

Kinase regulation of XIAP in Wnt signaling

By

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For my family

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Abstract

Regulation of XIAP by GSK3 during Wnt signaling

The Wnt signaling pathway plays essential roles in a wide variety of biological processes including early animal development, cell fate determination, cell proliferation, organogenesis, and stem-cell renewal. Deregulation of the Wnt pathway can lead to human disease (e.g. developmental defects and cancers). Our lab had previously demonstrated that X-linked Inhibitor of Apoptosis (XIAP) is required for Wnt signaling via a mechanism that involves XIAP-facilitated dissociation of Gro/TLE from TCF/Lef. We had shown that XIAP is recruited onto TCF/Lef complexes upon Wnt pathway activation and ubiquitinates Gro/TLE-bound TCF/Lef. Ubiquitinated Gro/TLE has decreased affinity for TCF/Lef, allowing beta-catenin to bind. The mechanism by which XIAP is recruited onto TCF/Lef is not known.

We found that XIAP is phosphorylated by GSK3, a known component of the Wnt pathway. Using mass spectrometry analysis with purified proteins, we identified two GSK3 phosphorylation sites on XIAP that are strongly phosphorylated. Mutational analysis of these two sites indicate that they are required by XIAP to fully activate Wnt signaling, as assessed by reporter assays in cultured mammalian cells and axis duplication assays in *Xenopus* embryos. Using purified proteins, we found that the XIAP phosphomutants have similar ubiquitination activity as wild-type XIAP. In cultured mammalian cells, however, the XIAP phosphomutants have a markedly decreased capacity to ubiquitinate Gro/TLE. We also showed that the phosphomutants also have a decreased affinity for Gro/TLE.

We propose a model in which phosphorylation is required for the interaction between XIAP and Gro/TLE to activate Wnt signaling. GSK3 phosphorylates XIAP at T180 and S239 to facilitate

its binding to Gro/TLE. XIAP can then ubiquitinate and remove Gro/TLE for subsequent binding of beta-catenin to form the transcriptionally active complex.

Chapter I: Wnt signaling and XIAP

Introduction

The Wnt signaling pathway plays an integral role in cell fate determination, embryonic development, cell proliferation, and adult tissue homeostasis. Misregulation of the pathway can lead to developmental defects and cancers in humans (Saito-Diaz, Chen et al. 2013).

Wnt proteins comprise a family of nineteen secreted glycoproteins that act as ligands that activate Wnt signaling (Komekado, Yamamoto et al. 2007). They are found in all metazoans from fruit flies to humans. Studies of the Wnt signaling pathway was initiated in the 1970s by Sharma and Chopra with the characterization of a *Drosophila* mutation that interfered with wing and haltere development leading to reduced or absent wings (Sharma and Chopra 1976). Based on the phenotype, the mutation was named “wingless” (*wg*). Further research by Wieschaus and Nusslein-Volhard found that *wg* played a fundamental role (e.g., segment polarity) in establishing the animal body plan (Nüsslein-Volhard and Wieschaus 1980). Subsequently, Nusse and Varmus performed a forward genetic screen to identify genes that could induce tumors in mice. Using the mouse mammary tumor virus (MMTV) to introduce insertions into the mouse genome, they identified *integration 1* (*int-1*), a locus whose disruption led to mammary tumorigenesis (Nusse, van Ooyen et al. 1984).

A cluster of cells called the Spemann organizer is responsible for patterning the body axis during *Xenopus* development (Spemann and Mangold 1924). Likewise, in zebrafish and chick, the analogous structures are the embryonic shield and Hensen’s node, respectively. These organizing regions represent important centers of induction that establish cell fate and the body plan during early animal development. In the *Xenopus* embryo, Wnt signaling is critical in the formation of

the Spemann organizer, which is formed on the future dorsum of the *Xenopus* embryo. Studies by Moon and McMahon showed that Int-1 mRNA injected into the ventral side of *Xenopus* embryos induced the formation of a second Spemann organizer, indicating that Wnt signaling played a critical role in organizer formation (McMahon and Moon 1989). *Xenopus* embryos injected with int-1 mRNA ultimately developed duplication of the dorsal axis and bifurcation of the neural plate, further demonstrating the importance of Int-1 in patterning vertebrate embryos. The recognition that wg is the *Drosophila* homolog of int-1 ultimately led to their simplification to *Wnt*.

These early discoveries revealed a central role of Wnt signaling in body patterning during development, and also suggested its potential role in oncogenesis (Polakis 2010). It is not surprising that Wnt signaling plays such an important role in animal development, given that its appearance accompanied the evolution of multi-cellular organisms. Consistent with this idea, genetic studies have shown that Wnt proteins appear to have undergone multiple rounds of duplication and differentiation, corresponding to an increase in the complexity of body plan during metazoan evolution.

The current model of the Wnt pathway

Wnt pathway activation can be simply described as the translocation of cytoplasmic beta-catenin (initially identified as the segment polarity gene *armadillo* in *Drosophila*) to the nucleus, where it can affect the transcription of Wnt-target genes. In the absence of a Wnt signal, beta-catenin is continuously synthesized and degraded by the beta-catenin destruction complex, which is composed of the scaffold proteins Axin and adenomatous polyposis coli (APC), and the kinases glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1) (Kimelman and Xu 2006). Upon binding of Wnt ligands to co-receptors Frizzled and LRP6, the activity of the beta-catenin

destruction complex is inhibited, thereby blocking the degradation of beta-catenin and allowing its accumulation in the cytoplasm. Accumulated beta-catenin then enters the nucleus to mediate transcription. Thus, Wnt pathway activation can be broadly divided into three distinct steps: 1) surface receptor activation, 2) inhibition of the beta-catenin destruction complex, and 3) formation of the beta-catenin co-activator complex initiating the transcription of Wnt-target genes.

Surface receptor activation

Wnt ligands are a family of secreted, lipid-modified cysteine-rich glycoproteins approximately 350-400 amino acids in length. In vertebrates, 19 Wnt ligands have been identified (Janda, Waghray et al. 2012). Post-translational modification of Wnt ligands plays important roles in their secretion and activity. N-glycosylation of Wnt ligands is crucial in the release of Wnt ligands from cells, and mutations that affect its glycosylation have been shown to block Wnt secretion (Komekado, Yamamoto et al. 2007). Wnt ligands have also been shown to undergo acylation with palmitate, which is required for their secretion and binding to Frizzled receptors (Willert, Brown et al. 2003). Palmitoylation of Wnt ligands occurs in the endoplasmic reticulum by the transmembrane O-acetyltransferase protein Porcupine (Port and Basler 2010). Loss of function Porcupine leads to the accumulation of Wnt ligands in the ER (Kadowaki, Wilder et al. 1996). Once palmitoylated, Wnt ligands pass through the trans-Golgi network where they bind Wntless (WI) and are transported to the plasma membrane for extracellular release.

Wnt ligands are capable of both short-range and long-range (morphogen) signaling. When released, Wnt ligands have been proposed to traverse through extracellular space via several mechanisms. These proposed mechanisms could be tissue-specific and influenced by the condition of the extracellular environment. Wnt ligands could travel in tissues by diffusion and interact with

cell-surface heparin sulfate proteoglycans (HSPGs), which facilitate the accumulation of extracellular ligands (Strigini and Cohen 2000). Many of these HSPG involved in binding Wnt ligands are attached to the cell surface by glycosylphosphatidylinositol (GPI) anchors. Another mechanism by which Wnt ligands may transverse long distances (characterized extensively in *Drosophila*) involves the internalization and then resecretion of Wnt ligands to establish a signal gradient (Pfeiffer, Ricardo et al. 2002).

The Frizzled (Fz) protein is a family of seven-pass transmembrane domain receptors, which share some structural similarity to G-protein-coupled receptors (GCPR) (Bhanot, Brink et al. 1996). LRP5 and LRP 6 are functionally-redundant single-pass transmembrane proteins that act in concert with Fz proteins to bind Wnt ligands (Skarnes, Pinson et al. 2000). Binding of Wnt ligands to Fz and LRP5/6 co-receptors results in the production of phosphatidylinositol (4,5)-bisphosphate (PIP₂) via a mechanism involving the cytoplasmic protein Disheveled, inducing the oligomerization and clustering of LRP5/6 (Pan, Choi et al. 2008).

Formation of oligomerized, “activated” LRP5/6 promotes the recruitment of Axin and associated members of the destruction complex to LRP5/6 (Mao, Wang et al. 2001). This initial recruitment of Axin (also known as the “initiation step” of receptor activation) results in phosphorylation of the intracellular domain (ICD) of LRP5/6 (Baig-Lewis, Peterson-Nedry et al. 2007). The ICD of the LRP5/6 receptor contains five PPPSPxS motifs, and each of these motifs is sufficient for pathway activation. The five motifs have been shown to act in a cooperative manner to mediate downstream signaling (Wolf, Palmby et al. 2007). Two kinases associated with the destruction complex, GSK3 and CK1, have been demonstrated to phosphorylate the PPPSPxS motifs in a sequential manner. Wnt binding induces phosphorylation by GSK3 and studies have also shown that CK1 primes the S/T clusters, outside of the PPPSPxS motif, for phosphorylation

by GSK3 (Davidson, Wu et al. 2005). In any case, phosphorylated LRP6 has high affinity for Axin and promotes further recruitment of Axin-bound GSK3, which in turn promotes further phosphorylation of LRP5/6. This positive feedback step is the “amplification step” in Wnt pathway activation. Phosphorylated LRP5/6 is able to directly inhibit GSK3 activity, thereby blocking beta-catenin phosphorylation and subsequent ubiquitin-mediated degradation by the proteasome.

Components and function of the beta-catenin destruction complex

In the absence of a Wnt signal, cytoplasmic levels of beta-catenin are maintained at a low level. This occurs through the beta-catenin destruction complex, which phosphorylates and targets beta-catenin for ubiquitin-mediated degradation by the proteasome. The beta-catenin destruction complex is composed of the scaffolding proteins Axin and APC, and the kinases GSK3 and CK1. Axin is the critical and limiting component in the destruction complex (Lee, Salic et al. 2003). The interaction between Axin and APC, beta-catenin, and GSK3 has been determined at atomic resolution by x-ray crystallography (Spink, Polakis et al. 2000). Cellular levels of Axin are generally low, and it has been proposed that this serves to separate Wnt from other pathways which share common components (e.g. GSK3, CK1, and beta-catenin), thus providing specificity. Axin levels have been shown to be highly regulated by PARsylation, which promotes its ubiquitin-mediated degradation (Huang, Mishina et al. 2009).

GSK3 is a serine/threonine protein kinase that has been shown to be involved in many different signaling pathways (Forde and Dale 2007). While GSK3 has both positive (acting at the level of LRP5/6) and negative (acting at the level of the destruction complex) effects on Wnt signaling, antagonizing GSK3 activity is central to all current models of Wnt signaling mechanisms. CKI consists of a family of serine/threonine kinases. CKI family members have also been shown to be

widely involved in other signaling pathways (Price 2006). The CKI family proteins have very similar catalytic domains, but differ in their C-terminal non-catalytic tails, which vary in length and have been implicated in regulating the catalytic activity of the different isoforms (Price 2006). Within the destruction complex, CKI provides the priming phosphorylation of beta-catenin for subsequent phosphorylation by GSK3 (Liu, Li et al. 2002).

The other scaffold protein in the destruction complex is adenomatous polyposis coli (APC), a large protein of around 300 kDa. APC is a negative regulator of Wnt signaling and its mutation has been identified in familial adenomatous polyposis (FAP), a familial precancerous syndrome associated with an extremely high risk (nearly 100%) for colorectal cancer (Kinzler, Nilbert et al. 1991). APC binds beta-catenin, GSK3, and Axin, and loss of APC leads to elevated levels of beta-catenin and constitutive Wnt pathway activation. The mechanism of action of APC in the Wnt pathway is an area of active research. It has been proposed to inhibit Wnt signaling via several distinct mechanisms, including 1) retaining beta-catenin in the nucleus (Hamada and Bienz 2004), 2) targeting the destruction complex to the cell cortex where E3 ligase for beta-catenin resides (Bienz, Yu et al. 1999), and 3) blocking the dephosphorylation of beta-catenin (Su, Fu et al. 2008).

The continual synthesis and degradation of cytoplasmic beta-catenin can be described as a “futile cycle”. Such futile cycles are thought to impart increased sensitivity in response to physiological changes (Samoilov, Plyasunov et al. 2005). Axin, the rate-limiting protein in the complex, plays a critical role in nucleation of the destruction complex. Binding of beta-catenin in the presence of both APC and Axin greatly enhances the phosphorylation of beta-catenin by CKI at residue serine 45. This phosphorylation creates a priming site to allow subsequent sequential phosphorylation of beta-catenin by GSK3 at residues threonine 41, serine 37, and serine 33 (Amit 2002). GSK3 has been shown to phosphorylate Axin, increasing its affinity for beta-catenin (Luo,

Peterson et al. 2007). CKI and GSK3 have also been shown to phosphorylate APC to increase its affinity for beta-catenin. Enhanced binding of phosphorylated APC to beta-catenin has been proposed to facilitate the removal of phosphorylated beta-catenin from Axin in order to allow the rebinding of non-phosphorylated beta-catenin to the destruction complex (Su, Fu et al. 2008). Thus, in this manner, the destruction complex can rapidly catalyze multiple rounds of beta-catenin phosphorylation. The exact timing and order of addition of the components involved in the formation of the destruction complex is not well understood. Regardless, beta-catenin, when phosphorylated at serine 33 and 37, is recognized by beta-TRCP, a subunit of the SCF ubiquitin ligase complex (Jiang and Struhl 1998). Binding to beta-TRCP-SCF catalyzes the polyubiquitination of beta-catenin and its subsequent degradation by the proteasome.

Upon Wnt pathway activation, the beta-catenin destruction complex is inhibited, leading to increased levels of cytoplasmic beta-catenin. Various models have been proposed by which receptor activation leads to inhibition of beta-catenin degradation. One model posits that Wnt activation leads to the dissociation of the components in the beta-catenin destruction complex. A possible mechanism is via the action of the GSK3 binding protein (GBP), which has been shown to compete with Axin for GSK3 binding (Yost, Farr et al. 1998). Knockout studies of GBP in mice, however, do not result in a Wnt phenotype. Another model suggests that the destruction complex remains intact and is localized to the Fz/LRP6 receptor, where phospho-LRP6 directly inhibits GSK3 activity (Hendriksen, Jansen et al. 2008). A third model proposes that Wnt signaling leads to inhibitory phosphorylation of GSK3 at serine 9 that inhibits its kinase activity (Yokoyama and Malbon 2007). Another model states that the beta-catenin destruction complex scaffold protein Axin is degraded upon Wnt signaling (Cselenyi, Jernigan et al. 2008). Because Axin is the rate-limiting component of the destruction complex, its degradation is likely to impact beta-catenin

turnover. Finally, a fifth model suggests that sequestration of GSK3 into multivesicular bodies (MVB) occurs upon Wnt signaling, thereby inhibiting the phosphorylation and degradation of beta-catenin by GSK3 (Taelman, Dobrowolski et al. 2010). Multiple lines of evidence support these models. Furthermore, many of these models are not mutually exclusive. Regardless of the model, the effect of destruction complex inhibition is the accumulation cytoplasmic beta-catenin upon Wnt pathway activation

Formation of the nuclear co-activator complex

Translocation of beta-catenin to the nucleus occurs rapidly following the rise of cytoplasmic beta-catenin levels upon Wnt activation. The beta-catenin protein does not contain any recognizable nuclear localization signal (NLS) and its mechanism of nuclear entry remains unclear. Beta-catenin does not utilize classic import factors such as RanGTPase (Fagotto 1996). However, the armadillo repeats of beta-catenin are structurally similar to importin-beta HEAT repeats and might play a role in interacting with the nuclear pore complex (Kutay 1997). On the other hand, it is also possible that Wnt signaling regulates beta-catenin nuclear export as there are numerous nuclear proteins shown to interact with beta-catenin and could serve as an anchor for nuclear retention (Cong and Varmus 2004).

In the nucleus, in the absence of Wnt activation, T cell specific factor (TCF) family proteins interact with Groucho/transducin-like enhancer (Gro/TLE) to form a co-repressor complex that inhibits the transcription of Wnt target genes. There are four mammalian TCF members, namely TCF1-4. TCF proteins bind at the DNA consensus sequence CCTTTG(T/A)(T/A), termed Wnt-responsive elements (WRE). WREs regulate about 300-400 gene collectively (Hatzis, van der Flier et al. 2008). Upon Wnt pathway activation, translocated beta-catenin displaces Gro/TLE and binds

to TCF, transforming the TCF co-repressor complex to a co-activator complex and promoting the transcription of Wnt target genes. The transition from a Wnt co-repressor complex to a co-activator complex is facilitated by X-linked inhibitor of apoptosis (XIAP), an E3 ligase that plays an integral part in the removal of Gro/TLE to promote the binding of beta-catenin to TCF (described in more detail below).

Transcription via the beta-catenin-TCF complex is facilitated by multiple nuclear factors. For example, the B-cell CLL/lymphoma 9 (BCL9) protein binds to TCF and Pygopus (Pygo) (Thompson, Townsley et al. 2002). Pygo contains a plant homology domain (PHD) that interacts with dimethylated histone 3 lysine 4. It has been postulated that the formation of a core transcriptional complex consisting of TCF, beta-catenin, BCL9, and Pygo is required for efficient transcription of Wnt target genes (Schwab, Patterson et al. 2007).

Role of ubiquitination in the Wnt pathway

Protein turnover represents a major mechanism in virtually all cellular processes. The turnover, or their levels and half-lives, of proteins with key cellular functions such as DNA replication, cell cycle progression, metabolism and apoptosis are tightly regulated for proper cellular function (Ciechanover 2009). In addition, proteins that are misfolded upon synthesis or damaged afterwards need to be targeted for degradation to circumvent disrupting other factors in the pathways that they normally participate in.

In the Wnt pathway, the degradation of beta-catenin plays a central role in controlling Wnt pathway activation, and degradation of beta-catenin occurs via the ubiquitin pathway. Ubiquitin is a small protein (76 kDa) that is covalently attached to the proteins targeted for proteasomal

degradation. Ubiquitin has also been shown to function in conjunction with autophagy, lysosomes, and other proteases to carry out regulated degradation of intracellular proteins.

In addition to regulating protein stability, ubiquitin can also change the activity and/or localization of a targeted protein in a process that is often dependent on the type and number of ubiquitin linkages employed (Peng, Schwartz et al. 2003). The ubiquitination of proteins is initiated via the action of the E1 activating enzyme, which catalyzes the formation of a thioester bond between a cysteine on the E1 and the C-terminal glycine of ubiquitin in an ATP-dependent manner. The E1 enzyme subsequently catalyzes the transfer of ubiquitin to the active site of the E2 conjugating enzyme. Lastly, the E2 catalyzes the transfer of ubiquitin to a lysine residue on the target protein in a process facilitated by an E3 ubiquitin ligase. E3 ubiquitin ligases are the specificity component of the ubiquitination reaction (Varshavsky 2006). There are hundreds of E3 ligases, which can be divided into two major families based on the presence of two distinct domains: 1) the Really Interesting New Gene (RING) and 2) the Homologous to E6AP Carboxy Terminus (HECT) domains (Pickart and Eddins 2004). Ubiquitination of substrates are directly catalyzed by HECT E3 ligases, and the ubiquitin is transferred from the E3 to the substrate. For RING E3s, the E2- ubiquitin conjugate is bound by the E3 ligase, which indirectly mediates the transfer of ubiquitin from the E2 to the substrate lysine. Ubiquitin can be added to the target protein in a chain-like fashion (polyubiquitination), or as a single moiety on the target protein (monoubiquitination). There are seven internal lysine residues (K6, 11, 27, 29, 33, 48, 63) present in the ubiquitin protein, each of which can be used to form distinct ubiquitin chain linkages that confer different functions (Behrends and Harper 2011). For example, beta-catenin is ubiquitinated by the SCF beta-TRCP E3 ligase (upon its phosphorylation by GSK3 and CK1) via K48-linked polyubiquitin chains, which targets it for degradation via the proteasome pathway (Aberle, Bauer

et al. 1997). In addition to SCF beta-TRCP, beta-catenin is regulated by another E3 ligase, c-Cbl, in the nucleus. Wnt activation promotes the translocation of c-Cbl to the nucleus where it ubiquitinates beta-catenin to inhibit the transcription of Wnt target genes (Chitalia, Shivanna et al. 2013). In contrast to the negative regulation of beta-catenin by SCF beta-TRCP and c-Cbl, ubiquitination of beta-catenin by the EDD E3 ligase promotes beta-catenin stability and nuclear translocation. Axin, the rate-limiting component of the destruction complex, has also been shown to be degraded in an ubiquitin-mediated fashion. Tankyrase is an poly(ADP-Ribose) polymerase that ADP-ribosylates Axin. The poly(ADP-ribose) moiety on Axin is recognized by RNF146, a poly(ADP-ribose)-directed E3 ligase, that ubiquitinates Axin and targets it for degradation (Huang, Mishina et al. 2009). The Wnt pathway protein, Disheveled, has also been shown to be ubiquitinated by the E3 ligases NEDD4L and KLHL12, increasing its turnover, decreasing its steady-state levels, and inhibiting Wnt signaling (Huang, Langelotz et al. 2009). Recently, the transmembrane RING E3 ligases, zinc and ring finger 3 and ring finger 43 (ZNRF3/RNF43), have been shown to regulate Frizzled and LRP6 homeostasis by catalyzing their ubiquitin-mediated degradation (Hao, Xie et al. 2012). The activities of these two E3 ligases are directly inhibited by the secreted protein, R-Spondin, upon its binding to the stem cell marker, LGR5 (Hao, Xie et al. 2012). Thus, nearly all major components of the Wnt pathway are regulated by the ubiquitin system, which control their levels and/or activities.

XIAP

X-linked inhibitor of apoptosis (XIAP) is an E3 ligase that was originally identified by its homology to genes in the genome of baculovirus (Liston, Fong et al. 2001). The baculovirus homologs of XIAP were themselves identified in genetic screens for suppressors of cell death (Uren, Pakusch et al. 1996). XIAP has been widely studied for its role in regulating apoptosis.

Members of the cellular inhibitor of apoptosis (cIAP) protein family contain one to three baculovirus IAP repeats (BIRs), which are 70-residue zinc-binding domains. XIAP contains three BIRs that can directly bind to caspase-3, -7, and -9 to inhibit their proteolytic activity (Galban and Duckett 2010). To date, XIAP is the only IAP family member that binds to the active site of caspases to inhibit their activities (Eckelman, Salvesen et al. 2006). Another key feature of IAP is the RING finger motif, which confers E3 ligase activity. The RING domains of IAP proteins (located at the C-terminal ends of the proteins) are much more similar in sequence to each other than to the RINGs of other E3 ligases (Galban and Duckett 2010). Upon viral infection, the RING domain has been found to enhance the antiapoptotic activity of IAPs (Suzuki, Nakabayashi et al. 2001).

XIAP itself can be degraded through the ubiquitin-proteasome pathway and is capable of autoubiquitination in a manner that is dependent on its RING domain (Lotocki, Alonso et al. 2003). However, the types and degree of ubiquitination on XIAP itself are not clearly understood, although K48 linkage has been proposed as the candidate modification. The association between XIAP autoubiquitination and cell death is not clearly defined. For example, it is not known if autoubiquitination occurs as a result of cell death or whether autoubiquitination and degradation of XIAP occur prior to cell death. Studies into XIAP function have also been complicated by the fact that XIAP-deficient mice are healthy and exhibit no obvious defects in apoptosis (Harlin, Reffey et al. 2001). It has been proposed that other IAPs, such as cIAP1 and cIAP2, could compensate for XIAP function in the XIAP-deficient mice (Olayioye, Kaufmann et al. 2004).

The mechanism by which XIAP is autoubiquitinated is also incompletely understood. It is possible that autoubiquitination involves other factors, whereby binding induces a conformational change in XIAP that allows for its autoubiquitination. Affinity purification approaches have

identified a number of proteins that bind XIAP, and they are diverse in sequence and structure (Vaux and Silke 2003). However, they all contain a tetrapeptide domain known as the IAP-binding motif (IBM), which confers the ability to bind XIAP. One of the best characterized IBM-containing protein is Smac (Diablo). Smac is released from the mitochondria in response to apoptotic signals, such as cytochrome c. Smac has also been shown to bind to XIAP and neutralize its apoptosis-inhibiting activities (Du, Fang et al. 2000). Other IBM-containing peptides have been shown to induce XIAP turnover in a RING-dependent manner. Studies have shown that cIAPs can also ubiquitinate XIAP (Galbán, Hwang et al. 2009). Further studies are needed to elucidate the regulation of XIAP turnover.

In addition to autoubiquitination, XIAP can also ubiquitinate other proteins. For example, XIAP can ubiquitinate apoptosis-inducing factor (AIF) and promote its degradation (Wilkinson, Wilkinson et al. 2007). XIAP has also been shown to ubiquitinate Survivin, in a mechanism requiring the XIAP-associated factor-1 (XAF1) (Arora, Cheung et al. 2007). Surprisingly, studies have also shown that XIAP can ubiquitinate Smac, whose binding induces the turnover of XIAP itself. It is therefore very possible that XIAP and its associated proteins exist in a dynamic equilibrium.

Beyond its role in apoptosis, increasing evidence indicate that XIAP can participate in a variety of signaling pathways. For example, there is a growing body of literature showing that XIAP regulates NF- κ B signaling. A number of studies have shown that XIAP overexpression is able to activate NF- κ B reporter activity, and that the RING domain is required for such activity (Barkett, Xue et al. 1997). A number of substrates in the NF- κ B have been proposed to be targets of XIAP ubiquitination. TGF-beta Activating Kinase-1 (TAK1), a serine threonine kinase, is a major component of the NF- κ B signaling and has been shown to form a complex with and be

ubiquitinated by XIAP (Hofer-Warbinek, Schmid et al. 2000). Another XIAP substrate is Copper Metabolism (Murr1) Domain Containing 1 protein (COMMD1), which plays a role in the ubiquitination of chromatin-bound NF- κ B proteins to suppress NF- κ B signaling. It has been hypothesized that XIAP activates NF- κ B dependent transcription by negatively regulating the activity of COMMD1 (Burstein, Hoberg et al. 2005).

The identification of XIAP as a regulator of Wnt signaling

Previous models of Wnt signaling proposed that, upon Wnt signaling, beta-catenin displaces the co-repressor, Gro/TLE, from TCF. In this model, elevated levels of nuclear beta-catenin is sufficient to displace Gro/TLE by mass action (Daniels and Weis 2005). Other studies, however, have shown that the increase in beta-catenin levels upon Wnt pathway activation can be as small as two-fold when compared to the basal state (Goentoro and Kirschner 2009). Thus, it is difficult to envision that beta-catenin can displace Gro/TLE from TCF via a simple competitive mechanism, and there is likely to be a facilitated mechanism by which Gro/TLE is removed from TCF upon Wnt signaling.

Taking advantage of the conserved nature of the Wnt pathway, Dr. Alison Hanson in our lab conducted an RNAi screen in *Drosophila* S2 cells to identify new E3 ligases that could regulate Wnt signaling. One gene identified as a potential positive regulator of Wnt signaling was DIAP1 (*Drosophila* inhibitor of apoptosis 1). XIAP is the mammalian homolog of DIAP1, and our lab proceeded to characterize the role of XIAP in Wnt signaling. In cultured human cells, knocking down XIAP with siRNA inhibited ligand-induced Wnt activation. XIAP is required for Wnt signaling *in vivo*, and knocking XIAP using morpholino oligonucleotides resulted in axis patterning defects consistent with Wnt inhibition. XIAP functions downstream of the beta-catenin

destruction complex, as evidenced by the observation that knocking down XIAP inhibits Wnt activation by the addition of GSK3 inhibitors. A candidate approach was then undertaken to determine the Wnt component that interacted with XIAP. Gro/TLE was identified as a potential candidate (Hanson, Wallace et al. 2012).

Subsequent studies showed that XIAP also binds TCF upon Wnt activation. Ubiquitination assays demonstrated that XIAP ubiquitinates Gro/TLE *in vitro* using purified components and in cultured mammalian cells, and ubiquitinated Gro/TLE exhibited decreased binding affinity to TCF4. These important studies led to a new model for nuclear activation of the Wnt pathway whereby, upon Wnt pathway activation, XIAP is recruited onto TCF. Bound XIAP ubiquitinates Gro/TLE to decrease its affinity for TCF, thereby facilitating the binding of beta-catenin to TCF (Hanson, Wallace et al. 2012).

A major unanswered question in the proposed model is how XIAP is recruited to the Gro/TLE –TCF complex upon Wnt signaling. A hint of how this process may be controlled came from Dr. Hanson's studies that a Wnt pathway activator, lithium (inhibits GSK3), is capable of promoting the association of XIAP with TCF (Hanson, Wallace et al. 2012). Previous studies have shown that GSK3 and XIAP interact through the latter's RING domain (Sun, Meares et al. 2009). Thus, GSK3 may directly regulate the recruitment of XIAP onto TCF upon Wnt pathway activation.

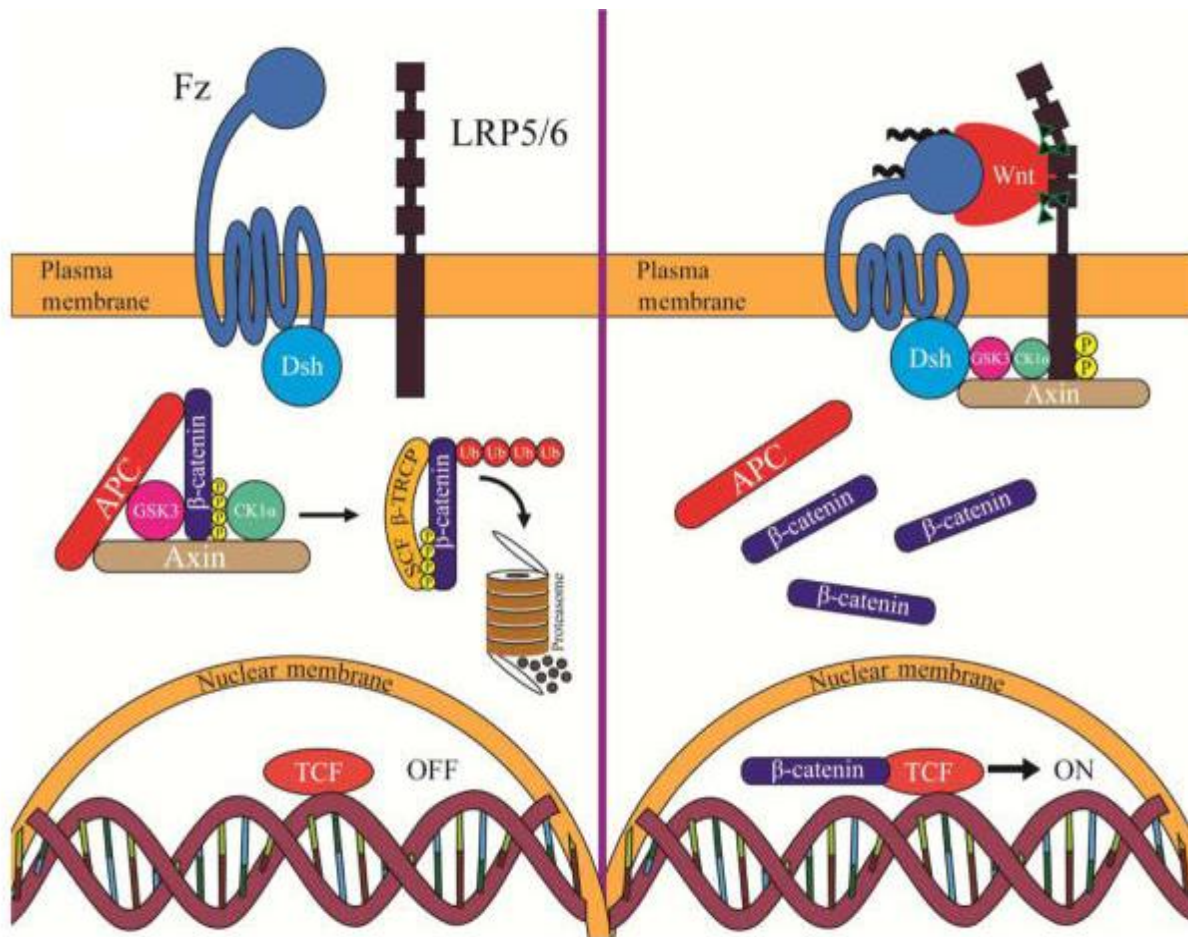


Figure 1.1. The current model of Wnt signaling. Left panel: In the absence of Wnt, cytoplasmic β -catenin forms a complex with APC, Axin, GSK3, and CK1 α . β -Catenin is phosphorylated by CK1 α and subsequently phosphorylated by GSK3. The phosphorylated form of β -catenin is recognized by the E3 ubiquitin ligase SCF β -TRCP, which targets β -catenin for proteasomal degradation. In the absence of nuclear β -catenin, Wnt target genes are repressed. Right panel: In the presence of Wnt ligand, a receptor complex forms between Fz, LRP5/6, and Wnt. The recruitment of Dsh by Fz leads to LRP5/6 phosphorylation by CK1 α and GSK3 followed by recruitment of Axin to LRP5/6. The latter disrupts Axin-mediated phosphorylation/degradation of β -catenin, leading to accumulation of β -catenin in the cytoplasm and its translocation to the nucleus, where it acts as a transcriptional co-activator with TCF to activate Wnt-responsive target genes. Figure from (Saito-Diaz, 2013).

Chapter II: Materials and Methods

Expression and purification of XIAP

BL21 bacterial cells were transformed with MBP-XIAP or MBP-XIAP phosphomutant plasmids and grown in a 37°C shaking incubator. IPTG (300 μ M) was then added when the OD of the culture reached 0.8. Flasks were then moved to an 18°C shaking incubator for an overnight induction. Cultures were then spun down, pellets were collected, and cells lysed by sonication in Tris-NaCl-phenylmethylsulfonyl fluoride (TNP) buffer (50mM Tris-HCl, 150mM NaCl, 2mM EDTA, 1mM phenylmethylsulfonyl fluoride (PMSF), 0.1% Triton X-100). Lysates were centrifuged at 12,000g at 4°C for 10 minutes and supernatant collected. Supernatants were then incubated with amylose beads on a shaking platform for two hours at 4°C. The beads were washed with ten column volumes of TNP buffer three times and then MBP-bound protein was eluted with TNP buffer containing 1% maltose. Eluted proteins were further purified on a Mono Q anion-exchange column using the AKTA FPLC apparatus (GE Healthcare). Fractions containing the MBP fusion proteins were collected and concentrated using Amicon Ultra Centrifugal Filter Units (Millipore) by spinning at 3,000 rpm for twenty minutes, to a final concentration of approximately 1 μ g/ μ l in TNP buffer. Samples were then aliquoted and stored at -80°C until use.

XIAP *in vitro* phosphorylation assay

For radioactive kinase assays, 2 μ g of XIAP was added, along with 250 units of GSK3 (New England Biolabs), 5 mM of cold ATP, 5 μ Ci of [³²P] γ ATP in 1X kinase reaction buffer (50mM Tris-HCl, 10mM MgCl₂, 5mM dithiothreitol (DTT), pH 7.5). Reactions were incubated at 30 °C for one hour, and reactions processed for SDS-PAGE followed by autoradiography.

Mass spectrometry analysis

A kinase reaction was performed as described for the *in vitro* phosphorylation reaction except no radioactive ATP was added. The reactions were then subjected to SDS-PAGE, and the gel stained with coomassie. The band corresponding to XIAP was then excised and subjected to liquid chromatography-mass spectrometry (LC-MS) to analyze for XIAP peptides enriched for phosphorylation using methods as previously described (Chaturvedi, Asim et al. 2014).

Mutagenesis

Forward and reverse primers containing the desired mutation were generated. Briefly, polymerase chain reaction (PCR) was performed to generate plasmids containing the mutation using the KOD polymerase in a 50ul reaction as previously described (Laible and Boonrod 2009). PCR products were digested with Dpn1 to destroy the template plasmid. Dpn1-treated reactions were then used to transform DH5 α cells. Colonies were grown, plasmids isolated, and plasmids sequenced for the presence of the appropriate mutation.

Immunofluorescence

Human embryonic kidney 293 cells (HEK293) were grown on coverslips coated with fibronectin, fixed in 3.7% formaldehyde, and permeablized. Samples were then incubated with primary antibody 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.

In vitro ubiquitination assay

In vitro ubiquitination assays were carried out in 20 ul reactions using the Ubiquitin Thioester/Conjugation Initiation Kit (Boston Biochem). Briefly, E1 ubiquitin-activating enzyme (1 ug), E2 UbcH5a (1ug), ubiquitin (1ug) and myc-TLE3 (2ug, generated from an *in vitro* transcription-translation reaction) (Promega) were assembled, and samples incubated on a TOMY shaker in a 30°C incubator for 30 minutes. Reactions were terminated with the addition of sample buffer, and the ubiquitinated products subjected to SDS-PAGE followed by immunoblotting for myc (Muratani and Tansey 2003).

In vivo ubiquitination assay

HEK293 cells were transfected with HA-TLE3, myc-XIAP, and His-Ubiquitin. Cells were treated with MG132 at 10uM for 4 hours prior to lysis. Lysates were sonicated at maximum for three pulses, one second each, and centrifuged at 12,000g for 10 minutes at 4°C. Supernatants were incubated with Ni-NTA agarose beads at room temperature for three hours. His-tagged proteins were eluted and processed for SDS-PAGE followed by immunoblotting for HA (Muratani and Tansey 2003).

TLE3-XIAP binding assay

HEK293 cells were transfected with HA-TLE3 and myc-XIAP. Cells were treated with MG132 at 10uM for 4 hours prior to lyses in non-denaturing lysis buffer (NDLB; 100mM Tris-HCl pH7.4, 500mN NaCl, 5mM EDTA, 1% (w/v) Triton X-100, and 1mM PMSF) plus ubiquitin aldehyde (250 ng/ul). Lysates were collected and centrifuged at 12,000g for ten minutes at 4°C. Anti-myc agarose beads were then added to the supernatant and samples incubated overnight at 4°C. Beads

were then washed three times with ten bead volumes of NDLB, bound proteins eluted with sampler buffer, and eluted proteins processed for SDS-PAGE and immunoblotting.

TOPFlash reporter assay

HEK293 STF (Super TOPFlash) cells containing stably transfected Wnt reporter were incubated with Wnt3a-conditioned or control media 24 hours post transfection. After 48 hours, cells were lysed with 1X Passive Lysis Buffer (Promega) and luciferase activity determine with Steady Glo following the manufacturer instruction (Promega). CellTiter-Glo (Promega) was used to normalize luciferase activities. Statistical analysis was performed using the Student's t test. A value of $p < 0.05$ was considered statistically significant.

Axis duplication assay

Xenopus embryos were *in vitro* fertilized, de-jellied, cultured, and injected with mRNA as previously described (Peng 1991). Embryos were assessed for complete or partial duplication and statistical analysis was performed using Fisher's exact test. A value of $p < 0.05$ was considered statistically significant. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Vanderbilt University Medical Center and were in accordance with their policies.

Immunoblotting

For immunoblotting, cells were typically lysed in non-denaturing lysis buffer (NDLB). Lysates were centrifuged at 12,000g for ten minutes at 4°C, and supernatants collected for SDS-PAGE. After transfer, PVDF membranes were blocked in TBS with 5% milk and incubated with primary

antibodies overnight at 4°C. Membranes were then washed with TBS three times and incubated with secondary antibodies at room temperature for 2 hours.

Plasmids

pCS2-myc-XIAP, pCS2-myc-XIAP Δ RING, pCS2-myc-TLE3, pCS2-HA-TLE3, pMAL-XIAP, pMAL-XIAP Δ RING, pGEX-TEV-GST were provided by the Ethan Lee lab. pMT107 (His-Ub) was a gift from Bill Tansey at Vanderbilt University. pCS2-myc-XIAP T180A, pCS2-myc XIAP S239A, pMAL-myc-XIAP T180A, and pMAL-myc XIAP S239A were generated using PCR-based mutagenesis techniques, using the following primers:

T180A: Forward-5'-ggccagactatgctcacctagccccaagagag-3', Reverse-
5'ctctcttggggctaggtgagcatagtctggcc-3'.

S239A: Forward-5'-ttgggccggaatcttaatattcgagctgaatctgatgctgtg-3', Reverse-
5'cacagcatcagattcagctcgaatattaagattccggcccaa-3'

Chapter III: Results

XIAP is a GSK3 substrate *in vitro*

One study has shown that XIAP binds GSK3 (Sun, Meares et al. 2009). It was unknown, however, whether XIAP was phosphorylated by GSK3. In order to determine whether XIAP is a kinase substrate of GSK3, we performed a radioactive *in vitro* kinase reaction using purified GSK3 and MBP-tagged XIAP. Because MBP is also a substrate for XIAP, the MBP tag of the MBP-XIAP fusion was cleaved using TEV-protease. As shown in Figure 1, XIAP is phosphorylated by GSK3. In order to determine potential GSK3 sites on XIAP, we used the Eukaryotic Linear Motif (ELM) Resource Program (a protein motif and peptide modification analysis tool), and found that XIAP was predicted to contain eight putative GSK3 phosphorylation sites distributed evenly across the protein (Figure 2). In order to experimentally identify sites on XIAP that are phosphorylated by GSK3, we performed an *in vitro* kinase reaction with purified MBP-XIAP and GSK3, and processed the reaction for liquid chromatography-mass spectrometry (LC-MS). From the LC-MS results, we identified two major sites that were subsequently validated: threonine 180 (T180), which is located within the BIR1 domain, and serine 239 (S239), which is located in the region between the BIR1 and BIR2 (Figure 3).

XIAP phosphomutants exhibit altered Wnt activity in cultured human cells

In order to determine whether the T180 and S239 sites of XIAP play a role in XIAP function in the Wnt pathway, we performed site-directed mutagenesis (to mutate these sites to alanines) to generate the XIAP mutants, XIAP T180A and S239A. We tested their effects in a Wnt reporter cell line, HEK293 STF. As previously shown, overexpression of XIAP has no observable effect on Wnt signaling as detected by the Wnt reporter assay (Figure 4). Similarly, we

did not detect any observable effect on Wnt signaling when the XIAP T180A and S239A mutants were overexpressed. We previously showed that knocking down XIAP blocked Wnt signaling, demonstrating its requirement for Wnt signal transduction. Our incapacity to detect activation of the Wnt pathway in cultured mammalian cells may be due to the fact that XIAP is not normally a limiting Wnt component in the absence of Wnt pathway activation. It is possible, however, that XIAP may become limiting when the Wnt pathway is activated. We tested this possibility, and showed that overexpression of XIAP potentiates the effect of Wnt3a (Fig. 4). The lack of increased beta-catenin levels above that of Wnt3a is consistent with the function of XIAP in the nucleus, downstream of the beta-catenin destruction complex. In contrast, we did not observe any enhancement of Wnt3a-mediated reporter activity when the XIAP T180A and S239A mutants were overexpressed. In fact, we observed inhibition of Wnt activity (compared to vector control) when the XIAP T180A and S239A mutants were overexpressed. Thus, phosphorylation of T180 and S239 on XIAP appear to play a role in the capacity of XIAP to enhance Wnt signaling, and XIAP T180A and S239A phosphomutants may be working in a dominant-negative fashion to inhibit maximal Wnt pathway activation.

XIAP phosphomutants do not have altered cellular localization

XIAP is localized predominantly to the cytoplasm, albeit there is a small XIAP pool that resides in the nucleus. Because phosphorylation has been shown to regulate the subcellular localization of proteins, we performed immunolocalization of the XIAP phosphomutants to determine if they were altered when compared to the wild-type protein. We transfected wild-type myc-XIAP, myc-XIAP T180A, and myc-XIAP S239A into HEK293 cells and performed immunostaining with an anti-myc antibody (Figure 5). We found there was no difference between

the cellular localization of wild-type XIAP and the XIAP phosphomutants, in that the mutants remained in the cytoplasm

XIAP phosphomutants affect axis duplication in *Xenopus* embryos

Next we aimed to assess the developmental functions of XIAP phosphomutants *in vivo*. Induction of secondary axis formation in *Xenopus* embryos is a powerful *in vivo* readout for Wnt signaling. We previously demonstrated that morpholino knockdown of XIAP resulted in ventralized embryos, and that injection of mRNA encoding XIAP induced secondary axis formation in *Xenopus* embryos. These studies demonstrated that XIAP plays a critical role in dorsal-anterior patterning in *Xenopus* embryos, a process regulated by Wnt signaling.

To test the effect of the XIAP mutants on axis formation, we injected *Xenopus* embryos with mRNAs encoding the XIAP T180A and S239A phosphomutants. We found that the XIAP phosphomutants were able to induce secondary axis formation to a similar extent as wild-type XIAP (Figure 6A). We observed potentiation when wild-type XIAP mRNA was co-injected with Xwnt8 mRNA into *Xenopus* embryos. We next tested if the XIAP phosphomutants were able to similarly potentiate co-injected Xwnt8. We found that the XIAP phosphomutants demonstrated much less activity than wild-type XIAP (Figure 6A). In the case of the XIAP T180A phosphomutant, co-injection of Xwnt8 resulted in a lower activity when compared to injection of Xwnt8 alone. These studies indicate that *in vivo*, in *Xenopus* embryos, similar to what was observed in cultured human cells, the XIAP T180A and S239A phosphomutants have impaired capacities to potentiate Wnt signaling. In the case of XIAP T180A, the phosphomutant also appear act in a dominant negative fashion.

The XIAP phosphomutants retain their capacity to ubiquitinate Gro/TLE *in vitro* but not in cultured human cells

We have previously shown that XIAP ubiquitinate TLE3 *in vitro* and in cultured mammalian cells. To directly test whether the decreased capacity of XIAP phosphomutants to potentiate Wnt signaling is due to their decreased capacity to ubiquitinate Gro/TLE, we expressed and purified recombinant wildtype XIAP and the XIAP T180A and S239A phosphomutants from bacteria and tested their capacity to ubiquitinate TLE3 in an *in vitro* ubiquitination assays (Fig. 7). We found that *in vitro*, the XIAP phosphomutants were able to ubiquitinate Gro/TLE to a similar degree as wild-type (Figure 8). Next we tested if there was a difference in the ubiquitination activities between wild-type XIAP and the XIAP phosphomutants in cultured human cells. HEK93 cells were transfected with HA-TLE3, His-ubiquitin, and either wild-type XIAP or XIAP phosphomutants. As negative control, the XIAP Δ RING mutant, which lacks the RING domain necessary for its E3 ligase activity, was also transfected. His-ubiquitinated proteins were isolated under denaturing conditions by nickel affinity purification, and HA-TLE3 was detected by immunoblotting (Fig. 9). We found that, as predicted, wild-type XIAP promoted the ubiquitination of HA-TLE3, as evidenced by the higher molecular weight species of TLE3. Neither of the phosphomutants, however, promoted the ubiquitination of TLE3 and they were indistinguishable from the XIAP Δ RING control. Thus, in contrast to the purified *in vitro* situation, the XIAP T180A and S239A phosphomutants have a decreased capacity to promote the ubiquitination of TLE3 in cultured cells. These differences in the activities of the XIAP mutants between the reconstituted purified and the cell-based ubiquitination assays may be due to the fact that in cells, in contrast to the *in vitro* reconstituted system, wild-type XIAP is phosphorylated. These studies highlight the

importance of XIAP phosphorylation in regulating its capacity to ubiquitinate Gro/TLE, and that the XIAP T180A and S239A phosphomutants do not have reduced intrinsic catalytic activity.

XIAP phosphomutants exhibit decreased affinity for TLE3 in mammalian cells

The decreased capacity of XIAP to ubiquitinate TLE3 in cultured cells could be due to their decreased catalytic activity or decreased affinity for TLE3. In order to test the latter possibility, we performed co-immunoprecipitation studies between the XIAP phosphomutants and TLE3. Cells were transfected with HA-TLE3 and either wild-type myc-XIAP or myc-XIAP phosphomutants. XIAP and XIAP phosphomutants were pulled-down with anti-myc agarose beads and immunoblotted for HA-TLE3 (Fig. 10). We found that, in contrast to TLE3 and wild-type XIAP, the interaction between TLE3 and XIAP was significantly reduced with the XIAP T180A and S239A mutants. Furthermore, the addition of Wnt3 did not significantly enhance the interaction between TLE3 and the XIAP mutants (Fig.10). These results indicate that the decreased capacity of the XIAP phosphomutants to ubiquitinate TLE3 is due, in part, to their decreased binding to TLE3.

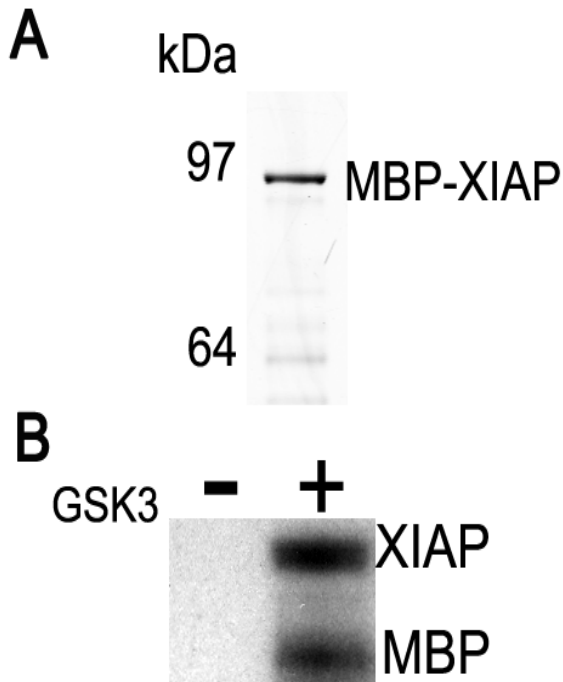


Figure 2.1. XIAP is a substrate of GSK3 in vitro. (A) Coomassie stain of purified XIAP-MBP. XIAP-MBP was expressed and purified from bacteria. (B) Autoradiography of phosphorylated XIAP. Kinase reaction using TEV-protease cleaved XIAP-MBP was incubated at 37°C for 1 hour. Reactions were analyzed by SDS/PAGE followed by autoradiography.

MOD GSK3 1	TFNSFEQS LENSATQS RSESDAVS TFGTWIYS LVRTTEKT TEKTPSLT ISGSNYKS QDESSQTS	2-9 [A] 98-105 [A] 238-245 [A] 271-278 [A] 352-359 [A] 356-363 [A] 399-406 [A] 423-430 [A]	- - 238-245 271-278 352-359 - - -	GSK3 phosphorylation recognition site	cytosol, nucleus	...((ST))...[ST]
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Figure 2.2. Putative GSK3 phosphorylation sites on XIAP. Sites were identified using the Eukaryotic Linear Motif (ELM) Resource Program.

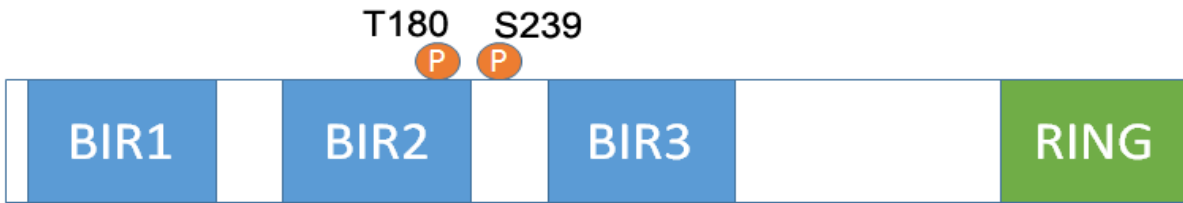


Figure 2.3. LC-MS analysis identifies T180 and S239 on XIAP as sites of phosphorylation by GSK3 in vitro.

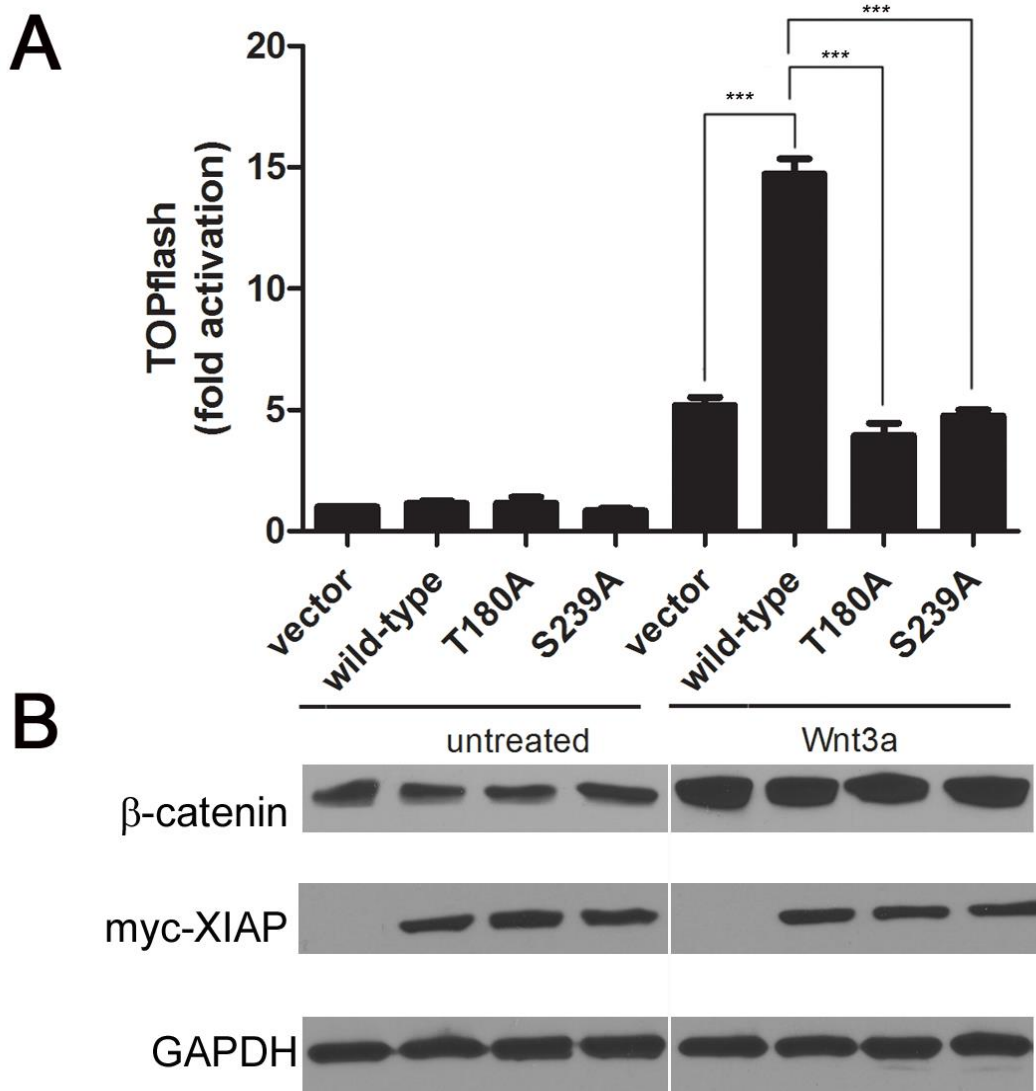


Figure 2.4. Overexpression of XIAP T180A and S239A phosphomutants has impaired capacities to potentiate Wnt signaling in cultured human cells. (A) Wnt signaling in HEK293 STF cells is potentiated upon overexpression of wild-type XIAP but not by overexpression of XIAP T180A and S239A phosphomutants. HEK293 cells were transfected with XIAP constructs for 24 hours and followed by treatment with vehicle or Wnt3a media. Reporter activity was assessed 72 hours post-transfection. Graph shows mean \pm SD of TOPflash normalized to cell titer.***p value <0.0001 . (B) Immunoblotting shows beta-catenin stabilization by Wnt3a treatment but not by overexpressing XIAP constructs.

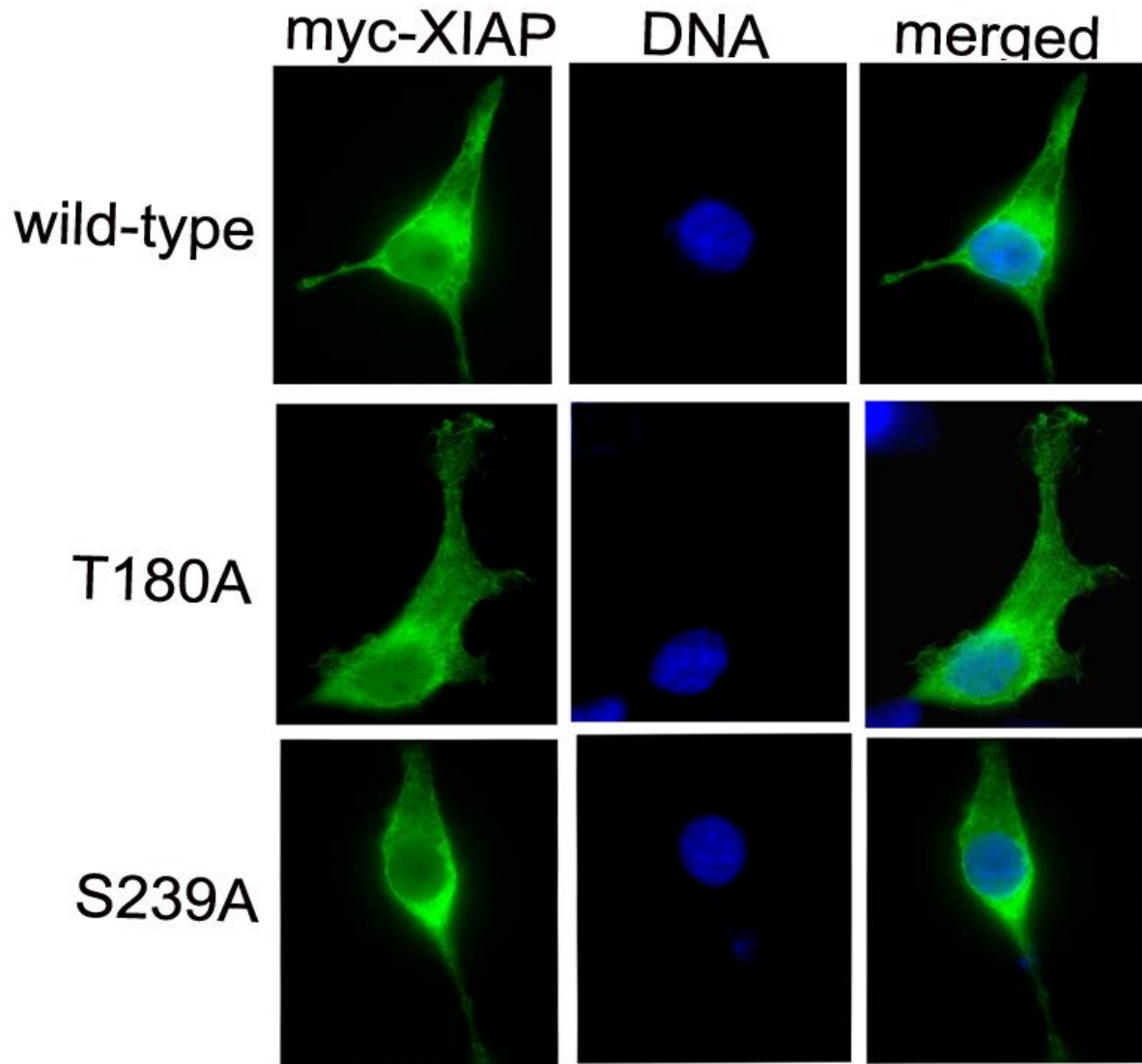


Fig 2.5. XIAP phosphomutants show similar localization to wild-type XIAP. HEK 293 STEF cells were transfected with wild type myc-XIAP, myc-XIAP T180A, and myc-XIAP T239A. Cells were fixed and stained for myc. DNA is stained with DAPI.

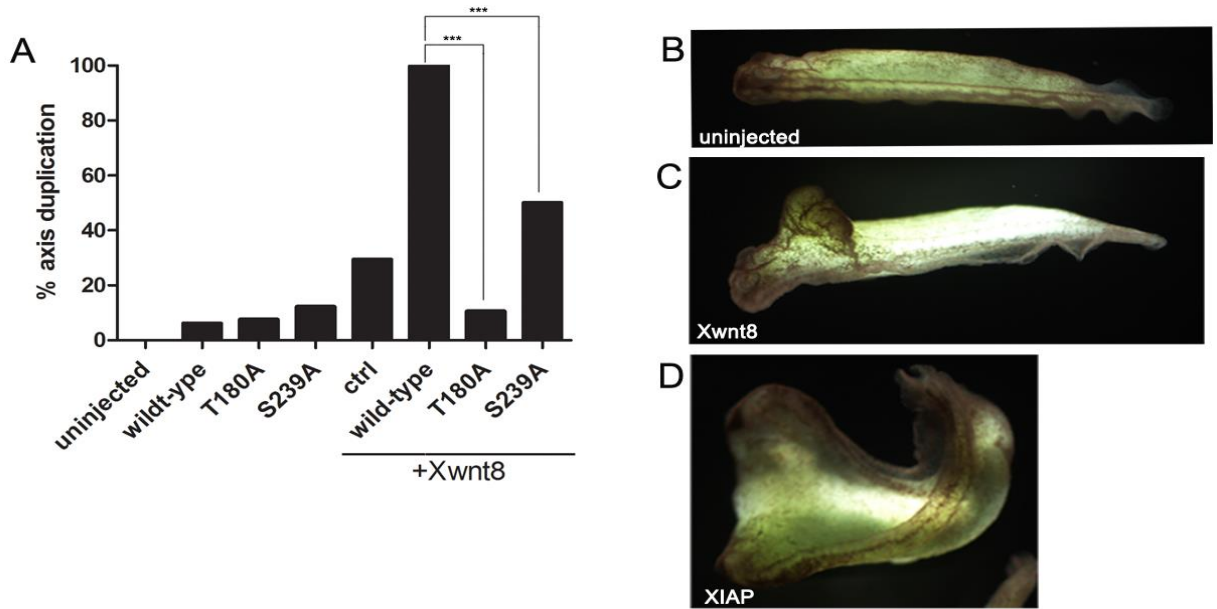


Figure 2.6. XIAP phosphomutants have decreased capacity to induce secondary axis formation in combination with Xwnt8 in Xenopus embryos. Xenopus embryos were injected in one ventral blastomere at the 4-cell stage with mRNAs (2 ng each) encoding XIAP, XIAP T180A, or XIAP S239A with or without 1 ng of Xwnt8. Embryos were allowed to develop for 48 hours and prior to analysis for axis duplication. (A) XIAP phosphomutants induced axis duplication to a similar extent as wild-type XIAP. Co-injection of XIAP mRNA significantly potentiated Xwnt8-induced axis duplication in contrast to the XIAP phosphomutants. ***p value < 0.001 versus wild-type + Xwnt8. (B-D) Representative images of the axis duplication assay. Representative pictures of embryos showing (B) uninjected control, (C) Xwnt8 injected, and (D) wild-type XIAP injected embryos.

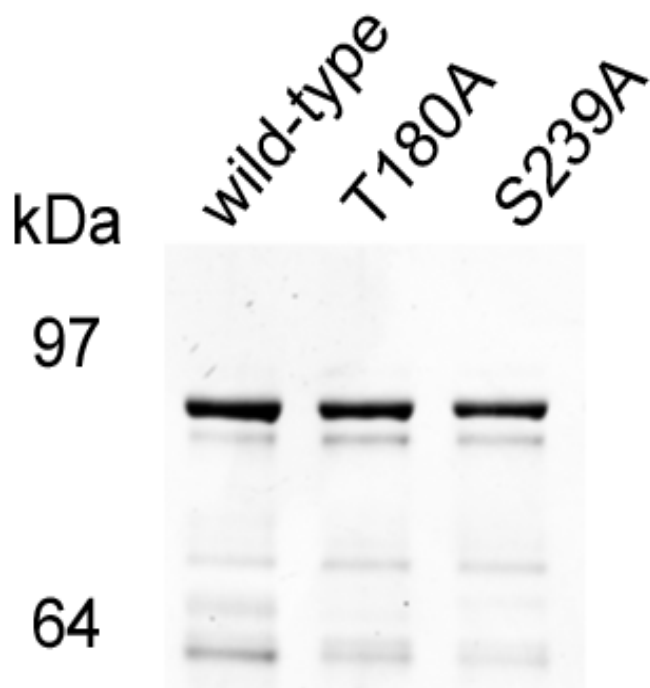


Figure 2.7. Purified MBP fusion of XIAP, XIAP T180A, and XIAP T239A. MBP fusions of wild-type and XIAP phosphomutants were expressed and purified from bacteria by amylose affinity resin followed by Mono Q anion exchange chromatography. Proteins (1 ug each) were run on SDS-PAGE followed staining with Coomassie Brilliant Blue.

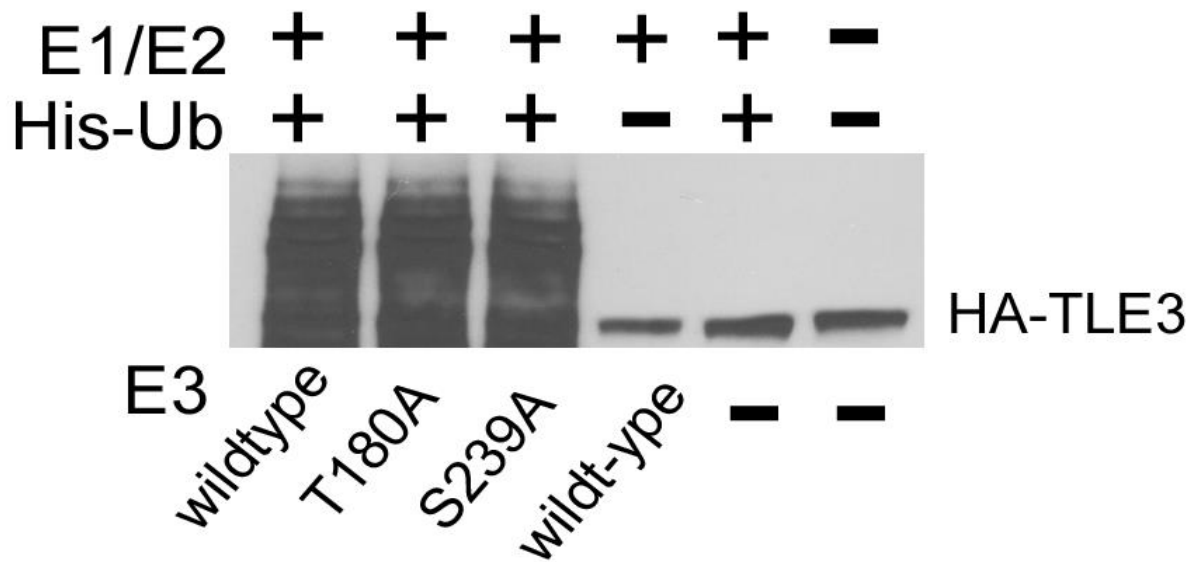


Figure 2.8. XIAP T180A and T239A ubiquitinate TLE3 in vitro to a similar extent as wild-type XIAP. In vitro translated HA-TLE3 was incubated with recombinant XIAP constructs and other ubiquitination components as indicated and visualized by immunoblotting with anti-HA antibody.

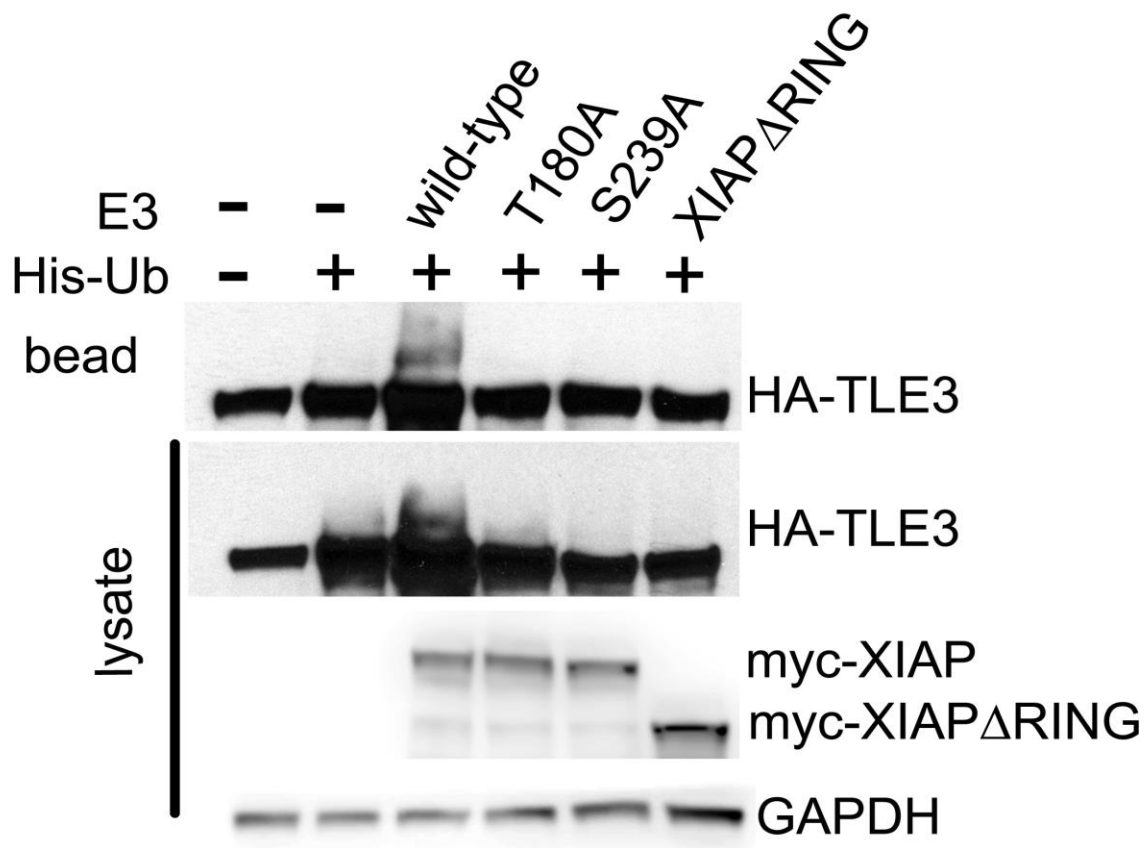


Fig 2.9. XIAP T180A and S239A have reduced capacity to ubiquitinate TLE3 in cultured human cells. HEK293 cells were transfected as indicated, lysed under denaturing conditions, and His-Ub modified proteins isolated by nickel affinity purification. XIAP and TLE were detected by immunoblotting with anti-myc and anti-HA antibodies, respectively.

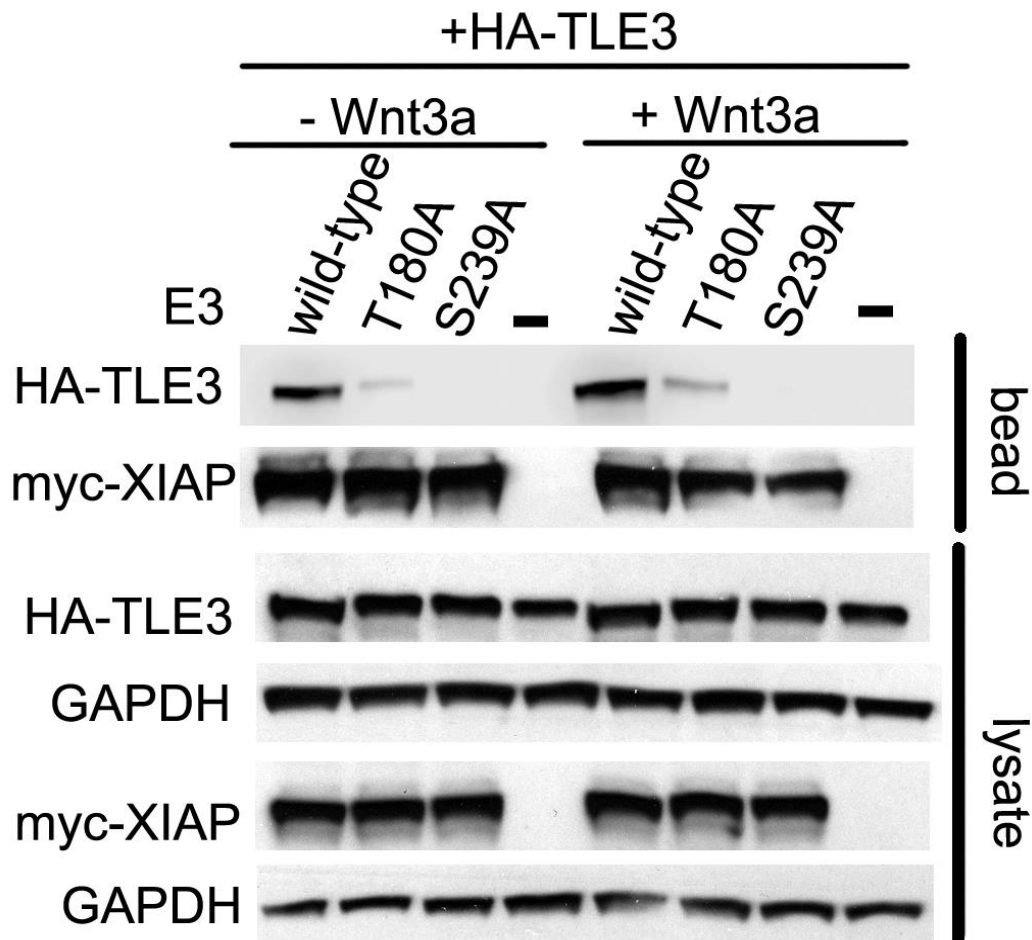


Figure 2.10. XIAP phosphomutants have a decreased affinity for TLE3. HEK293 cells were transfected as indicated with myc-XIAP and HA-TLE3 in the presence or absence of Wnt3a. Cells were lysed and XIAP immunoprecipitated with anti-myc antibody. Co-immunoprecipitated TLE3 was detected by anti-HA antibody.

Chapter IV: Discussion and future directions

Discussion

How the Wnt transcriptional complex is converted from a repressor complex into an activator complex is not well understood. Previous studies have shown that nuclear beta-catenin levels increase only modestly (2-6 fold) upon Wnt pathway activation (Goentoro and Kirschner 2009). Given that the affinity between Gro/TLE and TCF/Lef is in the low-mid nanomolar range, such an increase in beta-catenin levels is unlikely to be sufficient for beta-catenin to displace Gro/TLE via direct competition. Previously, we showed that upon Wnt activation, XIAP is recruited onto Wnt gene promoters to ubiquitinate Gro/TLE bound TCF/Lef, decreasing its affinity for TCF/Lef (Hanson, Wallace et al. 2012). Because XIAP can ubiquitinate Gro/TLE whether or not Gro/TLE is bound to TCF/Lef on Wnt gene promoters, our model proposes that XIAP also prevents free nuclear Gro/TLE from binding TCF/Lef. Regardless, we propose this Wnt signaling circuitry provides a mechanism to dampen transcriptional noise without a corresponding loss in sensitivity. It is not clear how Gro/TLE ubiquitination is regulated, and we speculate that there exists a deubiquitinase that may facilitate the binding of Gro/TLE to TCF/Lef in the Wnt unstimulated state. Such cycles of mono-ubiquitination and de-ubiquitination have been observed with other transcriptional activators including Smad4, p53, and FoxO (van der Horst, de Vries-Smiths et al. , Dupont, Mamidi et al.).

A major question that arises from our proposed model is how XIAP is recruited to the TCF/Lef transcriptional complexes in a Wnt dependent manner. Our preliminary studies indicate that inhibiting GSK3 with lithium was sufficient to promote XIAP recruitment to TCF. We therefore hypothesized that phosphorylation may play a role in regulating XIAP activity. Our

current data suggests a model in which XIAP phosphorylation is crucial for the interaction between XIAP and Gro/TLE to allow full Wnt activation (Figure 1).

We now know that cellular regulatory mechanisms provide specific and robust responses to external stimuli, and posttranslational modifications often provide a dynamic way to regulate protein activity and protein-protein interactions (Nishi, Hashimoto et al. 2011). Such dynamic regulation can be achieved through the reversible and the fast kinetics of post-translational modifications (Johnson 2009). It has been shown that a majority of proteins in a cell contains at least a few phosphosites, and that regulation through phosphorylation is very common. Phosphorylation has been shown to induce conformational changes through allosteric mechanisms, and has been demonstrated to modulate the association and dissociation of protein assemblies in numerous signaling pathways (Gsoner, Futschik et al. 2008).

Because XIAP is involved in cellular pathways other than Wnt signaling, it is possible that phosphorylation at different sites on XIAP confers specificity for its interaction with different binding partners. In the present study, we found that phosphorylation at T180 and S239 of XIAP increases its affinity of XIAP for Gro/TLE and is required for maximal Wnt activity in cultured human cells. In *Xenopus* embryos, we showed that wild-type and XIAP phosphomutants were able to induce axis duplication at a similarly low rate (~10-15%). When co-injected with Xwnt8, however, the differences between wild-type XIAP and the XIAP phosphomutants became evident: wild-type XIAP potentiated the activity of Xwnt8 whereas the XIAP phosphomutants had no effect (S239A) or inhibited (T180A) the activity of Xwnt8. These *in vivo* results parallel our findings in cultured cells and indicate that the XIAP phosphomutants are acting in a manner reminiscent of partial agonists in drug-receptor interaction (Hoyer and Boddeke 1993).

It remains to be determined why phosphorylation of XIAP on T180 and S239 is required for full Wnt activity. Previous studies have shown that beta-catenin interacts with adapter proteins such as Pygo within the co-activator complex to promote maximal transcription (Townsend, Cliffe et al. 2004). In the nucleus, the tumor suppressor APC has been proposed to aid in the switch between the transcriptional activation and repression by binding beta-catenin and facilitating its dissociation from the promoter (Sierra 2006). It is possible that XIAP interacts with these adaptor proteins via these phosphosites. Thus, XIAP phosphomutants could have altered interaction with these nuclear adaptor proteins. Finally, there is strong evidence that a cyclic pattern of alternating co-activator and co-repressor complex recruitment to Wnt gene promoters is required for maximal Wnt transcriptional activation (Sierra 2006). This cycling may represent a necessary resetting of the transcriptional complex after each round of transcription. Studies from our lab have shown that XIAP binding and dissociation from Wnt gene promoters parallel this cyclic pattern. It is possible that phosphorylation at S239 and T180 is important for cycling on and off the promoter of Wnt target genes. Thus, the XIAP phosphomutants are able to undergo a single round of Wnt activation (low activation), but are impaired in cycling through multiple rounds of activation (high activation). This possibility could be tested by performing kinetic ChIP assays to monitor the cycling of XIAP phosphomutants on and off the promoter.

Future directions

My studies have provided new and important insight into the function of XIAP in the Wnt pathway and its regulation by phosphorylation. I have shown that phosphorylation of XIAP is required for its full function in the Wnt pathway. Major questions, however, remain regarding the nature and mechanism of XIAP phosphorylation. For example, in order to obtain maximal XIAP activity in the Wnt pathway, does the phosphorylation at positions T180 and S239 have to be reversible? We can test this possibility by generating XIAP phosphomimetics mutants at positions T180 and S239, and test if these mutants function similarly to wild-type XIAP or to the alanine mutants.

The XIAP phosphomutants appear to retain full catalytic activity as demonstrated in our *in vitro* ubiquitination assays. In our cell-based assays, however, the mutants have impaired capacity to ubiquitinate TLE3, consistent with its decreased affinity for the substrate. It would be interesting to know whether the XIAP phosphomutants similarly have decreased activity towards a known XIAP substrate in another pathway (e.g. Smac). To test this possibility we could perform cell-based ubiquitination assays using Smac. If we do not detect any difference in the capacity of the XIAP phosphomutants to ubiquitinate Smac, in contrast to TLE3, it would suggest that these phosphorylation sites are involved in regulating XIAP substrate specificity.

To date, we have not been able to demonstrate that the T180 and S239 sites are phosphorylated *in vivo* by mass spectrometry. This may be due to the fact that the percentage of the total XIAP pool that participates in Wnt signaling is relatively small. A multitude of factors, including occupancy, charge, and hydrophobicity, can affect the chromatographic properties and ionization efficiencies and thus the ease of detection of phosphorylated peptides in cells

(Dephoure, Gould et al. 2013). Alternatively, the phosphorylation sites on XIAP are labile and may turn over rapidly, negating proper detection via mass spectrometry.

One way to overcome these hurdles is to develop antibodies that recognize the phosphorylated T180 and S239 sites. These antibodies will be helpful in determining 1) whether phosphorylation of XIAP is regulated by Wnt signaling, 2) whether XIAP is phosphorylated in the cytoplasm or nucleus, 3) whether the phosphorylated/dephosphorylated state of XIAP parallels its cycling on and off Wnt gene promoters, and 4) whether phosphorylation of XIAP at T180 and S239 is mediated by GSK3 *in vivo*.

Proteins often undergo cycles of ubiquitination and deubiquitination to modulate their activities. Removal of ubiquitin may be required for cycling Gro/TLE back onto TCF to reconstitute the co-repressor complex during Wnt signaling and upon termination of the Wnt signal. The deubiquitinase (DUB) that counteracts the activity of XIAP on Gro/TLE is not known. Unlike E3 ligases, which number in the hundreds, there are only about eighty DUBs. Therefore, an RNAi screen in cultured mammalian cells could be performed to identify DUBs that, when knocked down, would increase the extent of ubiquitinated Gro/TLE by XIAP using the His-Ubiquitin assay.

Previous studies from our lab have shown that the N-terminal Q domain of Gro/TLE contains the site of interaction with XIAP. However, the region of XIAP that binds Gro/TLE has not been mapped. It is possible that the region of XIAP that binds Gro/TLE lies within a region that spans T180 and S239. It is equally possible that phosphorylation at these sites induces conformation changes in an allosteric fashion to affect binding at distant domains. Regardless, given that we can readily express and purify XIAP and that XIAP can be readily

coimmunoprecipitated with TLE3, a simple binding assay could be developed to determine the region of XIAP that interacts with Gro/TLE.

XIAP is overexpressed in a majority of human cancers, and drugs that target XIAP are currently in clinical trials. Our studies demonstrate that XIAP potentiates Wnt signaling. These results suggest that XIAP misregulation may further contribute to enhance Wnt signaling in cancers with inappropriate Wnt pathway activation. Our previous studies showed that XIAP regulate the Wnt pathway downstream of 90% of all Wnt mutations in cancer (e.g. APC and beta-catenin). Thus, inhibiting the activity of XIAP may be one way to target cancers due to mutations in the Wnt pathway. Results from our current studies may be helpful in devising more specific, targeted approaches for inhibiting the Wnt activity of XIAP. Most of the drugs targeting XIAP have focused on its BIR domains. Our data suggests that the T180 and S239 phosphosites on XIAP are required for full Wnt activation. Small molecule inhibitors that target these regions may be selective for inhibiting the function of XIAP in Wnt signaling, but leaving intact other physiological functions of XIAP.

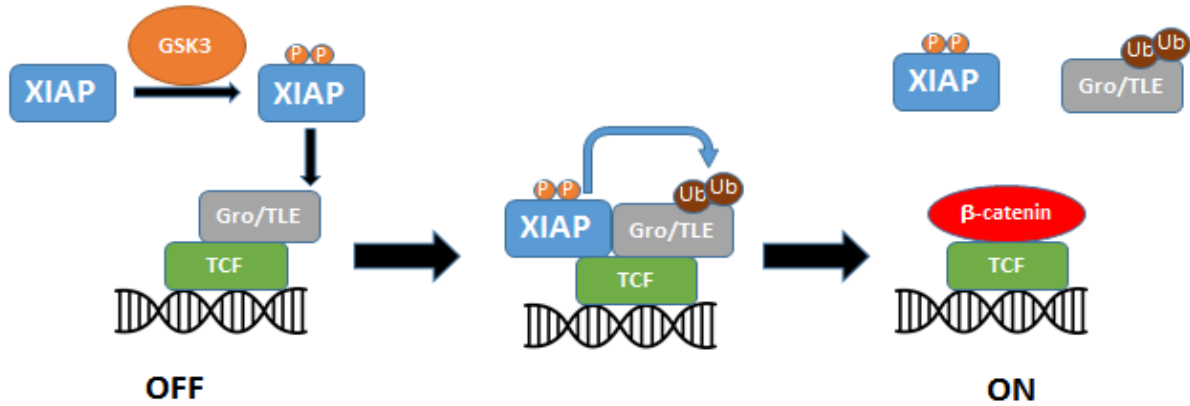


Figure 3.1. GSK3 phosphorylation is required for the interaction between XIAP and Gro/TLE. GSK3 phosphorylates XIAP at T180 and S239 to facilitate its binding to Gro/TLE. XIAP can then ubiquitinate and remove Gro/TLE for subsequent binding of beta-catenin to the promoter.

Appendix A: Screen for small molecule modulators of major embryonic pathways

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Introduction

In this review, we will discuss the unique biological properties of the *Xenopus* egg extract system and the advantages it offers for screening small molecular modulators of complex biological pathways. We will discuss molecular pathways that have been reconstituted using *Xenopus* egg extracts and small molecule screens that have been performed using these assays. Finally, we will describe our recent studies using *Xenopus* egg extracts to identify small molecule modulators of the Wnt pathway and how this approach could be similarly adapted to other embryonic signaling pathways such as Hedgehog and Notch.

Over the past decade, there has been a great expansion in drug discovery efforts within academia as evidenced by the emergence of a new discipline known as Chemical Biology (Mitchison 1994). With the advent of high throughput approaches and the development of faster and cheaper technologies, industry and academia have tremendous resources and opportunities to identify novel drugs at an accelerated pace. Traditionally, pharmacological agents have been identified through enzymatic assays using purified components (Lindsay 2003, Overington, Al-Lazikani et al. 2006). Such “targeted screening” approaches, which have been the focus of drug discovery over the past decade or so, offer a number of advantages. First, targeted biochemical screens represent the most direct way to identify drugs based on our current understanding of a molecular target or event. Second, enzymatic assays using purified components can often be readily adapted for high throughput screening (HTS); combined with well-established

colorimetric, fluorescent, and luminescent readouts, experimental set-up is often relatively straightforward. Third, with purified components, drug target identification is not a limitation. Fourth, the recent advent of increasingly sophisticated technologies has allowed investigators to acquire very precise kinetic data. Such information can facilitate the grouping of drugs into subclasses based on mechanism even at initial stages of screening and can reveal subtle drug effects as well.

Targeted screening, however, has some major limitations. The most obvious is that the investigator is restricted to the originally hypothesized target; thus, there is no potential for uncovering novel targets. Furthermore, it is exceedingly difficult to predict which compounds will work *in vivo* or even whether the targeted molecule will be an effective therapeutic target in the first place. An alternative approach to targeted drug screening is to screen for a specific phenotype (Mitchison 1994, Yeh and Crews 2003). Phenotypic screens can be performed in systems ranging from cultured cells to whole organisms. In fact, in recent years, a majority of compounds receiving FDA approval were discovered through phenotypic screens (Swinney and Anthony). These require no *a priori* knowledge of the direct target of compounds, only knowledge of the desired phenotype. Representative phenotypic screens include those based on *C. elegans* morphology, gastrulation in zebrafish, and mitosis in cultured mammalian cells (Peterson and Fishman 2004, Kwok, Ricker et al. 2006, DeMoe, Santaguida et al. 2009). Screening in such complex systems pre-selects for compounds that are cell/organism permeable, reach their target, and induce a desired effect *in vivo*; as such, hits are proven to be active *in vivo* from the start. The identification of novel drug targets can lend to important insights into the biological process in question.

As with targeted screening, there are also major drawbacks to phenotypic screening. Living systems are much more difficult to manipulate in HTS format and require significantly more

manual set-up. Phenotypes can often require time-consuming manual inspection or sophisticated algorithms for image analysis. Drug incubation times are longer (i.e. on the order of hours to days) such that screens require significantly more time to perform. The longer time course also increases the likelihood that the desired phenotypes may occur via non-direct mechanisms. Finding the optimal dose of drug to screen is another challenge: too low of a drug concentration can give false negative results, whereas too high of a drug concentration can produce toxicity with a valid drug candidate. Perhaps the major limitation of phenotypic screens, however, is the difficulty of drug target identification, which can represent a formidable challenge.

The *Xenopus* egg extract system

Cell extract-based screens, such as those performed using *Xenopus* egg extract, represents an ideal bridge between targeted and phenotypic screens. *Xenopus* egg extracts offers a powerful cell-free system to study complex biological pathways. A multitude of cellular events can be recapitulated in extracts (discussed below). In contrast to targeted screens, enzymes can be monitored in their native milieu that allows for appropriate post-translational modifications and regulation. Thus, using *Xenopus* egg extract, drugs that target enzymes directly as well as those that target unknown cofactors can be identified. In contrast to phenotypic-based screens, enzymes can be studied in a homogenous biochemical environment with minimal well-to-well, cell-to-cell, or animal-to-animal variation. The protein composition of the extract system can be readily altered via addition or depletion of individual components. Wild-type or mutant proteins can be added to extracts at precise concentrations to test their effects. Assay times range from only minutes to hours, and the option of preparing extracts that do not undergo transcription/translation decreases the number of potential mechanisms of action of drug library hits. Importantly, target identification is simplified due to the biochemical tractability of extracts.

Exactly what is *Xenopus* egg extract? *Xenopus* egg extract is essentially highly active cytoplasm that can be obtained in large quantities suitable for biochemical studies (King, Peters et al. 1995, Verma, Peters et al. 2004). *Xenopus* egg extract is prepared by a centrifugation step that disrupts the plasma membrane and releases the cytoplasmic fraction in an essentially undiluted form (Fig. 1). Because *Xenopus* eggs are maternally loaded with all the necessary components needed for early embryogenesis, *Xenopus* egg extract contains cytoplasmic proteins, organelles, amino acids, lipids, and nucleotides at or near physiological levels. The following biological processes have been studied in the *Xenopus* egg extract system: cytoskeletal dynamics, nuclear assembly and import, apoptosis, post-translational modifications, ubiquitin metabolism, cell cycle progression, and signal transduction pathways (Mitchison and Kirschner 1984, Dabauvalle and Scheer 1991, Glotzer, Murray et al. 1991, Murray 1991, Theriot, Rosenblatt et al. 1994, Salic, Lee et al. 2000, Verma, Peters et al. 2004, Chan and Forbes 2006, Kornbluth, Yang et al. 2006, Maresca and Heald 2006, Shennan 2006, Tutter and Walter 2006). In typical preparations of *Xenopus* egg extract, biochemical pathways remain largely intact and can be readily assayed in high throughput screens. *Xenopus* egg extract can be altered using standard biochemical approaches. Recombinant or *in vitro*-translated proteins can be added to the extract to test the effects of increased concentrations of particular components. Alternatively, because *Xenopus* egg extract retains a high capacity for translating mRNAs, synthetic mRNAs can be added to the extract to translate the desired proteins to high levels, thereby circumventing the need for generating recombinant proteins. For loss of function studies, antibodies can be used to immunodeplete specific proteins. Alternatively, dominant-negative versions of proteins can be added either as recombinant proteins or as mRNAs. Below, we describe the reconstitution of complex biological pathways using *Xenopus* egg extract. Table 1 lists the pathways that have been successfully screened in *Xenopus*

egg extract to identify small molecule modulators. Table 2 lists small molecule compounds that have been validated using *Xenopus* egg extract.

Reconstitution of complex biological pathways in *Xenopus* egg extracts

Cell cycle

The *Xenopus* egg extract system has been particularly valuable in elucidating mechanistic details of the cell cycle. A fertilized *Xenopus* egg undergoes many rounds of synchronous cell divisions in the absence of cell growth, thereby subdividing a single-celled egg into a similarly sized embryo of ~4,000 cells within the first eight hours of development. Proteins and mRNAs required for cell division are maternally loaded into the egg so as to allow cell divisions to take place without the need for transcription. In groundbreaking experiments performed over the past few decades, *Xenopus* egg extract has been used to reconstitute major cell cycle events: entry into mitosis, cyclin degradation, mitotic spindle assembly, and chromosome segregation.

Work by Murray and Kirschner demonstrated that addition of Cyclin B is sufficient to drive *Xenopus* egg extract into mitosis and that fluctuations in Cyclin B levels largely mediate progression through the embryonic cell cycle (Murray and Kirschner 1989, Murray, Solomon et al. 1989, Glotzer, Murray et al. 1991, Murray 1991). Using this same system, the detailed molecular basis for Cyclin B degradation was uncovered (Murray and Kirschner 1989, Murray, Solomon et al. 1989, Glotzer, Murray et al. 1991, Murray 1991). Studies in *Xenopus* egg extract identified Cyclin B and Cdk1 as the molecular components of Maturation Promoting Complex (Lohka, Hayes et al. 1988). Two decades later, studies in this system were key to formulating a biochemical and mathematical description of the mechanism by which Cyclin B and Cdk1 impart an oscillatory nature to the cell cycle (Pomerening, Kim et al. 2005).

Using this system, ubistatin was identified by King and colleagues as a cell cycle inhibitor in a small molecule screen of >100,000 compounds (Verma, Peters et al. 2004). Cyclin B is degraded upon exit from mitosis in a process mediated by the Anaphase-Promoting Complex, an E3 ubiquitin ligase. For a HTS assay, Cyclin B was fused to luciferase and added to extract. Its proteolysis was then monitored to identify compounds that blocked its degradation. Subsequent experiments in a purified biochemical system showed that ubistatin inhibited cell cycle progression by blocking the binding of ubiquitylated substrates to the proteasome (Verma, Peters et al. 2004).

Nuclear assembly and disassembly

Elucidation of the mechanism underlying nuclear assembly and disassembly has been greatly facilitated by the development of an *in vitro* system using *Xenopus* egg extract. The nuclear membrane serves to physically separate the genomic DNA from the cytoplasm. The nuclear pore complex mediates the trafficking of macromolecules between the nucleus and cytoplasm. *Xenopus* egg extract contains large amounts of disassembled nuclear components including an abundance of nuclear pores. Pioneering work by Lohka and Masui demonstrated that incubation of chromatin with *Xenopus* egg extract spontaneously induced the formation of a nuclear structure around demembrated sperm nuclei (Lohka and Masui 1983, Lohka and Masui 1984). Nuclei formed *in vitro* in this manner are indistinguishable from eukaryotic nuclei observed in cultured cells and organisms (as visualized by phase contrast and immunofluorescence microscopy). Using this *in vitro* system, nuclear import activity can be readily measured by assaying for accumulation of substrates within the reconstituted nuclei (Newmeyer, Finlay et al. 1986, Finlay, Newmeyer et al. 1987). *Xenopus* egg extract was also shown to reconstitute nuclei using purified lambda DNA as template (Newport 1987). This breakthrough allowed for identification of discrete intermediates in chromatin assembly.

In contrast to interphase extract, which promotes nuclear assembly, mitotic *Xenopus* egg extract promotes nuclear disassembly (Lohka and Masui 1983, Newport and Spann 1987). Mitotic *Xenopus* egg extract can be prepared from unactivated eggs in the presence of a calcium chelator or addition of recombinant cCclin B to drive interphase extract into mitosis (Newport 1987, Murray and Kirschner 1989). Addition of intact nuclei to mitotic extract results in nuclear envelope breakdown and vesicularization, lamin solubilization, and chromosome condensation. Thus, *Xenopus* egg extract represents a powerful biochemical system to nuclear pores (Chan and Forbes 2006).

DNA replication and Repair

Although studies in yeast have provided critical insights into proteins involved in the initiation of DNA replication, our understanding of the biochemical mechanisms and temporal events of eukaryotic DNA replication has been driven in large part by work using *Xenopus* egg extract. A large stockpile of material that is capable of supporting a rapid and complete round of chromosomal DNA replication is present within *Xenopus* egg extract, thus making it a powerful system to study this process. This *in vitro* system has been used in pioneering studies on the initiation of DNA replication (Blow and Laskey 1986). Demembrated sperm chromatin added to *Xenopus* egg extract undergoes a single round of semiconservative DNA replication. DNA is replicated efficiently but is only observed upon efficient nuclear assembly (Blow and Sleeman 1990). Evidence that structural components of the nucleus are essential for replication initiation comes from studies in which lamins are immunodepleted; nuclear assembly is not inhibited, but the nuclei formed are not capable of initiating DNA replication (Newport, Wilson et al. 1990, Meier, Campbell et al. 1991). Subsequent advances have led to the development of modified *Xenopus* egg extract capable of replicating chromosomal DNA in the absence of nuclei (Walter,

Sun et al. 1998). Such extract supports the replication of small DNA (e.g. plasmids). This feature has made it possible to study the effects of DNA topology and sequence on DNA replication. Studies using such modified *Xenopus* egg extract along with studies using traditional “nuclear assembly” egg extract have provided detailed insights into the formation of the pre-replication complex and its mechanism of activation (Arias and Walter 2004).

It is imperative for the survival of an organism that genomic integrity is preserved. To accomplish this goal, eukaryotic cells utilize numerous types of DNA damage and replication checkpoints. Biochemical studies using *Xenopus* egg extract have helped to identify the components and mechanisms involved in these checkpoints. For example, the functions of key regulators of the DNA checkpoint pathways in the mammalian DNA damage response, such as ATM and ATR, have been illuminated using *Xenopus* egg extract (Garner and Costanzo 2009). Using the *Xenopus* egg extract system, Walter and Newport demonstrated that uncoupling of MCM helicase (a helicase essential for DNA replication) and DNA polymerase in response to UV irradiation is necessary for checkpoint activation (Walter and Newport 2000).

Microtubule polymerization

Xenopus eggs, which are abundant in proteins involved in regulation of microtubules, have been instrumental in our understanding of microtubule dynamics (Shirasu, Yonetani et al. 1999). Microtubule ends undergo phases of polymerization (“rescue”) and depolymerization (“catastrophe”) in a stochastic fashion in a process known as “dynamic instability” that was first coined by Mitchison and Kirschner based on their studies of *Xenopus* egg extract (Mitchison and Kirschner 1984). The biochemical tractability of the *Xenopus* egg extract system has led to the

identification of numerous microtubule-associated proteins that modulate microtubule polymerization (Desai and Mitchison 1997).

The ability to reconstruct a complete, functional mitotic spindle in a test tube using *Xenopus* egg extract has been a watershed for the biochemical interrogation of spindle formation and function (Desai, Murray et al. 1999). During mitosis, microtubules undergo dramatic reorganization to mediate sister-chromatid separation during cell division. Because the cell cycle state can be synchronized in *Xenopus* egg extract, this system has been invaluable in our understanding of the role of microtubules in the formation of the mitotic spindle and chromosome segregation (Sawin and Mitchison 1991, Hannak and Heald 2006). The addition of fluorescent tubulin to *Xenopus* egg extract made it feasible to perform time-lapse image analysis of the mitotic spindle to study its dynamic properties (Sawin and Mitchison 1991, Sawin and Mitchison 1991). Using *Xenopus* egg extract, Rebecca Heald in the lab of Eric Karsenti showed that a bipolar spindle can spontaneously assemble around DNA-coated beads, suggesting that bipolarity is an intrinsic property of microtubules that form around chromatin during mitosis (Heald, Tournebize et al. 1996). Finally, taking advantage of the capacity for *Xenopus* egg extract to support mitotic spindle formation, a high throughput screen for inhibitors of mitotic spindle assembly led to the identification of the compound diminutol (Wignall, Gray et al. 2004).

Actin dynamics

The actin cytoskeleton has been successfully reconstituted using *Xenopus* egg extract. As with microtubules, actin nucleation and the rate of polymerization are regulated by numerous actin-binding proteins (Cameron, Giardini et al. 2000). Our understanding of actin dynamics and its interacting partners has been advanced by the successful reconstitution of actin in egg extract

(Theriot, Rosenblatt et al. 1994, Ma, Cantley et al. 1998, Rohatgi, Ho et al. 2000). *Listeria monocytogenes* is an intracellular bacterium that utilizes an actin-based mechanism in which an elongated structure containing actin filaments (“comet tail”) is assembled to propel the bacterium within the host cell. Using *Xenopus* egg extract, Theriot and colleagues were able to observe the actin-based motility of *Listeria monocytogenes* (Theriot, Rosenblatt et al. 1994). Depleting *Xenopus* egg extract of actin depolymerizing factor (ADF)/cofilin (XAC) resulted in increased length of the comet tail, demonstrating that XAC is involved in actin filament turnover during comet tail formation (Rosenblatt, Agnew et al. 1997).

Xenopus egg extract is an ideal system to dissect the components of signal transduction pathways that regulate the dynamic properties of actin. The actin cytoskeleton is regulated by a diverse set of membrane-proximal cues that recruit and activate Wiskott Aldrich Syndrome Protein (WASP) family members, which associate with the Arp2/3 complex to initiate the polymerization of new actin filaments. Using *Xenopus* egg extract, Cdc42, a member of the Rho family of GTPases, and phosphatidylinositol 4, 5-bisphosphate have been shown to activate the Arp2/3 complex through WASP to induce actin assembly (Ma, Cantley et al. 1998, Rohatgi, Ho et al. 2000). Taking advantage of this system, the Kirschner group purified Toca-1, a member of the evolutionarily conserved PCH protein family, from *Xenopus* egg extract and demonstrated that it is required for Cdc42-mediated activation of WASP (Ho, Rohatgi et al. 2004). The fluorescence of pyrene-labeled actin monomers increases 20- to 30-fold upon polymerization, and provides a fast and quantitative assay for actin polymerization. Taking advantage of this property, an HTS was performed in *Xenopus* egg extract that identified a cyclic peptide (Wiskostatin) that maintains WASP in an autoinhibitory state via an allosteric mechanism (Peterson, Lokey et al. 2001).

Apoptosis

Programmed cell death (apoptosis) has been successfully reconstituted using *Xenopus* egg extract (Deming and Kornbluth 2006). Apoptosis is mediated by the activation of a cascade of serine proteases of the caspase family (Spencer and Sorger 2011). These activated caspases ultimately impinge on a large number of cellular targets, thereby leading to cell death. In *Xenopus* egg extract, apoptosis occurs spontaneously when the extract is allowed to incubate at room temperature for an extended period of time (Newmeyer, Farschon et al. 1994). Alternatively, apoptosis can be induced in *Xenopus* egg extract upon addition of purified cytochrome c to extract depleted of the mitochondrial fraction. In such a system, markers of apoptosis (e.g. caspase activation, chromatin condensation, and nuclear fragmentation) can be readily monitored. Using this system, the Kornbluth group demonstrated a link between decreased nutrient status, caspase activation, and apoptotic cell death (Nutt, Margolis et al. 2005). *Xenopus* egg extract thus represents a powerful tool to study apoptosis.

The Wnt pathway

Overview of the Wnt pathway

The Wnt pathway controls many aspects of embryonic development and tissue maintenance (Logan and Nusse 2004). Wnt dysregulation leads to various developmental defects and has been linked to many types of cancer in humans (Logan and Nusse 2004, Clevers 2006). In the absence of the Wnt ligand, cytoplasmic β -catenin levels are kept low by the β -catenin destruction complex, which is composed of Axin, adenomatous polyposis coli (APC), glycogen synthase kinase 3 (GSK3), and casein kinase 1 α (CK1 α) (MacDonald, Tamai et al. 2009). Axin is a scaffolding protein that brings together the destruction complex components and is the limiting

factor in β -catenin degradation. CK1 α primes β -catenin for subsequent phosphorylation by GSK3, targeting β -catenin for ubiquitin-mediated proteolysis. Wnt signaling is initiated when Wnt ligands bind Frizzled receptors (Fz) and the co-receptor, low-density lipoprotein-related receptor 5/6 (LRP5/6). As a consequence, the β -catenin destruction complex is recruited to the plasma membrane, and β -catenin degradation is inhibited. Cytoplasmic accumulation of β -catenin leads to its translocation into the nucleus, where it turns on Wnt target gene transcription by activating T-cell factor/lymphoid enhancer factor (Tcf/Lef) family proteins. In the absence of β -catenin, Tcf/Lef acts as a transcriptional repressor when bound to Groucho. In the presence of β -catenin, Groucho is displaced and Tcf/Lef interacts with other nuclear proteins to initiate efficient transcription of Wnt target genes (Daniels and Weis 2005).

The Wnt pathway is constitutively active in many human cancers, particularly colorectal cancer (Kinzler and Vogelstein 1996). In the intestinal epithelial cells of familial adenomatous polyposis patients, the APC gene is inactive or defective (Kinzler, Nilbert et al. 1991, Nishisho, Nakamura et al. 1991). Consistent with a role for Wnt signaling in transformation of epithelial cells, loss of APC in mice also leads to the formation of intestinal polyps (Su, Kinzler et al. 1992). Remarkably, over 80% of sporadic colorectal cancers have mutations in APC and 10% in β -catenin (Kinzler, Nilbert et al. 1991). To date, all of the mutations in the Wnt pathway characterized in colorectal cancer lead to abnormal accumulation of β -catenin and chronic activation of the Wnt pathway. In addition to being the initiating event in colon cancer formation, continual Wnt pathway activation is thought to be required for maintenance of late-stage colon cancers (Pinto and Clevers 2005). Inhibition of Wnt signaling by RNAi or expression of a dominant-negative Tcf/Lef in colon cancer cell lines reduces their growth and reverses the epithelial-mesenchymal transition.

Aberrant Wnt signaling is also involved in cancers such as hepatocellular carcinoma, ovarian cancer, prostate cancer, and Wilms tumor (MacDonald, Tamai et al. 2009). Thus, inhibition of Wnt signaling may represent an effective therapeutic modality for the treatment of many common human cancers. Currently, there are no small compounds in late clinical trials or in clinical use that inhibit the Wnt pathway.

Use of *Xenopus* egg extract system for studying the Wnt pathway

Xenopus embryos have played an important role in our understanding of the Wnt pathway and its role in early vertebrate development (Hoppler 2008). The pathway is activated in the dorsal side of the embryo, an important step in organizing the tissue axis of the embryo. Ectopic expression of Wnt induces a secondary dorsal axis, and inhibition of the pathway reduces dorsalization of the embryo (Hoppler 2008). This phenotypic readout is simple and clear, and it has been used with great success to study the roles of Wnt components.

Xenopus egg extract is a biochemically tractable, *in vitro* system that has been used to reconstitute cytoplasmic aspects of the Wnt pathway (Salic, Lee et al. 2000). The regulated degradation of β -catenin, which is central to Wnt signaling, has been reconstituted using cytoplasmic egg extract. Extract prepared from *Xenopus* eggs is transcriptionally inactive, which is likely due to inaccessibility of the assembled chromatin to RNA polymerase II (Newport and Kirschner 1982, Newport and Kirschner 1982, Toyoda and Wolffe 1992, Barton, Madani et al. 1993). In contrast, *Xenopus* egg extract has a high capacity for translation and can readily translate exogenously added mRNA (Minshall, Blow et al. 1989, Murray and Kirschner 1989, Murray, Solomon et al. 1989, Glotzer, Murray et al. 1991, Matthews and Colman 1991, Murray 1991). The translational capacity of extract can be inhibited by addition of cycloheximide or by simply freeze-

thawing the extract (Murray and Kirschner 1989, Murray, Solomon et al. 1989, Glotzer, Murray et al. 1991, Murray 1991). Thus, β -catenin stability in extract can be directly assessed without complications from changes in its steady-state levels (Salic, Lee et al. 2000).

In addition, known components of the Wnt pathway, such as Dsh and LRP6, can be added as purified proteins directly into the system to effect changes in the kinetics of β -catenin degradation, thereby allowing for quantitative analysis of the Wnt pathway (Salic, Lee et al. 2000, Cselenyi, Jernigan et al. 2008). Using a biochemical approach that involved depleting and supplementing various Wnt components in *Xenopus* egg extract, the regulation of β -catenin turnover and the role of APC-axin- β -catenin interactions were examined; these data were used to develop a mathematical model of the Wnt pathway (Salic, Lee et al. 2000, Lee, Salic et al. 2003).

HTS screening of the Wnt pathway using *Xenopus* extract

Previous studies have shown that activation of the Wnt pathway promotes degradation of Axin and stabilization of β -catenin; conversely, inhibition of the Wnt pathway promotes stabilization of Axin and turnover of β -catenin (Mao, Wang et al. 2001, Lee, Salic et al. 2003, Tolwinski, Wehrli et al. 2003, Kofron, Birsoy et al. 2007, Cselenyi, Jernigan et al. 2008). Because Axin and β -catenin turnover represent independent readouts for Wnt signal transduction, and their stabilities are regulated in opposite directions, measuring changes in both of their levels represents a powerful approach to monitor Wnt pathway activity. Addition of a recombinant form of the intracellular domain of LRP6, which had been previously shown to promote degradation of Axin and stabilization of β -catenin, resulted in activation of the Wnt pathway (Cselenyi, Jernigan et al. 2008, Thorne, Hanson et al. 2010).

A high throughput screen using *Xenopus* egg extract to identify small molecules that inhibit the Wnt pathway was recently undertaken (Thorne, Hanson et al. 2010, Thorne, Lafleur et al.). To facilitate detection of β -catenin and Axin levels in a high throughput format, β -catenin and Axin proteins were fused to firefly and Renilla luciferase, respectively. The high throughput screen identified pyrvinium, an FDA-approved drug, as an inhibitor of the Wnt pathway. Inhibition of the Wnt pathway by pyrvinium was validated by *in vivo* studies (Thorne, Hanson et al. 2010). Injection of pyrvinium into developing *Xenopus* embryos induced ventralization and blocked Xwnt8-induced secondary axis formation, confirming that the compound was active in an organism. Further studies showed that pyrvinium was active in *Drosophila* and *C. elegans*, indicating that the molecular target of pyrvinium was conserved across phyla (Thorne, Hanson et al. 2010). Reconstitution studies using purified components ultimately identified casein kinase 1 α (CK1 α) as the cellular target of pyrvinium and suggested that pyrvinium may allosterically activate CK1 α . Further studies have subsequently shown that pyrvinium is biologically active in mouse models (Saraswati, Alfaro et al.).

Future studies of other embryonic signaling pathways using *Xenopus* egg extract

The Wnt, Hedgehog, and Notch pathways play critical roles in regulating cell fate during the embryonic development of metazoans (Logan and Nusse 2004, Andersson, Sandberg et al. , Ingham, Nakano et al.). Not surprisingly, these signaling pathways are required to maintain stem renewal and homeostasis in adult organs. Together, these pathways are hypothesized to be inappropriately regulated in the vast majority of solid human tumors (Beachy, Karhadkar et al. 2004, Ranganathan, Weaver et al.). Recent evidence strongly suggests the existence of a population of stem cells (cancer stem cells) capable of generating all of the cell types found in a given tumor (Dick 2009, Frank, Schatton et al.). Given their roles in stem cell renewal and

differentiation, it has been predicted that targeting these three pathways would have a major impact on regenerative medicine and cancer therapy. Currently, there are no drugs for general use in the clinic that target Wnt, Hedgehog, or Notch signaling pathways.

There are strong structural and regulatory parallels between Wnt, Hedgehog, and Notch signaling pathways (Fig. 2). All three pathways ultimately impinge on a transcription factor so as to regulate a program of gene expression that alters cellular behavior and fate. Many of the genes regulated by these three pathways are involved in cellular growth and proliferation (Barker and Clevers 2006, Katoh and Katoh 2009, Ranganathan, Weaver et al.). For all three pathways, proteolysis of a critical transcription factor occurs via the ubiquitin-proteasome system. In the case of Wnt and Hedgehog, proteolysis of the transcription factor occurs in the absence of ligand and is inhibited in a ligand-dependent manner (Logan and Nusse 2004, Robbins and Hebrok 2007, MacDonald, Tamai et al. 2009, Ingham, Nakano et al.). For Notch signaling, proteolysis occurs in a ligand-dependent manner to generate Notch intra-cellular domain (NICD), the active transcription factor. Growing evidence, however, suggests that ubiquitin-mediated turnover may limit the activity of the liberated NICD (Hubbard, Wu et al. 1997, Oberg, Li et al. 2001, Wu, Lyapina et al. 2001, Andersson, Sandberg et al.). Thus, as with the Wnt and Hedgehog pathways, proteolysis may represent a mechanism for regulating Notch signaling. As described above for the Wnt pathway, small molecules that modulate the the turnover of transcription factors that mediated Hedgehog and Notch signaling could similarly represent attractive strategies for targeting these two pathways. A brief description of the Hedgehog and Notch pathways is given.

The Hedgehog pathway

Hedgehog ligands are synthesized as precursor proteins that undergo autocleavage to generate the active ligands. In the absence of Hedgehog, the twelve-transmembrane protein Patched (Ptch) represses the activity of the seven-transmembrane protein Smoothed (Smo). Binding of Hedgehog to its receptor, Ptch, relieves inhibition of Smo activity by Ptch via a largely uncharacterized mechanism. Smo, which is the rate-limiting step in Hedgehog signaling, regulates the activity of all known downstream effectors upon binding of Hedgehog to Ptch (Ogden, Ascano et al. 2004). Hedgehog signaling ultimately alters the activity and stability of members of the GLI family of transcription factors in a manner that is tightly regulated by a large molecular weight protein complex (Fig. 2) (Robbins and Hebrok 2007, Ingham, Nakano et al.). The GLI proteins subsequently regulate a large number of Hedgehog target genes including *Cyclin D1*, *Myc*, and *Bcl-2* (Katoh and Katoh 2009).

Dysregulation of the Hedgehog pathway has been implicated in multiple types of human solid tumors. *GLI1* was initially identified as a gene that is amplified in malignant glioma and was subsequently shown to be upregulated in other malignant cancers such as basal cell carcinoma (Kinzler, Bigner et al. 1987, Dahmane, Lee et al. 1997, Green, Leigh et al. 1998). Ptch mutations have been implicated in basal cell carcinoma, in particular Gorlin syndrome, a rare inherited disease (Hahn, Wicking et al. 1996, Johnson, Rothman et al. 1996). The most clinically advanced drug that targets the Hedgehog pathway, the Smo inhibitor GDC-0449, is currently in clinical trials and has been shown to be effective for the treatment of basal cell carcinoma (Rubin and de Sauvage 2006). Resistance to GDC-0449, however, due to mutations in Smo that block its binding to the drug, has been reported (Yauch, Dijkgraaf et al. 2009). Patients with mutations in Smo (or elevated GLI) would likely benefit from compounds that target the Hedgehog pathway further downstream.

The Notch pathway

Notch receptors are single-pass type I transmembrane glycoproteins (Kopan and Ilagan 2009, Andersson, Sandberg et al.). Notch ligands (Delta/Delta-like, Serrate/Jagged, Lag-2) are themselves single-pass transmembrane receptors. Thus, activation of the Notch pathway requires close juxtaposition of cells. An unusual aspect of Notch signaling is that binding of Notch receptor to its ligand induces successive proteolytic events. The first cleavage, dependent on ligand activation, is mediated by an ADAM metalloprotease and occurs in the extracellular domain to generate a membrane-anchored Notch extracellular truncation (NEXT) fragment (Bozkulak and Weinmaster, 2009)(van Tetering et al., 2009). This NEXT fragment is a substrate for a gamma-secretase, which cleaves the protein within the transmembrane domain. The freed Notch ICD (NICD) subsequently translocates into the nucleus to initiate transcription of Notch target genes.

Blocking gamma secretase activity has been the primary focus for development of therapeutics against the Notch pathway, and several gamma secretase inhibitors (GSIs) are in early clinical trials (Wolfe 2009). Side effects of GSIs, however, including debilitating diarrhea resulting from treatment-induced differentiation of mucus-producing Goblet cells, represent a major hurdle for their further development. The carboxy terminus of NICD contains a PEST domain, which regulates its intracellular stability. The importance of this sequence is suggested by the identification of mutations within this domain in certain Notch-driven tumors (Weng, Ferrando et al. 2004, Westhoff, Colaluca et al. 2009). These mutations are predicted to increase NICD steady-state levels, thereby resulting in enhanced pathway activation. Interestingly, mutations that alter NICD stability have been shown to be resistant to the effects of GSIs (O'Neil, Grim et al. 2007). This observation suggests that promoting the degradation of NICD may be an alternative strategy for inhibiting Notch-driven tumors with stabilizing mutations of NICD.

Summary

Xenopus egg extract represents an attractive system that combines the advantages of a phenotypic screen (not limited by preconceived hypothesis as to the appropriate drug target) with the tractability of a biochemical approach (more quantitative and reproducible) for high throughput screening. As discussed herein, the *Xenopus* egg extract system has been exploited to reconstitute a wide range of complex biological processes. *Xenopus* egg extract has been successfully used in high throughput screens (or further validation) for small molecule modulators of the cell cycle, microtubule polymerization, and actin polymerization (Tables 1 and 2).

We have previously shown that the Wnt pathway can be reconstituted in *Xenopus* egg extract and successfully screened for small molecular modulators of β -catenin degradation. Similar to the Wnt pathway, other “embryonic” pathways (e.g. Hedgehog and Notch) play critical roles in early metazoan development. A common feature of all three is that proteolysis of key transcriptional mediators controls the extent of signaling through the pathway. Thus, as for the Wnt pathway, a screening strategy using *Xenopus* egg extract represents an attractive approach to identify small molecule modulators of the Hedgehog and Notch pathways. Evidence for the feasibility of such an approach comes from our recent studies showing that the transcriptional mediators of the Hedgehog (Gli1) and Notch (NICD) pathways readily undergo robust degradation in *Xenopus* egg extract (Thorne, Lafleur et al.).

As with any biochemical approach, an obvious drawback to using the *Xenopus* extract system to screen for inhibitors of Wnt, Hedgehog, and Notch pathways is that one cannot screen for modulators of cellular morphology. In addition, because one is limited to screening for cytoplasmic events of these pathways, it is not feasible to screen for compounds that selectively

modulate activation of particular receptor subtypes or other events at the level of the receptor (e.g. cell-to-cell communication). Specific transcriptional programs are ultimately initiated upon activation of the Wnt, Hedgehog, and Notch signaling pathways. To date, however, a system in which transcriptional responses can be readily measured in a high throughput fashion has not been developed. Regardless, the roles of Hedgehog and Notch pathways, like the Wnt pathway, have been well characterized during *Xenopus* embryonic development; thus, *in vivo* validation of compounds can also be readily performed using *Xenopus* embryos. Although we have focused on screening for small molecule modulators of pathways in which proteolysis plays a central role, we expect that other signaling pathways could similarly be interrogated using *Xenopus* egg extract.

Figures

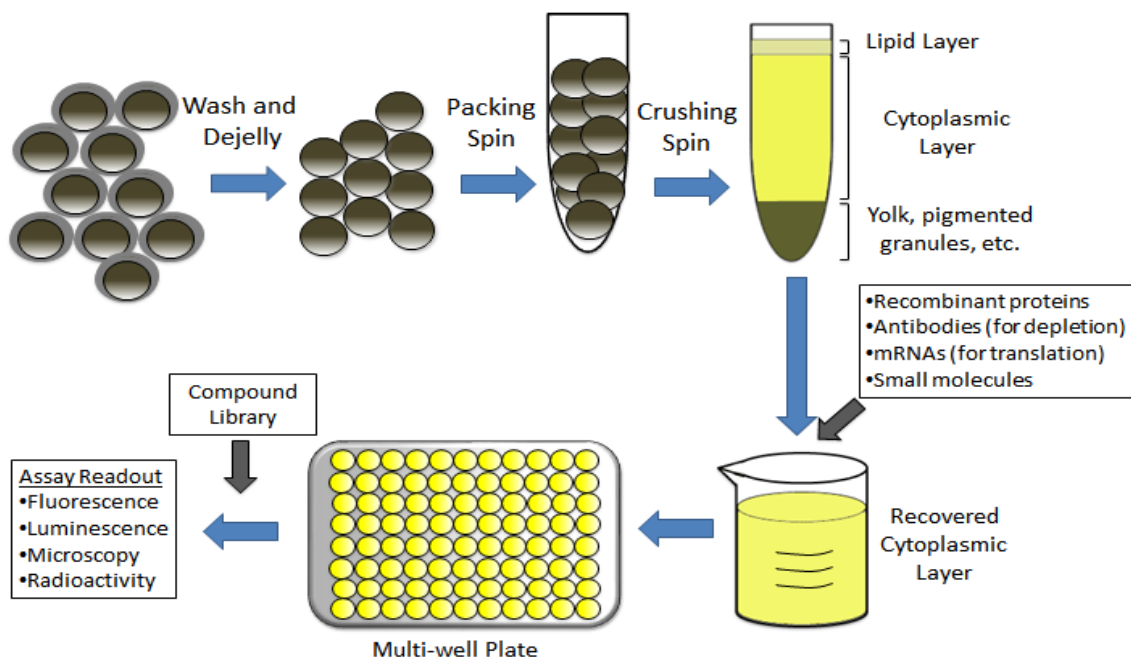


Figure A.1. Preparation of *Xenopus* egg extract for HTS screening. *Xenopus* females are injected with human chorionic gonadotropin to induce egg laying into containers filled with buffer. Eggs are collected and the jelly coat removed by treatment with 2% cysteine. The dejellied eggs are washed and subjected to a packing spin (~100 x g) to remove excess buffer. The packed eggs are then subjected to a crushing spin (>15,000 x g) that separates the crushed egg components into three distinct layers: lipid, cytoplasmic, and pigmented granule/yolk. The cytoplasmic layer is collected and can be used for biochemical studies or for HTS. Recombinant proteins can be added directly to the egg extract; alternatively, if recombinant proteins are not readily available, mRNA encoding the desired proteins can be added instead. For the latter, the egg extract must be freshly prepared because frozen extract loses its capacity to translate mRNAs. To immunodeplete specific proteins from extract, antibodies linked to resin can be added and removed. Finally, a specific compound can be added to perturb the signaling pathway followed by screening for other compounds that either synergize with it or block its effects. Screen readouts may include fluorescence with GFP constructs, luminescence with luciferase constructs, or radioactivity using scintillation proximity assays. Microscopic analysis of cellular structures (e.g. mitotic spindle, nuclear envelope, chromosomes, etc.) can also be performed in a high throughput manner with appropriate imaging analysis software.

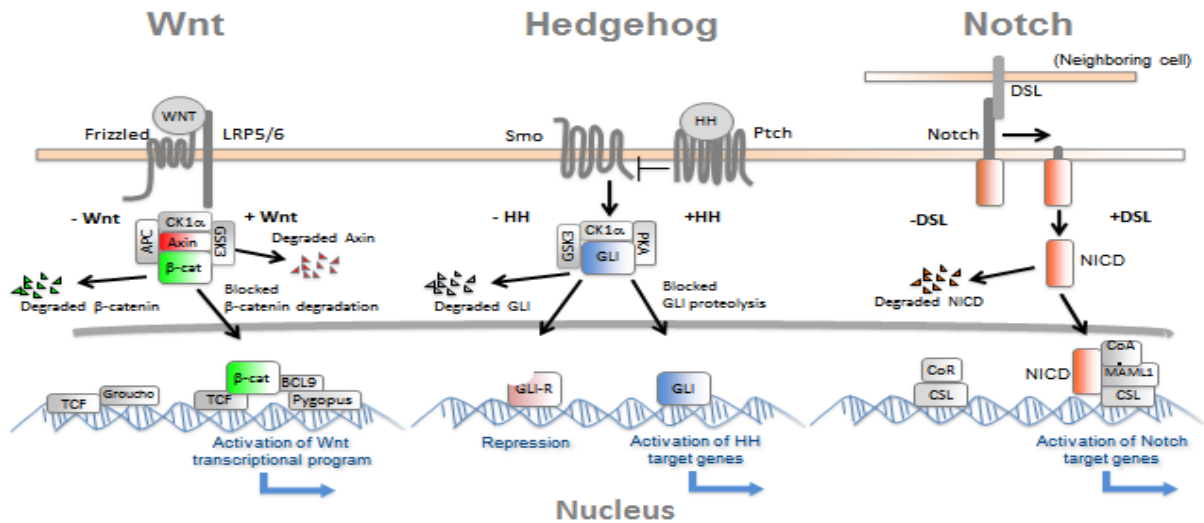


Figure A.2. Schematic of the Wnt, Hedgehog, and Notch signaling pathways highlighting cytoplasmic degradation of key transcriptional mediators. For Wnt signaling, the transcriptional coactivator, β -catenin, is degraded in the absence of a Wnt signal, whereas the scaffold protein, Axin, is stable. Binding of Wnt ligands to the Frizzled and LRP5/6 coreceptors results in inhibition of β -catenin degradation whereupon it enters the nucleus. Transcription factors such as TCF, BCL9, and Pygopus form a complex with nuclear β -catenin that leads to activation of a Wnt transcriptional program. In contrast, Axin degradation is stimulated upon Wnt pathway activation. Members of the GLI family of transcriptional factors are the mediators of canonical Hedgehog (HH) signaling. In the absence of HH, GLI proteins are degraded or converted to a lower molecular weight form (GLI-R), which acts as a transcriptional repressor. Proteolysis of GLI occurs in a cytoplasmic complex containing several kinases including glycogen synthase kinase 3 (GSK3), casein kinase 1 α (CK1 α), and protein kinase A (PKA). The binding of HH to its receptor, Patched (Ptch), relieves inhibition of the seven membrane-spanning protein, Smoothed (Smo), by Ptch via an unknown mechanism. The uninhibited Smo protein subsequently inhibits GLI proteolysis, promoting accumulation of the full-length form and subsequent activation of HH target genes. Notch signaling is initiated upon binding of the transmembrane Notch protein to the Delta/Serrate/LAG-2 (DSL) family of plasma transmembrane ligands present in the membrane of adjacent cells. Binding results in a series of proteolytic cleavage events that ultimately release the Notch intracellular domain (NICD) into the cytoplasm followed by its translocation into the nucleus. In the absence of NICD, the CBF1/Suppressor of Hairless/LAG1 (CSL) family of DNA binding proteins associates with corepressors (CoR) to inhibit Notch target gene transcription. Nuclear NICD interacts with CSL and the transcriptional coactivator Mastermind (MAML1) to recruit transcriptional coactivators (CoA) to initiate transcription of Notch target genes.

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