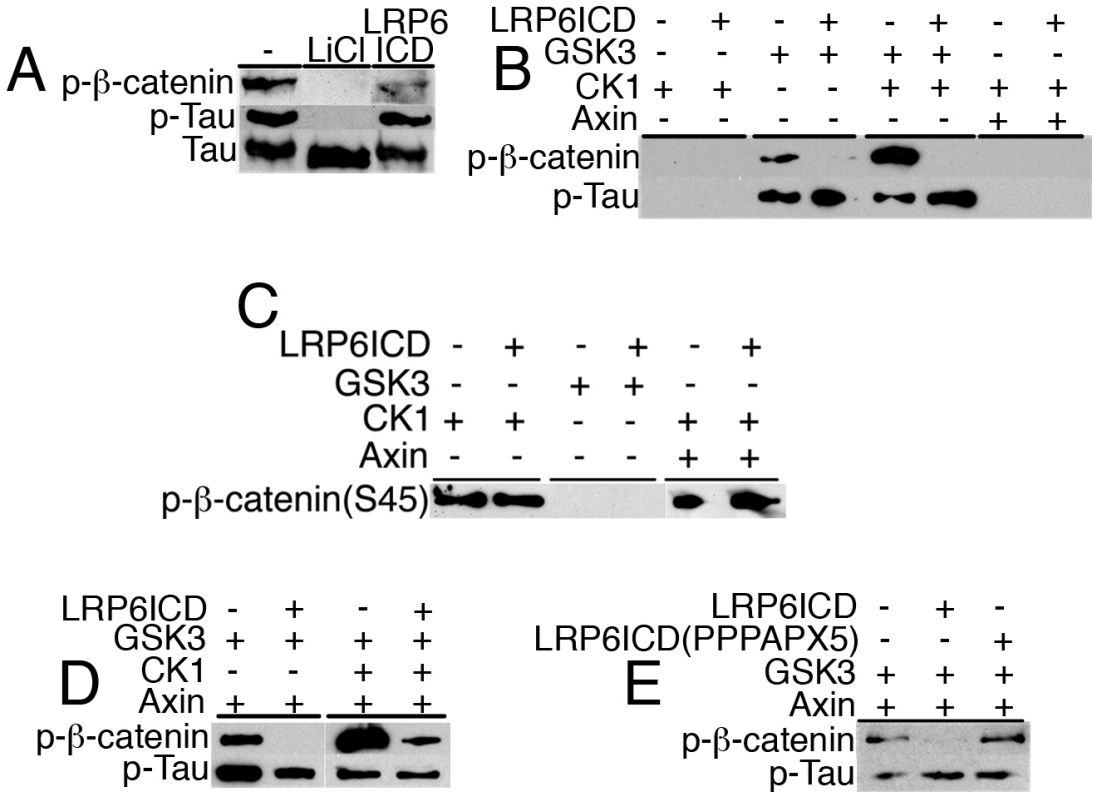

Figure 3.7 LRP6ICD directly and specifically inhibits GSK3's phosphorylation of β-catenin. (A) LiCl (50 mM) inhibits phosphorylation of β-catenin P33/37/41 and exogenous Tau P396 by GSK3, whereas LRP6ICD inhibits phosphorylation of β-catenin but not Tau by GSK3. Extract was supplemented with GSK3 to 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.

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 whether 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 3.7 B,C,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 3.7 B,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 3.7B). In addition, CK1γ'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 3.7B,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 3.7E), 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) (Mi and Johnson, 2005). 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: (i) requirement for PPPSP serines (Tamai et al., 2004) (Zeng et al., 2005), (ii) specificity for inhibition of β-catenin and not Tau phosphorylation (Figure 3.11A), and (iii) inhibition of β-catenin phosphorylation by GSK3 but not CK1 (Liu et al., 2002). Thus, we believe these studies recapitulate distinct properties of LRP6 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 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 ≈50% of cells (Figure 3.8). 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 3.13). In addition, the individual fusion proteins, when 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 ≈15% of cells (Figure 3.8). 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

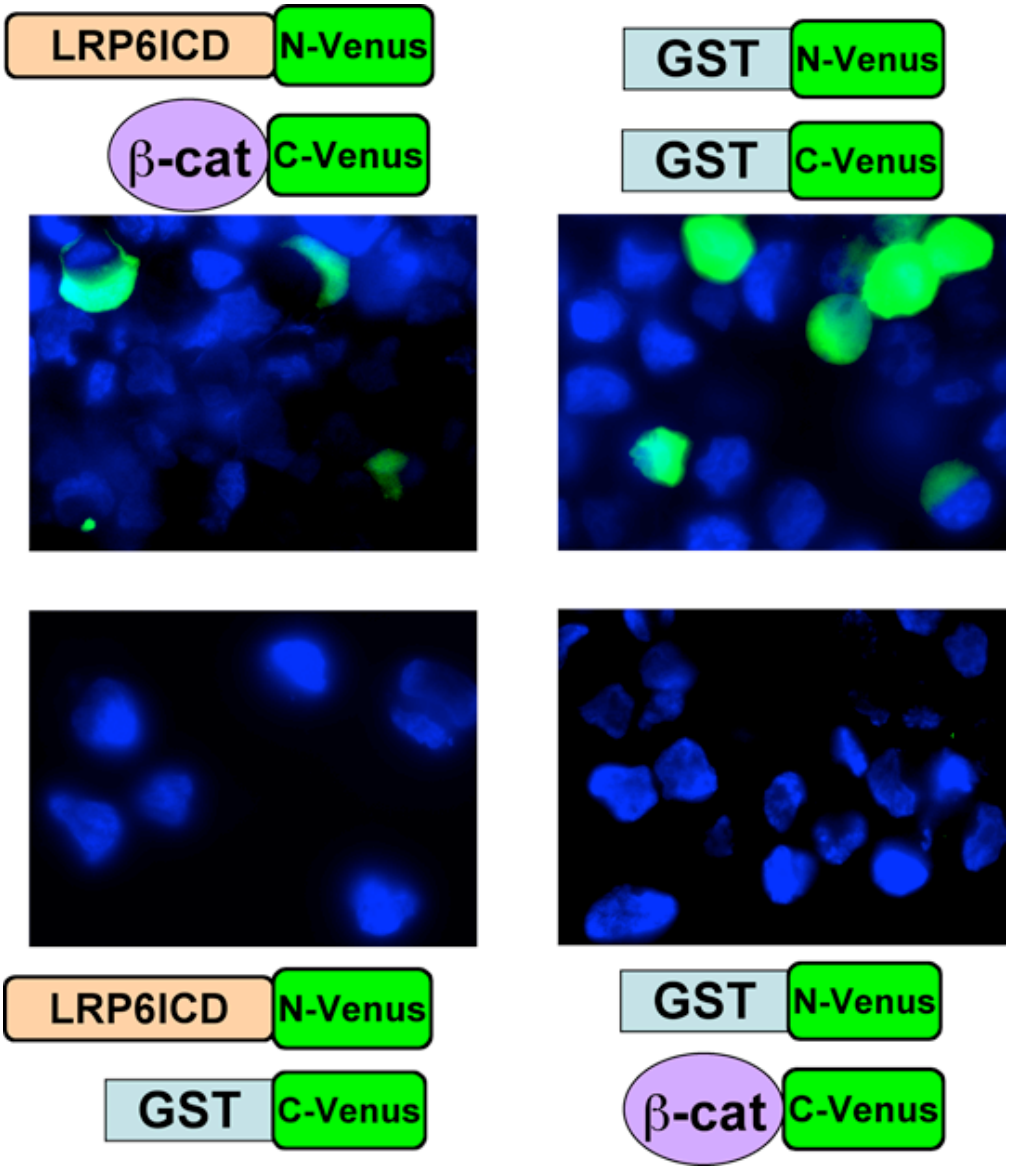

Figure 3.8 BiFC analysis of LRP6ICD and β-catenin interaction *in vivo*. Fluorescence is detected in HEK293 cells transfected with plasmids encoding LRP6ICD-YN plus β-catenin-YC and GST-YN plus GST-YC (positive control). No fluorescence is detected in cells transfected with plasmids encoding LRP6ICD-YN 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.

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. We also find that LRP6 requires intact PPPSP motifs to directly inhibit GSK3's phosphorylation of β-catenin. Thus, we propose the following working model. 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 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.

Because Axin is the limiting factor in β-catenin destruction complex formation, we predict that Axin degradation (although 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 that both LRP5/6-mediated inhibition of β-catenin phosphorylation and stimulation of Axin degradation contribute significantly to Wnt/β-catenin signaling. 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).

Future Directions

Mechanism for direct inhibition of GSK3 by LRP6

We have shown that LRP6ICD can inhibit GSK3 kinase activity towards β-catenin *in vitro*. A recent study has found that activation of the Wnt pathway results in recruitment of dephosphorylated β-catenin to the membrane (Hendriksen et al., 2008). We also know that Axin is recruited to LRP6 at the membrane in response to Wnt signaling (Mao et al., 2001b), and that LRP6 PPPSP domains are required for Axin binding (Tamai et al., 2004). We have found that LRP6 PPPSP are also required for inhibition of GSK3 kinase activity towards β-catenin. This has led us to propose that interaction between LRP6 and Axin brings LRP6 in close proximity to β-catenin and GSK3 allowing for inhibition of β-catenin phosphorylation. However, evidence suggests that the PPPSP motifs on LRP6 are phosphorylated by GSK3 indicating that the substrate (LRP6) of the kinase (GSK3) once phosphorylated inhibits the kinase. This suggests that significant structural changes take place after LRP6 is phosphorylated that results in its capability to inhibit GSK3 activity. Additionally, Axin may be important for the mechanism of LRP6 inhibition of GSK3. Structural analysis of the phosphorylated

LRP6-GSK3 complex (with Axin and/or β-catenin) will provide significant insight into the mechanism of LRP6 inhibition of GSK3 activity.

Mechanism for Axin degradation

Consistent with previously proposed models for LRP6 signaling, we demonstrate that LRP6ICD stimulates ubiquitin-mediated degradation of IVT Axin and Axin2 (Mao et al., 2001b; Tolwinski et al., 2003). We also found that LRP6ICD induces phosphorylation of Axin and that λ-phosphatase reverses the LRP6ICD-mediated phosphorylation. Total Axin signal is decreased after treatment with LRP6ICD even after λ-phosphatase treatment, consistent with LRP6ICD mediating Axin degradation. Since evidence suggests that Axin is a limiting component of the destruction complex (Lee et al., 2003), modulation of Axin levels can have a strong effect on pathway activity. However, the mammalian tissue culture experiments, Axin degradation lags behind β-catenin stabilization indicating that regulation of Axin stability is not a primary means of stabilizing β-catenin (Liu et al., 2005; Willert et al., 1999; Yamamoto et al., 1999). Due to Axin's role as a tumor suppressor, targeting Axin protein stability could be advantageous in treating colon cancer. Identification of Axin's E3-ubiquitin ligase would provide insight into the mechanism of Axin degradation. Additionally, it would beneficial to conduct a mutagenesis analysis of the candidate lysines in Axin that are ubiquitinated in response to LRP6 activation.

LRP6ICD as a tool to identify small molecule inhibitors of Wnt/β-catenin signaling.

Since LRPICD can stabilize β-catenin and induce Axin degradation in *Xenopus* egg extracts, a high throughput screen was designed to indentify small compounds that could reverse the effects of LRP6ICD on the both the stability of Axin and β-catenin (Cselenyi et al., 2008). This is in effort to identify novel compounds for therapeutic treatment of conditions resulting from over-activation of Wnt/β-catenin signaling. To monitor Axin and β-catenin protein stability at the same time Axin was tagged with *Renilla* luciferase and β-catenin was tagged with firefly luciferase. The reciprocal stability of β-catenin (increased) and Axin (decreased) in response to LRP6-mediated signaling provides a simple strategy for identified false positive small molecules such that compounds that interfere with energy metabolism would reduce both firefly and *Renilla* luminescent signals. Conversely, general inhibitors of protein degradation (e.g. proteasome inhibitors) would block both β-catenin and Axin degradation, leading to coincident enhancement of both firefly and *Renilla* luciferase signals. Using an FDA approved drug library from the National Institute of Neurological Disorders at the Harvard Medical School Institute for Chemistry and Cell Biology (ICCB) about 38 chemical inhibitors and activators were identified (Curtis Thorne (CT), unpublished results). One of these chemical inhibitors identified, WS-30, potentially inhibits Wnt/β-catenin signaling in *Xenopus* egg extract, mammalian culture cells, *Xenopus* embryos, *Drosophila* embryos, and in *C. elegans* (CT, unpublished results). In mammalian culture cells, WS-30 inhibits Wnt3a-mediated luciferase activity in a dose-dependent manner with an EC_{50} of ~10 nM. This small molecule can inhibit β-catenin degradation, stimulate Axin degradation, as well as stimulate Pygopus (a nuclear factor required for β-

catenin-mediated transcription) degradation indicating that either WS-30 targets each of these proteins individually, or it targets a protein that affects each of the proteins' stability. The latter option was found to be the case in that WS-30 binds to and stimulates casein kinase 1 alpha (CK1α) kinase activity, resulting in Axin degradation, β-catenin stabilization, and Pygopus degradation (CT, unpublished results). Additional studies for this small molecule as a potential therapeutic agent are ongoing.

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