REGULATION OF CANONICAL WNT SIGNALING BY UBIQUITYLATION

By

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For my family

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LIST OF ABBREVIATIONS

AES, Amino-terminal Enhancer of Split

APC, Adenomatous Polyposis Coli

Arm, Armadillo

BIR, Baculovirus Inhibitor of Apoptosis Protein Repeat

BMP, Bone Morphogenetic Protein

C-terminal, Carboxy-terminal

ChIP, Chromatin immunoprecipitation

CHX, Cycloheximide

CKIα, Casein Kinase I α

CSN, COP9 signalasome

DIAP1, Drosophila Inhibitor of Apoptosis 1

Dkk, Dickopff

Dsh, Dishevlled

dsRNA, double stranded RNA

DUB, De-ubiquitylase

E1, E1 activating enzyme

E2, E2 conjugating enzyme

E3, E3 ligase

ER, Endoplasmic reticulum

Fz, Frizzled

GSK3, Glycogen Synthase Kinase 3

GST, Glutathione S-Transferase

Gro, Groucho

HA, hemagglutinin

HDAC, Histone Deacetylase

HEK293, Human Embryonic Kidney 293

Hh, Hedgehog

Int1, Integration 1

K, Lysine

KLHL12, Kelch-like 12

KO, Knock Out

Lef, Lymphoid Enhancer Factor

LRP5/6, Low-Density Lipoprotein Receptor-Related Protein 5/6

MBP, Maltose Binding Protein

MLL, Mixed Lineage Leukemia

MO, Morpholino

NDLB, Non-Denatureing Lysis Buffer

NKD, Naked

N-terminal, Amino-terminal

ODE, Ordinary Differential Equation

P, Proline

PEV, Position Effect Variegation

Pol β, Polymerase β

RING, Really Interesting New Gene

RNAi, RNA interference

RT-PCR, Reverse Transcriptase Polymerase Chain Reaction

S, Serine

SCF, Skp1-Cullin-F-box

SDS/PAGE, Sodim Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis

sFRP, secreted Frizzled-Related Protein

siRNA, short-interfering RNA

T, Threonine

TCF, T-cell Factor

TGF-β, Transforming Growth Factor-β

TLE, Transducin-Like Enhancer of Split

Ub, Ubiquitin

Ubp64E, Ubiquitin-specific protease 64 E

USP, Ubiquitin Specific Protease

Wg, Wingless

WIF, Wnt Inhibitory Factor

WRE, Wnt Responsive Element

WT, Wild Type

XIAP, X-linked Inhibitor of Apoptosis Protein

CHAPTER I

INTRODUCTION TO WNT SIGNALING AND THE UBIQUITIN SYSTEM

Introduction

The canonical Wnt signaling pathway is a highly conserved cell signaling pathway present in all metazoans that regulates many fundamental processes during development and maintains tissue homeostasis in adults. Misregulation of this pathway results in a variety of disease states in humans, including cancer. Wnt signaling is initiated upon Wnt ligand binding to its two co-receptors Frizzled (Fz) and low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6), which leads to stabilization and nuclear translocation of the main cytoplasmic effector β-catenin. Once in the nucleus, β-catenin converts the Wnt transcription factor TCF/Lef from a transcriptional repressor into an activator to initiate a Wnt-specific transcriptional program. The Wnt pathway is heavily regulated by ubiquitylation, a post-translational modification in which the small protein ubiquitin is covalently attached to target proteins by a series of enzymes. In this chapter, I begin with an introduction to cellular communication, Wnt signal transduction, and ubiquitylation to provide a background for understanding the studies I present in Chapters III-V. In Chapter III, I describe a RNAi screen I performed to identify novel ubiquitin system components involved in regulating Wnt signaling and in Chapters IV and V I describe the identification of a novel E3 ubiquitin ligase and

de-ubiquitylase (DUB) involved in Wnt signal transduction, respectively. I summarize these collective findings in Chapter VI.

Signal Transduction: How Cells Communicate

The discovery of “animalcules” by Anton van Leeuwenhoek and of “cells” by Robert Hooke in the later half of the 17th century provided the foundation for the first unifying “cell theory” put forth nearly 200 years later by the botanist Matthias Jakob Schleiden and the zoologist Theodore Schwann (Mazzarello, 1999). Based on their work in which they discovered that both plants (Schleiden, 1838) and animal tissues (Schwann, 1839) are composed of many individual cells, Schwann published a treatise in which he proposed that all living things are composed of cells and that cells are the fundamental units of life (Schwann, 1839). This was a pivotal moment in the history of biology as it indicated that organisms are “republics of living elementary units” (Mayr, 1982) and that by studying the “elementary units” (i.e. cells) one could thus learn how whole “republics” (i.e. whole plants and animals) form and function. These findings quickly led to a reductionist approach to studying biology with a central focus on discovering how the minimal units of life (cells) function individually and in cooperation.

Much has been learned about the function of cells since their initial discovery, but many fundamental questions still remain, including: What constitutes a cell? How does it function? How do cells respond to their

environment? How do cells interact with other cells to form more complex life forms? Initially, cells were thought to consist merely of a cell wall, cytoplasm, and a nucleus (Mazzarello, 1999). We now know animal cells are separated from their external environment by a lipid bilayer (the plasma membrane) and contain water, multiple organelles, DNA, RNA, proteins, lipids, sugars, and ions that all interact in exquisitely complicated ways to ensure cells are able to survive and appropriately respond to their environment (Alberts, 2002). While there is still much more to be learned about the make-up of a cell, there is even more to be learned about how cells are able to respond to an ever-changing environment and able to interact and communicate with multiple other cells to form multi-cellular organisms.

One of the major ways cells respond to their environment and communicate with other cells is through a process referred to as "signal transduction" (Gomperts, 2009). Signal transduction is the process by which cells receive input from their surroundings (i.e. a "signal") that can be in the form of light, temperature, chemicals, protein ligands, or physical forces that bind, or otherwise affect the function of, cell surface receptors, which then transmit the signal intracellularly (Gomperts, 2009). Cell surface receptors are typically proteins that span the plasma membrane (transmembrane proteins) and contain an extracellular domain that is able to interact with the extracellular environment and an intracellular domain that is able to transmit the signal intracellularly. Once a signal is received by a cell surface receptor it initiates a cascade of intracellular

biochemical reactions that result in a variety of events depending on the signal, including changes in cellular metabolism, motility, gene transcription and even initiation of cell death (Gomperts, 2009).

Single-celled organisms contain mostly two-component signaling systems to allow for appropriate environmental responses (Stock et al., 2000), whereas multi-cellular organisms (metazoa) require more complex signal transduction pathways to coordinate the development and maintenance of multiple cell types and tissues (Gerhart, 1999). In fact, it is widely believed that the evolution of intercellular communication (cell-to-cell communication vs environment-to-cell communication) is what initially allowed the development of multi-cellular animals and plants (Alberts, 2002). It is now clear that intercellular communication via signal transduction pathways is essential for coordinating the embryonic development of all animals (Gerhart, 1999; Pires-daSilva and Sommer, 2003).

Despite the vast array of cell-types, tissue-types and morphologies found in the animal kingdom, it is estimated that only 17 signal transduction pathways exist to produce such diversity (Gerhart, 1999). Even more striking is that only a few of the 17 total signal transduction pathways found in metazoa are repeatedly used during embryonic development. This indicates that these few core, conserved pathways are utilized in many different ways to produce the abundance of phenotypes found throughout the animal kingdom (Gerhart, 1999; Pires-daSilva and Sommer, 2003). These core pathways include: Wnt, Hedghog (Hh), Notch, transforming growth factor β (TGF-β), receptor tyrosine

kinase (RTK), Janus kinase (JAK)/signal transducer and activator of transcription (STAT), and nuclear hormone pathways. A major remaining challenge for biologists is to define how these signaling pathways function, how they are employed during metazoan development to generate such diverse phenotypic outcomes, and how their misregulation leads to both aberrant development and to disease states in adults.

Historical Perspective: Wnt Signaling

The Wnt signal transduction pathway is critical for the development of all multi-cellular organisms and is highly conserved from the most basal metazoan, *Amphimedon queenslandica* (a demosponge), to humans (Adamska et al., 2010; Gerhart, 1999; Richards and Degnan, 2009). The Wnt pathway was discovered more than 30 years ago through a series of events that highlight its importance in both development and disease.

In 1976 Sharma and Chopra reported a *Drosophila melanogaster* mutant they named Wingless (Wg) because it lacked wings (Sharma and Chopra, 1976). A few years later Eric Wieschaus and Christiane Nusslein-Volhard performed their Nobel Prize-winning mutagenesis screen for segment polarity genes in *Drosophila* in which they showed Wg was required for proper segmentation of the early embryo (Nusslein-Volhard and Wieschaus, 1980). Together, these findings indicated an important role for Wg in *Drosophila* development. In 1982, Roel Nusse and Harold Varmus reported that the Mouse

Mammary Tumor Virus integrates upstream of a novel proto-oncogene they called Integration 1 (Int1) (Nusse and Varmus, 1982). Five years later it was discovered that Wg was the *Drosophila* ortholog of the mouse *Int1* gene (Cabrera et al., 1987; Rijsewijk et al., 1987). Subsequently the two names were combined into the mnemonic "Wnt;" a term which reflects its role in both fly development and carcinogenesis in mice (Nusse et al., 1991). Around the same time, it was shown that injection of *Int1* mRNA into *Xenopus laevis* embryos could induce a second body axis, demonstrating an important role for Int-1 in frog development in addition to its functions in flies and mice (McMahon and Moon). Together, these findings indicated that the Wnt family of proteins are highly conserved across phyla and play important roles in both embryonic development and cancer.

After Nusslein-Volhard and Wieschaus published their initial pioneering *Drosophila* mutagenesis screen for segment polarity regulators in which they identified Wg in 1980 (Nusslein-Volhard and Wieschaus, 1980), numerous papers followed that reported more *Drosophila* mutants with early embryonic patterning defects. Many of these mutants turned out to be components of the Wnt signaling pathway including Armadillo (the *Drosophila* ortholog of β-catenin) (Riggleman et al., 1990; Wieschaus and Riggleman, 1987), Dishevelled (Dsh) (Perrimon and Mahowald, 1987), Shaggy (the *Drosophila* ortholog of glycogen synthase kinase 3 (GSK3)) (Siegfried et al., 1992), and Frizzled (Fz) (Bhanot et al., 1996). These will be discussed further below.

In addition to the extensive work on the role of Wg in *Drosophila* development, much work was also performed in *Xenopus* embryos to further confirm a critical developmental role for Wnt signaling. The initial finding that injection of *Int1* mRNA into the ventral blastomeres of *Xenopus* embryos was sufficient to induce a second body axis (McMahon and Moon, 1989) was a monumental discovery as it finally provided a molecular explanation for the work of Spemann and Mangold who had shown 65 years earlier that transplantation of dorsal tissue to the ventral region of amphibian embryos resulted in twinned axes (Spemann, 1924). After their discovery of a dorsal head "organizer" developmental biologists had struggled for years trying to identify the organizer-inducing signal emanating from the dorsal tissue. With the discovery of Wnt and its ability to induce a twinned axis, this inducing signal had finally been found and made it clear that Wnt signaling has a profound effect on frog development. Since then, almost all Wnt pathway components have been validated through *Xenopus* axis specification studies.

These early studies laid the foundation for further work in multiple model systems showing that Wnt signaling plays critical roles in all aspects of development and in adult stem cell maintenance (Reya and Clevers, 2005). Thus, it is no surprise that misregulation of this pathway results in a variety of disease states in humans from birth defects to cancer (MacDonald et al., 2009). The most well-characterized link between misregulated Wnt signaling and disease is found in colorectal cancer where over 85% of patients have a mutation

in adenomatous polyposis coli (APC), a negative regulator of Wnt signaling (Kinzler and Vogelstein, 1996). Mutation of APC results in hyperactive Wnt signaling, leading to un-regulated cell growth and tumor formation (the role of APC in Wnt signaling will be discussed further below). In addition to the well-established role of Wnt signaling in colorectal cancer, numerous other cancer types have now been shown to exhibit misregulated Wnt signal transduction, including hepatocellular carcinoma, lung cancer, skin cancer, prostate cancer, breast cancer, and Wilms' tumor (Klaus and Birchmeier, 2008; Polakis, 2007). Wnt pathway mutations are also known to cause a variety of developmental defects including tetra-amelia (defect in limb formation), bone density defects, tooth agenesis, and defects in eye vascularization (MacDonald et al., 2009)(see also The Wnt Homepage: wnt.standford.edu). In order to understand these various diseases and to design rational therapies with which to treat them, a detailed understanding of the molecular mechanisms of Wnt signal transduction is required.

Current Model of Wnt Signaling

Wnt protein family members are able to activate both "canonical" and "non-canonical" Wnt signal transduction pathways. My work focuses exclusively on "canonical," or β-catenin-mediated, Wnt signaling so I will only discuss this pathway. The key feature of canonical Wnt signaling is the constant synthesis and degradation of the main cytoplasmic effector β-catenin (Figure 1.1). In the

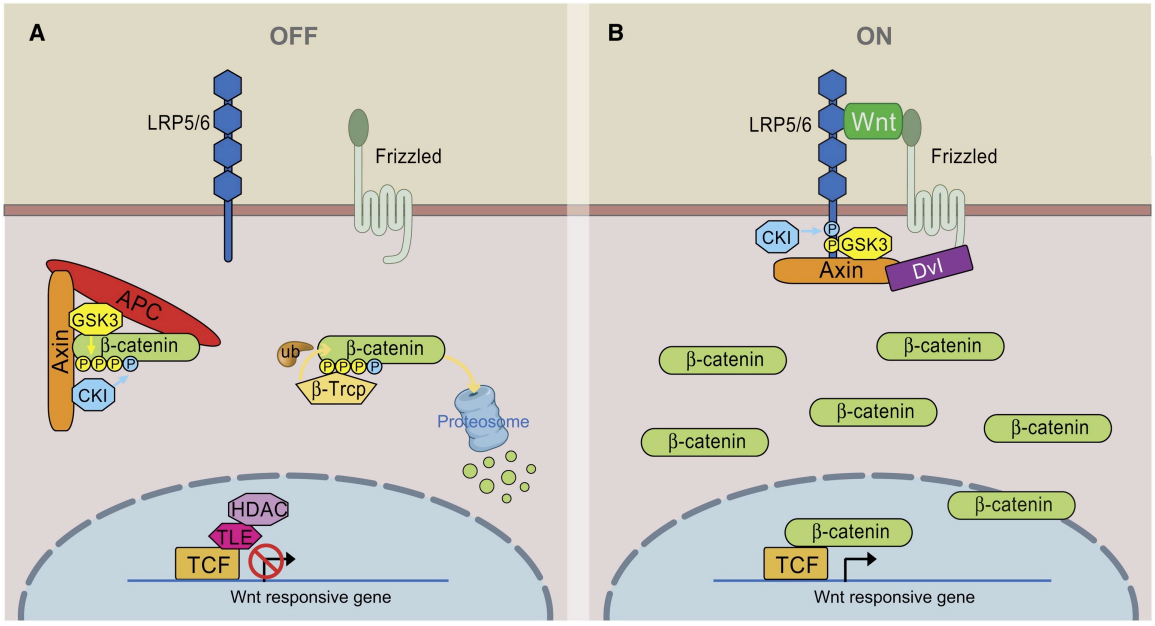


Figure 1.1. Schematic of Canonical Wnt Signaling.
(A) In the absence of Wnt ligand, cytoplasmic β-catenin is bound by the β-catenin destruction complex, composed of Axin, APC, CK1α and GSK3. Within this complex, β-catenin is first phosphorylated by CK1α, which primes for GSK3 phosphorylation. Phosphorylated β-catenin is recognized by the E3 ligase, $SCF^{\beta\text{-}TRCP}$, which polyubiquitylates β-catenin targeting it for proteasome-mediated degradation. In the nucleus, Wnt target genes are repressed by Groucho/TLE and associated HDACs. (B) In the presence of Wnt ligand, Wnt binds the co-receptors Fz and LRP5/6, which leads to membrane recruitment of Dvl and Axin. Axin-associated GSK3 and CK1α phosphorylate LRP5/6, which inhibits the activity of the β-catenin destruction complex. As a result, β-catenin levels rise in the cytoplasm and β-catenin translocates to the nucleus where it binds TCF/Lef to activate Wnt target gene transcription. Figure from (Macdonald et al., 2009).

absence of a Wnt signal, β-catenin is constitutively degraded by a "β-catenin destruction complex" composed of the scaffolds APC and Axin and the kinases GSK3 and casein kinase I alpha (CK1α) (Behrens et al., 1998; Gao et al., 2002). Within this complex β-catenin is phosphorylated by CK1α at serine 45, which primes for GSK3 phosphorylation at serines 33 and 37, and threonine 41 (Amit et

al., 2002; Liu et al., 2002). Phosphorylated β-catenin is then recognized by the E3 ubiquitin ligase Skp1-Cullin-F-box $(SCF)^{\beta\text{-}TRCP}$, which polyubiquitylates β-catenin targeting it for degradation by the 26S proteasome (Latres et al., 1999; Liu et al., 1999). Thus, in the absence of a Wnt signal, cytoplasmic levels of β-catenin are kept low.

The Wnt family of proteins, for which the pathway is named, are secreted lipid-modified glycoproteins that participate in both cell-to-cell communication and long-range signaling by acting as morphogens to pattern the development of various tissues (MacDonald et al., 2009; Port and Basler, 2010). At present, 19 different Wnt family members have been identified in mammals. Wnts are ~350-400 amino acids in length and contain an N-terminal signal sequence that targets them to the secretory pathway where they are N-linked glycosylated and cysteine and serine palmitoylated (Komekado et al., 2007; Takada et al., 2006; Willert et al., 2003). Once secrected, Wnt proteins reach their target cell by way of lateral diffusion involving heparan sulfate proteoglycans or via cytoneme projections from receptor cells, or can travel up to 20 cell diameters by forming soluble micelles, binding soluble lipid-binding proteins, as part of lipoprotein particles, or by traveling on exosomes (Port and Basler, 2010). Wnt proteins can be prevented from binding their receptors by a number of secreted molecules. The secreted Frizzled-related protein (sFRP) family of proteins and Wnt inhibitory factor (WIF) bind to Wnt and antagonize its ability to interact with Frizzled (Fz) (Bovolenta et al., 2008), while the Dickkopf (Dkk) family and Wise/Sclerostin

(SOST) family prevent Wnt binding and activation of the co-receptor LDL-receptor related proteins 5 and 6 (LRP5/6) (Itasaki et al., 2003; Semenov et al., 2005; Semenov et al., 2001). Wnt agonists of the Norrin and R-spondin families also exist that stimulate Fz-LRP5/6 activity either independent of or in coordination with Wnts, respectively (Kazanskaya et al., 2004; Xu et al., 2004).

Once a Wnt ligand has traversed the extracellular space and avoided any potential inhibitors, it will arrive at its target cell where it can bind its two co-receptors LRP5/6 and Fz, which are both required for pathway activation. There are 10 Fz family members in mammals, which are all seven-pass transmembrane receptors, while both LRP5 and LRP6 contain a single transmembrane domain (He et al., 2004; Malbon, 2004). Data generated thus far suggest a model in which Wnt binding induces the formation of a LRP5/6-Fz complex. Close association of the two receptors appears to be important as synthetically fusing LRP5/6 and Fz together in cultured cells is sufficient to activate the pathway (Holmen et al., 2005), but such endogenous receptor association upon Wnt stimulation has not been well-established.

Upon Wnt ligand binding, a key event in LRP5/6 receptor activation is phosphorylation of each of its five PPPSPxS motifs found in its intracellular domain (Tamai et al., 2004). Surprisingly, the kinases involved in LRP5/6 phosphorylation are the same kinases involved in β-catenin degradation: GSK3 and CKI, although in this case CKIγ is involved instead of CKIα. In the case of LRP5/6, it is thought that GSK3 serves as the priming kinase by phosphorylating

the serine in the PPPSP motifs, which then induces xS phosphorylation by CKIγ (Davidson et al., 2005; Zeng et al., 2005). Thus, GSK3 has both negative and positive roles in Wnt signal transduction. Phosphorylation of the PPPSPxS motifs recruits cytoplasmic Axin/GSK3 complexes to LRP5/6 upon Wnt stimulation, thus enhancing GSK3-mediated phosphorylation of LRP5/6 (Davidson et al., 2005; Tamai et al., 2004; Zeng et al., 2005). Additionally, CKIγ also phosphorylates a conserved S/T cluster outside of the PPPSPxS motifs in LRP5/6, which induces GSK3 binding (Davidson et al., 2005). Thus, multiple mechanisms exist to recruit additional GSK3 to LRP5/6 in response to Wnt stimulation in order to amplify the signal.

Fz function is required for phosphorylation of LRP5/6 upon Wnt ligand binding (Zeng et al., 2008). In the presence of a Wnt signal, the cytoplasmic scaffold Dishevelled (Dsh) becomes phosphorylated and associates with the C-terminal tail of Fz (Umbhauer et al., 2000; Wong et al., 2003). As Dsh and Axin can interact and polymerize through their DIX domains (Schwarz-Romond et al., 2007), it has been postulated that Fz-bound Dsh recruits the Axin-GSK3 complex to the plasma membrane to initiate LRP5/6 phosphorylation by GSK3 (Zeng et al., 2008). This has led to a model involving both an “initiation” and “amplification” step in Wnt signal transduction where Fz recruitment of Dsh and the Axin/GSK3 complex functions to initiate a Wnt signal by phosphorylating the PPPSPxS motifs and S/T sites in LRP5/6, while the phosphorylated PPPSPxS

and S/T-mediated recruitment of more Axin and GSK3 serve to amplify the signal (Baig-Lewis et al., 2007).

The mechanism by which receptor activation leads to β-catenin destruction complex inhibition is not well understood. Multiple mechanisms have been proposed, all of which ultimately result in the inhibition of GSK3's ability to phosphorylate β-catenin. While dissociation of the destruction complex has been proposed as a potential mechanism (Liu et al., 2005a), solid evidence for this is lacking and, in fact, recent studies have shown that the complex remains intact and co-localizes with Fz and LRP5/6 soon after Wnt stimulation (Bilic et al., 2007; Hendriksen et al., 2008; Mao et al., 2001; Yamamoto et al., 2006). More recent evidence suggests that translocation of the entire destruction complex to the plasma membrane may lead to direct inhibition of GSK3 activity by LRP5/6 (Cselenyi et al., 2008; Piao et al., 2008; Wu et al., 2009). This is consistent with the finding that de-phosphorylated β-catenin is present on phosphorylated LRP6-bound Axin soon after Wnt stimulation (Hendriksen et al., 2008). Degradation of Axin has also been proposed as an important event in β-catenin stabilization upon Wnt signaling (Kofron et al., 2007; Tolwinski et al., 2003; Yamamoto et al., 1999), as Axin is the limiting component in the destruction complex (Lee et al., 2003). Thus, affecting Axin levels would be predicted to have a profound effect on destruction complex formation. However, β-catenin is stabilized prior to Axin degradation (Liu et al., 2005a; Willert et al., 1999; Yamamoto et al., 1999). Thus, it is likely that GSK3 activity within the destruction complex is rapidly and directly

inhibited by LRP5/6 at the membrane upon Wnt ligand binding, and that Axin degradation serves as a subsequent step to prevent further destruction complex formation.

Once the β-catenin destruction complex is inhibited, β-catenin is no longer phosphorylated by GSK3 and, thus, no longer ubiquitylated and degraded by $SCF^{\beta\text{-}TRCP}$. Consequently, cytoplasmic β-catenin levels rapidly increase due to the unopposed constitutive synthesis of β-catenin (Bryja et al., 2007; Liu et al., 2005a). Elevated β-catenin translocates to the nucleus through a poorly understood process potentially involving the GTPase Rac1 (Wu et al., 2008). APC and Axin have been implicated in exporting β-catenin out of the nucleus while the co-activators Pygopus and BCL9 (see below) have been implicated in nuclear retention of β-catenin, but none of these proteins have been shown to affect the rate of export or import indicating they only play roles in the retention, and not shuttling, of β-catenin (Cong and Varmus, 2004; Henderson and Fagotto, 2002; Krieghoff et al., 2006). Thus, it remains to be determined how β-catenin is trafficked in and out of the nucleus.

Once in the nucleus, β-catenin binds the TCF/Lef family of DNA-binding transcription factors to activate Wnt target gene transcription (Arce et al., 2006). There are four TCF/Lef family members in mammals: TCF1, Lef1, TCF3, and TCF4. All TCF/Lef family members bind the consensus sequence CCTTTGWW (W indicates either T or A), known as the Wnt responsive element (WRE), found

in the promoters of Wnt target genes. In the absence of a Wnt signal TCF/Lef serves as a transcriptional repressor by binding the Groucho/TLE family of transcriptional co-repressors. Groucho is the *Drosophila* homolog of the human transducin-like enhancer of split (TLE) family of proteins, of which there are five: TLE1-4 and a truncated isoform named amino-terminal enhancer of split (AES) (Gasperowicz and Otto, 2005). All TLE family members can interact with all TCF/Lef family members to mediate repression (Brantjes et al., 2001). It is thought that Groucho/TLE proteins mediate repression by binding to TCF/Lef and recruiting histone deacetylases (HDACs), which compress chromatin locally, as well as by forming oligomeric structures, which mediate long-range chromatin condensation (Buscarlet and Stifani, 2007; Jennings and Ish-Horowicz, 2008).

The prevailing model for how TCF/Lef is turned from a transcriptional repressor into a transcriptional activator involves the direct displacement of Groucho/TLE by β-catenin through competition for overlapping binding sites on TCF/Lef (Daniels and Weis, 2005). This model was proposed based primarily on *in vitro* data using purified proteins in which it was found that β-catenin and Groucho/TLE bind TCF/Lef in a mutually exclusive manner. However, this model was never tested *in vivo*. The work I present in Chapter IV indicates that turning TCF/Lef from a repressor into an activator *in vivo* involves more than a simple competition between β-catenin and Groucho/TLE. I provide evidence indicating that mono-ubiquitylation of Groucho/TLE by the E3 ubiquitin ligase XIAP is

required to remove Groucho/TLE from TCF/Lef to allow β-catenin-TCF/Lef complex formation and Wnt-mediated transcriptional activation.

Upon TCF/Lef binding, β-catenin nucleates a transcriptional activation complex consisting of Pygopus, BCL9, p300/CBP and TRRAP/TIP60 histone acetyltransferases, MLL1/2 histone methyltransferases, the SWI/SNF family of ATPases for chromatin remodeling, Mediator for transcription initiation, and the PAF1 complex for transcription elongation and histone modifications (Mosimann et al., 2009; Willert and Jones, 2006). This β-catenin-mediated multi-protein complex functions to activate the transcription of an estimated 300-400 Wnt target genes, which regulate many cellular processes including cell survival, proliferation, and differentiation (Hatzis et al., 2008). In addition to β-catenin's role as a transcriptional activator, recent evidence indicates that β-catenin-TCF/Lef complexes can function as transcriptional repressors by binding to both canonical WREs and to a novel TCF binding element, AGAWAW (Blauwkamp et al., 2008; Theisen et al., 2007). To add even more complexity to β-catenin-mediated transcriptional regulation, it has been shown that β-catenin can interact with a number of other DNA-binding transcription factors besides TCF/Lef to activate or repress transcription of even more genes (e.g. Smad4, MyoD, c-Jun, and RAR, among many others) (MacDonald et al., 2009). Thus, it is clear that Wnt-mediated β-catenin stabilization and nuclear translocation has a profound

effect on total cellular gene expression and, thus, on the overall physiology of the cell; most of which remains to be discovered.

Historical Perspective: The Ubiquitin System

The discovery of the ubiquitin system highlights the importance of asking basic, and sometimes unpopular, questions in scientific discovery, such as: how do proteins degrade in the cell? In the decades prior to the discovery of the ubiquitin system, most scientists were fascinated by the discovery of DNA and how genes are transcribed and translated into proteins, while very little attention was paid to the stability of proteins once they had been synthesized. At that time, it was generally thought that proteins were long-lived, static molecules. Thus, very few scientists were interested in, or even believed in, the concept of protein degradation (Ciechanover, 2009; Varshavsky, 2006). Afterall, why would the cell expend so much energy to synthesize a protein just to degrade it? The idea that proteins might be in a dynamic state of synthesis and degradation was first proposed about 70 years ago when Rudolf Schoenheimer showed that only 50% of the ^{15}N-labeled tyrosine he administered to rats was recovered in the urine, and that the rest had been deposited in the rat's tissues, indicating that protein synthesis had occurred. Additionally, he found an equivalent amount of protein nitrogen excreted, indicating, for the first time, that protein degradation had taken place in the rat (Schoenheimer, 1942).

The idea that proteins turn over was not well accepted until the discovery of the lysosome in the mid-1950s (De Duve et al., 1953; Gianetto and De Duve, 1955). After the discovery of the lysosome, it was assumed that all proteins were degraded in this cellular compartment, but three important discoveries indicated the existence of non-lysosomal-mediated protein degradation: 1.) Differing protein half-lives, as it was predicted that proteins degraded by lysosomal proteases should be degraded at the same rate, but this was not found to be the case (Goldberg and St John, 1976; Schimke and Doyle, 1970), 2.) The fact that proteins were still degraded in the presence of lysosomal inhibitors, indicating there must be an alternative mode of protein degradation in the cell (Knowles and Ballard, 1976; Neff et al., 1979), and 3.) A paradoxical energy requirement for protein degradation, which was not expected to be necessary for lysosomal protease-mediated protein degradation (Mandelstam, 1958; Simpson, 1953; Steinberg and Vaughan, 1956). Regardless of these obvious inconsistencies, most scientists still believed that proteins were degraded in the lysosome and that the mystery of protein degradation had been solved by the discovery of this intracellular organelle.

A big breakthrough in support of non-lysosomal-mediated protein degradation came when Rabinovitz and Fisher observed that abnormal hemoglobin is degraded in rabbit reticulocytes, which do not contain lysosomes (Rabinovitz and Fisher, 1964). Subsequently, two groups independently prepared cell-free rabbit reticulocyte lysates in which they showed degradation of

abnormal hemoglobin was ATP-dependent and occurred optimally at neutral pH (unlike in the lysosome where protein degradation occurs optimally at an acidic pH) (Etlinger and Goldberg, 1977; Hershko, 1978). It was with this newly prepared rabbit reticulocyte lysate that Aaron Ciechanover, Avram Hershko and Irwin Rose performed their Nobel-Prize winning experiments in which they purified and characterized all of the main components of the hitherto unidentified "ubiquitin system" (described in more detail below): the small protein ubiquitin that is covalently attached to substrate proteins by a sequence of events involving an E1 (activating enzyme), E2 (conjugating enzyme), and E3 (ligating enzyme), as well as ubiquitin hydrolases, which cleave ubiquitin from target proteins (reviewed in (Ciechanover, 2009; Varshavsky, 2006)). These initial discoveries, along with the many others that followed, proved unequivocally that protein degradation occurs outside of lysosomes in a very complex and highly regulated manner. We are just beginning to understand the immense impact of this groundbreaking work.

Current Model of The Ubiquitin System

We now know the ubiquitin system regulates many fundamental cellular processes including the cell cycle, endocytosis, the immune response, development, and cell signaling pathways. This broad regulation occurs through "ubiquitylation" of proteins, a term which refers to the post-translational modification of proteins in which the small (76 amino acid), highly conserved

protein ubiquitin is covalently attached to target proteins in the form of monomers or polymers. The addition of ubiquitin to target proteins changes the activity, localization, or stability of the target protein depending on which type of ubiquitin modification is added (reviewed in (Hershko and Ciechanover, 1998; Pickart, 2001, 2004)).

Three enzymes catalyze the process of ubiquitin conjugation in sequence (Figure 1.2) (reviewed in (Hershko and Ciechanover, 1998; Pickart, 2001, 2004)). First, an E1 activating enzyme activates ubiquitin in an ATP-dependent manner and subsequently forms a thiolester bond between a cysteine residue in its active site and the carboxy-terminal glycine residue of ubiquitin. Next, the E1 catalyzes the transfer of the ubiquitin molecule to the active site cysteine of an E2 conjugating enzyme. Finally, the E2 catalyzes the transfer of ubiquitin from itself onto a lysine residue of the target protein by way of an E3 ubiquitin ligase. There are between several hundred to over a thousand E3 ligases in the human genome that fall into one of two major families: the *R*eally *I*nteresting *N*ew *G*ene (RING) and *H*omologous to *E*6AP *C*arboxy *T*erminus (HECT) families. RING E3s catalyze the transfer of ubiquitin from the E2 to the target protein by serving as bridges to bring the lysine residue of the target protein close to the E2-ubiquitin intermediate, thereby increasing the probability of reaction. HECT E3s form a thiolester intermediate with ubiquitin before it is transferred to the target protein (Pickart and Eddins, 2004). Once one ubiquitin molecule has been covalently attached to a lysine residue on the target protein, multiple ubiquitin

molecules can be added in succession through a poorly understood mechanism to produce a ubiquitin polymer consisting of many covalently-linked ubiquitin molecules (polyubiquitylation) (Hochstrasser, 2006).

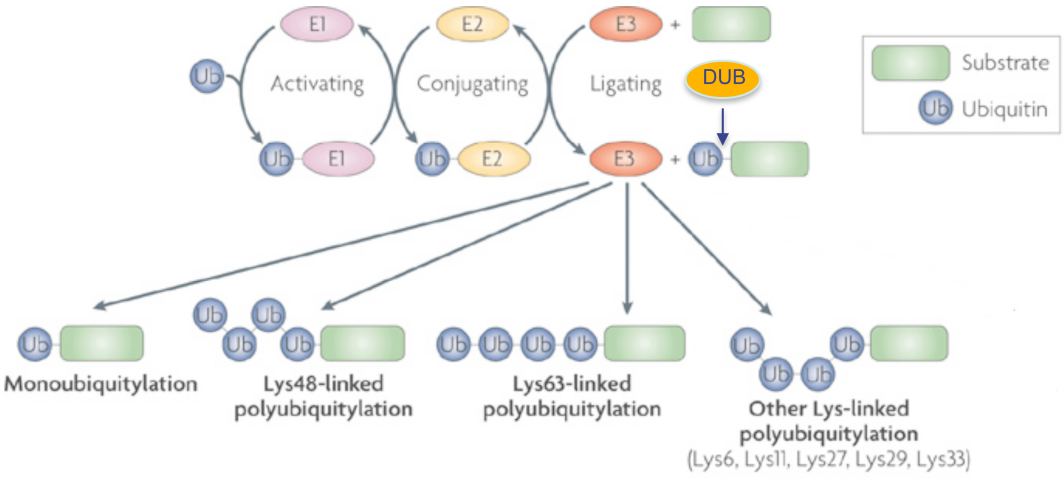


Figure 1.2. Schematic of the ubiquitin system. Figure adapted from (Dikic et al., 2009).

Ubiquitin contains seven internal lysine residues, each of which can be used for ubiquitin conjugation resulting in the formation of different lysine-linked ubiquitin chains (K6, K11, K27, K29, K33, K48, K63) (Behrends and Harper, 2011; Peng et al., 2003). The best understood polymer is the K48-linked ubiquitin chain, which typically marks the target protein for degradation by the 26S proteasome (Thrower et al., 2000). K63-linked chains typically do not mark a protein for proteasomal degradation, but rather activate specific proteins for

DNA repair, signal transduction, endocytosis, etc. (Pickart and Fushman, 2004; Sun and Chen, 2004). Target proteins can also be covalently attached to a single ubiquitin molecule at one lysine residue (monoubiquitylation) or at multiple lysine residues (multi-monoubiquitylation), resulting in different effects on target protein function such as regulating sub-cellular localization or the recruitment of ubiquitin-binding proteins (d'Azzo et al., 2005; Welchman et al., 2005).

The process of ubiquitin conjugation can be reversed by cleavage of the isopeptide bond between ubiquitin and the lysine residue of the target protein by deubiquitylating enzymes (DUBs) (Figure 1.2). This results in the release of free ubiquitin and free enzyme and reverses the effects of the ubiquitin modification (reviewed in (Amerik and Hochstrasser, 2004; Komander et al., 2009; Nijman et al., 2005)). There are approximately 79 functional DUBs in the human genome, most of which are cysteine proteases that contain a highly conserved cysteine residue in their active sites. DUBs fall into one of five subclasses based on their ubiquitin-protease domains: ubiquitin-specific proteases (USPs, 58 total), ubiquitin C-terminal hydrolases (UCHs, 4 total), Otubain proteases (OTUs, 14 total), Machado-Joseph disease proteases (MJDs, 5 total), and one class of metalloproteases called JAMM (JAB1/MPN/Mov34 metalloenzyme, 14 total). It is thought that DUBs regulate a limited number of substrates by recognizing either specific ubiquitin polymers or monomers (substrate specificity) and/or the target protein to which the ubiquitin moiety is attached (target specificity), giving DUBs two mechanisms by which to target specific sets of proteins (Nijman et al., 2005).

According to the current ubiquitin system model, the two enzymes that confer substrate specificity to the system are the E3 ligases and the DUBs. Thus, it is these two classes of enzymes that are likely to play specific roles in regulating cellular functions. Indeed, many E3s and DUBs have now been identified as key Wnt signaling regulators.

Regulation of Wnt signaling by The Ubiquitin System

Ubiquitylation plays a critical role in regulating Wnt signal transduction, most notably by regulating cytoplasmic levels of β-catenin, the key component of the pathway. The first E3 ubiquitin ligase (E3) identified for β-catenin was the multi-subunit E3 Skp1-Cullin-F-box $(SCF)^{\beta\text{-}TRCP}$, which recognizes phosphorylated β-catenin in the β-catenin destruction complex and targets it for proteasomal degradation (Aberle et al., 1997; Jiang and Struhl, 1998). Thus, $SCF^{\beta\text{-}TRCP}$ is critical for keeping cytoplasmic levels of β-catenin low in the absence of a Wnt signal. More recent findings indicate the existence of an additional E3 for β-catenin, Siah-1, which mediates K11-linked polyubiquitylation of β-catenin upon genotoxic stress (Liu et al., 2001; Matsuzawa and Reed, 2001). In response to DNA damage, it is thought that activated p53 induces the expression of Siah-1, which can ubiquitylate β-catenin independent of its phosphorylation status and independent of $SCF^{\beta\text{-}TRCP}$. Thus, it appears that cytoplasmic levels of β-catenin can also be directly affected by cellular stress through upregulation of Siah-1.

Jade-1 is an additional recently discovered E3 for β-catenin that appears to mainly regulate its nuclear levels (Chitalia et al., 2008). Like $SCF^{\beta\text{-}TRCP}$, Jade-1 only recognizes GSK3-phosphorylated β-catenin, but unlike $SCF^{\beta\text{-}TRCP}$ Jade-1 functions mostly in the nucleus and ubiquitylates β-catenin in both the absence and presence of Wnt signaling. At present Jade-1 mediated β-catenin regulation has only been observed in kidney tissues. It remains to be determined if this is a more general Wnt signaling regulatory mechanism.

In addition to the critical regulation of β-catenin levels by ubiquitylation, the two β-catenin destruction complex scaffolding proteins Axin and APC are also both regulated by the ubiquitin system. As discussed above, various groups have observed Axin degradation and, because it is a limiting component in the β-catenin destruction complex, Axin degradation has been proposed to be a critical Wnt signaling event. Until recently, however, the proteins involved in regulating Axin stability remained elusive. Axin was first shown to be parsylated by tankyrase 1 and 2, which was determined to be required for its ubiquitylation and degradation (Huang et al., 2009a). Subsequently a DUB, USP34, was discovered to regulate Axin stability, presumably by reversing the effects of an E3 ubiquitin ligase that had not been identified (Zhang et al., 2011). Just recently, an E3 ligase for Axin was discovered, RNF146, which recognizes parsylated Axin and targets it for proteasomal degradation (Zhang et al., 2011). Thus, three key

components for regulating Axin levels have been identified indicating regulating Axin stability is a critical event in Wnt signal transduction.

It has been known for some time that APC is ubiquitylated and degraded by the proteasome (Choi et al., 2004), but no E3 for APC has been identified. Recently the DUB, USP15, has been implicated in protecting APC from degradation as part of the COP9 signalasome (CSN) (Huang et al., 2009b). The CSN has been reported to bind to $SCF^{\beta\text{-}TRCP}$ to enhance its activity towards phosphorylated β-catenin. Thus, it appears that USP15 functions to stabilize APC in the destruction complex to allow for efficient degradation of β-catenin via the combined effects of CSN and $SCF^{\beta\text{-}TRCP}$. Another DUB, Trabid, has been shown to interact with and to remove K63-linked polyubiquitin chains from APC, although the functional consequence of both the addition of K63-linked chains to APC and their removal remains to be determined (Tran et al., 2008).

Wnt signaling events at the plasma membrane are also regulated by ubiquitylation. Most notably, the amount of the two co-receptors, LRP5/6 and Fz, available for initiation of Wnt signaling on the cell surface are regulated by components of the ubiquitin system. In the case of LRP5/6, mono-ubiquitylation serves as a quality control step to ensure the receptor is palmitoylated and properly folded before it exits the endoplasmic reticulum (ER) (Abrami et al., 2008). If LRP5/6 is not palmitoylated it becomes mono-ubiquitylated and retained in the ER. Neither the E3 that adds the mono-ubiquitin moiety onto LRP5/6 or the DUB that removes it have been identified. Recent work showed that Fz is also

modified by ubiquitin conjugation; a modification that results in translocation of Fz to the lysosome where it is degraded (Mukai et al., 2010). Mukai and colleagues identified a DUB, USP8, that removes the ubiquitin modification from Fz to prevent its lysosomal targeting and degradation, thereby increasing the amount of Fz on the cell surface available for Wnt signaling. The E3 ligase that targets Fz for lysosomal degradation remains to be identified.

Downstream of the two co-receptors lies the cytoplasmic protein Dsh, which has been shown to be ubiquitylated by the Kelch-like 12 (KLHL12)-Cullin3 E3 ligase complex in response to Wnt stimulation (Angers et al., 2006). Ubiquitylation of Dsh by KLHL12 leads to its proteasomal degradation. Thus, KLHL12 serves as a negative regulator of Wnt signaling. In addition to its regulation by KLHL12, Dsh is also regulated by K63-linked polyubiquitylation, which appears to positively regulate Dsh function in the Wnt pathway. The K63-linked ubiquitin chains are removed by the DUB CYLD (Tauriello et al., 2010); a process that inhibits Wnt signaling. It remains to be determined which E3 conjugates the K63-linked polyubiquitin chains onto Dsh and what effect this modification has on Dsh activity.

Clearly, the ubiquitin system is intimately involved in regulating Wnt signal transduction. Although numerous E3s and DUBs have now been identified as key Wnt regulators, at the time I began my thesis work only the E3 ligases for β-catenin ($SCF^{\beta\text{-}TRCP}$) and Dsh (KLHL12-Cullin3) had been identified. No other E3s or DUBs had been identified for the other components of the pathway that were

known to be ubiquitylated. Thus, I performed a targeted RNAi screen in *Drosophila* S2 cells to identify novel E3 ligases and DUBs involved in the regulation of Wnt signal transduction, which I describe in Chapter III. Identification and characterization of novel E3 ligases and DUBs involved in Wnt signaling will greatly enhance our understanding of how this important pathway is regulated by the complex ubiquitin system and potentially lead to the design of novel therapeutics with which to treat Wnt-driven diseases.

CHAPTER II

MATERIALS AND METHODS

Drosophila dsRNA Generation and S2 Cell RNAi Screen

Verified or predicted E3 ligases were identified using the "Termlink" function on www.flybase.org. The following search terms were used: ubiquitin protein ligase activity, E3, and ubiquitin ligase complex. We pooled the search results to give a final list of 146 E3 ligases, of which we were able to screen a subset of 122 of these clones. We used the *Drosophila* Gene Collection (DGC) to isolate plasmid cDNAs encoding each E3 ligase (see Figure 3.1A). T7 and T3 RNA polymerase promoters were added to the 5' and 3' end of each cDNA, respectively, via PCR using primers specific for the vector, similar to the methods described in (Clemens et al., 2000). Primer sequences are as follows for E3 ligases in pOTB7/pOT2: Forward-5'-CAGAGATGCATAATACGACTCACTATAGGGAGATTAGGTGACACTATAGAAC T-3', Reverse-5'-CCAAGCCTTCAATTAACCCTCACTAAAGGGAGAAAGCCCGCTCATTAGGCG GGTTAAA-3'

For E3 ligases in pBSSK/pFlc1: Forward-5'-CAGAGATGCATAATACGACTCACTATAGGGAGACGACTCACTATAGGGCGA AT-3', Reverse-5'-

CCAAGCCTTCAATTAACCCTCACTAAAGGGAGATTAACCCTCACTAAAGGGA ACAAAA-3'.

dsRNA was synthesized in an *in vitro* transcription reaction using mMessage mMachine (Ambion) according to manufacturer's instructions using T3 and T7 RNA polymerases, purified using RNeasy Mini Kit (Qiagen), and added to *Drosophila* S2 reporter cells stably transfected with a Wg TOPflash luciferase transcriptional reporter and a vector containing a constitutively expressed LacZ gene (gift from R. Nusse, Stanford). The S2 reporter cells were incubated with dsRNA for 72 hrs prior to incubation for 24 hrs in Wg-conditioned media. Cells were lysed in 1X Passive Lysis Buffer (Promega), and luciferase and β-galactosidase activities were measured using Steady Glo and β-Glo Assays (Promega), respectively. Luciferase activity was normalized to β-galactosidase activity (a measure of cell number).

Plasmids and Purified Proteins

pCS2-XIAP, pCS2-myc-XIAP, pCS2-HA-XIAP, pCS2-myc-cIAP1, pCS2-myc-cIAP2, pCS2-myc-TLE3, pCS2-myc-TLE3-Q, pCS2-myc-TLE1, pCS2-myc-AES, pCS2-HA-SMAC, and pMAL-XIAP, pCS2-USP47, pCS2-USP47mut, pCS2-GFP-USP47, were all generated using standard PCR-based cloning strategies. The following plasmids were purchased from Addgene and described previously (Lewis et al., 2004; Yang et al., 2000): pEBB-XIAP, pEBB-XIAPΔRING (1-351), pGEX-XIAP, pGEX-XIAPΔRING. The following plasmids were generous gifts:

pEBB-XIAPcasp-mut (D148A/W310A) (C. Duckett, University of Michigan), pMT107 (His-Ub) (W. Tansey, Vanderbilt University), pMP-SUMO-H_6-Groucho-Q (A. Courey, UCLA), pGEX-TCF4 (J. Eid, Vanderbilt University), pcDNA3-HA-TLE3 (A. Kispert, Hannover Medical School), pHR-myc-β–TRCP-1 (S. Elledge, Harvard Medical School), pCMV-Script-Smad4 (D. Beauchamp, Vanderbilt University) and TP1-Luc and NotchICV (S. Huppert, Vanderbilt University). TK-Renilla (Promega) and TOPflash (Korinek et al., 1997) were described previously. GST-XIAP, GST-XIAPΔRING (Lewis et al., 2004), SUMO-H_6-Groucho-Q (Kuo et al., 2010), and GST-TCF4 (Poy et al., 2001) were purified as described previously. MBP-XIAP was expressed in bacteria and purified according to manufacturer's instructions (New England Biolabs).

Cell Lines and Transfections

HEK293, HeLa, SW480, and L and L-Wnt3a cell lines were purchased from the American Type Culture Collection. Wg-secreting cells were purchased from the *Drosophila* Genomics Resource Center. HEK293 CMV-Luc was reported previously (Thorne et al., 2010). The following cell lines were gifts: HEK293 STF (J. Nathans, Johns Hopkins University), HCT116 XIAP WT and HCT116 XIAP KO (B. Vogelstein, Johns Hopkins University), S2 reporter cells (R. Nusse, Stanford University). Mammalian cell lines were cultured in DMEM plus 10% (v/v) FBS and antibiotics. *Drosophila* S2 cells were cultured in Schneider's medium plus 10% (v/v) FBS. DNA transfections were performed with Lipofectamine 2000

transfection reagent (Invitrogen) according to the manufacturer's protocol. siRNA transfections were performed using Dharmafect-1 (HEK293 and HeLa cells) or Dharmafect-4 (HCT116 and SW480 cells) according to the manufacturer's protocol with the following siRNA constructs: XIAP siRNA#1: 5'-AAGUGGUAGUCCUGUUUCAGCUU-3', XIAP siRNA#2: 5'-GGUAAGAACUACUGAGAAAUU-3', USP47 siRNA#1: 5'-UUGUUCACCAUCUUUAUCUdTdT-3',
USP47 siRNA#2: 5'-AAAUGCUAUAGCUUUCUUCdTdT-3',
or siGENOME Non-Targeting siRNA #5 (Thermo Scientific Dharmacon).

Reporter Assays

For cell-based luciferase assays, cells were plated and transfected with siRNA or DNA, as described above. L cell-conditioned media or Wnt3a-conditioned media was added to HEK293 STF cells 24 hrs after transfection. Cells were lysed 48 hrs after transfection with 1X Passive Lysis Buffer (Promega) and luciferase activity measured with Steady Glo (Promega). Luciferase activities were normalized to viable cell number using the CellTiter-Glo Assay (Promega). TOPflash experiments in HCT116 and SW480 cells were normalized to co-transfected *Renilla* gene expression. TP1 reporter assay was performed in HEK293 cells as previously described (Huppert et al., 2005). All graphs were made using Prism 4 (GraphPad Software, Inc.). Statistical analysis was

performed using the Student's *t* test. A value of $p < 0.05$ is considered statistically significant.

Ubiquitylation Assays

In vitro ubiquitylation assays were carried out in 20 ul reactions using the Ubiquitin Thioester/Conjugation Initiation Kit (Boston Biochem) and the following: 1 uM UbcH5a (Boston Biochem); 2.5 ug GST-XIAP, GST-XIAPΔRING, or MBP-XIAP; and 1 mM DTT. I*n vitro*-translated myc-TLE3 or HA-TCF4 or recombinant SUMO-H_6-Groucho-Q were used as substrates. Reactions were carried out at 30°C for 90 min and stopped by addition of sample buffer. Reaction products were resolved by SDS/PAGE and visualized by immunoblotting. *In vivo* ubiquitylation assays were performed using the His-tagged ubiquitin method as previously described (Salghetti et al., 1999).

Gel Filtration

Gel filtration was performed using an AKTA FPLC apparatus with Superose 6 or Superdex 200 columns (GE Healthcare). The following standards were used for calibration: thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B_{12} (1.35 kDa) (Bio-Rad). *In vitro*-translated and ubiquitylated myc-TLE3 and myc-TLE3-Q were chromatographed at 4°C in 50 mM Tris (pH 7.4) and 200 mM NaCl, as described previously for SUMO-H_6-Groucho-Q (Kuo et al., 2010).

Antibodies

The following antibodies were used for immunoprecipitation, immunoblotting and immunofluorescence: anti-β-catenin (BD Transduction Labs), anti-GAPDH (Abcam), anti-HA (3F10, Roche), anti-XIAP (R&D Systems (immunoprecipitation) and BD Transduction (immunoblotting)), anti-TLE3 (M-201, Santa Cruz), anti-Myc (9E10), anti-TCF4 (Cell Signaling Technologies), anti-Flag (M2, Sigma), anti-His (Novagen), anti-USP47 (Bethyl), anti-β-TRCP (Zymed, 37-3400), and anti-Smad4 (Santa Cruz, sc7966).

Immunoblots, Immunoprecipitations, and GST Pull-Downs

For immunoblots, cells were lysed in non-denaturing lysis buffer (NDLB) (50 mM Tris-Cl (pH 7.4), 300 mM NaCl, 5 mM EDTA, 1% (w/v) Triton X-100, protease inhibitor cocktail (Roche)) and soluble fractions were obtained. For cycloheximide (CHX)-chase experiments, cells were treated with 50 ug/ml CHX for the indicated time. For co-immunoprecipitations, cells were lysed in NDLB supplemented with 250 ng/ml ubiquitin aldehyde. Lysates were diluted to 1 mg/ml with NDLB and incubated with antibody O/N with rotation at 4°C followed by addition of Protein A/G beads (Santa Cruz) for 2 hrs. Beads were then washed five times with NDLB. Bound proteins were eluted from beads with protein sample buffer and analyzed by SDS-PAGE/immunoblotting. For *in vitro* binding assays, GST or GST-TCF4-bound glutathione beads were diluted into binding buffer (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% NP-40, 1 mM DTT, and protease inhibitor

cocktail) and incubated with *in vitro* translated myc-TLE3 or myc-TLE-Q or recombinant MBP-XIAP for 2 hrs with rotation at 4°C. Beads were washed five times for 10 min at RT in binding buffer, and proteins eluted with sample buffer and analyzed by SDS-PAGE/immunoblotting.

***Xenopus laevis* Studies**

Xenopus embryos were in vitro fertilized, dejellied, cultured, and injected as previously described (Peng, 1991). Morpholinos with the following sequences were purchased from Gene Tools. Standard Control MO: 5'-CCTCTTACCTCAGTTACAATTTATA-3', XIAP MO#1: 5'-GCATGTCATCTCCTCTTTAAATACG-3', XIAP MO#2: 5'-GGAACCACAACCTTCCTACCGGCTC-3', USP47 MO: 5'-GCTGACTCTCTTCTCCAGGCCTCAT-3'. Capped *Xwnt8*, *XIAP*, and *USP47* mRNA were generated using mMessage mMachine (Ambion) according to manufacturer's instructions. Animal caps were excised from stage 9 embryos, cultured until stage 11, and RT-PCR of *Siamois*, *Xnr3*, *Chordin*, and *ODC* transcripts was performed as described (Cselenyi et al., 2008). *In situ* hybridization analysis was performed as described (Harland, 1991) using a probe against *Xenopus* USP47 using Sp6 polymerase for the antisense strand. For whole embryo sectioning and staining, embryos were formalin fixed, processed, embedded into paraffin and stained with hematoxylin and eosin for histological analysis. Statistical analysis was performed using the Fisher's exact test. A

value of $p < 0.05$ is considered statistically significant. All the work performed on *Xenopus* embryos was approved by the Institutional Animal Care and Use Committee (IACUC) at Vanderbilt University Medical Center and was in accordance with their policies and guidelines.

Immunofluorescence

Cells were grown on fibronectin-coated coverslips, fixed in 3.7% formaldehyde, permeabilized, incubated with primary antibody (anti-myc 1:1000, anti-XIAP 1:200, anti-TLE3 1:200, anti-β-catenin 1:1000) followed by secondary antibodies conjugated to Cy3 or Alexa 488, and mounted in ProLong Gold with DAPI (Invitrogen). Cells were visualized using a Cascade 512B camera mounted on a Nikon Eclipse TE2000-E confocal microscope.

Real-Time RT-PCR

HEK293 cells were transfected with siRNA as described above and incubated for 24 hrs. Cells were then serum starved (0.5% FBS) for 16 hrs and incubated with Wnt3a-CM for 24 hrs. Total RNA was isolated using RNAeasy RNA extraction kit (Qiagen) and cDNA generated using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, ABI). Real-time RT-PCR assays were performed in quadruplicate using TaqMan Gene Expression Master Mix (ABI), gene specific TaqMan TAMRA probes (ABI), and an ABI 7000 sequence detection system. The following *AXIN2* primer sequences were used. Forward: 5'-

GTCCAGCAAAACTCTGAGGG-3', Reverse: 5'-CTGGTGCAAAGACATAGCCA-3'.

CHAPTER III

RNAi SCREEN TO IDENTIFY NOVEL UBIQUITIN SYSTEM COMPONENTS INVOLVED IN WNT SIGNALING

Introduction

As I discussed in Chapter I, the Wnt signaling pathway is heavily regulated by ubiquitylation, but not many of the ubiquitin system components (i.e. E3 ligases and DUBs) involved in Wnt pathway regulation had been identified at the time I began my graduate studies. Thus, I designed a RNA interference (RNAi) screen in *Drosophila* S2 cells to identify novel E3s and DUBs involved in Wg/Wnt signaling. RNAi is a potent method for knocking down expression of specific mRNA molecules. This approach has been well established using the *Drosophila* S2 cell system (Clemens et al., 2000; Goshima et al., 2007), which has many advantages: 1) there are fewer genes in *Drosophila* than in mammalian systems, simplifying the screen; 2) uptake of dsRNA is very robust in S2 cells and does not require use of a transfection reagent; and 3) *Drosophila* dsRNA can be synthesized in the laboratory using *Drosophila* Gene Collection cDNA clones (which we have in our laboratory) and adding a second T7 polymerase promoter (within the primer) via PCR to generate full-length dsRNA for each gene of interest. This generally results in very efficient knockdown of target gene expression.

Thus, I designed a RNAi screen targeting all of the predicted E3 ligases and DUBs in the *Drosophila* genome (see Chapter II for details). I used the *Drosophila* Gene Collection to collect plasmid cDNAs encoding each DUB and E3 ligase. An additional T7 RNA polymerase promoter was added to the 3' end of the reverse strand of each cDNA via PCR, and dsRNA was synthesized in an *in vitro* transcription reaction using T3 and T7 RNA polymerases (Figure 3.1). The dsRNA was then purified and added to *Drosophila* S2 reporter cells (obtained from R. Nusse, Stanford University), which have been stably transfected with the well-characterized TOPflash luciferase transcriptional reporter and with a vector containing a constitutively expressed LacZ gene. The TOPflash reporter contains 8 TCF/LEF binding sites upstream of a minimal promoter, which drives expression of luciferase (Korinek et al., 1997). In these S2 reporter cells, luciferase activity is a measure of Wg signaling activity that can be normalized to β-galactosidase activity, which is a measure of cell number. The dsRNA was incubated with the S2 reporter cells for 72 h at which time Wg (the *Drosophila* Wnt homolog)-conditioned media was added to the cells in a 1:1 ratio. The cells were incubated for an additional 24 h, lysed, and luciferase and β-galactosidase activity were measured. Luciferase activity was then normalized to β-galactosidase activity to obtain the final results.

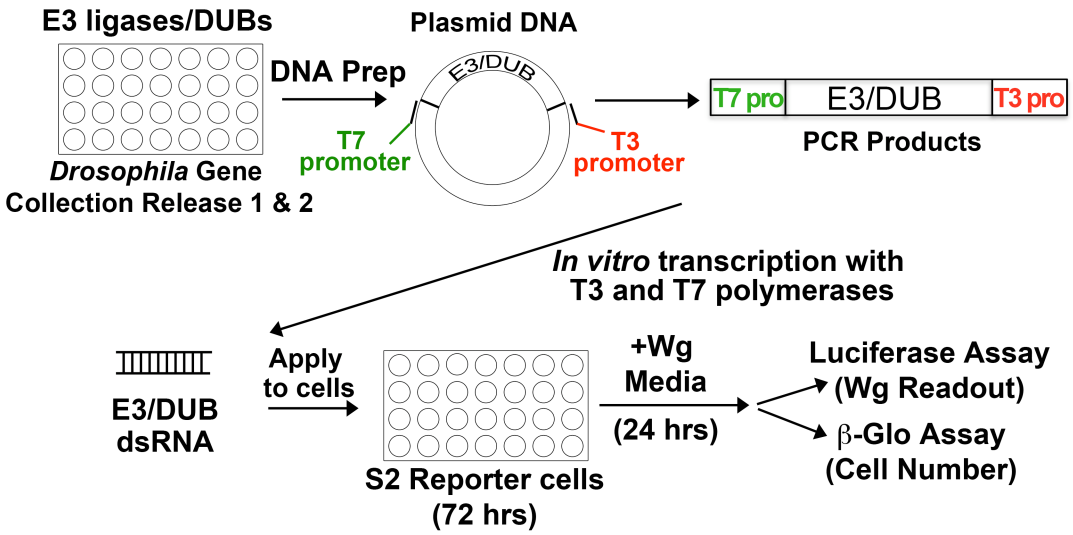


Figure 3.1. Schematic of *Drosophila* RNAi screen to identify E3 ligases and DUBs involved in regulating Wg/Wnt signlaling. See text and Chapter II (Materials and Methods) for details.

Results

Results of the *Drosophila* E3 ligase RNAi screen

In both the E3 ligase and DUB RNAi screen, Axin (a potent negative regulator of Wg signaling) and Armadillo (Arm, the *Drosophila* β-catenin homolog and potent positive regulator of Wg signaling) dsRNA served as controls to confirm effective knockdown in each experiment. Both the Axin and Arm controls are shown in Figure 3.2A, but the Axin control is not shown in the rest of the figures to make it easier to visualize differences between the samples treated with dsRNA targeting the E3s and the Wg-treated samples. Axin control dsRNA

significantly increased Wg signaling, while Arm control dsRNA significantly decreased Wg signaling in all samples, indicating that the dsRNA treatment was effective.

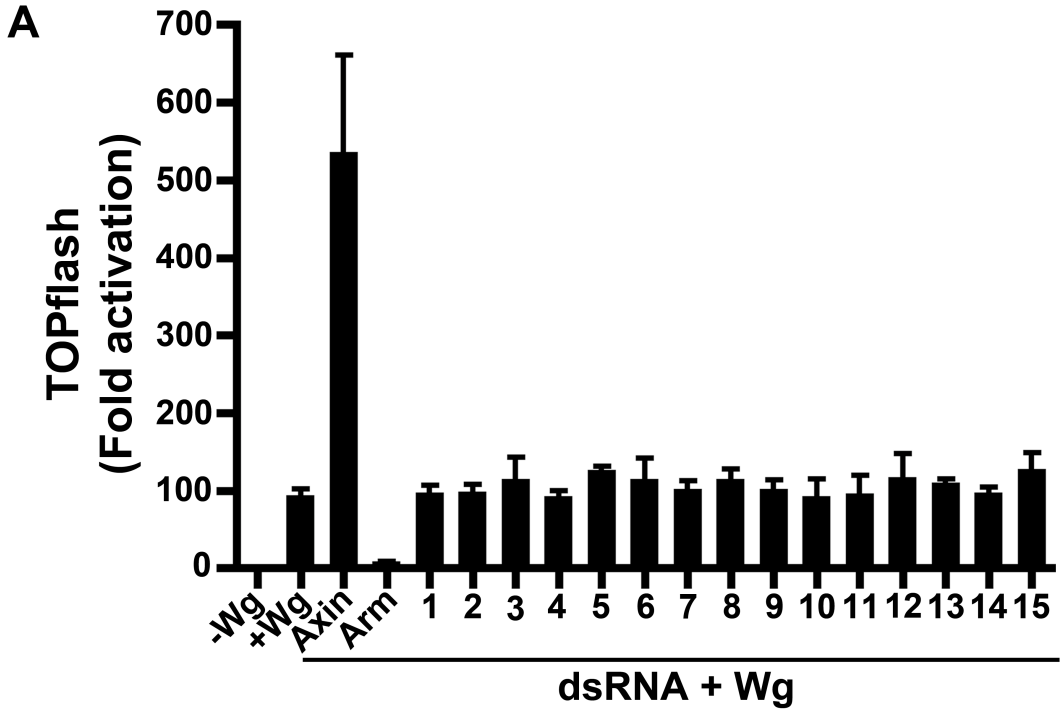
Of the 118 E3 ubiquitin ligases screened (Table 3.1 and Figure 3.2), the knockdown of one (number 112) was particularly potent in inhibiting Wg signaling, reducing TOPflash activity to a similar extent as knocking down Arm. dsRNA number 112 targets *Drosophila* Inhibitor of Apoptosis 1 (DIAP1), a well-characterized anti-apoptotic effector and a member of the evolutionarily conserved Inhibitor of Apoptosis protein (IAP) family (Srinivasula and Ashwell, 2008). I chose to pursue this "hit" further because it suggested an unexpected link between a classic IAP family member and regulation of the Wg signaling pathway. This result is described in detail in Chapter IV.

In addition to E3 number 112, two other interesting "hits" were recovered. One of these was number 52, which corresponds to the *Drosophila* gene Trithorax (Trx) (Figure 3.2E). Knockdown of Trx activates Wg signaling, indicating it is a negative Wg signaling regulator in S2 cells, however, the human homolog of Trx (Mixed-Lineage Leukemia (MLL), a methyltransferase) has now been shown to associate with β-catenin to promote histone H3 lysine 4 (H3K4) methylation to activate Wnt target gene transcription (Sierra et al., 2006). The discrepancy between my data and the data of Sierra et al. could simply be due to differences in regulation of Wg/Wnt signaling in *Drosophila* versus human cells. Additionally, it is not clear how a methyltransferase was included in the predicted

E3 ligase list obtained from www.flybase.org (see Chapter II for details). It may be due to the fact that MLL contains a zinc finger domain, which is found in RING domain-containing E3 ligases. Regardless of the fact that MLL is not an E3 ligase and that my data indicates its fly homolog is a negative Wg signaling regulator, the fact that knockdown of the gene affected Wg activity validated that I was able to identify Wg signaling regulators using this method.

The other interesting "hit" was E3 number 106, which corresponds to the *Drosophila* gene Something That Sticks Like Glue (SNAMA), whose human homolog is Retinoblastoma Binding Protein 6 (RBBP6). My results indicate SNAMA/RBBP6 is a negative regulator of Wg/Wnt signaling as its knockdown activates TOPflash activity (Figure 3.2H). Nothing has been published indicating a role for SNAMA/RBBP6 in Wg/Wnt signaling to date. The few studies that have been published on RBBP6 have failed to reach a conclusion regarding its function, but it has been strongly implicated in tumorigenesis and is required for development; two roles which have largely been attributed to its capacity to bind the tumor suppressor proteins p53 and pRB (Li et al., 2007; Motadi et al., 2011; Rowe et al., 2006; Simons et al., 1997). Interestingly, it has recently been predicted to be a transcriptional repressor, which is consistent with my data indicating it represses Wg signal transduction (Peidis et al., 2010). It will be interesting to determine if RBBP6 acts as a transcriptional repressor in the Wnt pathway and how this may or may not be influenced by its interaction with p53 or pRB.

There were other E3 ligases screened that affected Wg signaling either positively or negatively, but many do not have obvious human homologs. It will be interesting to see how many of them turn out to be *bona fide* Wnt signaling regulators.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TOPflash
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600
500
400
300
200
100
0
-Wg
+Wg
Axin
Arm
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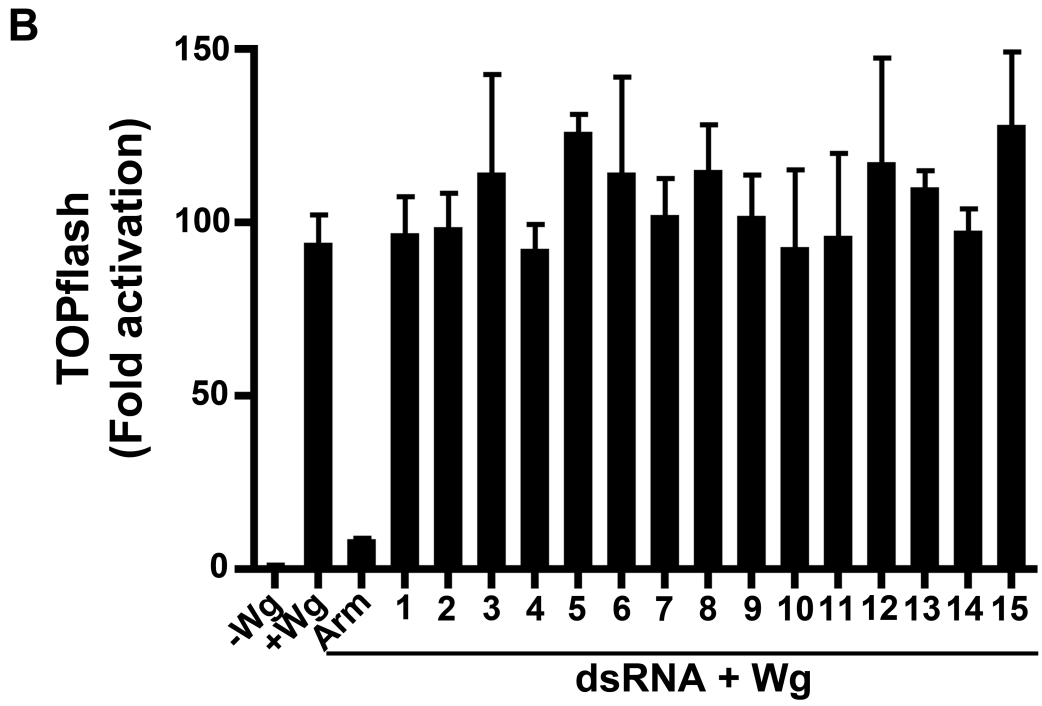
2
3
4
5
6
7
8
9
10
11
12
13
14
15
dsRNA + Wg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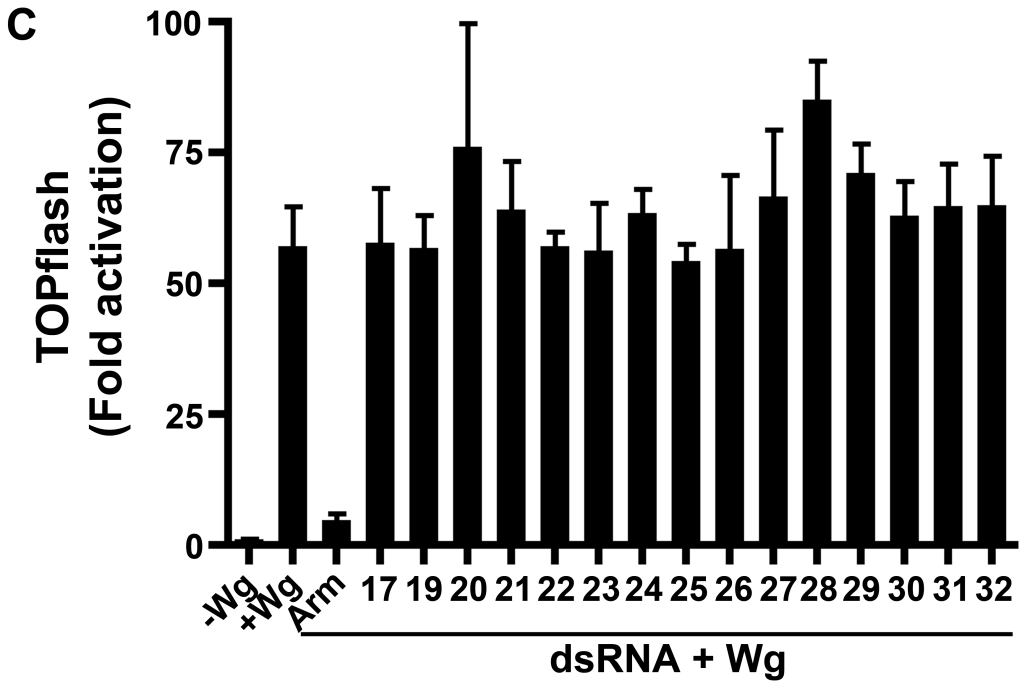
14
15
dsRNA + Wg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Arm
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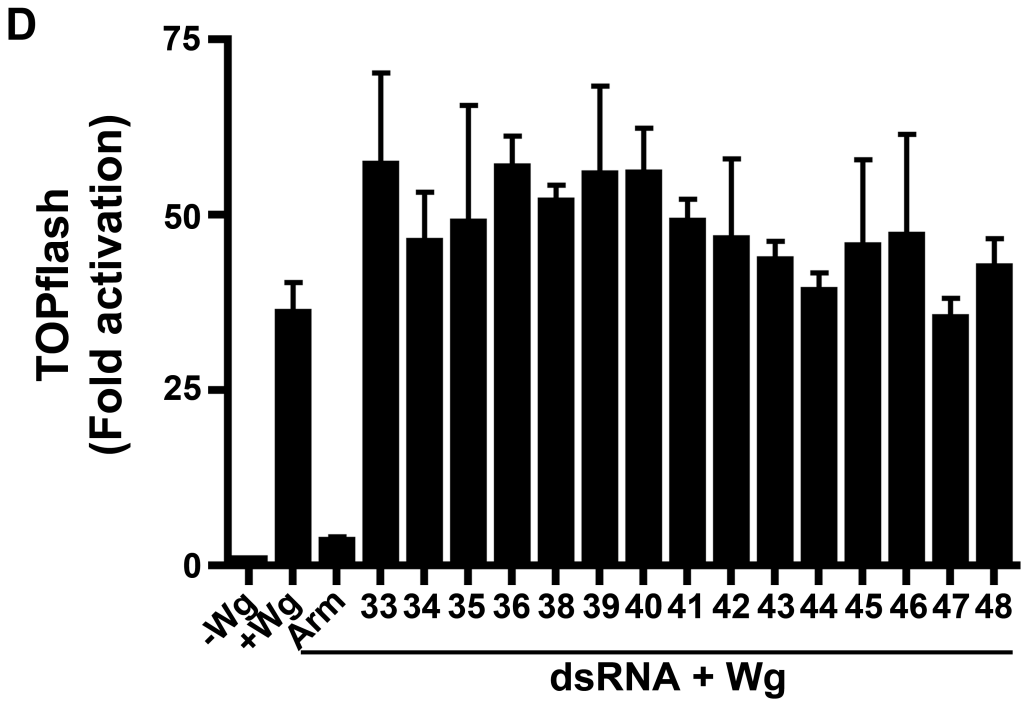
dsRNA + Wg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Arm
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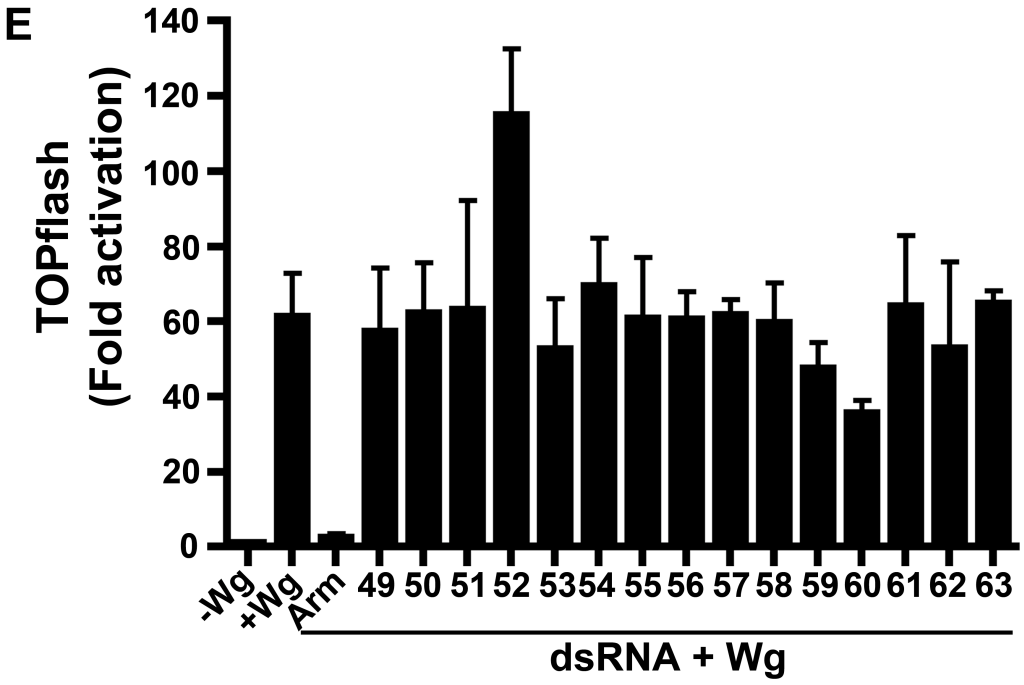
dsRNA + Wg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Arm
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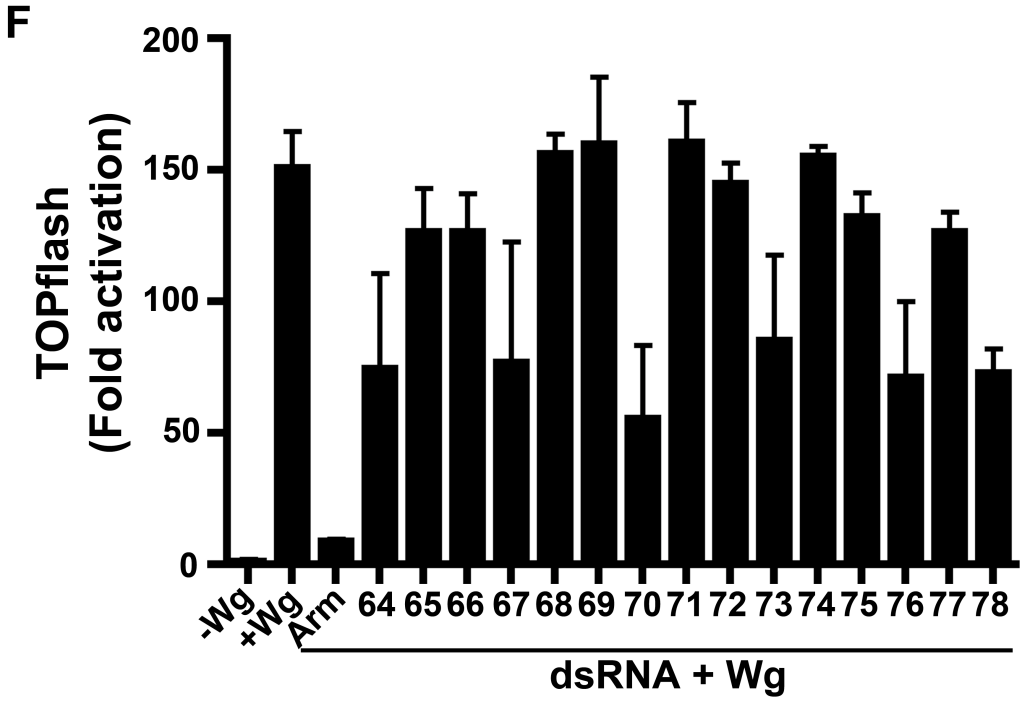
dsRNA + Wg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150
100
50
0
-Wg
+Wg
Arm
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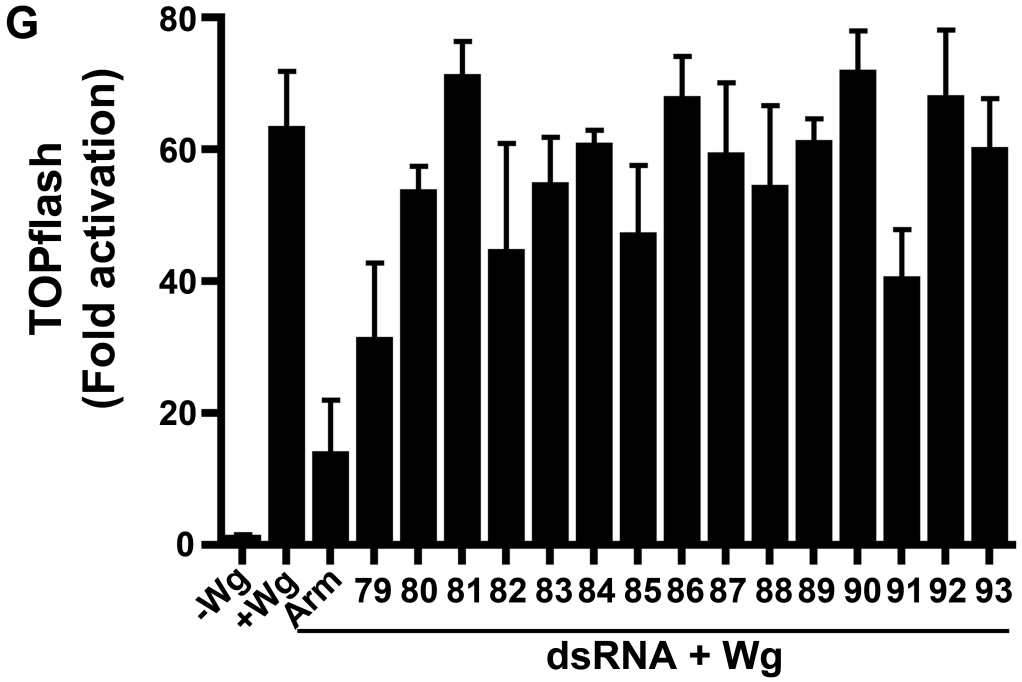
dsRNA + Wg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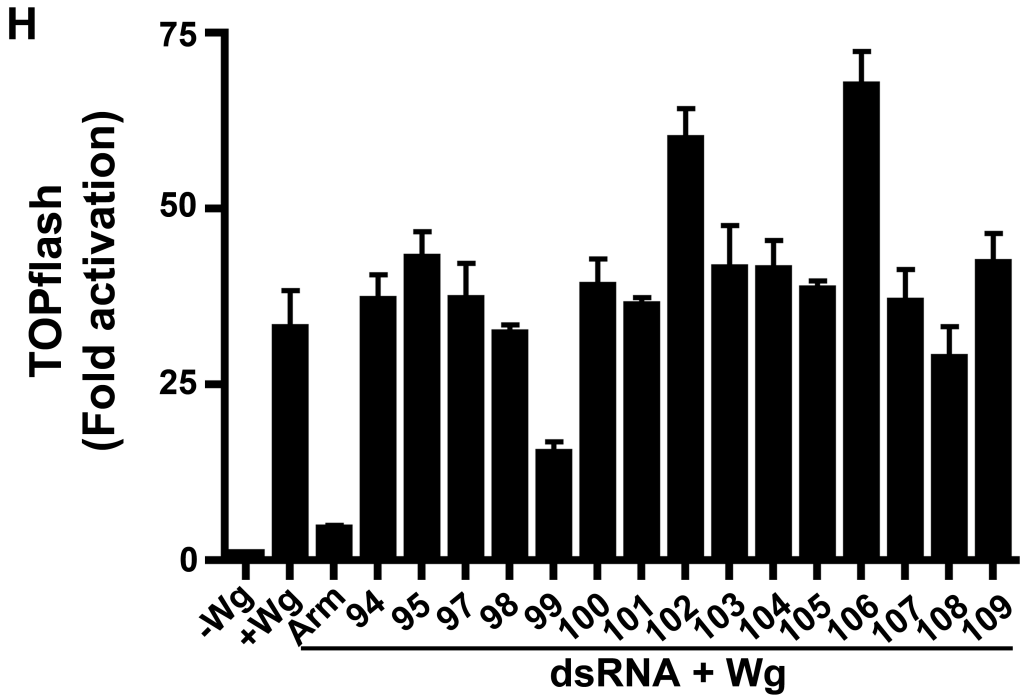
Arm
79 80 81 82 83 84 85 86 87 88 89 90 91 92 93
dsRNA + Wg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(Fold activation)
75
50
25
0
-Wg
+Wg
Arm
94 95 97 98 99 100 101 102 103 104 105 106 107 108 109
dsRNA + Wg

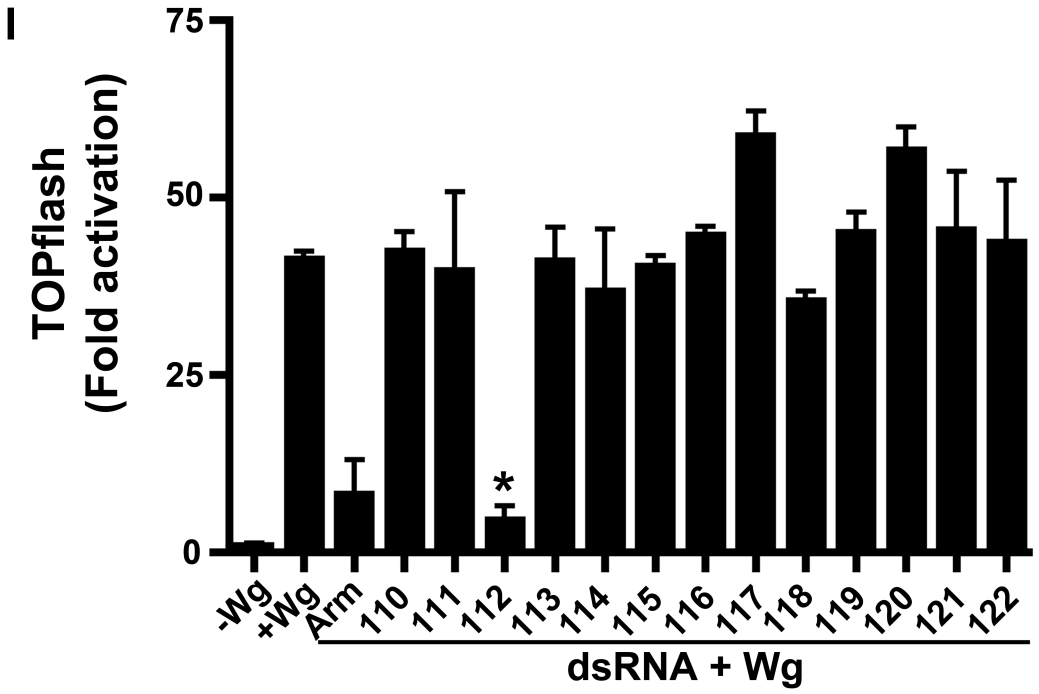


Figure 3.2. Results of *Drosophila* E3 ligase RNAi screen for Wg/Wnt signaling regulators.

(A-I) Results of TOPflash activity normalized to β-galactosidase activity relative to –Wg treatment. Mean ± standard deviation (SD) of each dsRNA treatment performed in triplicate is shown. Axin (A only) and Arm serve as controls for negative and positive regulators of Wg/Wnt signaling, respectively. Asterisk indicates the most potent "hit" identified in the screen, which is described further in Chapter IV.

E3 Collection #	FULL NAME	SYMBOL	CLONE ID	PLATE	ROW	COLUMN	VECTOR	Antibiotic	SIZE
1	CG31716	CG31716	RE04975	1	I	4	PFLC1	Amp	3500
2	CHIP	CHIP	RE01069	1	A	11	PFLC1	Amp	1278
3	l(3)73Ah	CG4195	RE11339	1	J	2	PFLC1	Amp	1964
4	ppa	CG9952	RE01138	1	A	15	PFLC1	Amp	3449
5	hyd	CG9484	RE13070	1	P	8	PFLC1	Amp	9074
6	CG11419	CG11419	RE25242	2	P	20	PFLC1	Amp	1100
7	Trim9	CG31721	RE22018	2	B	14	PFLC1	Amp	3105
8	cdc16	cdc16	RE28575	3	A	24	PFLC1	Amp	2583
9	CG5087	CG5087	RE40614	4	I	1	PFLC1	Amp	3943
10	CG9153	CG9153	RE53774	4	L	6	PFLC1	Amp	3818
11	ARIADNE	ari-1	RE69116	5	N	24	PFLC1	Amp	2672
12	CG2617	CG2617	RE60872	5	M	14	PFLC1	Amp	1263
13	Cul-5	CG1401	RE55959	5	G	3	PFLC1	Amp	3511
14	dx	CG3929	RE59350	5	G	8	PFLC1	Amp	3824
15	Mes-4	CG4976	RE61305	5	O	8	PFLC1	Amp	4585
16	Roc2	CG8998	RE61847	5	B	5	PFLC1	Amp	590
17	Vhl	CG13221	RH61560	9	K	3	PFLC1	Amp	1308
18	lack	CG4943	LD16661	10	C	11	pBS-SK-	Amp	4865
19	lmg	CG18042	AT07979	11	F	13	POTB7	Chlor	2956
20	CG1134	CG1134	AT15655	12	C	20	POTB7	Chlor	1239
21	CG12362	CG12362	AT17761	12	O	2	POTB7	Chlor	1962
22	CG17329	CG17329	AT18988	12	F	23	POTB7	Chlor	673
23	CG5071	CG5071	AT17603	12	M	6	POTB7	Chlor	2241
24	Roc1b	CG16988	AT21612	12	F	2	POTB7	Chlor	514
25	CG4238	CG4238	AT17882	12	O	12	POTB7	Chlor	3515
26	HERC2	CG11734	AT22791	12	L	2	POTB7	Chlor	15796
27	ariadne 2	ari-2	GH07166	13	J	18	POT2	Chlor	3002
28	CG11321	CG11321	GH08772	13	N	6	POT2	Chlor	8769
29	CG13030	CG13030	AT26312	13	I	23	POTB7	Chlor	1448
30	elfless	CG15150	AT24563	13	C	1	POTB7	Chlor	1166
31	Cbl	Cbl	LD46082	14	N	22	POT2	Chlor	3889
32	CG9014	CG9014	LP07794	15	B	15	POT2	Chlor	1359
33	skpF	CG12227	LP10147	15	J	11	POT2	Chlor	697
34	CG5604	CG5604	LP05936	15	G	10	POT2	Chlor	8344
35	Roc1a	CG16982	SD23839	16	I	12	POT2	Chlor	1168
36	CG5382	CG5382	GM05688	DGC.1	J	15	pBS SK-	amp	1200
37	Cul-3 (guftagu?)	CG11861		DGC.1	I	21	pBS SK-	amp	2725
38	Gbp	CG5519	LD02793	DGC.1	E	21	pBS SK-	amp	1774
39	CG4973	CG4973	LD35003	DGC.10	P	22	pOT2		1403
40	d4	CG2682	LD29238	DGC.10	J	9	pOT2		1766
41	Iap2	CG8293	LD34777	DGC.10	P	18	pOT2		2095
42	unk	CG4620	LD33756	DGC.10	L	16	pOT2		2710
43	CG10542	CG10542	LD35285	DGC.11	A	21	pOT2		2210
44	CG11982	CG11982	LD47007	DGC.11	F	18	pOT2		1571
45	CG13605	CG13605	LD44641	DGC.11	J	15	pOT2		2122
46	CG17033	CG17033	LD41235	DGC.11	I	14	pOT2		1554
47	CG17260	CG17260	LD44813	DGC.11	L	3	pOT2		1282
48	CG32486	CG32486	LD47625	DGC.11	L	8	pOT2		2347
49	mr (morula)	CG3060	LD45730	DGC.11	P	5	pOT2		1451
50	neur (neuralized)	CG11988	LD45505	DGC.11	N	19	pOT2		2656
51	shtd (shattered)	CG9198	LD37115	DGC.11	I	3	pOT2		1799
52	trx (trithorax)	CG8651	LD39445	DGC.11	A	2	pOT2		2552
53	CG11414	CG11414	SD03374	DGC.12	E	8	pOT2		1571
54	CG17019	CG17019	SD05126	DGC.12	K	22	pOT2		2855
55	CG5140	CG5140	GH03577	DGC.12	D	16	pOT2		1539
56	sip3	CG1937	GH11117	DGC.12	L	10	pOT2		2282
57	park (parkin)	CG10523	SD01679	DGC.12	M	5	pOT2		1569
58	CG32369	CG32369	GH21463	DGC.13	G	7	pOT2		2452
59	CG4030	CG4030	LD23155	DGC.13	G	20	pOT2		2311
60	CG6752	CG6752	LD30968	DGC.13	D	11	pOT2		2785
61	CG9772	CG9772	GM13370	DGC.13	O	15	pOT2		1975
62	Cul-2	CG1512	LD36177	DGC.13	J	13	pOT2		2817
63	dor	CG3093	SD04291	DGC.13	L	6	pOT2		3173
64	CG11261	CG11261	LD29662	DGC.14	G	24	pOT2		2210
65	CG15105	CG15105	GH06739	DGC.14	P	15	pOT2		4683
66	Cul-6	CG11261	LD29662	DGC.14	G	24	pOT2		2210
67	Gol (goliath)	CG2679	GH20973	DGC.14	E	15	pOT2		2586
68	msl-2	CG3241	GH22488	DGC.14	N	24	pOT2		3715
69	CG6923	CG6923	LD22771	DGC.15	E	19	pOT2		4531
70	CG9934	CG9934	SD06937	DGC.15	H	12	pOT2		4432

71	mib1	CG5841	SD05267	DGC.15	D	14	pOT2		4073
72	mib2	CG17492	GH28686	DGC.15	E	5	pOT2		3511
73	POSH	CG4909	LD45365	DGC.15	B	23	pOT2		3149
74	CG8184	CG8184	SD03277	DGC.15	P	11	pOT2		3685
75	Nedd4	CG7555	SD04682	DGC.15	B	24	pOT2		3641
76	ARCHIPELAGO	ago	CG15010	DGC.16	P	2	pOT2		5312
77	CG33144	CG33144	GH08706	DGC.16	A	11	pOT2		4533
78	CG5591	CG5591	GH27953	DGC.16	K	3	pOT2		3354
79	CG9086	CG9086	LD31957	DGC.16	P	12	pOT2		6381
80	Cul-4	CG8711	GM14815	DGC.16	K	19	pOT2		3314
81	rols	CG32096	GH15583	DGC.16	J	4	pOT2		5122
82	Topors	CG15104	LD43109	DGC.16	E	6	pOT2		4038
83	As	CG6190	LD21888	DGC.16	B	17	pOT2		3667
84	CG3356	CG3356	LP03102	DGC.16	G	12	pOT2		3917
85	CG32210	CG32210	SD01201	DGC.17	G	5	pOT2		5405
86	CG10981	CG10981	GM01182	DGC.2	A	3	pBS SK-	amp	1660
87	CG15011	CG15011	LD05244	DGC.2	O	11	pBS SK-	amp	2903
88	CG32350	CG32350	LD20292	DGC.2	F	4	pBS SK-	amp	2870
89	CG7376	CG7376	LD03886	DGC.2	K	23	pBS SK-	amp	2250
90	skpA	CG16983	HL01263	DGC.2	C	17	pBS SK-	amp	1533
91	Trc8	CG2304	LD08152	DGC.2	G	2	pBS SK-	amp	1493
92	cdc23	cdc23	LD09850	DGC.3	L	2	pBS SK-	amp	2245
93	CG13344	CG13344	GM02568	DGC.3	B	19	pBS SK-	amp	1925
94	CG15439	CG15439	LD18949	DGC.3	H	3	pBS SK-	amp	2617
95	CG1815	CG1815	LD02460	DGC.3	P	13	pBS SK-	amp	2862
96	CG31687	CG31687	LD09850	DGC.3	L	2	pBS SK-	amp	2245
97	Cul-1 (lin19)	CG1877	LD20253	DGC.3	H	19	pBS SK-	amp	2984
98	slmb	CG3412	LD08669	DGC.3	H	6	pBS SK-	amp	2534
99	CG17735	CG17735	HL01545	DGC.3	D	21	pBS SK-	amp	2452
100	Su(dx)	CG4244	LD10565	DGC.3	N	24	pBS SK-	amp	2901
101	CG16807	CG16807	LD12033	DGC.4	K	10	pBS SK-	amp	3332
102	CG2926	CG2926	LD09942	DGC.4	L	15	pBS SK-	amp	4435
103	CG2991	CG2991	LD08641	DGC.4	E	4	pBS SK-	amp	3147
104	CG9461	CG9461	GM01353	DGC.4	O	16	pBS SK-	amp	4150
105	CG11070	CG11070	LD34475	DGC.5	A	24	pOT2		3416
106	CG3231	CG3231	LD21643	DGC.5	E	12	pOT2		3937
107	CG7864	CG7864	GM14467	DGC.5	D	8	pOT2		1048
108	mei-P26	CG12218	GH10646	DGC.5	D	1	pOT2		5254
109	morgue	CG15437	GH02435	DGC.5	I	19	pOT2		1698
110	stc	CG3647	LD22726	DGC.5	I	8	pOT2		4067
111	CG2681	CG2681	GH02982	DGC.6	E	6	pOT2		1079
112	th (thread)	CG12284	GH15335	DGC.6	F	3	pOT2		1484
113	CG7081	CG7081	LD46714	DGC.7	M	7	pOT2		1041
114	poe	CG14472	LP02909	DGC.7	A	6	pOT2		1065
115	CG5555	CG5555	GH07062	DGC.8	J	3	pOT2		1976
116	CG8974	CG8974	GH14055	DGC.8	P	22	pOT2		1703
117	Sce	CG5595	LD23953	DGC.8	A	14	pOT2		1534
118	sina	CG9949	HL08111	DGC.8	C	21	pOT2		2280
119	Traf2	CG10961	GH01161	DGC.8	I	20	pOT2		2785
120	CG15141	CG15141	LD24839	DGC.8	G	12	pOT2		1688
121	CG11534	CG11534	GH12489	DGC.9	M	20	pOT2		1627
122	CG1909	CG1909	GH22690	DGC.9	P	24	pOT2		2405

DON'T HAVE	bon	CG5206							
DON'T HAVE	CG11360	CG11360							
DON'T HAVE	CG15800	CG15800							
DON'T HAVE	CG17048	CG17048							
DON'T HAVE	CG2709	CG2709							
DON'T HAVE	CG31053	CG31053							
DON'T HAVE	CG1392	CG1392							
DON'T HAVE	CG31807	CG31807							
DON'T HAVE	CG32581	CG32581							
DON'T HAVE	CG32847	CG32847							
DON'T HAVE	CG3639	CG3639							
DON'T HAVE	CG6613	CG6613							
DON'T HAVE	CG6688	CG6688							
DON'T HAVE	CG8419	CG8419							
DON'T HAVE	CG9941	CG9941							
DON'T HAVE	Dnr1	Dnr1							
DON'T HAVE	hiw (highwire)	hiw							
DON'T HAVE	ida								
DON'T HAVE	lt (light)								
DON'T HAVE	mat1								
DON'T HAVE	Mi-2								
DON'T HAVE	Psc								
DON'T HAVE	Su(z)2								
DON'T HAVE	CG3099	CG3099							

Table 3.1. List of the predicted E3 ligases in the *Drosophila* genome used for the RNAi screen.
The numbers in the left-hand column correspond to the numbers of the dsRNAs graphed in Figure 3.2. The corresponding gene names and symbols are indicated next to each number in the table. Information regarding the vector in which each cDNA is cloned as well as where each clone is located in the *Drosophila* Gene Collection (DGC) 1 and 2 is shown at right. The clones that are not found in the DGC, and thus were not screened, are noted at the end of the table.

Results of the *Drosophila* DUB RNAi screen

Of the 29 DUBs screened (out of the 30 predicted in the *Drosophila* genome) (Table 3.2 and Figure 3.3), two “hits” were recovered, both of which inhibit Wg signaling when knocked down, indicating they are Wg signaling activators. One of the DUB hits, Cylindromatosis (CYLD, and dsRNA number 11 in Figure 3.3A), was shown to be a Wnt pathway regulator by the Clevers

laboratory (Wnt Meeting, Berlin) at the time I performed this screen, further validating my approach for identifying *bona fide* Wg/Wnt regulators in my DUB RNAi screen. They have now published a paper indicating CYLD cleaves K63-linked polyubiquitin chains from Dsh, which inhibits Wnt signaling, as described in Chapter I (Tauriello et al., 2010). Thus, again, my results were opposite of those found in human cells as my data gathered in S2 cells indicated that CYLD is a positive Wg signaling regulator. Regardless of this discrepancy, my screen identified CYLD as a potential Wnt pathway regulator indicating I was able to identify novel Wnt pathway components using this method.

Because CYLD was already under investigation by another laboratory, I decided to focus on the other DUB hit, Ubiquitin Binding Protein 64 E (Ubp64E, and dsRNA number 15 in Figure 3.3A), whose human homolog is Ubiquitin Specific Peptidase 47 (USP47), a putative DUB that had no known function at the time I began my studies. Characterization of this DUB hit is described in Chapter V.

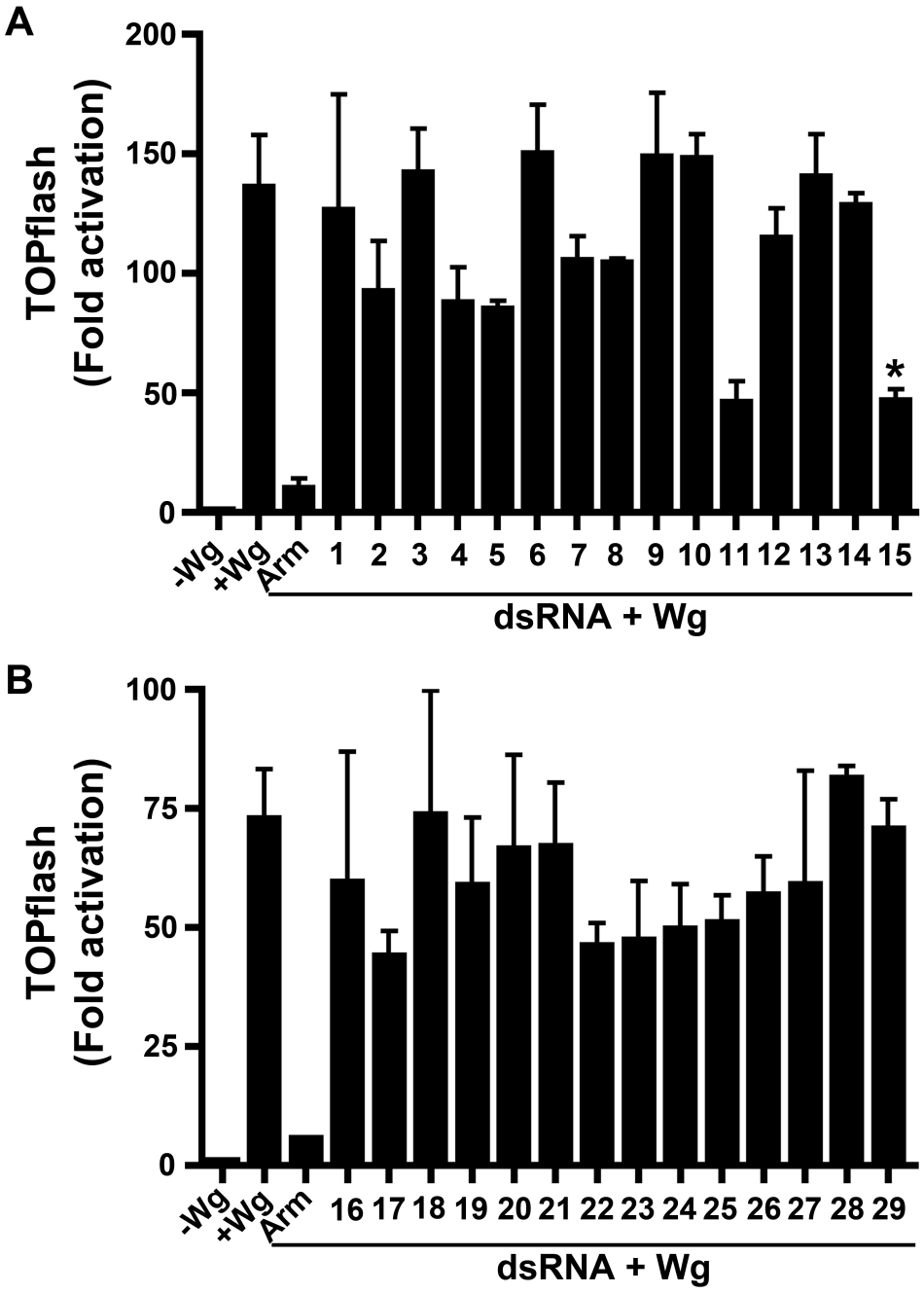


Figure 3.3. Results of *Drosophila* DUB RNAi screen for Wg/Wnt signaling regulators.

(A and B) Results of TOPflash activity normalized to β-galactosidase activity relative to –Wg treatment. Mean ± standard deviation (SD) of each dsRNA treatment performed in triplicate is shown. Arm serves as a control for positive regulators of Wg/Wnt signaling. Asterisk indicates the "hit" I chose to pursue and is described in Chapter V.

DUB Collection #	SYMBOL	Clone ID	Predicted Gene	Plate	Row	Column	Vector	Antibiotic	Size	cDNA Accession
1	CG8494	HL02756		3	E	18	pBS SK-	amp	1990	AY069300
2	isopep-T-3	LD10242	CG11025	3	L	24	pBS SK-	amp	2404	BT004899
3	CG7023	RE52890		4	H	2	PFLC1	Amp		
4	CG15817	LD22910		5	I	10	pOT2		4860	AF181650
5	CYLD	RE64280	CG5603	5	N	1	PFLC1	Amp		
6	faf	LD22582	CG1945	5	I	4	pOT2		5393	AF145677
7	Uch	GH02396	CG4265	5	I	13	pOT2		1068	AF145600
8	Uch-L3	LD24440	CG3431	5	E	9	pOT2		1153	AF132567
9	Ulp1	GH02751	CG12359	5	K	9	pOT2		3449	AF145608
10	CG12082	RE70722		6	E	13	PFLC1	Amp		
11	dp	SD02173	CG33196	7	G	24	pOT2		1245	AY122242
12	CG8445	GH01941		8	O	12	pOT2		2148	AY047515
13	CG1950	AT10439		11	D	20	POTB7	Chlor		
14	not	LD43147	CG4166	11	B	19	pOT2		2508	AY058707
15	Ubp64E	LD38333	CG5486	11	M	7	pOT2		3143	AY058672
16	CG3016	LD41827		13	N	17	pOT2		2314	AY069649
17	CG5384	LD40495		13	L	23	pOT2		1715	
18	CG5794	AT30546		13	D	15	POTB7	Chlor		
19	CG7288	LD38070		13	L	7	pOT2		1700	AY061442
20	Usp7	LD41613	CG1490	13	N	15	pOT2		2224	AY061459
21	CG8232	LD22095		14	B	19	pOT2			AY119601
22	CG14619	SD04280		15	B	10	pOT2		3450	AY069803
23	CG4165	LD34905		15	G	2	pOT2		4221	AY051846
24	CG5798	SD04548		15	B	16	pOT2		3208	AY122247
25	mule	LD40339	CG5505	15	K	18	pOT2		3515	AY051916
26	CG30421	GH27809		16	L	8	pOT2		5184	AY060809
27	CG32479	LD28815		16	N	6	pOT2		6352	AY122170
28	CG8334	SD15907		16	E	23	POT2	Chlor		
29	CG8830	LD36231		16	C	14	pOT2		3195	
30	ec		CG2904							

Table 3.2. List of the predicted DUBs in the *Drosophila* genome used for the RNAi screen.
The numbers in the left-hand column correspond to the numbers of the dsRNAs graphed in Figure 2.3. The corresponding gene names and symbols are indicated next to each number in the table. Information regarding the vector in which each cdna is cloned as well as where each clone is located in the *Drosophila* Gene Collection 1 and 2 is shown at right.

Discussion

Performing a targeted RNAi screen in *Drosophila* S2 cells provided two advantages over performing an RNAi screen in mammalian cells or over previously reported Wnt pathway RNAi screens: 1) there is less redundancy in

gene function in S2 cells than in mammalian cells, making it easier to identify genes that affect Wg/Wnt signaling, and 2) focusing solely on E3s and DUBs instead of the entire genome (as was done by (DasGupta et al., 2005)), reduced the amount of error that comes with handling a large number of samples as well as simplified the data analysis once the screen was completed. Additionally, the smaller number of samples allowed re-testing of the most promising hits in a time-efficient manner.

While no screen is perfect and false negatives and false positives are always identified, I deem a screen successful if it uncovers any novel biology. To this end, both the E3 and DUB RNAi screens I performed as described here were successful. The E3 ligase screen allowed me to identify DIAP1, whose human homolog is XIAP, as a novel key Wg/Wnt signaling regulator. My E3 screen did not identify the key Wg/Wnt signaling regulator Slimb/β-TRCP, indicating there are false negatives in my data set, but it did pick up Trithorax/MLL as a Wg/Wnt signaling component, which has now been shown to be a Wnt pathway regulator. Thus, I was able to identify a *bona fide* Wnt pathway regulator in my E3 RNAi screen.

Similarly, I identified the DUB Ubp46E, whose human homolog is USP47, as a novel Wg/Wnt pathway regulator in my DUB RNAi screen. I did not identify the other DUBs that have now been shown to be Wnt pathway regulators, as discussed in Chapter I, indicating there are also false negatives in my DUB data set. However, I did identify CYLD as a Wg/Wnt pathway regulator, which has now

been shown to be a *bona fide* Wnt pathway component indicating I was able to identify novel DUBs involved in regulating Wnt signaling.

While these screens identified other E3s and DUBs that remain to be characterized, I chose to focus on one E3 and one DUB for my thesis work. I will discuss the E3 ligase screen hit, XIAP, in detail in Chapter IV and the DUB screen hit, USP47, in Chapter V.

CHAPTER IV

XIAP MONO-UBIQUITYLATES GROUCHO/TLE TO PROMOTE CANONICAL WNT SIGNALING

Introduction

The canonical Wnt signaling pathway is present in all metazoans and regulates many developmental processes (Logan and Nusse, 2004; MacDonald et al., 2009). Misregulation of the Wnt pathway results in a variety of disease states in humans, including cancer. β-catenin is the main cytoplasmic effector in the Wnt pathway. In the absence of Wnt ligand, a β-catenin destruction complex, composed of Axin, glycogen synthase kinase 3 (GSK3), casein kinase I α (CKIα), and the tumor suppressor adenomatous polyposis coli (APC), promotes phosphorylation of β-catenin, targeting it for ubiquitin-mediated proteasomal degradation. Binding of a Wnt ligand to its two cell-surface receptors, Frizzled (Fz) and LDL receptor-related protein 5/6 (LRP5/6), results in inhibition of β-catenin phosophorylation and, thus, stabillzation of β-catenin. Stabilized β-catenin translocates to the nucleus where it binds to TCF/Lef to activate a Wnt-specific transcriptional program.

A critical nuclear event that occurs upon Wnt pathway activation is the β-catenin-mediated conversion of TCF/Lef from a transcriptional repressor to a transcriptional activator. In the absence of a Wnt signal, TCF/Lef is bound to the

Groucho (Gro)/TLE family of transcriptional co-repressors to repress transcription of Wnt target genes (Cavallo et al., 1998; Roose et al., 1998). This transcriptional repression is thought to involve recruitment of histone deacetylases by Gro/TLE to alter local chromatin structure as well as a role for Gro/TLE oligomerization, which promotes long-range chromosome condensation (Buscarlet and Stifani, 2007; Jennings and Ish-Horowicz, 2008). According to the current model of Wnt signaling, a pool of β-catenin that enters the nucleus upon Wnt pathway activation directly competes with Gro/TLE for TCF/Lef binding (Daniels and Weis, 2005). Once bound to TCF/Lef on chromatin, β-catenin recruits a co-activator complex, thereby converting TCF/Lef into a transcriptional activator.

The Wnt pathway contains many components that are known to be regulated by ubiquitylation (Tauriello and Maurice, 2010). E3 ubiquitin ligases for β-catenin (Jiang and Struhl, 1998; Marikawa and Elinson, 1998) and Dishevelled (Angers et al., 2006) have been reported, but little was known about other E3 ligases that regulate Wnt signaling at the time we began our study. Thus, we sought to identify novel E3 ligases involved in Wnt signaling to gain a better understanding of how the ubiquitin system regulates this pathway.

Here, we performed a targeted RNAi screen in *Drosophila* S2 cells to identify novel E3 ubiquitin ligases involved in Wingless (Wg, the *Drosophila* homolog of Wnt) signal transduction that led to our identification of *Drosophila* Inhibitor of Apoptosis 1 (DIAP1) as a critical Wg pathway component. We demonstrate that the human homolog, X-linked Inhibitor of Apoptosis (XIAP), is

similarly required for Wnt signaling in cultured human cells and *Xenopus* embryos, indicating evolutionary conservation of function. In response to Wnt pathway activation, we show that XIAP is recruited to TCF/Lef transcriptional complexes where it binds and ubiquitylates Gro/TLE, thereby decreasing the affinity of Gro/TLE for TCF/Lef. Together, our data reveal a mechanism by which ubiquitin-mediated removal of Gro/TLE from TCF/Lef bound to chromatin is required in order to allow assembly of β-catenin-TCF/Lef complexes that can initiate a Wnt-specific transcriptional program. In contrast to a direct displacement model in which β-catenin and Gro/TLE simply compete for TCF/Lef binding (Daniels and Weis, 2005), we provide evidence for a more intricate transcriptional switch in the Wnt pathway. This Wnt signaling circuitry, involving the coincident activities of β-catenin and XIAP, may explain why modest changes in β-catenin levels in response to Wnt ligand are sufficient to robustly activate the pathway.

Results

Drosophila RNAi screen identifies DIAP1 as a critical component of the Wingless signaling pathway

To identify novel E3 ubiquitin ligases involved in Wingless (Wg) signaling, we performed a genome-scale RNAi-based screen targeting E3 ubiquitin ligases in *Drosophila* S2 cells (Figure 4.1A, see Chapter II for details). Plasmids

encoding experimentally verified and predicted E3 ubiquitin ligases (122 clones total) were obtained from the *Drosophila* Gene Collection Release 1 and 2, and a PCR approach was used to generate linear cDNA products suitable for *in vitro* dsRNA synthesis. For the screen, dsRNA was added to a *Drosophila* S2R+ reporter cell line stably transfected with the Wg responsive TOPflash luciferase reporter (Korinek et al., 1997).

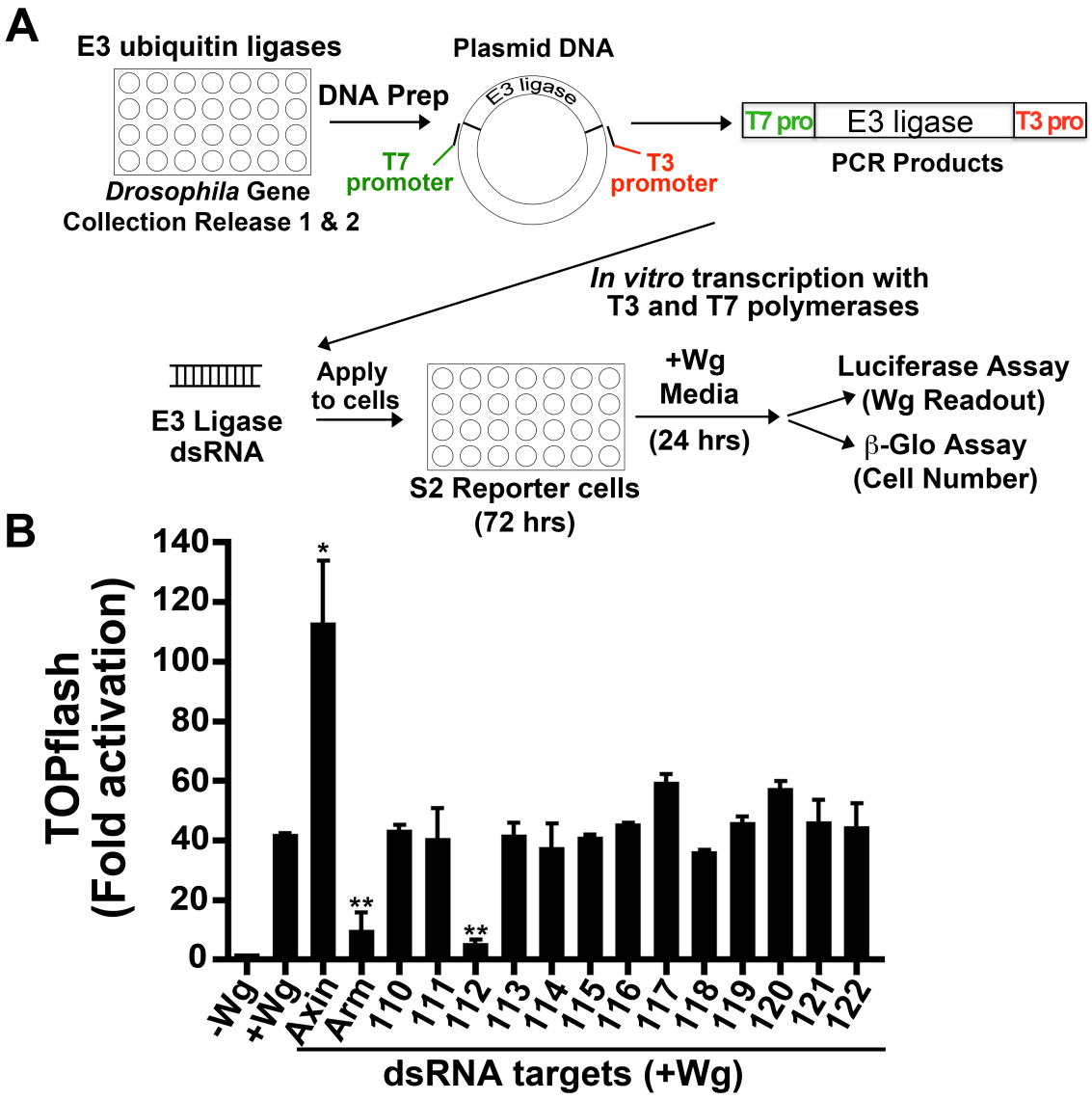


Figure 4.1. *Drosophila* S2 cell RNAi screen identifies the E3 ligase DIAP1 as a positive regulator of Wingless signaling.
(A) Schematic of RNAi screen to identify E3 ubiquitin ligases that regulate Wingless signaling in *Drosophila* S2 cells (see text for more details).
(B) Results of RNAi screen. Graph represents mean ± standard deviation (SD) of TOPflash normalized to cell number. Axin and Armadillo (Arm) dsRNA treatments were performed as controls. Results for DIAP1 dsRNA (112) and 12 additional dsRNAs included in the screen are shown. Results are representative of at least three experiments performed in quadruplicate. *p-value < 0.01, **p-value < 0.001 versus +Wg.

Axin (a potent negative regulator of Wg signaling) and Armadillo (Arm, the *Drosophila* β-catenin homolog and potent positive regulator of Wg signaling) served as controls to confirm effective knockdown by dsRNA treatment in our screen (Figure 4.1B). Of the 122 E3 ubiquitin ligases screened, the knockdown of one (no. 112) was particularly potent in inhibiting Wg signaling, reducing TOPflash activity to a similar extent as knocking down Arm. dsRNA no. 112 targets *Drosophila* Inhibitor of Apoptosis 1 (DIAP1), a well-characterized anti-apoptotic effector and a member of the evolutionarily conserved Inhibitor of Apoptosis protein (IAP) family (Srinivasula and Ashwell, 2008). We chose to pursue this hit further because it suggested an unexpected link between a classic IAP family member and regulation of the Wg signaling pathway.

XIAP is required for Wnt signaling in cultured mammalian cells

We next sought to determine if XIAP, the homolog of DIAP1, is similarly required for Wnt signaling in mammalian cells. We tested the effects of XIAP knockdown on Wnt3a-induced transcriptional activation using a human embryonic kidney (HEK) 293 cell line stably transfected with the TOPflash reporter (STF293) (Xu et al., 2004). Knockdown of XIAP with two independent short-interfering RNA (siRNA) constructs significantly blocked Wnt3a-induced TOPflash activation (Figure 4.2A), while having no observable effect on the activity of a constitutively active luciferase reporter (CMV-Luciferase) (Thorne et

al., 2010) or a Notch reporter (Minoguchi et al., 1997) (Figure 4.3), indicating specific

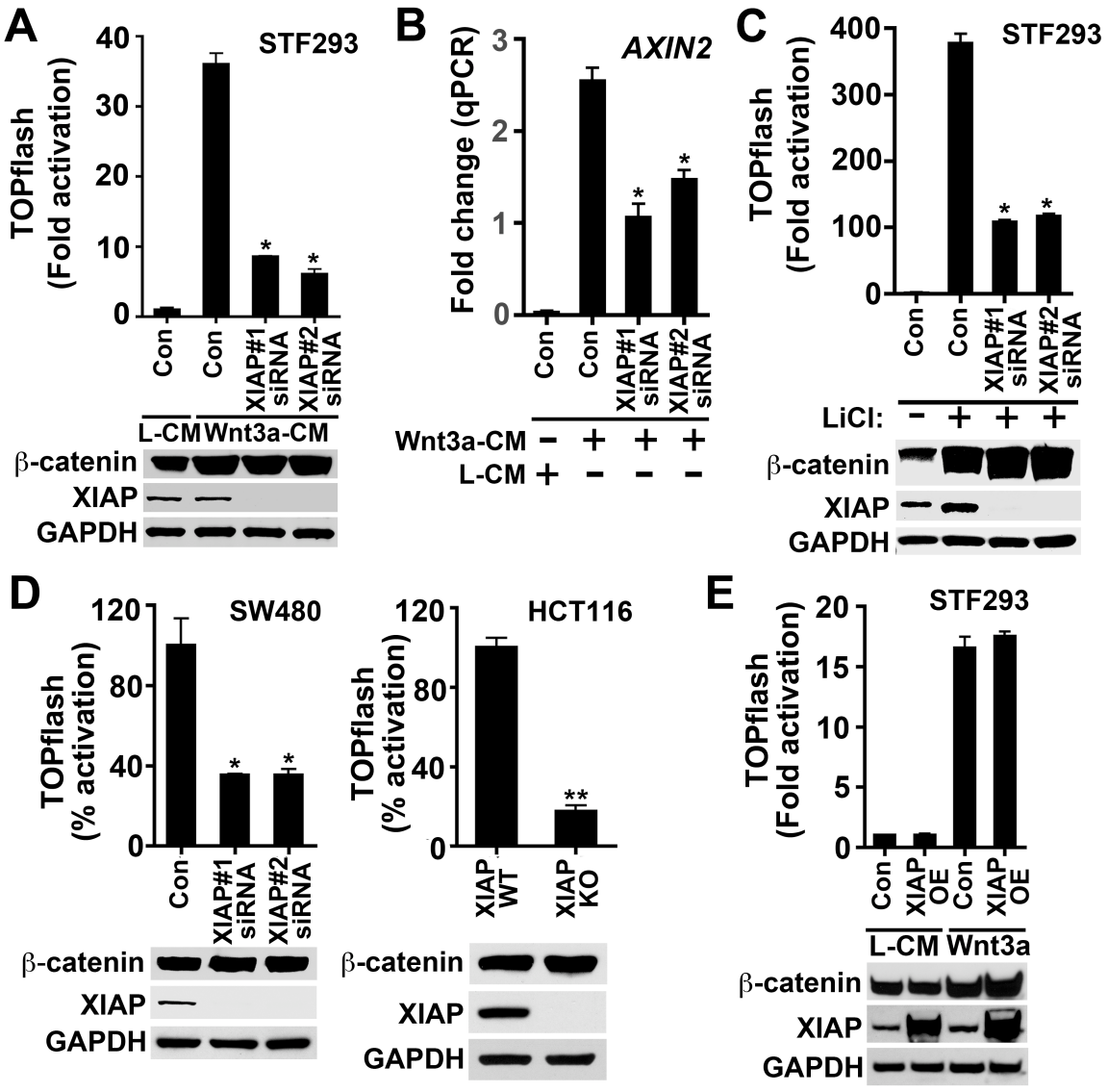


Figure 4.2. XIAP is required for Wnt signaling in cultured mammalian cells. (A and B) Knockdown of XIAP by siRNA inhibits Wnt signaling. (A) HEK293 STF (STF293) cells were transfected with non-targeting control (Con) or two independent XIAP siRNAs and treated with L cell (L-CM) or Wnt3a cell- (Wnt3a-CM) conditioned media for 24 hrs. Graph shows mean ± SD of TOPflash normalized to cell number. Immunoblotting confirmed knockdown of XIAP protein and Wnt3a-mediated stabilization of β-catenin. GAPDH is loading control. *p-value < 0.0001 versus Wnt3a-CM-treated Con. (B) Quantitative real-time RT-PCR of endogenous Wnt target gene, *AXIN2*, in HEK293 cells treated with XIAP siRNAs or non-targeting control. Graph is ratio of *AXIN2* to *PMMD1* mRNA (control). Results (mean ± SD) of four independent real-time RT-PCR reactions are shown. *p-value < 0.0005 versus Wnt3a-CM-treated Con.

(C and D) XIAP is required downstream of β-catenin stabilization for Wnt signaling in multiple cell types. (C) STF293 cells were transfected with XIAP siRNAs or non-targeting control and treated with 30 mM LiCl. Graph shows mean ± SD of TOPflash normalized to cell number. Immunoblots confirmed knockdown of XIAP protein and LiCl-mediated stabilization of β-catenin. GAPDH is loading control. *p-value < 0.0001 versus LiCl-treated Con. (D) Knockdown of XIAP inhibits Wnt signaling in the colon cancer lines SW480 and HCT116. SW480 cells were transfected with TOPflash and XIAP siRNAs or non-targeting control. XIAP-deficient HCT116 cells (XIAP KO) were transfected with TOPflash. Graphs show mean ± SD of TOPflash normalized to *Renilla* luciferase (transfection control). Immunoblots confirmed loss of XIAP protein. No observable change in β-catenin levels was detected. GAPDH is loading control. *p-value < 0.0005, **p-value < 0.0001 versus Con or XIAP WT.

(E) Overexpression (OE) of XIAP does not increase TOPflash activity. STF293 cells were transfected with XIAP and treated with L-CM or Wnt3a-CM for 24 hrs. Graph shows mean ± SD of TOPflash activity normalized to cell number. Immunobloting confirmed increased XIAP expression and Wnt3a-induced β-catenin stabilization. GAPDH is loading control. All TOPflash results are representative of at least three independent experiments performed in triplicate.

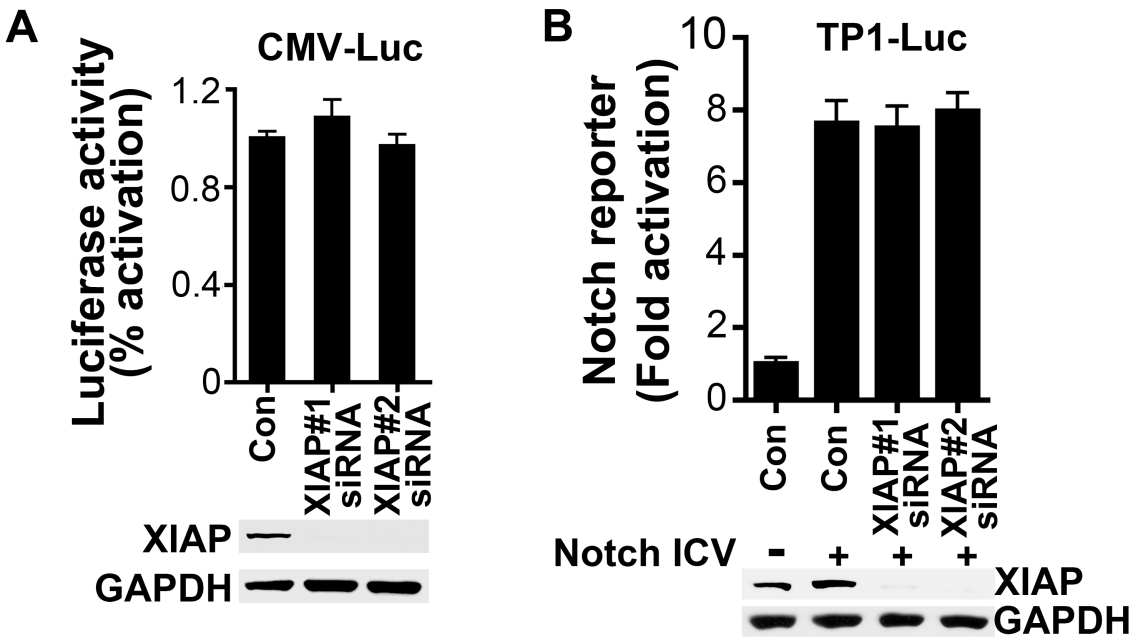


Figure 4.3. Knockdown of XIAP does not inhibit CMV-Luciferase or Notch signaling.

(A) HEK293 cells stably transfected with CMV-Luciferase (CMV-Luc) were transfected with non-targeting control (Con) or two independent XIAP siRNAs. Graph shows mean ± SD of CMV-luc normalized to cell number. Immunoblot confirmed knockdown of XIAP protein by XIAP siRNA but not control siRNA. GAPDH is loading control. Results represent at least three independent experiments performed in triplicate.

(B) To assess the effect of XIAP knockdown on Notch signaling, HEK293 cells were transfected with TP1-Luc (Notch reporter) and Notch intracellular domain (ICV) plus either non-targeting control (Con) or two independent XIAP siRNAs. Graph shows mean ± SD of reporter firefly luciferase activity normalized to *Renilla* luciferase (transfection control). Immunoblotting confirmed knockdown of XIAP. GAPDH is loading control. Results represent at least three independent experiments performed in triplicate.

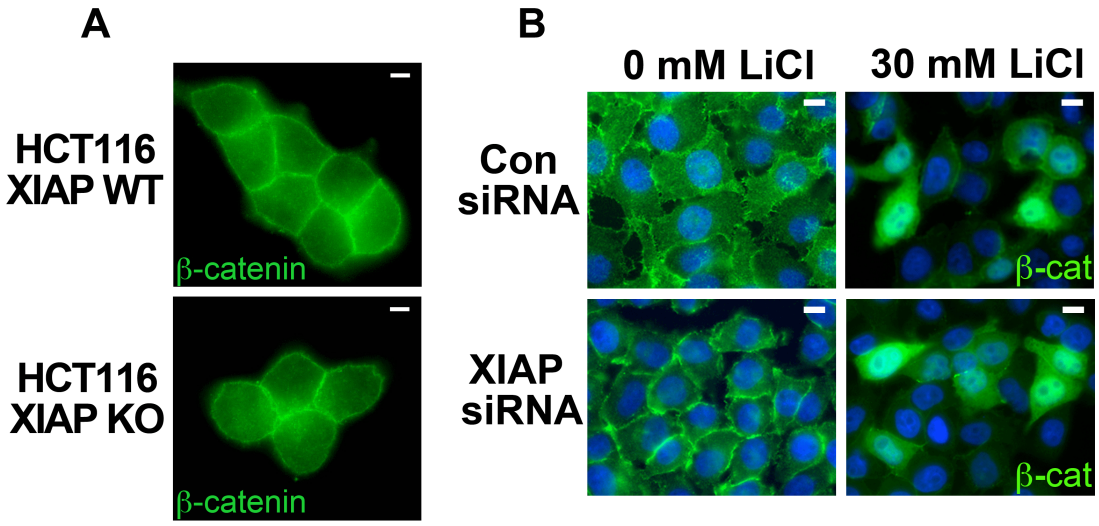


Figure 4.4. XIAP loss or knockdown has no effect on β-catenin levels or localization.
(A) A HCT116 cell line in which XIAP has been stably knocked out (HCT116 XIAP KO) and a corresponding wild-type control (HCT116 XIAP WT) were fixed, and immunostained for β-catenin. No change in β-catenin levels or localization were observed (greater than 300 cells scored per condition). Scale bars, 5 μm.
(B) HeLa cells were transfected with control (Con) or XIAP siRNA, treated with LiCl as indicated, and stained for β-catenin and DNA. 20.8% (33/158) of control-siRNA and 20.6% (32/155) of XIAP-siRNA cells treated with LiCl were positive for nuclear β-catenin. Scale bars, 5 μm.

inhibition of TOPflash activity. XIAP knockdown also inhibited Wnt3a-induced expression of endogenous *AXIN2* transcripts, further indicating that XIAP is required for Wnt-mediated transcriptional activation (Figure 4.2B).

Knockdown of XIAP did not change the levels of cytoplasmic β-catenin, suggesting that XIAP is functioning downstream of the β-catenin destruction complex (Figure 4.2A and 4.4). To further test this possibility, we investigated whether knockdown of XIAP could inhibit Wnt signaling in cells treated with lithium (which inhibits the destruction complex kinase GSK3). We found that knockdown of XIAP inhibited TOPflash activation in STF293 cells even when the β-catenin destruction complex is inhibited by lithium and elevated levels of β-catenin are present (Figure 4.2C).

The SW480 and HCT116 colon cancer cell lines have significantly decreased capacity to degrade β-catenin due to mutations in APC and β-catenin, respectively (Korinek et al., 1997; Morin et al., 1997). Both cell lines exhibit constitutively active, ligand-independent Wnt signaling. Knockdown of XIAP by RNAi inhibited Wnt signaling in SW480 cells but had no observable effect on β-catenin protein levels (Figure 4.2D). Wnt signaling was also significantly reduced without reduction in β-catenin levels in HCT116 cells deficient for XIAP (generous gift from B. Vogelstein, Johns Hopkins University). Furthermore, we did not detect any change in the localization of β-catenin in the absence of XIAP (Figure 4.4A) or when XIAP was knocked down by RNAi (Figure 4.4B).

In contrast to downregulation of XIAP, overexpression of XIAP had no observable effect on Wnt pathway signaling in the absence or presence of Wnt ligand, suggesting that XIAP is not a limiting Wnt pathway component in the cell lines we tested (Figure 4.2E). Together, these data indicate that XIAP is required

for activation of Wnt target genes in cultured mammalian cells and that it likely functions downstream of β-catenin stabilization.

XIAP is required for Wnt signaling in *Xenopus* embryos

To determine if XIAP is required for Wnt signaling in a developing organism, we investigated its role in dorsal-anterior structure formation in *Xenopus laevis* embryos; a process that is critically regulated by Wnt signaling (Heasman, 2006). Consistent with Wnt pathway inhibition, knockdown of XIAP by dorsal injection of two independent XIAP morpholinos inhibited dorsal-anterior structure formation and resulted in severely ventralized embryos (Figure 4.5A).

To more specifically examine whether the ventralized phenotype we observed was due to Wnt pathway inhibition, we tested the effect of XIAP knockdown on *Xwnt8*-induced secondary axes. There was a significant reduction in the number of secondary axes when *Xwnt8* mRNA was co-injected ventrally with XIAP morpholino versus control morpholino (Figure 4.5B). Additionally, co-injection of XIAP morpholino with *Xwnt8* mRNA into *Xenopus* animal caps significantly reduced expression of the *Xwnt8* target genes *Xnr3* and *Siamois*, further demonstrating a requirement for XIAP for Wnt signaling in *Xenopus* embryos (Figure 4.5C).

Ventral injection of *XIAP* mRNA induced partial secondary axes, consistent with Wnt pathway activation (McMahon and Moon, 1989). In contrast to the cultured mammalian cell results where overexpression of XIAP did not

observably affect Wnt signaling, overexpression of XIAP in *Xenopus* embryos was sufficient to ectopically activate the Wnt pathway (Figure 4.5D). Together, these results show that XIAP is required for Wnt signaling and dorsal axis formation during *Xenopus* development.

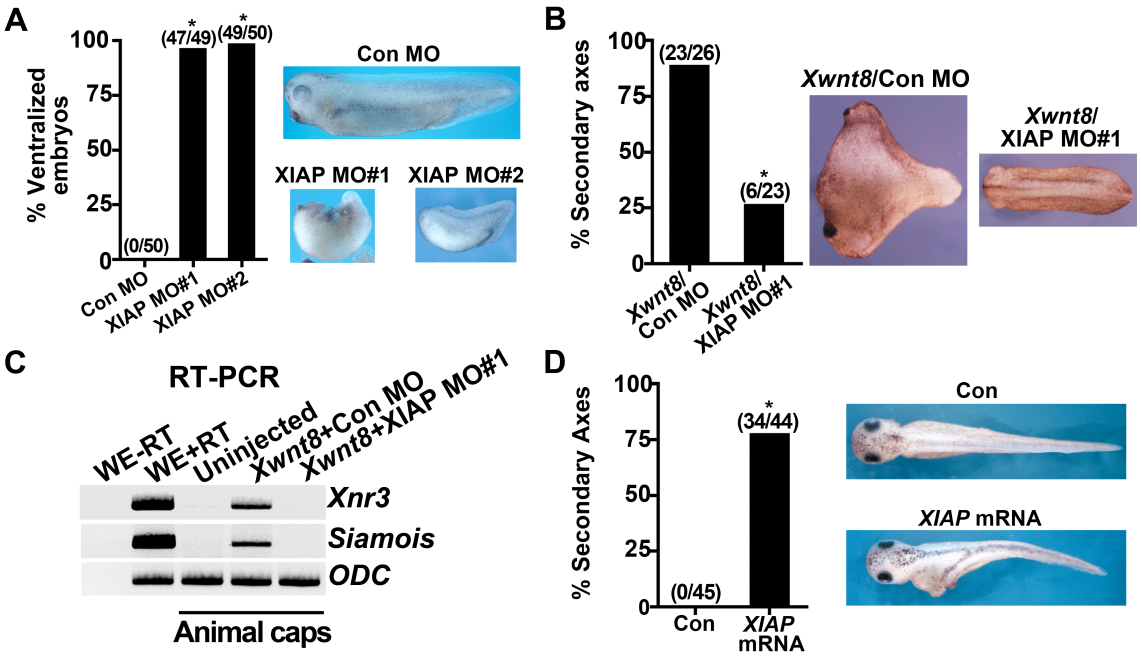


Figure 4.5. XIAP is required for Wnt signaling *in vivo*.
(A) XIAP is required in *Xenopus* embryos for dorsal-anterior patterning. Embryos (4-cell stage) were injected dorsally with control (Con MO) or two independent XIAP morpholinos (XIAP MO#1 and #2) (25 ng) and dorsal-anterior index (DAI) determined (Kao and Elinson, 1988). The percentage of ventralized embryos (DAI <= 2) is graphed on the left (absolute numbers above bars) with representative embryos on the right. *p-value < 0.0001 versus Con MO.
(B) Downregulating XIAP by morpholino injection inhibits Xwnt8-induced secondary axis formation. Embryos (4-cell stage) were co-injected ventrally with *Xwnt8* mRNA (0.7 pg) plus control or XIAP morpholino (25 ng). The percentage of embryos with secondary axis formation is graphed on the left (absolute numbers above bars) with representative embryos on the right. *p-value < 0.0001 versus *Xwnt8*/Con MO.
(C) XIAP is required for Xwnt8-induced expression of Wnt target genes in *Xenopus* ectodermal explants. Total RNA was extracted from animal caps co-injected with *Xwnt8* mRNA (0.7 pg) and control or XIAP morpholino (25 ng) and *Siamois* and *Xnr3* gene expression assayed by RT-PCR. WE = whole embryo control. RT = reverse transcriptase. *Ornithine decarboxylase* (*ODC*) is loading control.
(D) XIAP induces secondary axis formation. Embryos (4-cell stage) were injected ventrally with control (Con) or *XIAP* mRNA (2 ng) and allowed to develop. The percentage of embryos with secondary axis formation is graphed on the left (absolute numbers above bars) with representative embryos on the right. *p-value < 0.0001 versus Con.

XIAP binds and ubiquitylates Groucho/TLE

Having demonstrated that XIAP is required for Wnt signaling across phyla, our next aim was to elucidate the molecular mechanism of its function in the Wnt pathway. Based on our cultured mammalian cell studies, it is likely that XIAP acts in the Wnt pathway downstream of the destruction complex. Thus, we took a candidate approach and performed co-immunoprecipitation experiments with tagged XIAP and tagged versions of a majority of the known nuclear Wnt pathway components. Of all of the proteins screened, only the transcriptional co-repressor Transducin-like enhancer of split 3 (TLE3, the mammalian homolog of *Drosophila* Groucho) co-immunoprecipitated with XIAP (Figure 4.6A and data not shown). The interaction between XIAP and TLE3 was confirmed by demonstrating that endogenous XIAP co-immunoprecipitated with endogenous TLE3 in the absence and presence of Wnt stimulation (Figure 4.6B).

These results prompted us to ask where XIAP and TLE3 interact in the cell. XIAP is thought to be primarily a cytoplasmic protein, although it can be translocated to the nucleus under certain conditions (Liston et al., 2001; Russell et al., 2008), while TLE3 is found almost exclusively in the nucleus. We analyzed the subcellular localization of XIAP and TLE3 in the absence and presence of Wnt signaling and detected no observable changes in the localization of either protein upon Wnt stimulation (Figure 4.7A and data not shown). Notably, although the majority of XIAP is found in the cytoplasm, there is an observable

nuclear pool of endogenous XIAP (in the presence and absence of Wnt stimulation), which can be dramatically enhanced by overexpression of TLE3 (Figure 4.7B), indicating these two proteins can co-localize in the nucleus and that TLE3 levels can alter the population of XIAP in the nucleus.

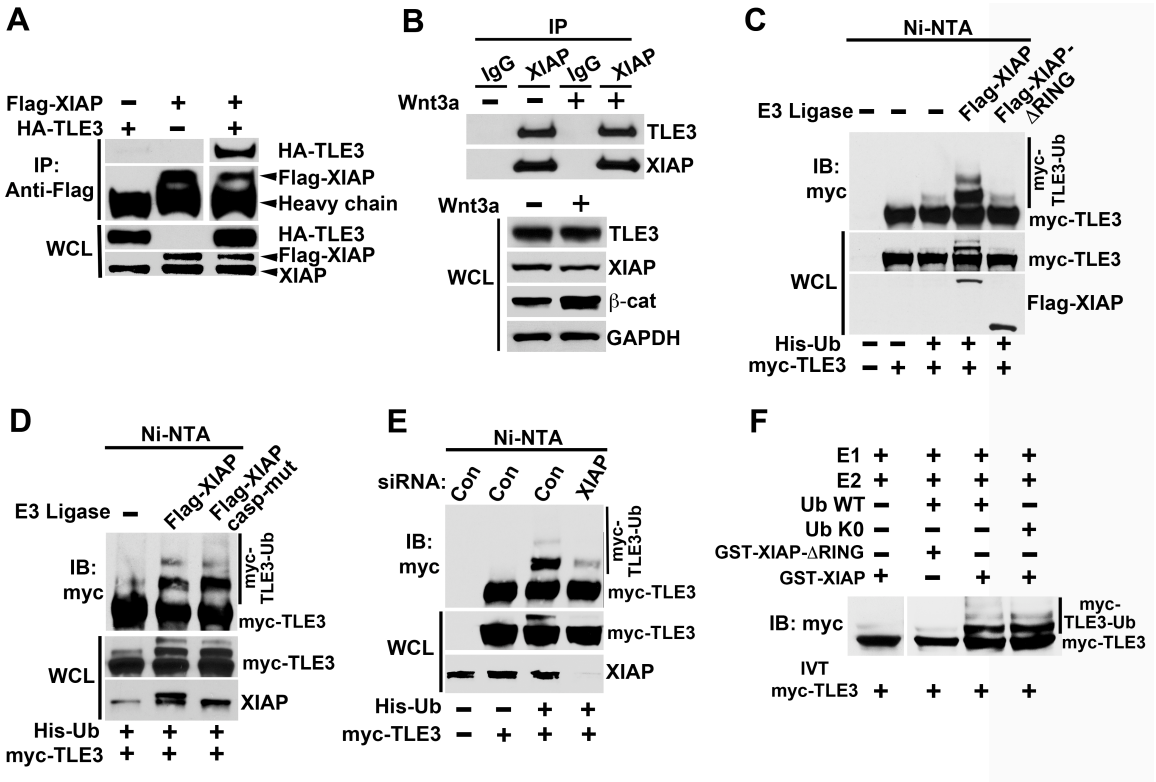


Figure 4.6. XIAP binds and ubiquitylates Groucho/TLE.
(A and B) XIAP interacts with Groucho/TLE3. (A) Tagged XIAP co-immunoprecipitates with tagged Groucho/TLE. HEK293 cells were transfected as indicated with Flag-XIAP and HA-TLE3 and treated with Wnt3a-CM. Cells were lysed and XIAP immunoprecipitated with anti-Flag antibody. Co-immunoprecipitated TLE3 was detected by anti-HA antibody. (B) Endogenous XIAP co-immunoprecipitates with endogenous TLE3. HEK293 cells were treated with L-CM or Wnt3a-CM for 3 hrs, lysed, and immunoprecipitated with anti-XIAP antibody. Co-immunoprecipitated proteins were detected by immunoblotting.
(C, D, and E) His-ubiquitylation assays. HEK293 cells were transfected as indicated, lysed under denaturing conditions, and His-Ub modified proteins isolated by nickel affinity purification. XIAP and TLE were detected by immunoblotting with anti-myc and anti-Flag antibodies, respectively. WCL = whole cell lysates. IB = immunoblot. (C) XIAP ubiquitylates Groucho/TLE3 in cultured mammalian cells. (D) XIAP ubiquitylates Groucho/TLE independent of its anti-apoptotic function. Flag-XIAP casp-mut is a mutant form of XIAP that cannot bind and inhibit caspases. (E) Knockdown of XIAP inhibits Groucho/TLE ubiquitylation.
(F) XIAP ubiquitylates Groucho/TLE *in vitro*. *In vitro*-translated myc-TLE3 was incubated in an *in vitro* ubiquitylation assay with recombinant proteins as indicated and visualized by immunoblotting with anti-myc antibody. Ub KO is a mutant form of ubiquitin in which all seven lysines have been mutated to arginines, allowing only for mono-ubiquitylation of substrates.

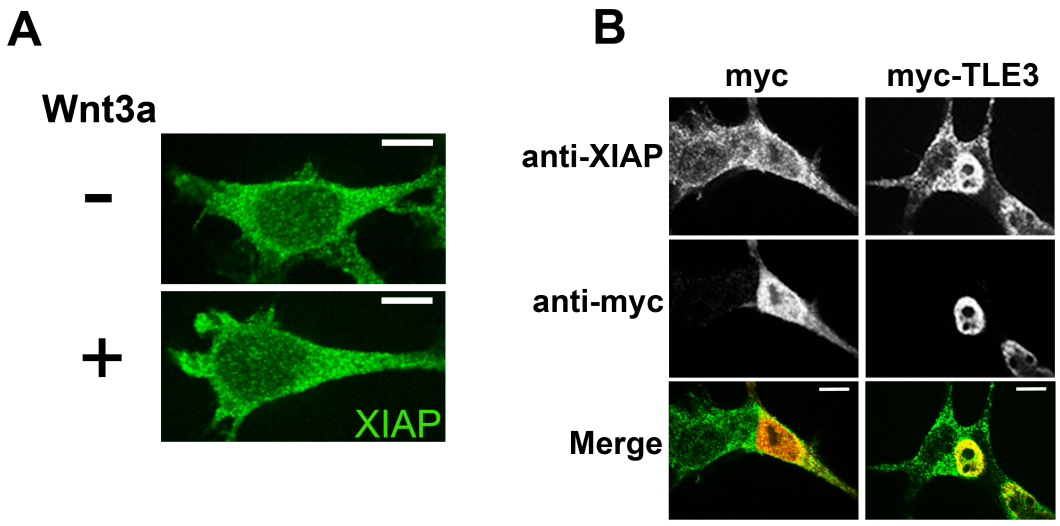


Figure 4.7. TLE3 promotes XIAP nuclear localization.
(A) Wnt stimulation does not alter nuclear localization of XIAP. HEK293 cells were treated with L-CM or Wnt3a-CM, fixed, and immunostained for XIAP. For each condition, greater than 300 cells were assessed. No change in XIAP staining was detected for any cells. Scale bars, 5 μm.
(B) TLE3 promotes nuclear localization of XIAP. HEK293 cells were transfected with myc or myc-TLE3 as indicated and stained for endogenous XIAP and myc. Greater than 300 cells were scored for each condition. All cells expressing myc-TLE3 showed enhanced XIAP nuclear staining. In contrast, no enhanced nuclear XIAP staining was detected in the myc control cells. Scale bars, 5 μm.

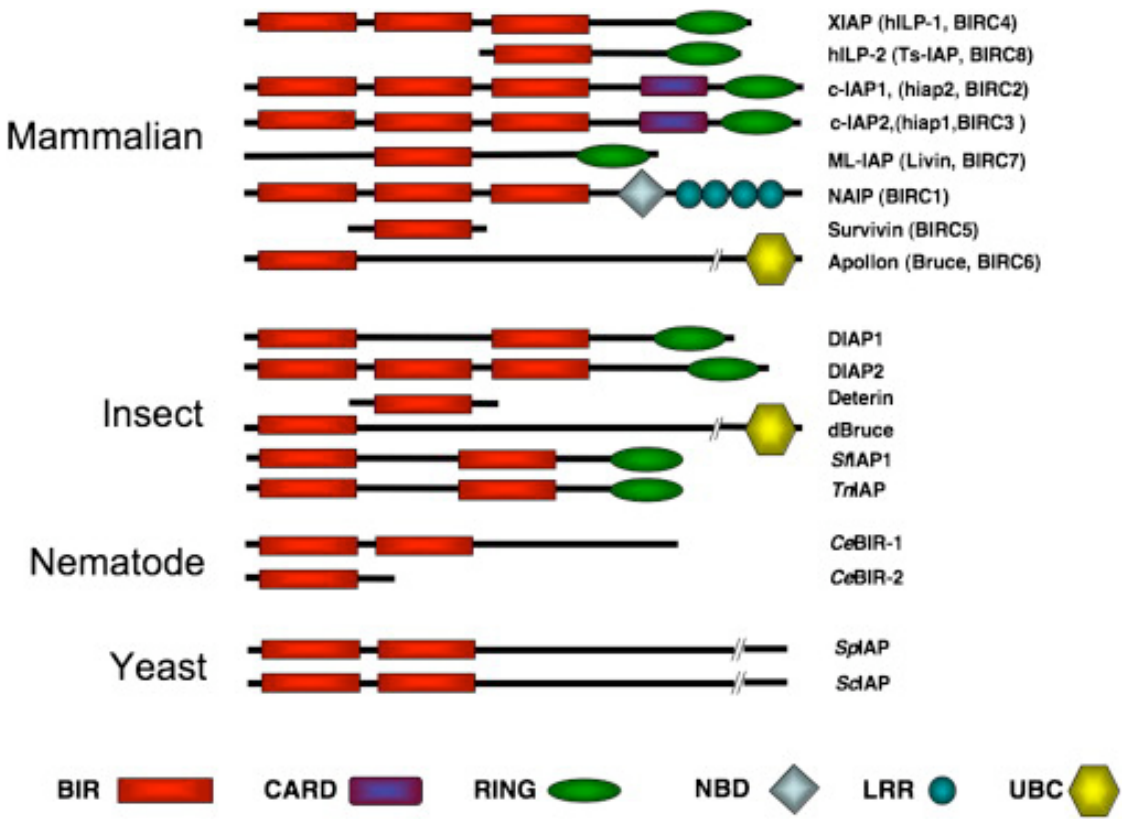


Figure 4.8. Domain Structure of the IAP Protein Family.
The characteristic BIR domains are indicated by red rectangles, CARD domains by purple rectangles, RING domains by green ovals, NBD domains by diamonds, LRR domains by teal circles, and UBC domains (conserved domains found in E2 ubiquitin-conjugating enzymes) by yellow hexagons. Diap1, Diap2, Deterin, and dBruce are *Drosophila* IAPs, while *Sf*IAP1 and *Tn*IAP are lepidopteran IAPs. IAP, inhibitor of apoptosis; XIAP, X-linked IAP; BIRC, baculoviral IAP repeat containing; hILP, human IAP-like protein; Ts-IAP, testis-specific IAP; c-IAP, cellular IAP; ML-IAP, melanoma-IAP; NAIP, neuronal apoptosis inhibitory protein; DIAP, *Drosophila* IAP; *Sf*IAP1, *Spodoptera frugiperda* IAP; *Tn*IAP, *Trichoplusia ni* IAP; *Ce*BIR-1,-2, *Caenorhabditis elegans* BIRC; *Sp*IAP, *Schizosaccharomyces pombe* IAP; *Sc*IAP, *Saccharomyces cerevisiae* IAP; BIR, baculoviral IAP repeat; CARD, caspase recruitment domain; NBD, nucleotide binding oligomerization domain; LRR, leucine rich repeat. Figure from (O'Riordan et al., 2008).

XIAP is a member of the highly conserved Inhibitor of Apoptosis (IAP) protein family (Figure 4.8). The IAP family was originally named after the discovery of the first family member, which was shown to be an inhibitor of apoptosis in baculovirus in 1994 (O'Riordan et al., 2008). For many years the IAP proteins have thus been studied primarily in terms of their anti-apoptotic functions, but it is becoming clear that IAPs have diverse roles in the cell in addition to inhibiting apoptosis (Galban and Duckett, 2010; O'Riordan et al., 2008; Srinivasula and Ashwell, 2008). Members of the IAP protein family contain two classic structural features: the baculovirus IAP repeat (BIR) and the Really Interesing New Gene (RING) domain. The BIR domain is the defining feature of IAP family members, with IAPs containing between one and three BIRs (Figure 4.8). It was originally thought that the BIR domains function to inhibit cell death by directly binding and inhibiting caspases, but it is now known that XIAP is the only family member that inhibits apoptosis through direct binding and inhibition of effector caspases 3, 7, and 9. The other IAP family members, of which there are seven in humans, are thought to bind caspases via their BIR domains, but are unable to directly inhibit caspase enzymatic activity (O'Riordan et al., 2008; Srinivasula and Ashwell, 2008).

In addition to the classic BIR domain, many IAPs contain a C-terminal RING domain. For many years the RING domain of IAPs was ignored because the function of RING domains was not known. Thus, most work tended to focus on the anti-apoptotic effects of IAPs via their BIR domains. It has since been

discovered that RING domains confer E3 ligase activity to their cognate proteins and, consequently, a variety of apoptotic and non-apoptotic functions for the RING domains of IAPs have now been uncovered (O'Riordan et al., 2008; Srinivasula and Ashwell, 2008). The most well characterized RING-containing human IAP is XIAP, which contains three BIR domains and a C-terminal RING domain (Figure 4.8) (O'Riordan et al., 2008). For years XIAP was studied primarily as an inhibitor of apoptosis via its BIR domains, but has now been shown to be involved in diverse cellular processes, many of which require its RING domain (Galban and Duckett, 2010). The RING domain of XIAP is even thought to be required for its anti-apoptotic activity via ubiquitylation and degradation of pro-apoptotic factors such as the XIAP inhibitor second mitochondria-derived activator of caspase (Smac) (MacFarlane et al., 2002). Additionally, it has been proposed that XIAP may ubiquitylate caspases, which does not lead to their proteasomal degradation, but rather, inhibits their enzymatic activity (Ditzel et al., 2008). Aside from these apoptotic functions, roles for XIAP and its RING domain have been identified in NF-κB and TGFβ signaling, innate immunity, cell division, and copper homeostasis (Galban and Duckett, 2010). Aside from Copper metabolism (Murr1) domain containing 1 (COMMD1), which is ubiquitylated by XIAP and subsequently degraded (Burstein et al., 2004), no other true “targets” for XIAP-mediated ubiquitylation in these non-apoptotic roles have been discovered.

Given the emerging role of XIAP and its RING domain in non-apoptotic functions, we sought to determine if XIAP is functioning as an E3 ligase for Gro/TLE in the Wnt pathway via its RING domain using a previously described assay (Salghetti et al., 1999). Cells were transfected with myc-TLE3, His-Ubiquitin, and either Flag-XIAP or Flag-XIAP-ΔRING. His-ubiquitylated proteins were isolated under denaturing conditions by nickel affinity purification, and myc-TLE3 was detected by immunoblotting. In the absence of XIAP, a band corresponding to the mono-ubiquitylated species of TLE3 was detected that is significantly enhanced when full-length XIAP, but not XIAP-ΔRING, was overexpressed, indicating the RING domain of XIAP is required for the enhanced ubiquitylation of TLE3 by XIAP (Figure 4.6C). Higher molecular weight species of TLE3 were also present in the whole cell lysate in the presence of full-length XIAP, but not XIAP-ΔRING, further supporting the His-Ubiquitin pull-down results.

To investigate whether the anti-apoptotic activity of XIAP is required for ubiquitylation of TLE3, we overexpressed a previously characterized form of XIAP with point mutations in each of its three BIR domains that abolish its capacity to bind and inactivate caspases (Lewis et al., 2004). Overexpression of this mutant form of XIAP enhanced the ubiquitylation of TLE3 to a similar extent as wild-type XIAP, indicating that the anti-apoptotic functions of XIAP are not required for its capacity to ubiquitylate TLE3 (Figure 4.6D). Overexpression of Second mitochondria-derived activator of caspases (Smac), a small peptide that binds to the BIR2 and BIR3 domains of XIAP and inhibits its anti-apoptotic

activity (Huang et al., 2003), similarly had no effect on the capacity of XIAP to ubiquitylate TLE3 (Figure 4.9). Based on these results, we conclude that the capacity of XIAP to enhance TLE3 ubiquitylation requires its C-terminal RING domain but is independent of its anti-apoptotic functions.

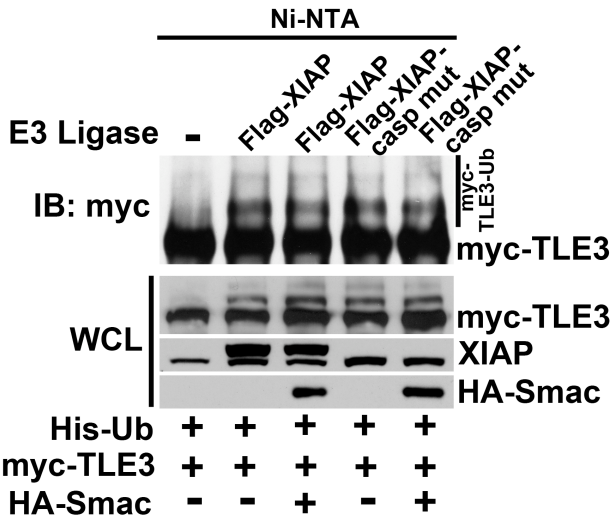


Figure 4.9. Overexpression of Smac does not inhibit XIAP-mediated Groucho/TLE ubiquitylation.
HEK293 cells were transfected as indicated and His-ubiquitylated proteins were isolated by nickel affinity purification.

XIAP has two closely related homologs, cellular Inhibitor of Apoptosis 1 and 2 (cIAP1 and cIAP2) (Figure 4.8). In some cases cIAP1 and cIAP2 have redundant activity with XIAP and have been shown to be capable of compensating for loss of XIAP (Srinivasula 2008). To determine if c-IAP1 or c-IAP2 are also capable of ubiquitylating TLE3, we performed an ubiquitylation assay in cells using tagged versions of both proteins. We found that, in contrast

to XIAP, neither c-IAP1 or c-IAP2 have the capacity to promote the ubiquitylation of TLE3 (Figure 4.10). This finding suggests that ubiquitylation of TLE3 is not a general property of IAP family members, but is specific to XIAP.

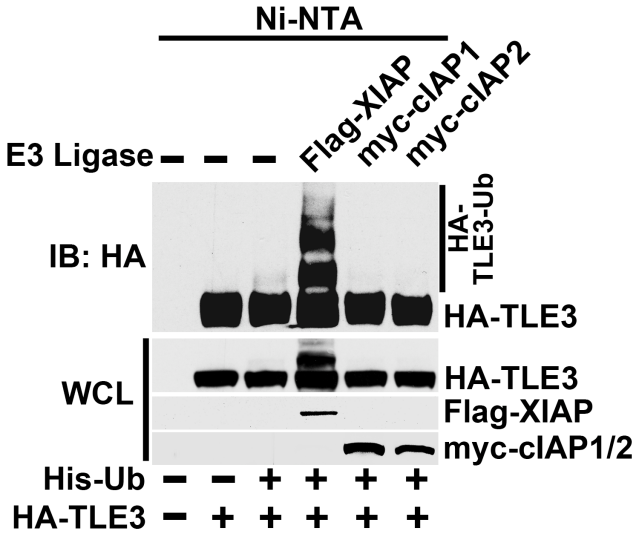


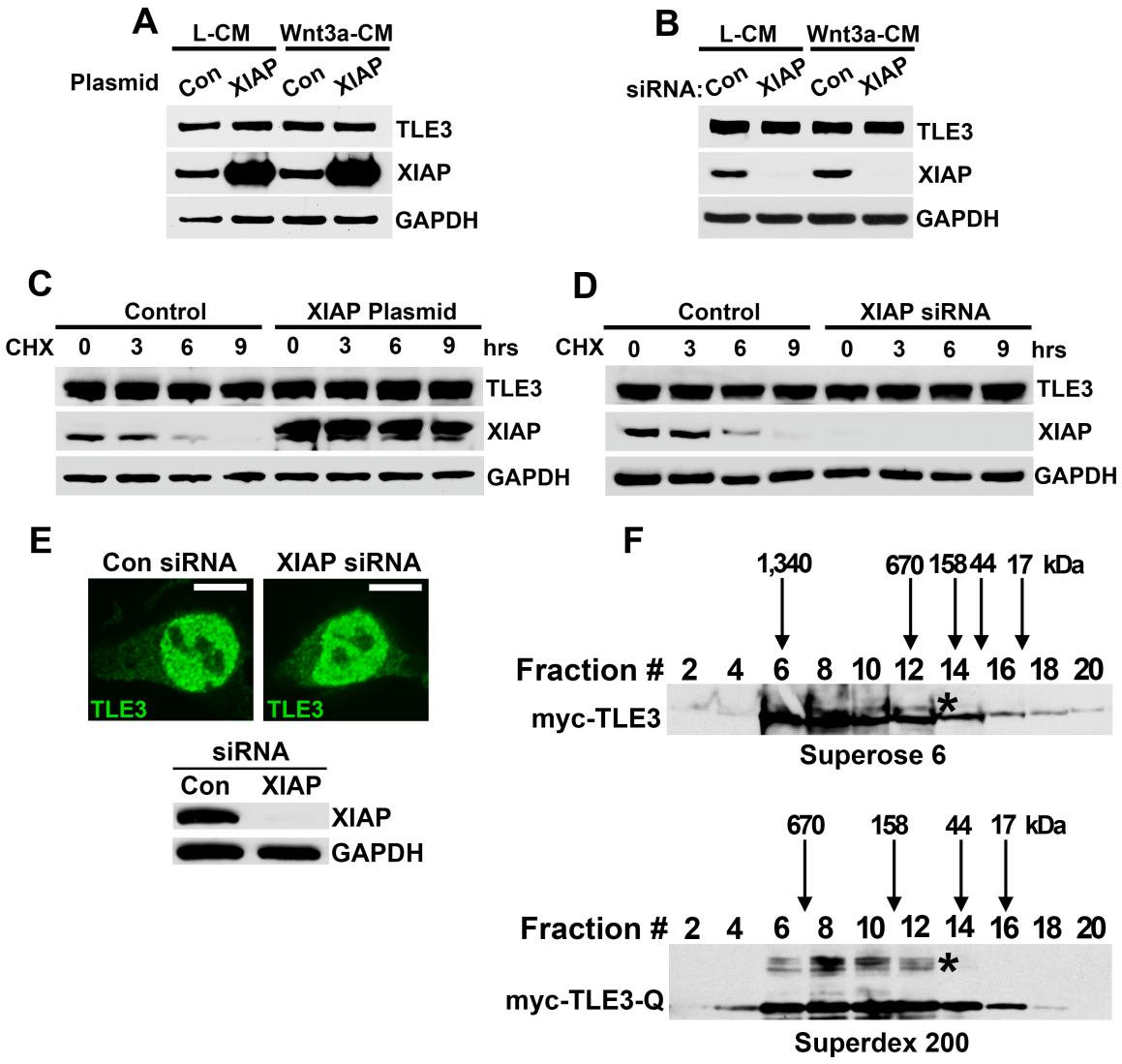
Figure 4.10. Overexpression of cIAP1 or cIAP2 fails to promote Groucho/TLE3 ubiquitylation.
HEK293 cells were transfected as indicated, lysed under denaturing conditions, and His-Ub modified proteins isolated by nickel affinity purification. TLE3 was detected by immunoblotting with anti-HA antibodies. XIAP, cIAP1, and cIAP2 were detected by immunoblotting with anti-Flag and anti-myc antibodies, respectively.

To investigate whether endogenous XIAP is required for TLE3 ubiquitylation, we knocked down XIAP in HEK293 cells using siRNA and performed an ubiquitylation assay using His-Ubiquitin and myc-TLE3 as described above. Our results show that the ubiquitylation of TLE3 is significantly decreased when XIAP is knocked down, indicating that endogenous XIAP is likely required for ubiquitylation of TLE3 in cells (Figure 4.6E).

To more directly determine if XIAP can bind and ubiquitylate TLE3, we performed an *in vitro* ubiquitylation assay using recombinant XIAP (Figure 4.6F). We find that recombinant XIAP, but not XIAP-ΔRING, is capable of ubiquitylating TLE3 *in vitro*. The addition of wild-type ubiquitin and KO ubiquitin (ubiquitin in which all lysines have been mutated to arginine, making this mutant incapable of forming polyubiquitin chains) resulted in identical patterns of ubiquitylated TLE3 species as detected by immunoblotting. In contrast, ubiquitylated TLE3 species were not detected in the absence of added ubiquitin. The XIAP-mediated *in vitro* ubiquitylation pattern of TLE3 is essentially identical to the ubiquitylation pattern of TLE3 we observed when XIAP is overexpressed in cultured cells (Figure 4.6C). These results suggest that XIAP has the capacity to directly ubiquitylate TLE3 via its RING domain and that it likely conjugates multiple monoubiquitin moieties onto TLE3 (a prominent single mono-ubiquitin moiety as well as a less-abundant second mono-ubiquitin moiety).

Ubiquitylation of Groucho/TLE does not affect its turnover, localization, or capacity to tetramerize

Having established that XIAP binds and ubiquitylates TLE3, we next asked how ubiquitylation of TLE3 might affect its function in the Wnt pathway. Ubiquitylation of proteins often serves as a signal for proteasomal degradation (Ravid and Hochstrasser, 2008). Thus, we sought to determine if gain or loss of XIAP has any effect on TLE3 levels. Neither overexpression nor siRNA knockdown of XIAP had any effect on the steady-state levels of TLE3 either in the presence or absence of Wnt stimulation (Figure 4.11A and B). Furthermore, overexpression and siRNA knockdown of XIAP had no effect on the turnover rate


A
L-CM
Wnt3a-CM
Plasmid
Con
XIAP
Con
XIAP
TLE3
XIAP
GAPDH
B
L-CM
Wnt3a-CM
siRNA:
Con
XIAP
Con
XIAP
TLE3
XIAP
GAPDH
C
Control
XIAP Plasmid
CHX
0 3 6 9 0 3 6 9 hrs
TLE3
XIAP
GAPDH
D
Control
XIAP siRNA
CHX
0 3 6 9 0 3 6 9 hrs
TLE3
XIAP
GAPDH
E
Con siRNA
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TLE3
TLE3
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Con
XIAP
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670
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17
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Fraction #
2 4 6 8 10 12 14 16 18 20
myc-TLE3
*
Superose 6
670
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44
17
kDa
Fraction #
2 4 6 8 10 12 14 16 18 20
myc-TLE3-Q
*
Superdex 200

Figure 4.11. Ubiquitylation of Groucho/TLE by XIAP does not affect its stability, nuclear localization, or capacity to tetramerize.
(A and B) Overexpression or knockdown of XIAP in cultured mammalian cells does not affect steady-state Groucho/TLE levels. HEK293 cells were transfected with vector control (Con) or XIAP expression plasmid (A) or with control (Con) or XIAP siRNA (B) as indicated, treated with L-CM or Wnt3a-CM, and immunoblotting performed.
(C and D) Overexpression or knockdown of XIAP does not affect the rate of Groucho/TLE turnover. HEK293 cells were transfected with vector control or XIAP expression plasmid (C) or with control or XIAP siRNA (D) followed by treatment with cyclohexamide (CHX). Cells were then harvested at the indicated time points for immunoblotting.
(E) Knockdown of XIAP does not affect Groucho/TLE nuclear localization. HEK293 cells were transfected with control (Con) or XIAP siRNA and immunostained for TLE3. No change in TLE3 localization was observed. Greater than 300 cells were scored per condition. Scale bars, 5 μm.
(F) Ubiquitylation of Groucho/TLE does not affect its capacity to tetramerize. *In vitro*-translated full-length TLE3 (Top) or TLE3-Q (Bottom) proteins (both myc-tagged) were ubiquitylated *in vitro* and resolved by gel filtration. Fractions were immunobloted with anti-myc antibody. Asterisks indicate ubiquitylated species. Elution profiles of protein standards are indicated by arrows. GADPH is loading control in (A-E).

of TLE3 (Figure 4.11C and D). The stabilization of XIAP seen in Figure 4.11C is likely due to overwhelming of the endogenous XIAP degradation machinery caused by the significant overexpression of XIAP. These results indicate that ubiquitylation of TLE3 by XIAP does not affect TLE3 stability.

Recent studies have shown a role for mono-ubiquitylation in regulating the subcellular localization of transcription factors (Dupont et al., 2009; Li et al., 2003; van der Horst et al., 2006). Thus, we examined whether siRNA knockdown of XIAP has any effect on the nuclear localization of TLE3. Prominent nuclear staining of TLE3 could be detected in both control and XIAP-siRNA cells (Figure

4.11E), indicating loss of XIAP does not cause a shift in the subcellular localization of TLE3. Overexpression of XIAP similarly does not result in a shift in the nuclear localization of TLE3 (data not shown).

TLE3 is a member of the highly conserved Gro/TLE family of proteins that function as transcriptional co-repressors in the Wnt pathway. Five isoforms of TLE proteins have been identified in humans (Gasperowicz and Otto, 2005). By performing the same ubiquitylation assay as described above (Figure 4.6C), we demonstrated that XIAP has the capacity to ubiquitylate all human TLE isoforms, including the truncated isoform Amino-terminal enhancer of split (AES) (Figure 4.12A). Because AES only contains the first 197 amino acids found in full-length TLE isoforms (including the N-terminal glutamine-rich (Q) and Glycine-Proline rich (GP) domains), this narrows the potential target sites of XIAP-mediated ubiquitylation to this region of the TLE proteins (Figure 4.12C). We further narrowed the potential XIAP target sites by demonstrating that recombinant XIAP can ubiquitylate recombinant *Drosophila* Groucho Q domain *in vitro* (Figure 4.12B). The Q domains of Gro/TLE proteins are highly conserved and contain eight lysines that are present in *Drosophila* Gro and all human TLE isoforms (Figure 4.12C and D). Thus, based on our results, XIAP likely targets one (or two) of the conserved lysines in the Q domain of the Gro/TLE proteins.

Mapping the potential XIAP-mediated ubiquitylation sites on Gro/TLE to the Q domain provided clues regarding the functional consequence of this modification. Gro/TLE proteins have been shown to homo-tetramerize through

their Q-domain, a property that is required for TCF/Lef binding and for mediating transcriptional repression (Gasperowicz and Otto, 2005; Jennings and Ish-Horowicz, 2008). Thus, we next tested if ubiquitylation of TLE3 by XIAP disrupts its capacity to tetramerize by assessing the hydrodynamic properties of *in vitro*-translated ubiquitylated and non-ubiquitylated species of TLE3. We analyzed the effect of XIAP-mediated ubiquitylation on both the N-terminal Q domain of TLE3 (TLE3-Q) and full-length TLE3 by performing gel-filtration analysis (Figure 4.11F). We found that the ubiquitylated forms of TLE3-Q and full-length TLE3 eluted in the same peak fractions as that of their non-ubiquitylated forms, indicating that ubiquitylation of TLE3 does not disrupt its capacity to tetramerize. The larger than expected apparent molecular weight of TLE3-Q and full-length TLE3 on gel filtration chromatography likely reflects an elongated tetrameric protein complex and has been previously reported (Kuo et al., 2010).

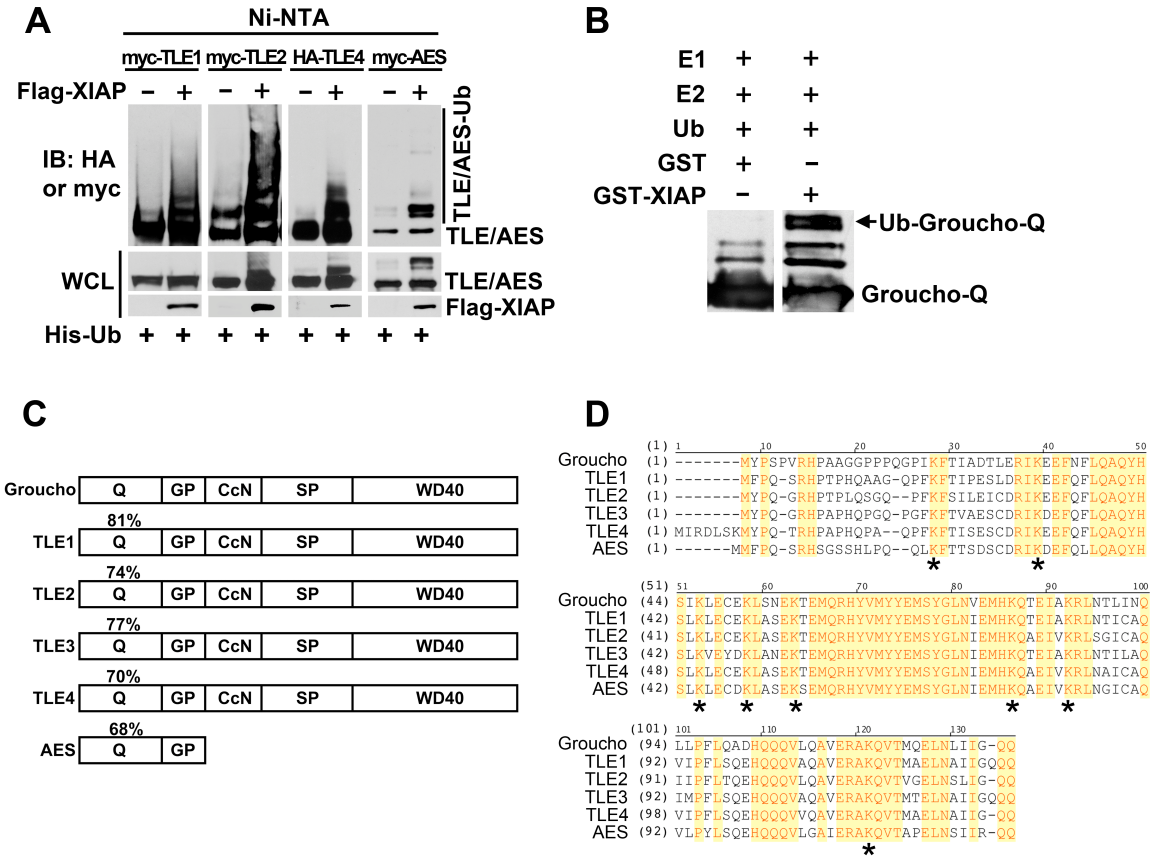


Figure 4.12. XIAP ubiquitylates all human TLE isoforms and *Drosophila* Groucho.

(A) XIAP ubiquitylates all human TLE isoforms. HEK293 cells were transfected as indicated, His-ubiquitylated proteins were isolated and analyzed by immunoblotting.

(B) XIAP ubiquitylates *Drosophila* Groucho Q domain. Recombinant SUMO-His_6-Groucho-Q was used in an *in vitro* XIAP ubiquitylation reaction.

(C) Cartoon of *Drosophila* Groucho and human TLE isoforms. Percentage identity of the Q-domains of human TLE/AES compared to the Q-domain of *Drosophila* Groucho is indicated.

(D) Amino acid sequence alignment of the *Drosophila* Groucho and human TLE Q domains is shown. Identical amino acids are shaded in yellow. Asterisks indicate conserved lysine residues.

Ubiquitylation of Groucho/TLE by XIAP disrupts its binding to TCF/Lef

Although we found that ubiquitylation of TLE3 did not interfere with its tetramerization in our hydrodynamic studies, we hypothesized ubiquitylation of TLE3 may interfere with its capacity to bind TCF/Lef as Gro/TLE interacts with TCF/Lef via its Q domain. To test this possibility, we ubiquitylated TLE3-Q and full-length TLE3 *in vitro* and assessed their capacity to bind TCF4. In contrast to their non-ubiquitylated forms, the ubiquitylated species of TLE3-Q and full-length TLE3 were not pulled down by recombinant GST-TCF4 protein, indicating that ubiquitylation of TLE3 inhibits its capacity to bind TCF4 (Figure 4.13A). To further confirm that TCF/Lef has reduced affinity for ubiquitylated Gro/TLE *in vivo*, myc-TLE3 and HA-TCF4 were transfected into cells (along with ubiquitin and XIAP to enhance TLE3 ubiquitylation) (Figure 4.13B). The presence of ubiquitylated or non-ubiquitylated TLE3 in HA-TCF4 immunoprecipitates was assessed by immunoblotting. In contrast to total cellular lysates, which contained a noticeable band representing ubiquitylated TLE3, only the non-ubiquitylated TLE3 co-immunprecipiated with TCF4, thereby providing further evidence that ubiquitylation of Gro/TLE inhibits its capacity to bind TCF/Lef.

These findings suggest that ubiquitylation of TLE3 by XIAP may be required during Wnt pathway activation to remove TLE3 from TCF/Lef, allowing subsequent β-catenin binding and transcriptional activation. To determine if XIAP might also bind TCF/Lef in the process of binding and ubiquitylating Gro/TLE, we performed an *in vitro* binding assay with recombinant XIAP and TCF4. We found

that XIAP is pulled down with TCF4, but not the control, indicating XIAP can directly bind TCF4 (Figure 4.13C). In contrast to Gro/TLE, however, we were unable to detect ubiquitylation of TCF4 by XIAP in an *in vitro* ubiquitylation assay (Figure 4.13D), indicating specificity of the E3 ubiquitin ligase activity of XIAP for Gro/TLE.

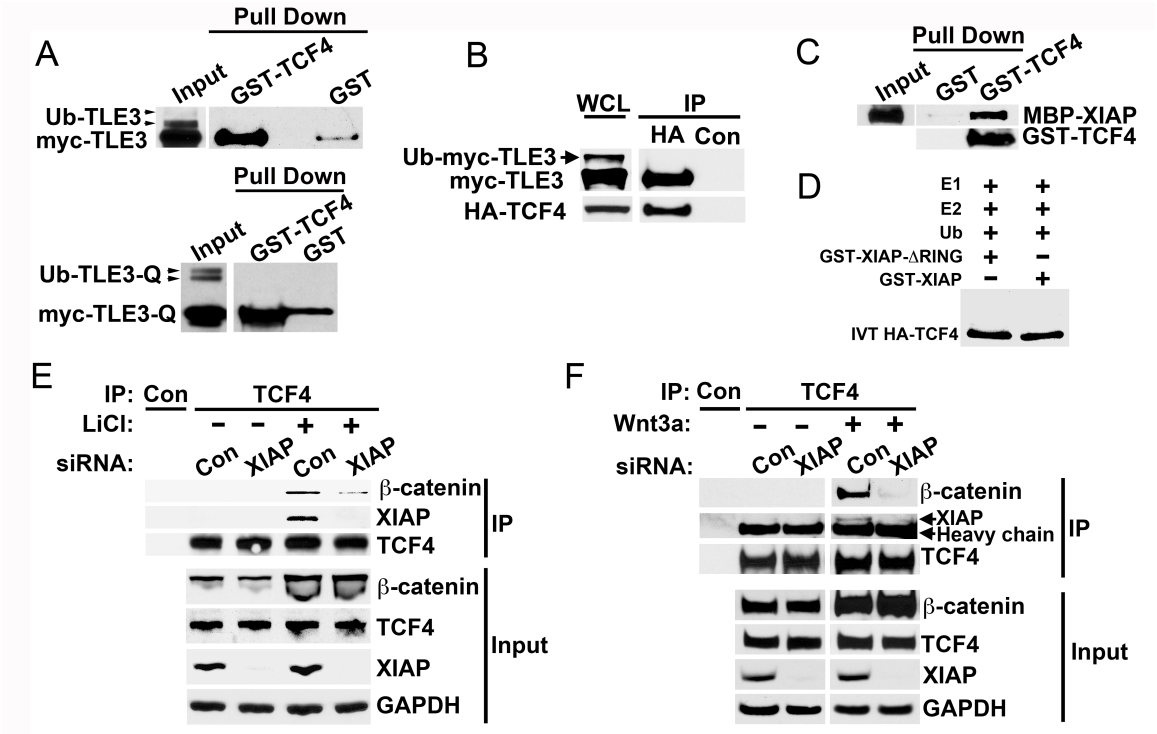


Figure 4.13. Ubiquitylation of Groucho/TLE by XIAP disrupts TCF/Lef binding.

(A) Ubiquitylation of Groucho/TLE disrupts TCF/Lef binding *in vitro*. *In vitro*-translated full-length myc-TLE3 (Top) or myc-TLE3-Q (Bottom) proteins were ubiquitylated *in vitro* and tested in an *in vitro* binding assay with recombinant GST-TCF4 or GST proteins immobilized on glutathione beads. myc-TLE3 was visualized by immunoblotting with anti-myc antibody. Arrowheads indicate ubiquitylated species.

(B) Ubiquitylation of Groucho/TLE disrupts TCF/Lef binding in cultured cells. HEK293 cells were transfected with Flag-XIAP, His-Ub, myc-TLE3 and HA-TCF4. HA-TCF4 was immunoprecipitated with anit-HA antibody and co-immunoprecipitated myc-TLE3 was detected by immunoblotting. IgG antibody (Con) was used as control.

(C) XIAP directly interacts with TCF4. An *in vitro* binding assay was performed using recombinant MBP-tagged XIAP and either recombinant GST-TCF4 or GST proteins immobilized on glutathione beads. XIAP was detected using an anti-XIAP antibody.

(D) XIAP fails to ubiquitylate TCF/Lef. An *in vitro* XIAP ubiquitylation assay was performed with *in vitro*-translated HA-TCF4 as the test substrate. In parallel, ubiquitylation of myc-TLE3 was observed (positive control, data not shown).

(E and F) XIAP is recruited to TCF/Lef and is required for efficient binding of TCF/Lef to β-catenin upon Wnt pathway activation. (E) HEK293 cells transfected with control (Con) or XIAP siRNA were treated with 30 mM LiCl for 3 hrs and endogenous TCF4 immunoprecipitated. Co-immunoprecipitated endogenous β-catenin and XIAP were detected by immunoblotting. (F) HEK293 cells were treated as in (E) except L-CM or Wnt3a-CM was added for 3 hrs.

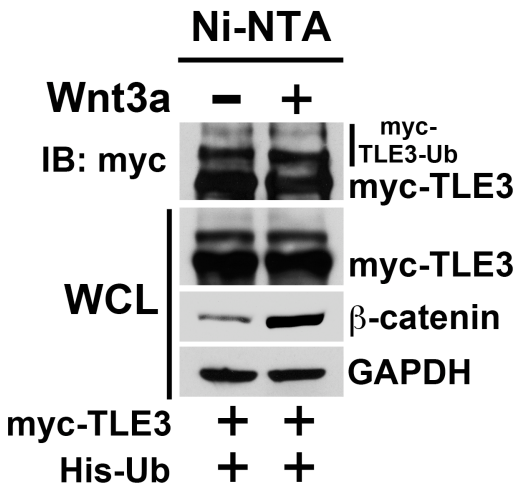


Figure 4.14. Wnt signaling does not increase TLE3 ubiquitylation.
HEK293 cells were transfected with myc-TLE3 and His-Ub and treated with L-CM or Wnt3a-CM. His-ubiquitylated proteins were isolated and analyzed by immunoblotting.

If XIAP ubiquitylates Gro/TLE in response to a Wnt signal in order to remove it from TCF/Lef, one might predict that ubiquitylation of Gro/TLE would increase upon Wnt signaling. We detected no change, however, in the degree of Gro/TLE ubiquitylation in response to Wnt stimulation (Figure 4.14). Because we also found that XIAP interacts with TLE3 in the absence or presence of Wnt signaling (Figure 4.6B), this would suggest that XIAP constitutively binds and ubiquitylates non-TCF-bound Gro/TLE in the nucleus. Thus, XIAP may regulate the nuclear pool of Gro/TLE that is available for binding TCF/Lef.

Given that XIAP binds TCF4, it is possible that XIAP may be recruited to the TCF/Lef transcriptional complex to specifically ubiquitylate TCF-bound

Gro/TLE upon Wnt pathway activation. To test whether XIAP is recruited to the TCF/Lef transcriptional complex in a Wnt-dependent manner, we performed co-immunoprecipitation assays in cells using an antibody against endogenous TCF4 (Figure 4.13E and F). In the absence of lithium (Figure 4.13E), XIAP was not detectable in TCF4 immunoprecipitates. Upon Wnt pathway stimulation by lithium, however, XIAP co-immunoprecipitated with TCF4. Wnt pathway activation normally leads to recruitment of β-catenin onto TCF/Lef. Accordingly, we observed endogenous β-catenin co-immunoprecipitating with TCF4 upon lithium treatment whereas no detectable β-catenin co-immunoprecipitated with TCF4 in the absence of lithium treatment. Significantly, knockdown of XIAP by siRNA reduced the amount of β-catenin that co-immunoprecipitated with TCF4 in the presence of lithium. These results were confirmed using Wnt3a-conditioned media, suggesting this is not due to global inhibition of GSK3 activity (Figure 4.13F). These data indicate XIAP is recruited to TCF4 transcriptional complexes in response to Wnt pathway activation and that XIAP is required for efficient recruitment of β–catenin to TCF4 transcriptional complexes.

Together, our data suggest a model (Figure 4.15) in which XIAP constitutively binds and ubiquitylates non-TCF-bound Gro/TLE in the nucleus, thereby limiting the amount of Gro/TLE available to form co-repressor complexes with TCF/Lef. In the presence of a Wnt signal, XIAP is recruited to TCF/Lef transcriptional complexes where it ubiquitylates Gro/TLE. Ubiquitylation of Gro/TLE decreases its affinity for TCF/Lef and allows for the efficient recruitment

and binding of the transcriptional co-activator β-catenin to TCF/Lef in order to initiate a Wnt-specific transcriptional program.

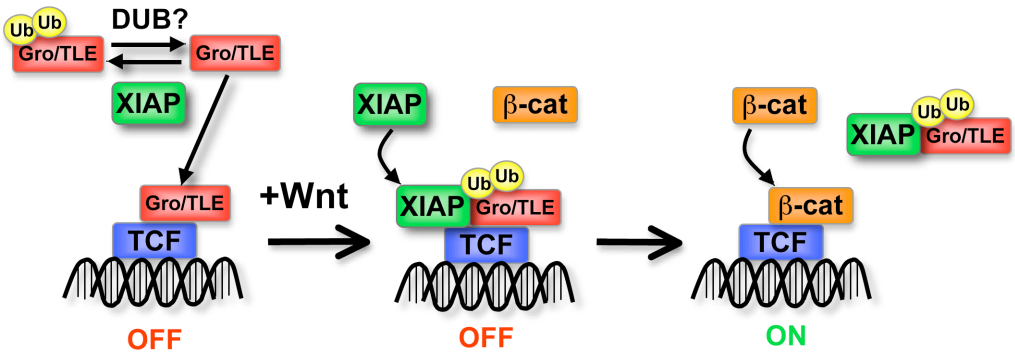


Figure 4.15. Model of XIAP-mediated regulation of Groucho/TLE in the Wnt pathway.
See text for details.

Summary

A key event in Wnt signaling is conversion of TCF/Lef from a transcriptional repressor to an activator, yet how this switch occurs is not well understood. Here, we report an unanticipated role for X-linked Inhibitor of Apoptosis (XIAP) in regulating this critical Wnt signaling event that is independent of its anti-apoptotic function. We identified DIAP1 as a positive regulator of Wingless signaling in a *Drosophila* S2 cell-based RNAi screen. XIAP, its vertebrate homolog, is similarly required for Wnt signaling in cultured mammalian cells and in *Xenopus* embryos, indicating evolutionary conservation of function. Upon Wnt pathway activation, XIAP is recruited to TCF/Lef where it mono-ubiquitylates Groucho/TLE: this

modification decreases the affinity of Groucho/TLE for TCF/Lef. Our data reveal a transcriptional switch involving XIAP-mediated ubiquitylation of Groucho/TLE that facilitates its removal from TCF/Lef, thus allowing assembly of β-catenin-TCF/Lef complexes and initiation of a Wnt-specific transcriptional program.

CHAPTER V

THE DUB USP47 IS REQUIRED FOR WNT SIGNALING

Introduction

The canonical Wnt signaling pathway regulates many fundamental processes during metazoan development and is critical for tissue homeostasis in the adult (Logan and Nusse, 2004; MacDonald et al., 2009). A key event in Wnt signal transduction is the stabilization of the cytoplasmic protein β-catenin. In the absence of a Wnt ligand, a β-catenin destruction complex, composed of Axin, glycogen synthase kinase 3 (GSK3), casein kinase I α (CKIα), and the tumor suppressor adenomatous polyposis coli (APC), promotes phosphorylation of β-catenin, targeting it for ubiquitin-mediated proteasomal degradation. Binding of a Wnt ligand to its two cell-surface receptors, Frizzled (Fz) and LDL receptor-related protein 5/6 (LRP5/6), results in inhibition of β-catenin phosophorylation and, thus, stabillzation of β-catenin. Stabilized β-catenin translocates to the nucleus where it binds to TCF/Lef to activate a Wnt-specific transcriptional program.

The Wnt pathway is heavily regulated by ubiquitylation (Tauriello and Maurice, 2010). At the time I began these studies, however, only the E3 ligases for β-catenin (Jiang and Struhl, 1998; Marikawa and Elinson, 1998) and Dishevelled (Angers et al., 2006) had been identified, and no deubiquitylases

(DUBs) in the Wnt pathway had been reported. Thus, I sought to identify novel DUBs involved in Wnt signaling to gain a better understanding of how the ubiquitin system regulates this pathway.

Here, I performed a targeted RNAi screen in *Drosophila* S2 cells to identify novel DUBs involved in Wingless (Wg, the *Drosophila* homolog of Wnt) signal transduction that led to the identification of Ubiquitin-specific protease 64 E (Ubp64E) as a critical Wg pathway component. I demonstrate that the human homolog, Ubiquitin Specific Protease 47 (USP47), is similarly required for Wnt signaling in cultured human cells and is required for primary body axis formation in *Xenopus* embryos, indicating evolutionary conservation of function. At the molecular level, I show that USP47 interacts with two Wnt pathway E3 ligases, β-TRCP and XIAP, although this interaction does not affect the stability of β-TRCP or XIAP. Together, these studies identify USP47 as a novel DUB involved in the regulation of Wnt signal transduction and provide insight into its potential mechanism of action.

Results

Drosophila RNAi screen identifies the DUB Ubp64E as a novel Wingless signaling component.

To identify novel de-ubiquitylases (DUBs) involved in Wingless (Wg) signaling, I performed a genome-scale RNAi-based screen targeting DUBs in

Drosophila S2 cells (Figure 5.1A, see Chapter II for details). Briefly, plasmids encoding experimentally verified and predicted DUBs (29 clones total) were obtained from the *Drosophila* Gene Collection Release 1 and 2, and a PCR approach was used to generate linear cDNA products suitable for *in vitro* dsRNA synthesis. For the screen, dsRNA was added to a *Drosophila* S2R+ reporter cell line stably transfected with the Wg responsive TOPflash luciferase reporter (Korinek et al., 1997).

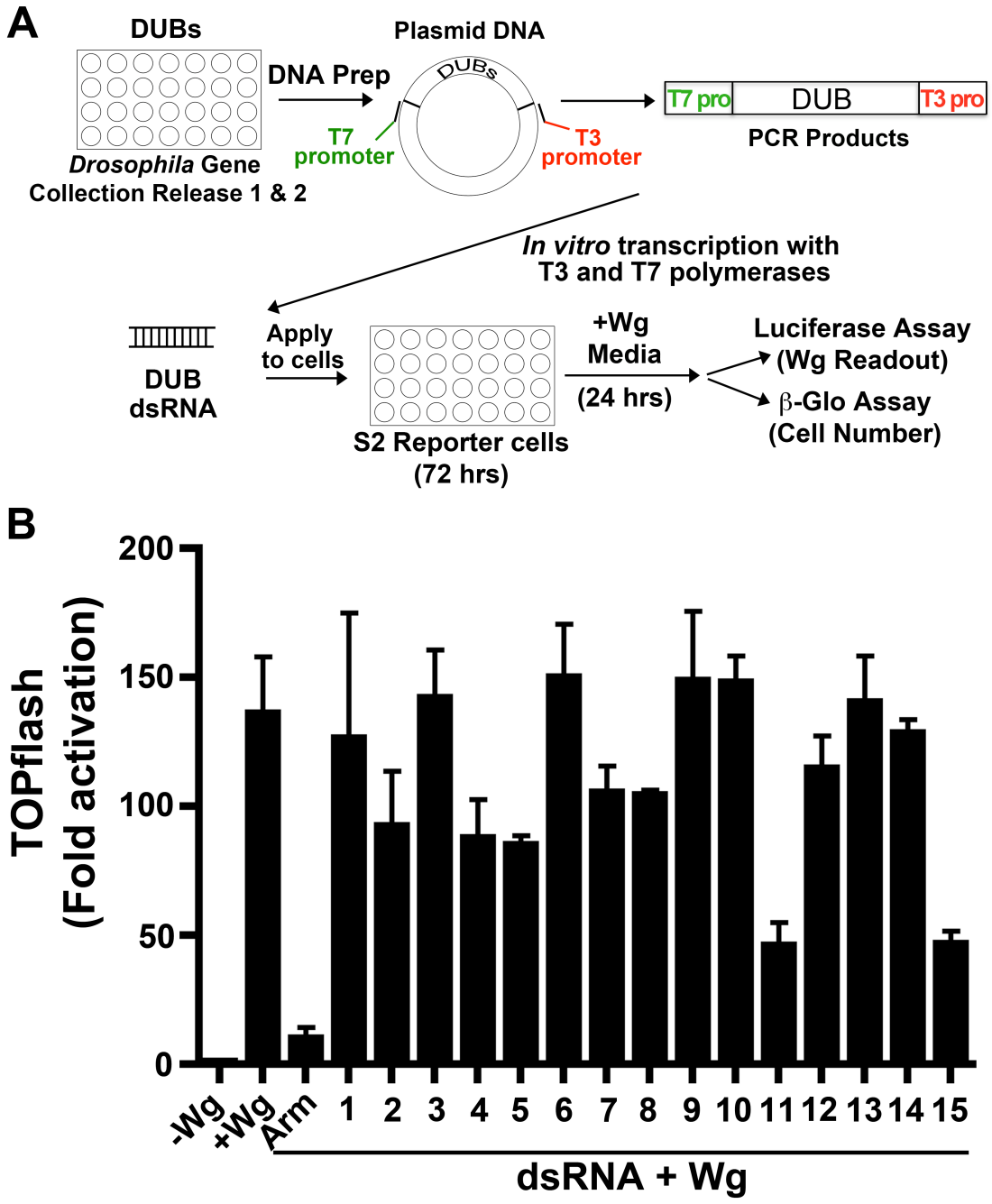

A
DUBs
Plasmid DNA
DNA Prep
DUBs
T7 promoter
T3 promoter
T7 pro
DUB
T3 pro
PCR Products
Drosophila Gene Collection Release 1 & 2
In vitro transcription with T3 and T7 polymerases
DUB dsRNA
Apply to cells
+Wg Media
(24 hrs)
Luciferase Assay (Wg Readout)
β-Glo Assay (Cell Number)
S2 Reporter cells (72 hrs)
B
TOPflash (Fold activation)
200
150
100
50
0
-Wg
+Wg
Arm
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
dsRNA + Wg

Figure 5.1. Drosophila RNAi screen identifies the DUB Ubp64E as a novel Wingless signaling component.
(A) Schematic of RNAi screen to identify DUBs that regulate Wingless signaling in *Drosophila* S2 cells (see text for more details).
(B) Results of RNAi screen. Graph represents mean ± standard deviation (SD) of TOPflash normalized to cell number. Axin and Armadillo (Arm) dsRNA treatments were performed as controls. Results for Ubp64E dsRNA (15) and 12 additional dsRNAs included in the screen are shown. Results are representative of at least three experiments performed in quadruplicate.

Axin (a potent negative regulator of Wg signaling) and Armadillo (Arm, the *Drosophila* β-catenin homolog and potent positive regulator of Wg signaling) served as controls to confirm effective knockdown by dsRNA treatment in the screen Of the 29 DUBs screened, the knockdown of two, no. 11 and no. 15, were particularly potent in inhibiting Wg signaling (Figure 5.1B). dsRNA no. 11 targets Cylandromatosis (CYLD), a DUB that had been reported as a Wnt signaling regulator at the "Wnt Singaling in Development and Disease Meeting" (Berlin, Germany, 2007). Thus, I decided to focus on the other DUB, no. 15, which encodes Ubiquitin-specific protease 64E (Ubp64E), a putative DUB that had no known function at the time I began this work.

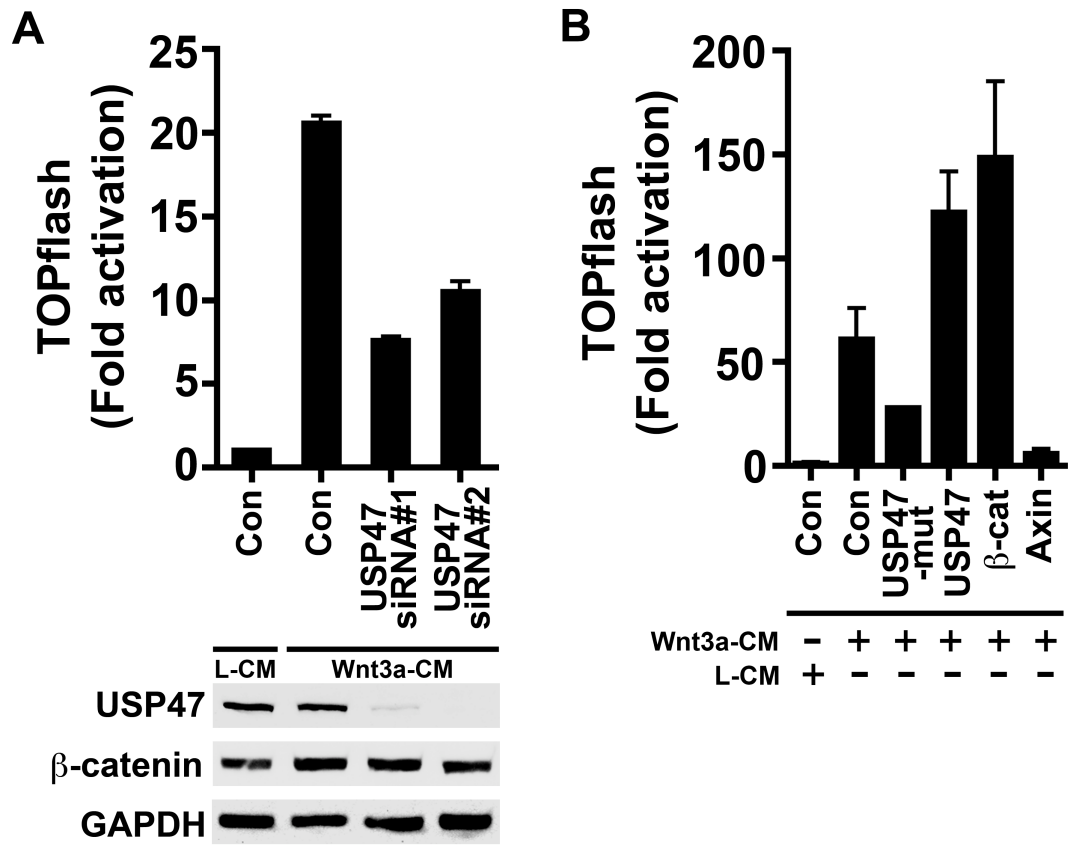


Figure 5.2. USP47 is required for Wnt signaling in cultured mammalian cells.

(A) Knockdown of USP47 by siRNA inhibits Wnt signaling. HEK293 STF (STF293) cells were transfected with non-targeting control (Con) or two independent USP47 siRNAs and treated with L cell (L-CM) or Wnt3a cell- (Wnt3a-CM) conditioned media for 24 hrs. Graph shows mean ± SD of TOPflash normalized to cell number. Immunoblotting confirmed knockdown of XIAP protein and Wnt3a-mediated stabilization of β-catenin. GAPDH is loading control.

(B) Overexpression (OE) of USP47 enhances TOPflash activity. STF293 cells were transfected with USP47 or USP47-mut and treated with L-CM or Wnt3a-CM for 24 hrs. Graph shows mean ± SD of TOPflash activity normalized to cell number.

USP47 is required for Wnt signaling in cultured mammalian cells.

To determine if USP47, the human homolog of Ubp64E, is similarly required for Wnt signaling in mammalian cells, I next tested the effects of USP47 knockdown on Wnt3a-induced transcriptional activation using a human embryonic kidney (HEK) 293 cell line stably transfected with the TOPflash reporter (STF293) (Xu et al., 2004). Knockdown of USP47 with two independent short-interfering RNA (siRNA) constructs blocked Wnt3a-induced TOPflash activation (Figure 5.2A), indicating that USP47 is required for Wnt-mediated transcriptional activation. Notably, knockdown of USP47 did not change the levels of cytoplasmic β-catenin, suggesting that USP47 is likely functioning downstream of the β-catenin destruction complex (Figure 5.2A).

In contrast to loss of USP47 function, overexpression of USP47 activated Wnt signaling in STF293 cells to a similar extent as β-catenin overexpression, while the catalytically dead USP47 mutant (in which the active site cysteine has been mutated to an alanine) inhibited Wnt signaling similar to overexpression of the potent negative regulator Axin (Figure 5.2B). This suggests that the DUB activity of USP47 is required for its capacity to activate Wnt signaling and that the catalytically dead USP47 mutant acts as a dominant negative Wnt signaling inhibitor. Together, these results indicate that the DUB activity of USP47 is required for Wnt signaling and that USP47 likely functions downstream of the β-catenin destruction complex in the Wnt pathway.

USP47 is localized in the cytoplasm

To determine where USP47 is found in the cell, I first expressed GFP-USP47 in HEK293 cells and visualized its localization using fluorescence microscopy (Figure 5.3A). GFP-USP47 is localized predominantly in the cytoplasm while the GFP control is found throughout the cell. The localization pattern of GFP-USP47 did not change in the presence of Wnt signaling (data not shown). Interestingly, GFP-USP47 is found at the leading edge of the cell in what appear to be lamellipodial structures (Alberts, 2002), although more specific co-staining (e.g. with actin) is needed to prove the leading edge structures are indeed lamellipodia. This localization pattern is consistent with the findings of Rorth and colleagues who showed that Ubp64E is critical for border cell migration during *Drosophila* oogenesis by regulating the stability of the transcription factor Slowborders (Slbo) (Rorth et al., 2000). Thus, Ubp64E and USP47 may play important roles in cell migration in both *Drosophila* and mammalian cells, respectively.

In addition to the fluorescence imaging studies, cellular fractionation and Western blot analysis also indicated that USP47 is found predominantly in the nucleus; a localization that does not change upon Wnt stimulation (Figure 5.3B). These findings indicate that USP47 is predominantly a cytoplasmic protein and that Wnt signaling does not affect its subcellular localization.

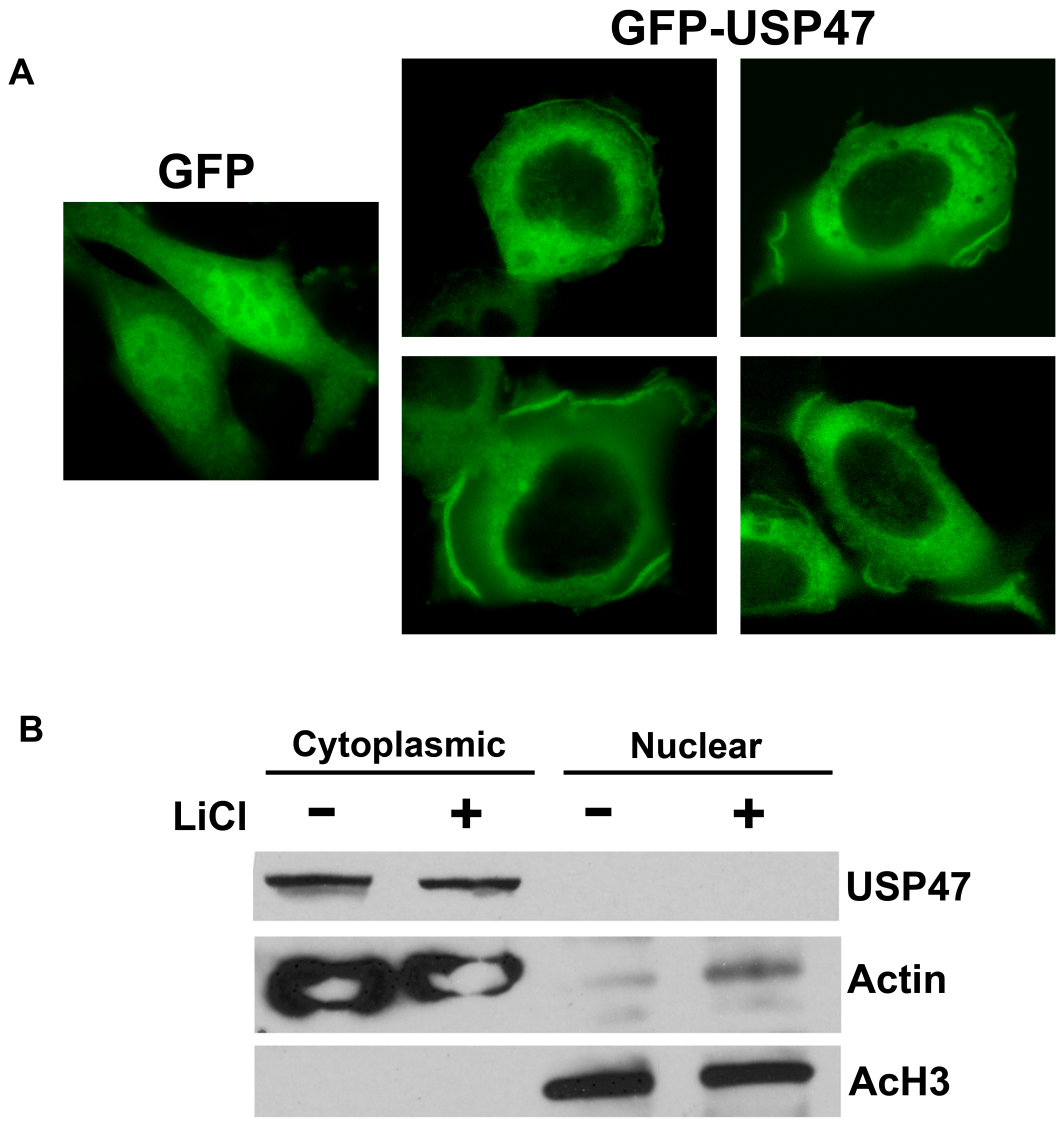


Figure 5.3. USP47 is located in the cytoplasm.
(A) HEK293 cells transfected with GFP-USP47 or GFP control were fixed and visualized with immunofluorescence microscopy.
(B) HEK293 cells were treated with LiCl (30 mM) as indicated for 24 hr, fractionated, and immunoblotted as indicated.

USP47 is expressed throughout *Xenopus* development

To determine if USP47 plays a critical role in Wnt signaling in a whole organism, I used the classic Wnt model system *Xenopus laevis*. Before performing loss or gain-of-function studies in *Xenopus*, however, I first determined when and where USP47 is expressed during *Xenopus* development. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis using primers specific for *Xenopus* USP47 (*X*USP47) showed that *XUSP47* is expressed at all developmental time points tested (Figure 5.4A).

In situ hybridization analysis with an anti-sense probe targeting *XUSP47* similarly showed that *XUSP47* is expressed at all time points tested during *Xenopus* development and that it is dynamically localized in a manner similar to the key Wnt component, β-catenin (Figure 5.4B-H) (β-catenin images from (DeMarais and Moon, 1992)). Figure 5.4B-E shows *XUSP47* is localized to the animal half of the early embryo. At the neurula stage (Figure 5.4F), *XUSP47* is primarily localized to the anterior (right) and posterior (left) ends of the embryo similar to *Xβ-catenin*, while at the tail bud stage (Figure 5.4G) *XUSP47* is localized to the branchial arches, the eye, and the posterior end of the embryo, also similar to *Xβ-catenin*. At the tadpole stage (Figure 5.4H), *XUSP47* is localized to the head and spinal cord, again similar to *Xβ-catenin*. Together, these studies indicate that *XUSP47* is expressed during *Xenopus* development in a manner that is consistent with USP47 being a Wnt signaling regulator.

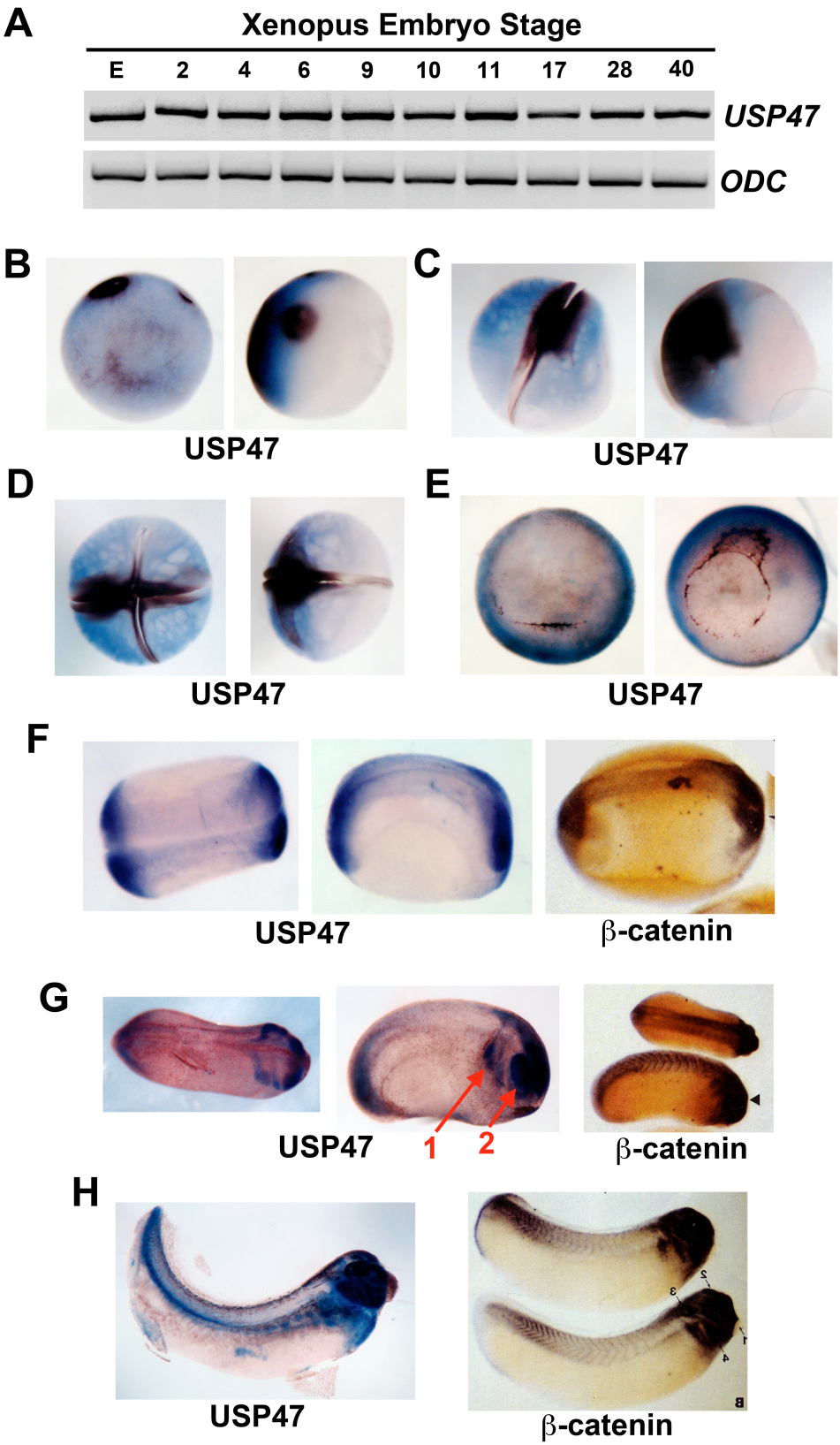

A
Xenopus Embryo Stage
E 2 4 6 9 10 11 17 28 40
USP47
ODC
B
USP47
C
USP47
D
USP47
E
USP47
F
USP47
β-catenin
G
USP47
1
2
β-catenin
H
USP47
β-catenin

Figure 5.4. USP47 is dynamically expressed throughout *Xenopus* development.
(A) RT-PCR analysis of *XUSP47* expression during *Xenopus* development. Embryos were collected at the indicated stages, RNA extracted, and RT-PCR performed. *Ornithine decarboxylase* (*ODC*) is loading control.
(B-H) *In situ* hybridization detects dynamic *XUSP47* expression throughout *Xenopus* development. (B) Egg, (C) 2-cell stage, (D) 4-cell stage, (E) Stage 10-11, (F) Neurula stage, (G) Tailbud stage, (H) Tadpole stage. Red arrows point to pharyngeal arches (1) and eye (2). Images of *Xβ-catenin In situs* are from (DeMarais and Moon, 1992).

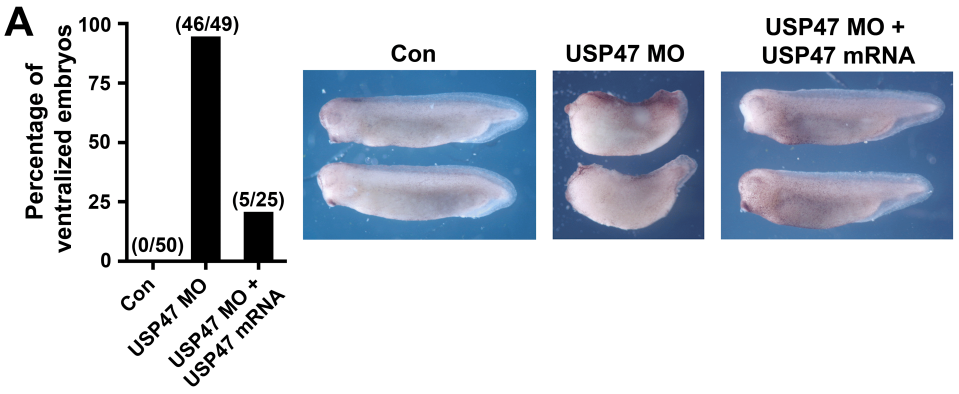
USP47 affects primary body axis formation in *Xenopus* embryos.

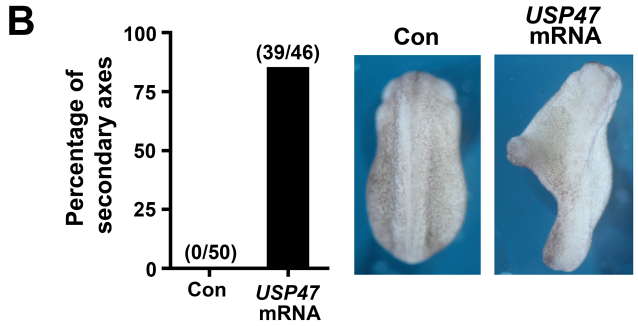
Once I had established that *XUSP47* is expressed in the early *Xenopus* embryo in a manner consistent with Wnt pathway regulation, I next tested if loss or gain of USP47 function is able to perturb primary body axis formation in *Xenopus* embryos; a process critically regulated by Wnt signaling (Heasman, 2006). Knockdown of USP47 by dorsal injection of a USP47 morpholino resulted in severely ventralized embryos, consistent with Wnt pathway inhibition (Figure 5.5A). This phenotype was rescued by co-injection of mouse *USP47* mRNA with the USP47 morpholino indicating the observed phenotype is specifically due to USP47 knockdown and not some other non-specific morpholino effect.

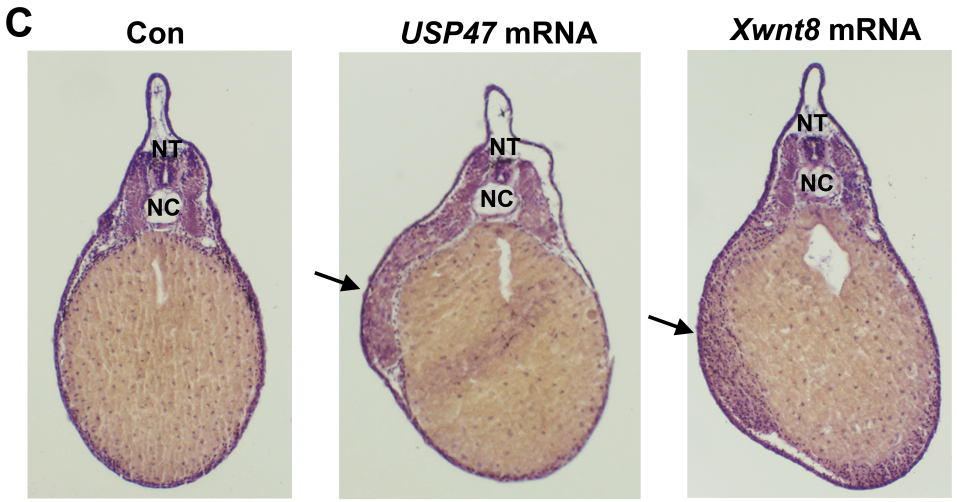
Overexpression of USP47 by ventral injection of *USP47* mRNA resulted in partial axis duplication, consistent with Wnt pathway activation (Figure 5.5B). To examine the partially duplicated axes in more detail, the embryos were sectioned and stained with hematoxylin and eosin (Figure 5.5C). These studies revealed that the primary tissue induced by *USP47* injection is mesodermal tissue and that

the partial secondary axes induced by *USP47* injection look nearly identical to those induced by injection of a small amount of *Xwnt8* mRNA, further indicating that overexpression of USP47 activates Wnt signaling in *Xenopus* embryos.

To further confirm that USP47 is capable of inducing ectopic head organizer formation, I tested the capacity of USP47 to induce the expression of the classic organizer marker Chordin. Injection of *USP47* mRNA into *Xenopus* animal caps induced the expression of *Chordin* to a greater extent than injecting a small amount of *Xwnt8* (a key organizer inducer) (Figure 5.5D). Co-injection of *Xwnt8* with *USP47* did not result in enhanced *Chordin* expression, indicating these two molecules do not synergize. Together, these results indicate that USP47 is required for proper axis formation and that USP47 is capable of ectopic organizer formation in *Xenopus* embryos; two features consistent with USP47 playing a critical role in Wnt signaling during *Xenopus* development.


A
Percentage of ventralized embryos
100
75
50
25
0
(0/50)
(46/49)
(5/25)
Con
USP47 MO
USP47 MO + USP47 mRNA
Con
USP47 MO
USP47 MO + USP47 mRNA


B
Percentage of secondary axes
100
75
50
25
0
(0/50)
(39/46)
Con
USP47 mRNA
Con
USP47 mRNA


C
Con
USP47 mRNA
Xwnt8 mRNA
NT
NC
NT
NC
NT
NC

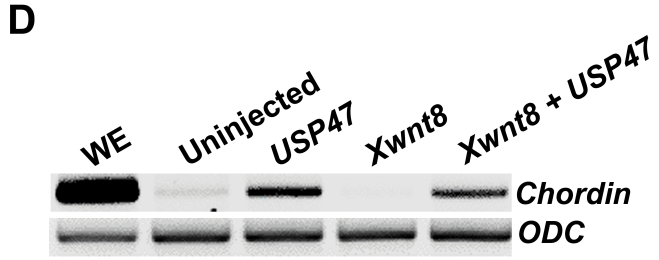

D
WE
Uninjected
USP47
Xwnt8
Xwnt8 + USP47
Chordin
ODC

Figure 5.5. Loss and gain of USP47 perturbs axis formation in *Xenopus* embryos.
(A) USP47 is required in *Xenopus* embryos for primary body axis formation. Embryos (4-cell stage) were injected dorsally with control (Con MO), USP47 morpholino (USP47 MO) alone (25 ng), or USP47 MO and USP47 mRNA (2 ng) and dorsal-anterior index (DAI) determined (Kao and Elinson, 1988). The percentage of ventralized embryos (DAI <= 2) is graphed on the left (absolute numbers above bars) with representative embryos on the right.
(B) USP47 induces secondary axis formation. Embryos (4-cell stage) were injected ventrally with control (Con) or *USP47* mRNA (2 ng) and allowed to develop. The percentage of embryos with secondary axis formation is graphed on the left (absolute numbers above bars) with representative embryos on the right.
(C) USP47 induces mesoderm formation. Embryos treated as in (B) were fixed, sectioned, and stained with hemotoxylin and eosin. NT = Neural Tube, NC = Notochord. Arrows point to induced mesoderm in both the *USP47*- and *Xwnt8*-injected embryos.
(D) USP47 induces expression of the head organizer marker Chordin in *Xenopus* ectodermal explants. Total RNA was extracted from animal caps injected with *Xwnt8* mRNA (0.35 pg), *USP47* mRNA (2 ng), or both and *Chordin* gene expression was assayed by RT-PCR. WE = whole embryo control. *Ornithine decarboxylase* (*ODC*) is loading control.

USP47 interacts with β-TRCP and Smad4, but does not affect their stability

Having demonstrated that USP47 regulates Wnt signaling across phyla, I next sought to elucidate the molecular mechanism of its function in the Wnt pathway. Work in *Drosophila* indicated that Ubp64E interacts with Slimb (the *Drosophila* homolog of β-TRCP, the E3 ligase that targets β-catenin for proteasomal degradation) (Bajpe et al., 2008). Thus, I tested if USP47 also interacts with β-TRCP in mammalian cells by overexpressing USP47 and myc-β–TRCP and performing a co-immunoprecipitation experiment in the presence and absence of Wnt stimulation. The results indicate that USP47 co-

immunoprecipitates with β-TRCP in both the absence and presence of Wnt

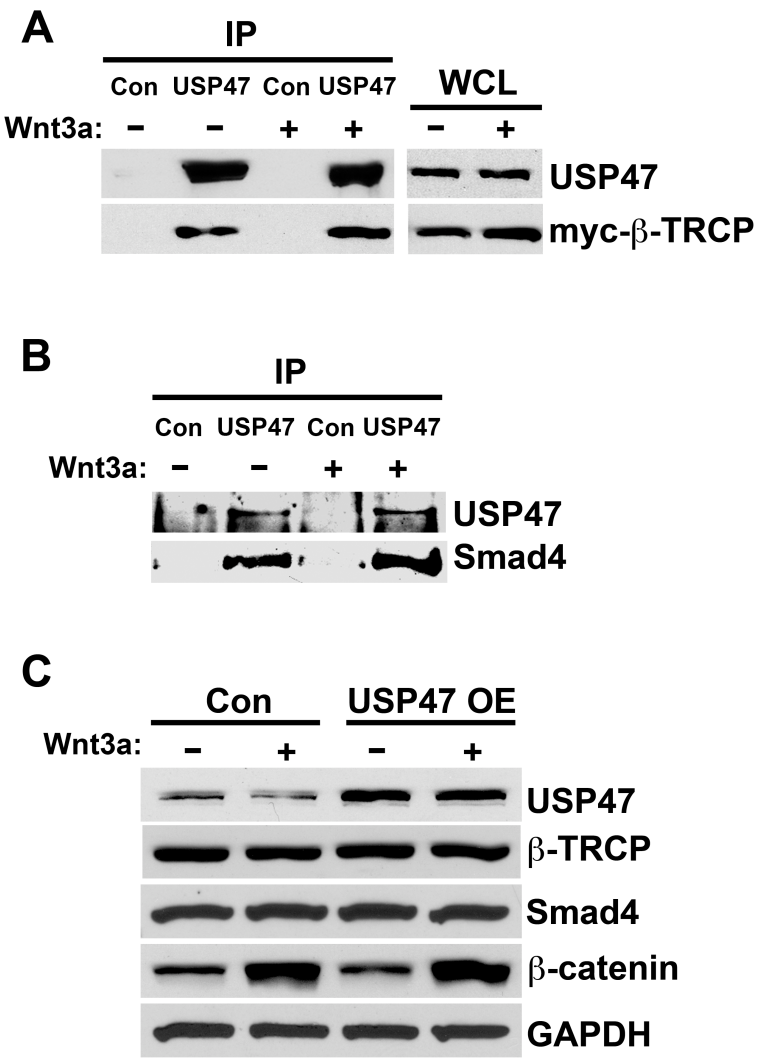


Figure 5.6. USP47 interacts with β-TRCP and Smad4.
(A) USP47 interacts with β-TRCP. HEK293 cells were transfected with USP47 and myc-β-TRCP and treated with Wnt3a-CM. Cells were lysed and USP47 immunoprecipitated. Co-immunoprecipitated β-TRCP was detected by anti-myc antibody.
(B) USP47 interacts with Smad4. HEK293 cells were transfected with Smad4 and USP47 and treated with L-CM or Wnt3a-CM for 3 hrs, lysed, and immunoprecipitated with anti-USP47 antibody. Co-immunoprecipitated Smad4 was detected by anti-Smad4 antibody.
(C) Overexpression of USP47 in cultured mammalian cells does not affect steady-state β-TRCP, Smad4, or β-catenin levels. HEK293 cells were transfected with vector control (Con) or USP47 expression plasmid as indicated, treated with L-CM or Wnt3a-CM, and immunoblotting performed.

signaling (Figure 5.6A). Peschiaroli and colleagues recently reported that USP47 interacts with β-TRCP via the β-TRCP WD40-repeat region (Peschiaroli et al., 2010), confirming that USP47 and β-TRCP are associated in mammalian cells.

That USP47 (a DUB) interacts with β-TRCP (an E3 ligase) is not surprising given that most E3s are associated with DUBs, which often function to regulate the stability of their partner E3s (Sowa et al., 2009). However, overexpression of USP47 does not have any observable effect on the steady state levels of β-TRCP (Figure 5.6C). Loss (Figure 5.2A) or gain (Figure 5.6C) of USP47 also has no effect on the steady state levels of the most well characterized Wnt pathway β-TRCP substrate, β-catenin. Thus, USP47 does not likely regulate the stability of β-TRCP or β-catenin in the Wnt pathway.

Given that USP47 interacts with β-TRCP, I next sought to determine if β-TRCP has other Wnt-relevant substrates in addition to β-catenin. One group has

shown that β-TRCP also ubiquitylates Smad4, targeting it for proteasomal degradation (Wan et al., 2004). This is interesting as work performed by Josh Smith in the Beauchamp laboratory (Vanderbilt University, personal communication) indicated that Smad4 inhibits Wnt signaling in colorectal cancer cells, potentially through downregulation of β-catenin transcription. This hypothesis was confirmed by a group showing that restoring Smad4 expression in SW480 cells (colorectal cancer cells containing a mutant, truncated APC protein) suppresses Wnt signaling by decreasing β-catenin expression and re-localizing β-catenin to the plasma membrane (Tian et al., 2009). These studies indicated the existence of cross-talk between Smad4 (traditionally thought of as a TGF-β or BMP pathway component) and Wnt signaling. Thus, I performed a co-immunoprecipitation experiment to determine if USP47 interacts with Smad4. For this, USP47 and Smad4 were overexpressed in the absence and presence of Wnt signaling. USP47 was immunoprecipitated and co-immunoprecipitated Smad4 was detected with anti-Smad4 antibody. The results indicate that USP47 interacts with Smad4 in both the absence and presence of Wnt signaling (Figure 5.6B), however, overexpression of USP47 has no observable effect on the steady state levels of Smad4, indicating that USP47 does not affect Smad4 stability (Figure 5.6C). Together, these findings indicate a possible role for β-TRCP or Smad4 as part of the molecular mechanism of USP47 in the Wnt pathway.

USP47 interacts with XIAP, but does not affect TLE3 or XIAP stability.

In addition to binding the E3 ligase β-TRCP, data I collected in a mass spectrometry screen to identify USP47 binding partners indicated that USP47 might also interact with the E3 ligase XIAP. One of the USP47 binding partners identified in the mass spectrometry screen was Apoptosis Inhibitory Factor (AIF), which has been shown to interact with XIAP, the E3 ligase that mono-ubiquitylates Groucho/TLE (see Chapter III) (mass spectrometry data not shown). Thus, I tested if USP47 associates with XIAP by performing a co-immunoprecipitation experiment with overexpressed, tagged versions of both proteins. Figure 5.7A shows that XIAP is immunoprecipitated with USP47 when both proteins are expressed together, but not when they are expressed alone,

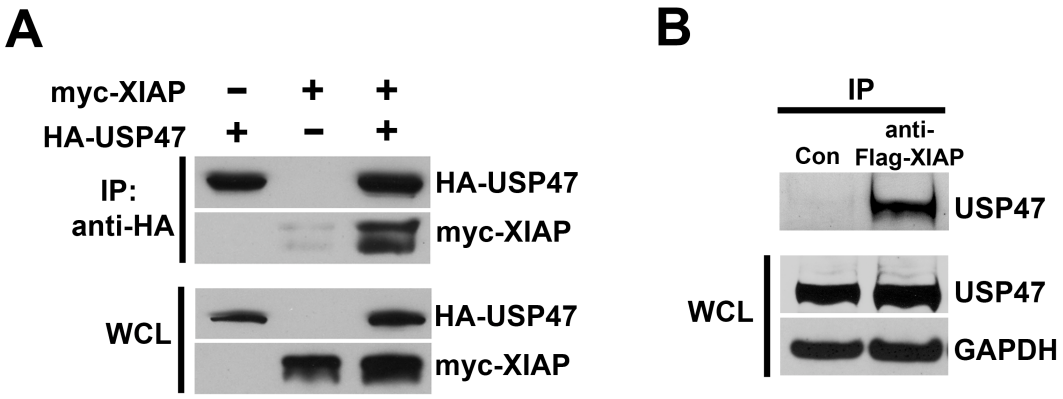


Figure 5.7. USP47 interacts with XIAP.
(A) Tagged USP47 co-immunoprecipitates with tagged XIAP. HEK293 cells were transfected as indicated with HA-USP47 and myc-XIAP and treated with Wnt3a-CM. Cells were lysed and USP47 immunoprecipitated with anti-HA antibody. Co-immunoprecipitated XIAP was detected by anti-myc antibody.
(B) Endogenous USP47 co-immunoprecipitates with tagged XIAP. HEK293 cells were transfected with Flag-XIAP, lysed, and immunoprecipitated with anti-XIAP antibody. Co-immunoprecipitated USP47 was detected with anti-USP47 antibody.

indicating a specific interaction. The interaction between USP47 and XIAP was confirmed by demonstrating that endogenous USP47 co-immunoprecipitates with tagged XIAP (Figure 5.7B).

Given that XIAP ubiquitylates Groucho/TLE, and that many DUBs regulate the stability of their partner E3s, I next sought to determine if loss of USP47 function affects the steady state levels of TLE3 or XIAP. Figure 5.8A shows that knockdown of USP47 with two independent siRNA constructs has no observable effect on the steady state levels of either TLE3 or XIAP. Overexpression of USP47 also has no observable effect on the steady state levels of TLE3 or XIAP

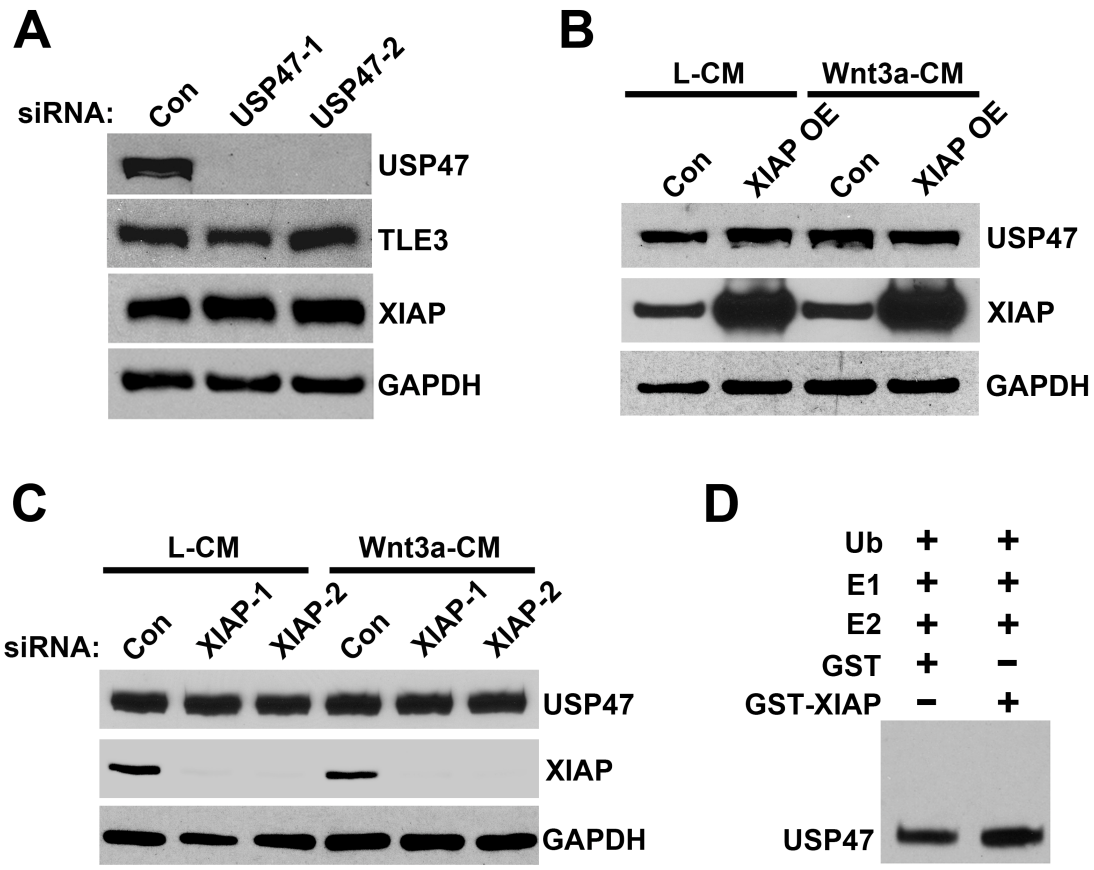


Figure 5.8. USP47 and XIAP do not affect the stability of each other.
(A) Knockdown of USP47 in cultured mammalian cells does not affect steady-state XIAP or TLE3 levels. HEK293 cells were transfected with non-targeting control (Con) or two independent USP47 siRNAs (USP47-1 and USP47-2) as indicated, and immunoblotting performed.
(B and C) Overexpression or knockdown of XIAP in cultured mammalian cells does not affect steady-state USP47 levels. HEK293 cells were transfected with vector control (Con) or XIAP expression plasmid (B) or with control (Con) or XIAP siRNA (C) as indicated, treated with L-CM or Wnt3a-CM, and immunoblotting performed.
(D) XIAP does not ubiquitylate USP47 *in vitro*. *In vitro*-translated USP47 was incubated in an *in vitro* ubiquitylation assay with recombinant proteins as indicated and visualized by immunoblotting with anti-USP47 antibody.

(data not shown). These data indicate that USP47 does not regulate the stability of the E3 ligase XIAP or its Wnt pathway substrate, Groucho/TLE.

To determine if gain or loss of XIAP may affect USP47 stability, I overexpressed XIAP (Figure 5.8B) or knocked down XIAP with two independent siRNA constructs (Figure 5.8C) and saw no observable changes in the steady state levels of USP47. These data indicate that XIAP does not affect the stability of USP47. These findings are consistent with the finding that XIAP does not ubiquitylate USP47 *in vitro* in the same assay in which XIAP efficiently ubiquitylates TLE3 (Figure 5.8D and see Chapter III, Figure 4.6F). These results indicate that USP47 is not a XIAP substrate.

Summary

Canonical Wnt signaling regulates many fundamental developmental processes and is misregulated in a variety of disease states in humans. The central event of Wnt signal transduction is the stabilization of the cytoplasmic protein β-catenin. When stabilized, β-catenin enters the nucleus to activate a Wnt-specific transcriptional program by binding to the Wnt transcription factor TCF/Lef. Here, we identified the de-ubiquitylase Ubp64E as a positive regulator of Wingless (the *Drosophila* Wnt homolog) signaling in a *Drosophila* S2 cell-based RNAi screen. USP47, its vertebrate homolog, is similarly required for Wnt

signaling in cultured mammalian cells and in *Xenopus* embryos, indicating evolutionary conservation of function. Our data indicate that USP47 likely functions at the level of transcription in the nucleus potentially through its interaction with the E3 ligases β-TRCP or XIAP or an as yet unidentified target.

CHAPTER VI

DISCUSSION AND FUTURE DIRECTIONS

Introduction

The work presented in this thesis describes the identification of two novel ubiquitin system components involved in regulating Wnt signaling: the E3 ligase XIAP and the de-ubiquitylase USP47. Here, I will discuss the results presented in the last two chapters and provide future directions. I have broken this chapter into two parts. In Part I I discuss the implications of XIAP as a Wnt pathway regulator and in Part II I discuss the implications of USP47 as a Wnt pathway regulator. I conclude with a general discussion of the significance of my findings.

Part I

Discussion

Conversion of the Wnt transcription factor TCF/Lef from a transcriptional repressor to a transcriptional activator is a critical event in Wnt signal transduction, yet our understanding as to how this switch occurs in cells is limited. The current model, based primarily on reconstitution studies using purified proteins, proposes direct displacement of the transcriptional co-repressor Gro/TLE by the co-activator β-catenin through competition for overlapping binding sites on

TCF/Lef (Daniels and Weis, 2005). Here, we provide evidence for a more finely tuned transcriptional switch that involves the facilitated removal of Gro/TLE from TCF/Lef upon its ubiquitylation by the E3 ligase XIAP.

Our data suggest a model (Figure 6.1) in which XIAP constitutively binds and ubiquitylates non-TCF-bound Gro/TLE in the nucleus, thereby limiting the amount of Gro/TLE available to form co-repressor complexes with TCF/Lef. In the presence of a Wnt signal, XIAP is recruited to TCF/Lef transcriptional complexes where it ubiquitylates Gro/TLE. Ubiquitylation of Gro/TLE decreases its affinity for TCF/Lef and allows for the efficient recruitment and binding of the transcriptional co-activator β-catenin to TCF/Lef in order to initiate a Wnt-specific transcriptional program.

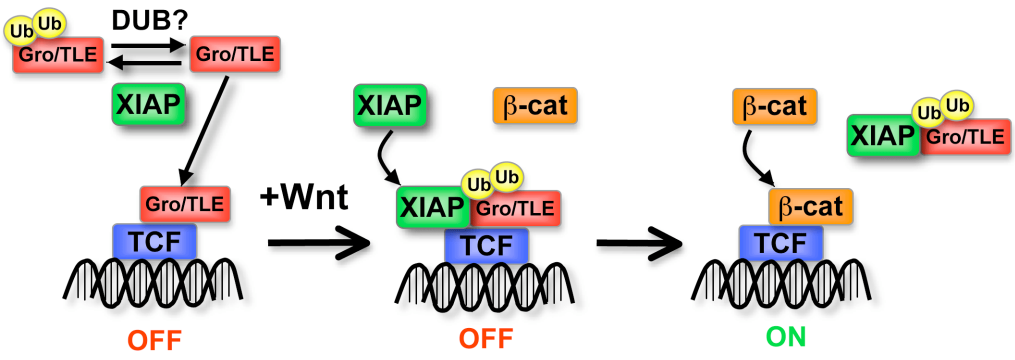


Figure 6.1. Model of XIAP-mediated regulation of Groucho/TLE in the Wnt pathway.
See text for details.

Our proposed model for Wnt-mediated transcriptional activation parallels the findings of Sierra and colleagues who proposed that inactivation of Wnt target gene transcription similarly occurs as a multi-step process (Sierra et al., 2006). Their data suggest that APC and β-TRCP (an E3 ligase) mediate the removal of β-catenin from Lef1 to allow for subsequent TLE1 binding. Together, these experiments, and our current study, have revealed that transcriptional activation and inactivation in the Wnt pathway are highly regulated processes.

β-catenin protein levels are tightly regulated in the cell via constant synthesis and degradation by the β-catenin destruction complex. Why, then, would a cell evolve an additional layer of regulation for Wnt transcriptional activation, as we propose here, as opposed to a mechanism driven simply by the bimolecular association between β-catenin and TCF/Lef? We propose that this Wnt signaling circuitry provides a mechanism to dampen transcriptional noise without a corresponding loss in sensitivity. Binding of Gro/TLE to TCF/Lef allows the system to be resistant to stochastic fluxes in β-catenin levels in the absence of Wnt pathway activation. In the presence of a Wnt signal, a coincident circuit involving nuclear accumulation of β-catenin and recruitment of XIAP to TCF/Lef is established. Such circuitry ensures that transcriptional activation only occurs upon Wnt ligand binding and provides an additional mechanism for reducing spontaneous activity. Sensitivity to a Wnt signal is maintained by the facilitated

removal of Gro/TLE from TCF/Lef, which ensures that even low levels of β-catenin would be sufficient to bind TCF/Lef and activate transcription.

Support for this model comes from a study demonstrating that β-catenin levels change only modestly (~2-6-fold) upon Wnt signaling in human cells and *Xenopus* embryos (Goentoro and Kirschner, 2009). It is unlikely that the modest accumulation of nuclear β-catenin that occurs upon Wnt pathway activation is sufficient to effectively displace Gro/TLE from TCF/Lef. This suggests that a facilitated mechanism for the removal of Gro/TLE is required prior to formation of a β-catenin-TCF/Lef complex.

In addition to its role in regulating the TCF/Lef transcriptional switch, our data indicate that XIAP may also regulate the nuclear pool of Gro/TLE that is available to form co-repressor complexes with TCF/Lef. Here, we find that XIAP is associated with Gro/TLE in the presence and absence of Wnt signaling. Additionally, whereas ubiquitylated Gro/TLE is readily observed in total cellular lysates, only the non-ubiquitylated form of Gro/TLE binds to TCF/Lef. This suggests a model in which XIAP functions to constitutively ubiquitylate free Gro/TLE to control the pool of Gro/TLE that can bind TCF/Lef. Our data also suggest the presence of an as yet unidentified de-ubiquitylase (DUB) that facilitates the removal of ubiquitin from Gro/TLE, which would allow TCF/Lef binding (Figure 6.1). This cycle of mono-ubiquitylation and de-ubiquitylation has been shown to regulate the activity of the transcriptional activators Smad4, p53, and FoxO (Dupont et al., 2009; Li et al., 2003; van der Horst et al., 2006). Our

study provides the first evidence for similar regulation of a transcriptional repressor, indicating this may be a general mechanism for transcription factor regulation in the cell.

Until recently, most studies have focused on transcriptional co-activator activity because it was generally believed that co-repressors are abundant proteins subject to little regulation. It is becoming clear, however, that co-repressor activity is highly complex and can be controlled through a variety of mechanisms (Cinnamon and Paroush, 2008; Perissi et al., 2010). Here, we show that the co-repressor Gro/TLE is regulated by ubiquitylation in a manner that may be Wnt-pathway specific. Gro/TLE has been shown to participate in transcriptional repression of multiple signaling pathways (Buscarlet and Stifani, 2007). The co-repressor function of Gro/TLE occurs locally through its binding to DNA-bound transcription factors (primarily via its C-terminal WD40 domain) and histone deacetylase recruitment, and globally via its N-terminal Q domain, which mediates oligomerization to alter chromatin structure and mediate long-range repression. Our finding that XIAP ubiquitylates Gro/TLE on its N-terminal Q domain (which disrupts TCF/Lef binding), but does not disrupt its capacity to oligomerize, suggests that XIAP modification of Gro/TLE may specifically affect its Wnt repressive function. This possibility is consistent with our observation that XIAP knockdown had no observable effect on Notch signaling. In the absence of Notch signaling, Gro/TLE normally binds to the Hairless protein to repress Notch target gene activation by the transcription factor, Suppressor of Hairless (Barolo

et al., 2002; Nagel et al., 2005). Binding to Hairless occurs via the C-terminal WD40 domain of Gro/TLE (Jennings et al., 2006). Thus, ubiquitin modifications of Gro/TLE on its N-terminal Q domain would not be expected to disrupt its interaction with Hairless in the Notch pathway or other pathways in which repression by Gro/TLE occurs via the WD40 domain or via Gro/TLE oligomerization.

The identification of XIAP as a novel Wnt pathway component provides a link between apoptosis and Wnt signaling and represents a way for the cell to coordinate both survival and proliferation. Wnt signaling has been shown to inhibit apoptosis and to be required for the expression of XIAP in cancer cells (Chen et al., 2001; Gandhirajan et al., 2010; Suzuki et al., 2004; Wang et al., 2010). Thus, XIAP may be part of a positive feedback loop involving Wnt pathway-induced proliferation and inhibition of apoptosis. Surprisingly, the XIAP knock-out mouse has no obvious apoptotic or Wnt phenotypes as would be expected given its important role in apoptotic inhibition and our findings indicating that XIAP is required for Wnt signaling in cultured human cells and in *Xenopus* embryos. Only exon 1 of XIAP was deleted in the knockout mouse (Harlin et al., 2001). Thus, it is possible that there is read through that permits expression of the C-terminus of XIAP, which includes the RING domain. Alternatively, there may be other IAP family members or other E3 ligases that can compensate for XIAP function when it is knocked out in the mouse.

Our findings may have important clinical implications as XIAP is upregulated in a majority of human cancers, and inhibitors of XIAP are currently in clinical trials (LaCasse et al., 2008). Drug development has been largely focused on developing small molecule and peptide Smac mimetics that bind to the BIR domains of XIAP to inhibit its anti-apoptotic function. Here, we show that the critical role XIAP plays in Wnt signaling depends on its E3 ligase RING domain and is distinct from its anti-apoptotic function. Our results predict that small molecules targeting the RING domain of XIAP, rather than its BIR domains, would represent more selective inhibitors of Wnt signaling. Alternatively, drugs targeting both the anti-apoptotic (caspase binding BIR domains) and pro-Wnt (E3 ligase RING domain) functions of XIAP (e.g. downregulating XIAP by RNA interference) may be particularly effective therapeutics against Wnt-driven cancers. Moreover, recent findings indicate that inducing apoptosis results in "compensatory proliferation" of surrounding surviving cells due to release of mitogenic signals (e.g. Wnt) from dying cells (Bergmann and Steller, 2010), further indicating that drugs targeting both aspects of XIAP function may be particularly effective anti-cancer therapies even in non-Wnt-driven tumors.

Future Directions

Like all scientific inquiry, these studies have raised more questions than they have answered. I will outline some of these important unanswered questions here.

Is XIAP required for Wnt signaling in the absence of Groucho/TLE?

To validate that Groucho/TLE is truly the Wnt pathway target of XIAP, it will be important to determine if XIAP is required for Wnt signaling in the absence of Groucho/TLE. If Groucho/TLE is not present to repress Wnt signaling, then XIAP should not be required for Wnt signal transduction. This experiment is complicated in mammalian cells as there are five Groucho/TLE isoforms. Thus, the simplest approach would be to knockdown Groucho in *Drosophila* S2 cells, as there is only one Groucho in flies, and ask if DIAP1 is still required for Wg signaling using the same assay that was used in my original RNAi screen. This is also complicated by the fact that DIAP1 is thought to be required for cell survival in S2 cells and, thus, when it is depleted, cells undergo apoptosis. I have found this to be the case to some extent. Thus, it may be necessary to knock down an effector caspase (i.e. DRONC) to prevent the S2 cells from undergoing apoptosis when DIAP1 is knocked down in order to answer this question.

What are the sites of XIAP-mediated ubiquitylation on Groucho/TLE?

I have narrowed the sites of XIAP-mediated ubiquitylation on Groucho/TLE to the N-terminal Q domain of Groucho/TLE, which contains eight totally conserved lysine residues. However, it will be important to determine which of the eight lysine residues in the Q domain are the XIAP targets.

Identifying the ubiquitylation sites on Groucho/TLE will allow the synthesis of Groucho/TLE lysine-to-arginine mutants that are unable to be ubiquitylated by XIAP. These mutants could then be used in experiments to determine if the non-ubiquitylated form of Groucho/TLE is a better repressor than the wild type form of Groucho/TLE. This could be accomplished by overexpressing the ubiquitylation mutant and wild type forms of Groucho/TLE in cultured cells and assaying their capacity to inhibit TOPflash activity. I would predict that the ubiquitylation mutant form of Groucho/TLE would be able to inhibit TOPflash at a lower concentration than the wild type form of Groucho/TLE. Alternatively, the kinetics of Groucho/TLE dissociation from TCF/Lef in response to Wnt stimulation could be tested via chromatin immunoprecipitation (ChIP) to determine if the ubiquitylation mutant Groucho/TLE cycles off of TCF/Lef slower than the wild type form, or if it does not dissociate from TCF/Lef at all. These experiments may be potentially complicated by the presence of wild type Groucho/TLE in the cell, however, as the mutant Groucho/TLE will be able to oligomerize with the wild type form, perhaps masking any defects in TCF/Lef dissociation. Thus, the cleanest experiment would involve eliminating all wild type forms of Groucho/TLE and then asking questions about the behavior of the ubiquitylation mutant form, which is a complicated task.

Mapping the ubiquitylation sites on Groucho/TLE will be useful for functional assays as just described as well as for determining how modification on specific residues might affect the structure and binding interfaces of

Groucho/TLE. At present, the structure of Groucho/TLE has not been solved as it is a hard protein to purify, but there are groups working on it. Once the structure of the Q domain of Groucho/TLE is known it will be very interesting to see where the modified lysine residues reside within that structure. Based on a protein structure predictor program, (http://swissmodel.expasy.org/) it is predicted that all eight lysine residues in the Groucho/TLE Q domain reside on the external surface of the molecule and would therefore be available for modification by XIAP. More information is required to know which residues are modified and how these modifications might specifically affect interactions between Groucho/TLE and TCF/Lef, but not affect Groucho/TLE oligomerization.

What are the concentrations of β-catenin and Groucho/TLE in the nucleus?

As discussed above, the prevailing model for how TCF/Lef is converted from a transcriptional repressor into an activator involves direct displacement of Groucho/TLE by accumulating nuclear β-catenin in response to Wnt stimulation (Daniels and Weis, 2005). However, recent evidence suggests there is not enough nuclear β-catenin present in response to Wnt stimulation to simply outcompete Groucho/TLE for TCF/Lef binding (Goentoro and Kirschner, 2009). My data show that Groucho/TLE must be removed from TCF/Lef before β-catenin can bind, further suggesting that β-catenin cannot directly displace Groucho/TLE on its own. In order to really prove that Groucho/TLE must be removed from TCF/Lef in order for β-catenin to bind, however, some rigorous

biochemical measurements and mathematical modeling must be performed. At present, the only precise measurement known for these three proteins in the cell is the K_d for the β-catenin-TCF/Lef interaction, which was reported to be about 20 nM (Daniels and Weis, 2005). Thus, the K_d for the Groucho/TLE-TCF/Lef interaction must be determined as well as the nuclear concentrations of Groucho, TCF/Lef and β-catenin in the absence and presence of Wnt stimulation. With these measurements, it will be possible to determine if, in fact, enough β-catenin enters the nucleus upon Wnt signaling to simply outcompete Groucho/TLE for TCF/Lef binding. This can also be tested theoretically with ordinary differential equations (ODEs) for the three proteins using the experimentally verified concentrations and K_d's for each protein to predict the relationship between them in the absence and presence of Wnt signaling. Such precise measurements and analysis will give much needed insight into this important nuclear Wnt signaling event.

How and when is XIAP recruited to the TCF/Lef transcriptional complex?

I have shown that XIAP is recruited to the TCF/Lef transcriptional complex upon Wnt stimulation. The obvious question is: how? Some insight comes from the fact that XIAP is recruited to TCF/Lef upon LiCl treatment, meaning inhibition of GSK3 is sufficient to recruit XIAP to TCF/Lef. This suggests that this event is controlled downstream of β-catenin stabilization in the Wnt pathway. Perhaps XIAP binds β-catenin and is recruited to TCF/Lef along with β-catenin and its

binding partners in order to displace Groucho/TLE; this possibility, and others, remain to be tested.

The more interesting questions are: what are the kinetics of XIAP-TCF/Lef association? When does XIAP come on to TCF/Lef and when does it come off? Is the binding of XIAP to TCF/Lef coordinated with Groucho/TLE removal and β-catenin association? Is XIAP really required for removal of Groucho/TLE from TCF/Lef? What happens when XIAP is knocked down by siRNA? Does Groucho/TLE still cycle off of TCF/Lef in response to Wnt or are the kinetics of Groucho/TLE removal slower? All of these important questions can be answered using ChIP assays to look at association and dissociation of multiple factors on endogenous Wnt target gene promoters in response to Wnt stimulation at different time points. Such studies have been beautifully performed in the laboratory of Katherine Jones (Sierra et al., 2006). We are now working with her laboratory to carry out these experiments to address these interesting questions.

Does loss of XIAP inhibit tumor formation?

One major outstanding question is: does inhibition of XIAP affect tumor growth or formation? Many drug companies are currently targeting XIAP to inhibit tumor growth in combination with chemotherapeutic agents based on its anti-apoptotic function. It would be very interesting to determine if the patients who are receiving an anti-sense XIAP oligonucleotide (which would knockdown

the entire protein and thus affect the pro-Wnt RING domain of XIAP) have decreased Wnt signaling in their tumors. Given that pharmaceutical companies are involved in all of the current XIAP inhibitor clinical trials, patient samples are not readily available. Thus, the next best option is to turn to the *APC*$^{Min/+}$ mouse model, which is a well-established system for studying the effects of aberrant Wnt signaling on intestinal tumorigenesis (Clarke, 2006), to see if loss of XIAP inhibits Wnt signaling and tumor growth in this context. This would be the most definitive way to test if XIAP inhibition may be a useful way to inhibit Wnt-driven tumor formation. If that looks promising, it would be interesting to see how effective XIAP inhibition might be in preventing the formation or growth of non-Wnt-driven tumors as well.

What is the DUB that opposes XIAP-mediated Groucho/TLE ubiquitylation?

The last major unanswered question raised by this work is: what is the DUB that opposes Groucho/TLE ubiquitylation by XIAP? I found that Groucho/TLE is constitutively ubiquitylated by XIAP, a modification that inhibits Groucho/TLE-TCF/Lef binding. This suggests the existence of a DUB that would function to remove the ubiquitin modification on Groucho/TLE to allow TCF/Lef binding. Considering that there are only approximately 79 human DUBs, a small functional screen could be performed in which XIAP, His-ub, Groucho/TLE, and one of each of the DUBs could be overexpressed in cultured mammalian cells and a His-ub assay performed as described above. Potential “hits” would be

DUBs that decrease the amount of XIAP-ubiquitylated Groucho/TLE. These hits could then be followed up with overexpression/knockdown studies to determine effects on Wnt signaling in cultured cells and *Xenopus* embryos, and, ultimately, to determine if they affect the pool of Groucho/TLE available for TCF/Lef binding, as would be predicted.

Part II

Discussion

Ubiquitylation plays an important role in regulating many Wnt pathway components. Prior to beginning this work, however, no DUBs in the Wnt pathway had been identified. At present, DUBs for TCF (Zhao et al., 2009), Axin (Zhang et al., 2011), APC (Tran et al., 2008), and Dsh (Tauriello et al., 2010) have now been reported that regulate either the stability or activity of these proteins. In this work, I identified USP47 as a novel DUB involved in the regulation of Wnt signaling and show that it associates with two Wnt pathway E3 ligases, β-TRCP and XIAP.

USP47 and β-TRCP

The finding that USP47 interacts with β-TRCP was very interesting given that β-TRCP is the primary E3 ligase responsible for regulating cytoplasmic β-catenin levels in the Wnt pathway. Given that many DUBs regulate the stability

of their cognate E3s, I expected to find that USP47 plays a role in stabilizing β-TRCP. However, my data, and that of Peschiaroli and colleagues show this is not likely the case (Peschiaroli et al., 2010). The work of Peschiaroli et al. also showed that β-TRCP has no effect on the stability of USP47 even though USP47 binds the substrate-recognition WD-40 domain of β-TRCP. Thus, USP47 and β-TRCP do not likely regulate the stability of each other.

Another possibility is that USP47 may affect the levels of the classic Wnt pathway β-TRCP substrate, β-catenin. Although USP47 does not appear to interact with β-catenin (data not shown), it is still possible that USP47 can affect the ubiquitylation or stability of β-catenin via its interaction with β-TRCP; however, my data indicate that USP47 does not affect the stability of β-catenin, ruling out this possibility.

The last apparent possibility for how USP47 may be affecting Wnt signaling via its interaction with β-TRCP is by affecting the ubiquitylation or stability of the β-TRCP substrate, Smad4. A paper published by Wan et al., showed that Smad4 is ubiquitylated by β-TRCP and subsequently degraded by the proteasome (Wan et al., 2004). Numerous papers have now shown that Smad4 can act as both a positive and negative regulator of Wnt signaling in different contexts (Li et al., 2011; Lim and Hoffmann, 2006; Romero et al., 2008; Tian et al., 2009). Thus, I sought to determine if USP47 might be affecting Wnt signaling by affecting Smad4 levels. Even though my data indicate that USP47

can interact with Smad4, it does not appear that USP47 has any effect on its stability.

Ubiquitylation of a protein can have many consequences besides targeting it for proteasomal degradation. Thus, even though USP47 does not observably affect the stability of β-TRCP, β-catenin, or Smad4, it is possible that USP47 may affect a non-degradative ubiquitin modification on these proteins, which could result in changes in their localization, binding partners, or activity. Most notably, mono-ubiquitylation of Smad4 has been shown to be critical in regulating its transcriptional activity by regulating its association with its transcriptional co-activator Smad2 and its nuclear localization (Dupont et al., 2009; Tian et al., 2009). Thus, it will be important to test whether USP47 affects non-degradative ubiquitin modifications on these proteins in the future, as described below.

USP47 and XIAP

In light of my discovery of XIAP as a new Wnt pathway component (see Chapter III), it was exciting to find that USP47 also interacts with this E3 ligase. My data indicate that USP47 is likely a positive Wnt regulator. Because XIAP is also a positive Wnt regulator, if USP47 affects XIAP function in the Wnt pathway, it must be doing so in a positive manner. This rules out the possibility that USP47 could be a DUB for XIAP's substrate, Groucho/TLE, as removing the XIAP-mediated ubiquitin modification on Groucho/TLE would be predicted to inhibit, not enhance, Wnt signaling (see Chapter III for details). This leaves the

possibility that USP47 might affect the ubiquitylation or stability of XIAP itself; however, my data do not show any observable effects on XIAP levels in either the presence or absence of USP47. There are also no observable changes in USP47 levels in the presence or absence of XIAP, ruling out the possibility that XIAP and USP47 regulate the stability of one another in the cell.

As discussed above, it is possible that USP47 affects a non-degradative, inhibitory ubiquitin modification on XIAP that must be removed to allow XIAP to ubiquitylate Groucho/TLE to promote Wnt signaling. This possibility remains to be tested as discussed below.

It is interesting to note the recently reported role for USP47 in regulating cell growth and survival, which is similar to the role of XIAP in promoting cell survival. Peschiaroli et al., reported significant decreases in cell growth and survival when USP47 was knocked down with siRNA, which was enhanced upon treatment with chemotherapeutic agents (Peschiaroli et al., 2010), much like in cells that are XIAP deficient (Engesaeter et al., 2011; Wang et al.). One explanation for this comes from the discovery that USP47 is a critical component of the DNA base excision repair process in cells by functioning as a DUB for polymerase β (Pol β) (Parsons et al., 2011). Parsons et al., show that USP47 rescues Pol β from ubiquitin-mediated degradation when it is needed in response to DNA damage. Thus, without USP47 there is not enough Pol β present in the cell and the cell becomes more susceptible to DNA damage resulting in decreased growth and survival. Another explanation is possible, however.

USP47 may affect XIAP function in the Wnt pathway (a possibility that remains to be determined) as well as its function as an inhibitor of apoptosis. Just as USP47 may be required for XIAP to effectively ubiquitylate Groucho/TLE, it may also be required for XIAP to effectively inhibit apoptosis. In support of this possibility, the *Drosophila* homolog of USP47, Ubp64E, has recently been shown to genetically interact with dcp-1 (one of the major effector caspases in the fly) (Kim et al., 2010), indicating USP47 may regulate apoptotic events in the cell, potentially through its interaction with XIAP. These interesting possibilities remain to be tested.

USP47 and Transcription

My findings indicate that USP47 is required downstream of the β-catenin destruction complex in the Wnt pathway as knockdown or overexpression of USP47 has no effect on β-catenin levels in the cell. Thus, even though USP47 appears to be predominantly a cytoplasmic protein based on my data and (Parsons et al., 2011), it is likely playing a role at the level of transcription in the nucleus in the Wnt pathway. This discrepancy in localization and function is not novel. XIAP is predominantly a cytoplasmic protein and β-TRCP's role in the Wnt pathway has largely been attributed to its cytoplasmic role of regulating β-catenin levels in the β-catenin destruction complex, but both XIAP and β−TRCP have newly discovered critical nuclear Wnt signaling functions. XIAP is recruited to TCF/Lef upon Wnt stimulation in order to ubiquitylate Groucho/TLE to allow β-

catenin binding (Chapter III), while β-TRCP appears to be required for the removal of β-catenin from the TCF/Lef complex to allow for association of Groucho/TLE (Sierra et al., 2006). What recruits XIAP, β-TRCP, and potentially USP47 to the nucleus is an important unanswered question.

That USP47 may be involved in transcriptional events in the Wnt pathway is consistent with the reported functions of its homolog, Ubp64E, in *Drosophila*. Ubp64E was originally identified as a strong dominant enhancer of position effect variegation (PEV) in *Drosophila* (Henchoz et al., 1996). PEV refers to the process of a gene becoming inactivated by random insertion next to heterochromatin, most often in peri-centrosomal regions (Girton and Johansen, 2008). In their study, Henchoz et al. showed that loss of Ubp64E increased the spread of heterochromatin in the *white* gene locus while gain of Ubp64E suppressed the spread of heterochromatin, indicating Ubp64E promotes chromosome de-condensation. Ubp64E was the first enzyme discovered to be either a suppressor or an enhancer of PEV as almost all other suppressors or enhancers identified at that time were transcription factors. To date, only one other DUB, USP22, has been identified as a modulator of PEV and an important regulator of chromatin dynamics (Fodor et al., 2010). USP22 is known to de-ubiquitylate Histone H2B, which is required for chromosome de-condensation (Zhang et al., 2008; Zhao et al., 2008). It remains to be determined how Ubp64E promotes euchromatin formation. One intriguing possibility is that Ubp64E disrupts Groucho/TLE function in coordination with XIAP.

In addition to the role of Ubp64E in PEV, it has also been shown to regulate the stability of two transcription factors: Slowborders (a transcriptional activator required for border cell migration in fly ovaries), and Tramtrack (a transcriptional repressor involved in fly eye development) (Bajpe et al., 2008; Rorth et al., 2000). Thus, even though USP47 is mostly found in the cytoplasm, much evidence exists to suggest one of its primary roles in the cell is to regulate chromosome dynamics and gene transcription. This implies that USP47 may be involved in regulating one of the many important transcriptional events in the Wnt pathway either through XIAP, β-TRCP, their substrates, or an as yet unidentified target.

Future Directions

The data presented in Chapter V are part of a work in progress. Thus, additional experiments must be performed to confirm and support the results as stated. I will briefly go over the important missing pieces required to interpret the current data before focusing on the broader, more interesting future directions of this project.

Is USP47 required for Wnt signaling in mammalian cells?

To confirm that USP47 is required for Wnt signaling in mammalian cells, it will be important to determine if USP47 is required for transcription of endogenous Wnt target genes such as *AXIN2* or *c-myc* via RT-PCR analysis of

USP47 siRNA-treated samples. Additionally, determining the requirement for USP47 in different cell lines is important to confirm that USP47 is required for Wnt signaling in multiple cell types. To confirm that USP47 is acting downstream of the β-catenin destruction complex, it will be necessary to determine if knockdown of USP47 via siRNA is able to inhibit LiCl-mediated activation of Wnt signaling. Lastly, to confirm that USP47 enhances Wnt signaling in mammalian cells, it will be necessary to repeat the overexpression studies and to immunoblot for both the wild type and mutant forms of USP47 to ensure they are being expressed in the cellular lysates.

Is USP47 required for Wnt signaling in Xenopus embryos?

While the data presented here strongly indicate that USP47 is required for Wnt signaling in *Xenopus* embryos, more rigorous studies must be performed to fully support this statement. First, it must be shown that knockdown of USP47 via injection of USP47 morpholino inhibits the formation of *Xwnt8*-induced secondary axes, which would show loss of USP47 specifically inhibits Wnt signaling. Second, effects of gain and loss of USP47 function on endogenous *Xwnt8* target gene expression (i.e. *Siamois* and *Xnr3*) in animal caps must be determined to show truly specific effects of USP47 on Wnt signaling in *Xenopus* embryos.

Does USP47 bind endogenous Smad4 and XIAP?

The co-immunoprecipitation experiment (Figure 5.6B) indicating overexpressed USP47 and Smad4 interact is weak and needs to be repeated in order to determine if USP47 really binds Smad4. Additionally, it would be best to test if endogenous USP47 and Smad4 can interact to determine if they normally form a complex in untreated cells. Given that USP47 and β-TRCP have now been reported to interact (Peschiaroli et al., 2010), that data does not need to be repeated. However, it would be good to show that endogenous USP47 interacts with endogenous XIAP to confirm the interaction of these two proteins as well.

Does USP47 affect the localization or ubiquitylation of β-TRCP, β-catenin, Smad4, XIAP or Groucho/TLE?

Given that USP47 does not affect the stability of β-TRCP, β-catenin, Smad4, XIAP, or Groucho/TLE the question remains: does USP47 affect either the localization or ubiquitylation of any of these proteins? Changes in protein localization can be assessed by overexpression or siRNA knockdown of USP47 followed by immunofluorescence microscopy using antibodies against the endogenous proteins where available. If no immunofluorescence amenable antibodies exist, tagged versions of proteins can be used instead. In addition, nuclear and cytoplasmic fractionation studies can be employed to determine if overexpression or knockdown of USP47 induces changes in the localization of each protein. These studies should also be performed in the presence and

absence of Wnt stimulation to determine if Wnt signaling has any effect on the subcellular localization of each protein.

The next step is to determine if USP47 might affect a non-degradative ubiquitin modification on any of the aforementioned proteins. There are currently no reports of non-degradative ubiquitin modifications on β-TRCP, β-catenin, or XIAP, however, that does not mean they do not exist. Smad4 and Groucho/TLE, on the other hand, are both critically regulated by non-degradative mono-ubiquitylation (Dupont et al., 2009)(Chapter IV). Thus, it will be important to determine if gain or loss of USP47 has any effect on the ubiquitylation status of each of these proteins by performing *in vivo* His-ubiquitylation assays when USP47 is overexpressed or knocked down via siRNA. If a change is observed in the ubiquitylation status of a protein, it will be important to determine what kind of ubiquitin modification it is using ubiquitin mutants and to show that USP47 can de-ubiquitylate that substrate both *in vivo* and *in vitro*.

Is USP47 involved in Wnt-mediated transcriptional events in the nucleus?

As discussed above, much evidence exists to suggest USP47 is an important regulator of transcriptional events in the cell and my data indicate that USP47 is likely functioning at the level of transcription in the Wnt pathway. Thus, it will be important to probe this interesting possibility. First, it will be interesting to determine if USP47 might be functioning with XIAP as part of the mechanism to disrupt Groucho/TLE-TCF/Lef binding. To this end, it will be necessary to test

whether USP47 is recruited to the TCF/Lef complex in response to Wnt signaling in the same manner as XIAP. This can be done by performing co-immunoprecipitation experiments or chromatin immunopreciptiation (ChIP) analyses looking at various time points after Wnt stimulation to see when USP47, XIAP, and Groucho/TLE might come on and off TCF/Lef on Wnt target genes and whether these events are coordinated.

Alternatively, perhaps USP47 functions as part of the mechanism to remove β-catenin from TCF/Lef via β-TRCP. This possibility could also be tested using ChIP analysis to determine if USP47 might cycle on and off of TCF/Lef in a manner similar to β-TRCP or β-catenin, which might give insight into its function within the Wnt transcriptional complex.

Lastly, it might be useful to examine Smad4-TCF/Lef interactions in the absence and presence of USP47 to determine if USP47 may affect the ability of Smad4 to form a transcriptional complex with TCF/Lef in the Wnt pathway. This can also be accomplished using both co-immunoprecipiation experiments and ChIP analysis in cultured cells in which USP47 has been either overexpressed or knocked down.

What is the USP47 substrate in the Wnt pathway?

After all of the above experiments have been performed, it is still possible that the substrate and molecular mechanism of USP47 will not be determined. If that is the case, more screening will be needed. Two approaches can be utilized

in this instance: 1. Purification of USP47 from cultured mammalian cells followed by mass spectrometry analysis to identify novel binding partners, or 2. A candidate approach in which USP47 is immunoprecipitated from cultured cells in the presence and absence of Wnt stimulation and assayed for interaction with all of the known nuclear Wnt components via immunoblotting. If a novel USP47 interactor is found via one of these methods, its stability, localization, and ubiquitylation status should be tested in the context of USP47 gain and loss of funciton to determine the likelihood that it is, in fact, a Wnt-pathway substrate of USP47.

Significance

Through a siRNA screen performed in *Drosophila* S2 cells I identified a novel E3 ligase and deubiquitylase involved in regulating Wg/Wnt signaling. Although the significance of the discovery of Ubp64E/USP47 as a Wg/Wnt signaling regulator remains to be determined, the significance of the identification of DIAP1/XIAP as a novel Wg/Wnt signaling component is readily apparent given the well-established role for XIAP in human cancer. Overexpression of XIAP has been observed in nearly every cancer type analyzed, including all 60 cell lines of the National Cancer Institute tumor cell line panel (Fong et al., 2000; Tamm et al., 2000). The most often-cited explanation for why XIAP is routinely upregulated in cancer cells focuses on the capacity of XIAP to inhibit apoptosis and, thus, prevent cancer cell death. While this is certainly part of the reason,

my studies, and those of others, provide evidence that cancer cells not only upregulate XIAP to prevent apoptosis, but also to increase proliferation (via Wnt signaling) and to promote metastasis (via NF-κB signaling) (Mehrotra et al., 2010).

In the work presented here, I identify XIAP as a critical Wnt signaling component. This finding provides a novel link between apoptosis and Wnt signaling and represents a way for a cell to coordinate both survival and proliferation within one protein. The requirement of XIAP for Wnt signal transduction ensures that Wnt-induced proliferation will only occur in non-apoptotic cells, as XIAP inhibits apoptosis. In fact, it has been reported that Wnt signaling promotes the expression of XIAP (Chen et al., 2001; Gandhirajan et al., 2010; Suzuki et al., 2004; Wang et al., 2010), thus forming a positive feedback loop within a cell involving Wnt pathway-induced proliferation and inhibition of apoptosis.

This positive feedback loop between Wnt signaling and XIAP may be part of the reason why many cancers exhibit hyperactive Wnt signaling (Reya and Clevers, 2005). Mutations that render the Wnt pathway constitutively active provide a potential cancer cell with a dual advantage: growth factor independent proliferation and enhanced cell survival, two traits critical for cancer formation (Hanahan and Weinberg, 2011). In colorectal cancer (CRC), the most well studied Wnt-driven cancer, it is thought that cancer cells must survive long enough to acquire a series of step-wise mutations that occur over a period of

decades in order for an invasive tumor to form (Figure 6.2)(Kinzler and Vogelstein, 1996). During this long progression from tumor initiation to invasion, there are many circumstances in which a cancer cell is exposed to harsh environments that would normally cause cell death. A successful cancer cell

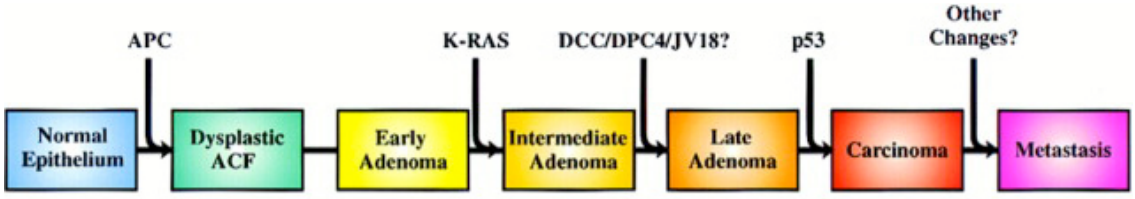


Figure 6.2. Genetic changes associated with colorectal tumorigenesis. APC mutations initiate the neoplastic process, and tumor progression results from mutations in the other genes indicated. Patients with Familial Adenomatous Polyposis inherit APC mutations and develop numerous dysplastic aberrant crypt foci (ACF), some of which progress as they acquire the other mutations indicated in the figure. K-RAS is an oncogene that requires only one genetic event for its activation. The other specific genes indicated are tumor suppressor genes that require two genetic events (one in each allele) for their inactivation. Chromosome 18q21 may contain several different tumor suppressor genes involved in colorectal neoplasia, with *DCC*, *DPC4*, and *JV18–1* genes proposed as candidates. A variety of other genetic alterations have each been described in a small fraction of advanced colorectal cancers. These may be responsible for the heterogeneity of biologic and clinical properties observed among different cases. Figure adapted from (Kinzler and Vogelstein, 1996).

must survive loss of cell-cell and cell-matrix attachments, hypoxia, overexpression of oncogenes, and massive DNA damage, in order to become a fully invasive and malignant tumor (Hanahan and Weinberg, 2011). Thus, constitutive Wnt signaling, and resultant XIAP expression, not only increases the proliferative potential of a cell, but it makes it more likely to survive long enough to acquire the other mutations needed to form a fully invasive tumor.

This dual role of Wnt signaling may partly explain why mutations in the Wnt pathway are most often the first "hit" in the progression of CRC (Figure 6.2). Why Wnt pathway activation most often occurs before Ras activation is unclear, based on this proposed hypothesis, as Ras also induces proliferation and has also been shown to upregulate XIAP expression to enhance cell survival (Liu et al., 2005b). Perhaps Wnt mutations are favored early to select for a stem cell-like cell as Wnt signaling has been shown to be required for maintaining "stemness" in the colon and "cancer stem cells" have been postulated as the major driving force in tumor initiation and progression (Beachy et al.; Reya and Clevers, 2005). Once a stem cell-like cell is selected, it may then be further transformed by an activating Ras mutation that has many oncogenic effects on the cell in addition to the myriad Wnt signaling effects.

The real question, then, is why do CRC cells, and cancer cells in general, so often lose p53 if XIAP is so often expressed at high levels, which presumably inhibits apoptosis? One possibility is that p53 is lost not because of its role as an apoptotic effector, but because of its many other tumor suppressor functions within cells. This appears to be the case given that loss of p53 had no effect on the frequency of apoptosis in $Apc^{Min/+}$ mouse early or late stage adenomas, leading the authors of this study to conclude that p53 is most likely lost either to prevent cellular senescence or to increase the angiogenic potential of cancer cells (Fazeli et al., 1997). Alternatively, or additionally, p53 may be lost to allow cell migration. In CRC, p53 is most often lost just prior to tumor invasion and

metastasis (Figure 6.2) suggesting that p53 may be functioning as a metastasis suppressor that must be lost in order for tumor invasion to occur. There is a lot of evidence to support this idea. p53 has been shown to regulate the expression of the important metastasis suppressors KIA1, Nm23, and E-cadherin (Marreiros et al., 2005; Mashimo et al., 1998; Roger et al., 2010). Thus, loss of p53 results in loss of expression of these genes, which significantly increases the metastatic potential of cancer cells. p53 has also been shown to prevent RhoA activation by Ras, suggesting an important function of p53 is to prevent Ras-mediated cell migration (Xia and Land, 2007). Thus, p53 may be lost in CRC, and other cancers containing anti-apoptotic activity, not because it increases apoptotic-resistance, but rather because losing p53 confers other important growth advantages to cancer cells.

Interestingly, a recent study showed that XIAP also directly promotes metastasis independently of its anti-apoptotic function. XIAP induces NF-κB signaling in cooperation with another IAP family member, Survivin, which leads to increased fibronectin expression, β1 integrin signaling, and activation of the cell motility kinases FAK and Src (Mehrotra et al., 2010). Thus, XIAP is a triple threat in terms of cancer: it is required for Wnt-induced proliferation, which is commonly found in many cancer types, it prevents cancer cell death by inhibiting apoptosis, and it directly promotes metastasis.

Given the multi-faceted role of XIAP in cancer, targeting XIAP would be predicted to be a particularly effective chemotherapeutic strategy. Indeed, many

drug companies are targeting XIAP and clinical trials with XIAP inhibitors are currently underway (Hunter et al., 2007; LaCasse et al., 2008). To date, however, much work has focused on inhibiting the anti-apoptotic BIR domains of XIAP, while my studies, and those of Mehrotra et al., reveal that the anti-apoptotic function of XIAP is dispensable for its Wnt pathway function and its ability to promote metastasis (Mehrotra et al., 2010). Thus, targeting the entire XIAP protein (e.g. with siRNA constructs) would be predicted to be a more effective chemotherapeutic strategy as loss of the entire protein would inhibit all of the oncogenic functions of XIAP. One pharmaceutical company, Aegera Therapeutics (Montreal, QC, Canada), has designed a XIAP anti-sense oligonucleotide (AEG35156) that has proven effective in multiple cancer types in pre-clinical studies including pediatric tumors, acute myeloid leukemia (AML), breast, ovarian, prostate, lung, and colon cancer (Holt et al., 2011; LaCasse et al., 2006; Shaw et al., 2008; Tamm, 2008). There are currently multiple ongoing clinical trials using AEG35156 as a single agent or in combination with other chemotherapy drugs (Tamm, 2008). The only results published from these studies so far are from a phase I/II clinical trial evaluating the effects of AEG35156 in patients with relapsed/refractory AML, which indicate that AEG35156 is very effective in this patient population in combination with idarubicin and cytarabine (Schimmer et al., 2009). It will be interesting to see how effective such XIAP antagonists are in multiple cancer types in the future.

In conclusion, the work presented here identifies a novel role for XIAP in

Wnt signaling, which provides further insight into why XIAP might be highly expressed in cancer cells. These findings also provide rationale for targeting the entire XIAP protein in cancer treatment as the role of XIAP in Wnt signaling and metastasis is independent of the anti-apoptotic functions of its BIR domains.

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