Novel Biochemical Regulatory Mechanisms of Developmental Signaling Pathways

By

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To my family

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CHAPTER I

INTRODUCTION TO DEVELOPMENTAL SIGNALING PATHWAYS

Introduction

The fundamental processes of metazoan development are widely conserved throughout the animal kingdom. Among the conserved components are two critical developmental pathways, the Wnt signaling pathway and the Notch signaling pathway. These pathways are similar in many ways, including ligand-dependent activation of the pathway in wild-type conditions, key transcriptional co-activators which form transcriptional activation complexes, and a downstream effector protein in which the stability of the protein is tightly regulated in order to regulate the transcriptional activity of the pathway. Because these developmental pathways regulate organismal growth and development, they can potentially be co-opted when key regulatory genes are mutated. Both the Wnt pathway and the Notch pathway are very often misregulated in human cancers. In this work, Chapter I sets the historical and scientific foundation for key mechanistic questions in both the Wnt and Notch pathways which are explored in greater mechanistic detail in Chapters III and IV. Chapters V and VI conclude with discussion and future directions from the findings presented in this document.

Cell Signaling and Signal Transduction of Developmental Pathways

Cell signaling is a form of cellular communication in which cells interact with their surroundings, process this information, and provided an appropriate response to these

signals. The process in which a cell recognizes, processes, and responds to a signal is called signal transduction.

The process of signal transduction regulates and coordinates all metazoan developmental processes. Metazoan organismal development is a highly regulated and coordinated series of signal transduction events between cells and other cells which cooperate to form a fully functional reproducing animal.

One of the earliest studies involving signal transduction and development comes from the work of Hans Spemann in the early 20th century. In this seminal work, Spemann, along with Hilde Mangold, showed that transplantation of the dorsal lip of the blastopore of an amphibian embryo beginning gastrulation onto the other side of another developmentally staged embryo resulted in the formation of two body axes in the grafted embryo, producing a mirror image twin. One of these axes was formed by the endogenous dorsal lip of the blastopore while the other was formed by the transplanted dorsal lip tissue. The transplanted dorsal lip induced the formation of a complete dorsal axis in a location that normally forms the ventral side of the embryo. Due to its inductive potential, the dorsal lip of the amphibian blastopore was termed the organizer (Spemann and Mangold, 1938; Spemann and Mangold, 2001). This landmark discovery provided major evidence that cells signal to each other and that these signals can induce cooperative growth and development. Because of this work, Spemann was awarded the Nobel Prize in Medicine or Physiology in 1935.

Currently, 18 signal transduction pathways have been identified (Gerhart, 1999). These signaling pathways typically have conserved structural mechanisms in order to transduce the signal. Some type of ligand is released into the extracellular environment

or expressed at the surface of the siganal-sending cell and binds to a cell surface receptor. This ligand-bound receptor gets activated. Then, the activated receptor transduces the signal intracellularly. Finally, intracellular signal transduction occurs through secondary messengers which send the signal through the cell via other messengers to ultimately induce a physiological response.

Current evidence suggests that 5 of the 18 currently known signaling pathways control developmental processes (Gerhart, 1999). As metazoans evolved multi-cellularity, a means of signaling between these multiple cells also evolved in order to facilitate communication between these cells. These cell-cell signaling pathways that evolved in response to multicellularity are the developmental signaling pathways that are conserved throughout all metazoans. Two of these developmental pathways, the Wnt/β-catenin signaling pathway and the Notch signaling pathway, will be described in more detail later in this document. This dissertation will focus primarily on Notch signaling.

The description of these signaling processes as “pathways” paints an inaccurate picture of the diversity and complexity of metazoan development. These signaling processes, rather than being discrete, independent “pathways”, are interconnected, forming a signaling “network”. Abundant evidence exists that there is crosstalk between these “pathways”, in which the activation or non-activation of a receptor of one pathway affects another pathway (van Amerongen and Nusse, 2009). In this chapter I will describe the history and importance of Wnt/β-catenin signaling, the history and importance of Notch signaling, and the evidence of cross-talk between the two that links them into a signaling network.

Historical Significance: Wnt Signaling in Development and Disease

The canonical Wnt/β-catenin signaling pathway plays a critical role in cell fate determination, cell proliferation, cell polarity, and cell death during embryonic development and in tissue homeostasis in adults. The Wnt pathway is named for its ligands, the Wnt family of secreted glycoproteins, was discovered nearly 40 years ago. The history of Wnt/β-catenin signaling highlights the roles of this pathway in both development and disease. Many of the details of Wnt/β-catenin signaling can be found in other reviews [reviewed in (MacDonald et al., 2009; Saito-Diaz et al., 2013)].

In 1976, Sharma and Chopra described a *Drosophila melanogaster* mutant which had absent or reduced wings and halteres, which they named *wingless (wg).* Based on the mutant phenotype, they hypothesized that the *wingless* locus played a critical role in development (Sharma and Chopra, 1976). This hypothesis was confirmed in 1980 when Wieschaus and Nusslein-Volhard identified *wg* as a segmentation gene in a *Drosophila* mutagenesis screen for gene required in segmentation(Nusslein-Volhard and Wieschaus, 1980). For this landmark discovery in developmental biology, Nusslein-Volhard and Wieschaus were awarded the Nobel Prize in Physiology or Medicine in 1995.

Several years later, Nusse and Varmus conducted a forward genetic screen to identify genes which could lead to tumorigenesis. They used mouse mammary tumor virus (MMTV) insertion sites and identified a locus termed *int-1*, short for integration-1, which induced mouse mammary tumors (Nusse et al., 1984; Nusse and Varmus, 1982). Later, comparative genomic studies identified *wg* and *int-1* as homologs, and the name

was merged into the mnemonic Wnt (Nusse et al., 1991). The injection of *int-1* in *Xenopus* embryos induced the formation of a secondary body axis, confirming the role of *int-1* as both an oncogene and a critical component of vertebrate early axis formation (McMahon and Moon, 1989a; McMahon and Moon, 1989b). These studies take together suggest that the Wnt proteins play a critical role in normal development as well as a critical role in carcinogenesis.

Drosophila mutagenesis screens (similar to the one described earlier from Nusslein-Volhard and Wieschaus) played an important role in identifying components of the Wnt/β-catenin signaling pathway (Nusslein-Volhard and Wieschaus, 1980). In the 15 years after that initial publication, key Wnt pathway components such as *armadillo* (the *Drosophila* homolog of β-catenin), *dishevelled* (Dsh), *shaggy* (the *Drosophila* homolog of glycogen synthase kinase 3, GSK3), *frizzled* (Fz), and *arrow* (the *Drosophila* homolog of LRP5/6) (Bhanot et al., 1996; Klingensmith et al., 1994; Riggleman et al., 1990; Riggleman et al., 1989; Siegfried et al., 1992; Wehrli et al., 2000) were identified.

The Wnt/β-catenin pathway was then linked to the formation of the Spemann-Mangold organizer referenced earlier in this chapter (Spemann and Mangold, 1938). Injection of *Wnt-1 and XWnt8* into *Xenopus* blastomeres induces a secondary axis due to a second organizer (Smith and Harland, 1991; Sokol et al., 1991). This secondary axis formation was also phenocopied using other Wnt/β-catenin pathway components (Dominguez et al., 1995; Fagotto et al., 1999; Guger and Gumbiner, 1995; He et al., 1995; Sokol et al., 1995). Many of these other components were identified by their effects on vertebrate development, such as Axin (Zeng et al., 1997), APC (Munemitsu et al., 1995; Rubinfeld et al., 1993), and the co-receptor LRP5/6 (Pinson et al., 2000;

Tamai et al., 2000; Wehrli et al., 2000). All of these major Wnt components can induce secondary axis formation in *Xenopus* embryos, and the axis duplication assay has emerged as a powerful validation tool to identify *bona fide* regulators of Wnt/β-catenin signaling.

Many developmental signaling pathways are also critical drivers of cell growth and cell-cell signaling in cancer. The Wnt/β-catenin signaling pathway is no exception. Perturbations in Wnt/β-catenin signaling lead to a large number of diseases, varying from congenital birth defects to multiple types of cancer [reviewed in (MacDonald et al., 2009)]. Perhaps the most well-known connection between Wnt/β-catenin signaling and cancer is a genetic lesion in the Wnt pathway component APC that occurs colorectal cancer. In familial adenomatous polyposis (FAP), a form of hereditary colorectal cancer (Kinzler et al., 1991; Nishisho et al., 1991), patients missing one copy of APC lose their second copy of APC and develop benign polyps at an early age. These polyps then develop other mutations and lead to invasive colon carcinoma. Later, loss of both APC alleles was linked to over 80% of sporadic, nonhereditary colorectal cancers (Kinzler and Vogelstein, 1996). Misregulated Wnt/β-catenin signaling was then found in many other types of cancers, including liver cancer, skin cancer, lung cancer, Wilms' Tumor, breast cancer, prostate cancer, and others [reviewed in (Klaus and Birchmeier, 2008) and (Saito-Diaz et al., 2013)]. Developmental genetic defects can also result from misregulated Wnt/β-catenin signaling (Boyden et al., 2002; Gong et al., 2001; Lammi et al., 2004; Niemann et al., 2004; Toomes et al., 2004; Xu et al., 2004). Understanding the molecular mechanisms governing Wnt/β-catenin signaling is critical towards both

understanding the pathophysiological effects of Wnt/β-catenin misregulation and designing therapeutics against the Wnt/β-catenin signaling pathway.

The Current Model of the Wnt/β-catenin Signaling Pathway

Wnt signaling promotes a variety of cellular responses in development, physiology, and disease. The original hypothesis was that Wnt signaling promotes these responses by activating different transcriptional target genes in different cellular contexts. This pathway, in which Wnt signaling activates specific transcriptional target genes, was previously referred to as “canonical” Wnt signaling. I have referred to it as Wnt/β-catenin signaling to distinguish it from other Wnt-mediated pathways. Other Wnt-mediated pathways signal cytoplasmic changes involving the action cytoskeleton (Wnt/PCP pathway) and intracellular calcium stores (Wnt/Ca^{2+} pathway). These other pathways may be regulated by the tyrosine kinase receptors ROR and RYK (Nusse, 2008). In recent years, even the simplicity of the two pathway model has been questioned (van Amerongen et al., 2008). These other pathways are outside the scope of this document. For all intents and purposes, every reference to Wnt signaling refers to Wnt/β-catenin signaling.

The Wnt/β-catenin signaling pathway, fundamentally, results in the cytoplasmic protein β-catenin entering the nucleus to modulate transcription. When Wnt ligand is not bound, β-catenin is continually degraded by the β-catenin destruction complex. The destruction complex consists of the scaffold proteins Axin and APC and the protein kinases GSK3 and Casein Kinase 1 (CK1) [Figure 1.1 (Saito-Diaz et al., 2013)].

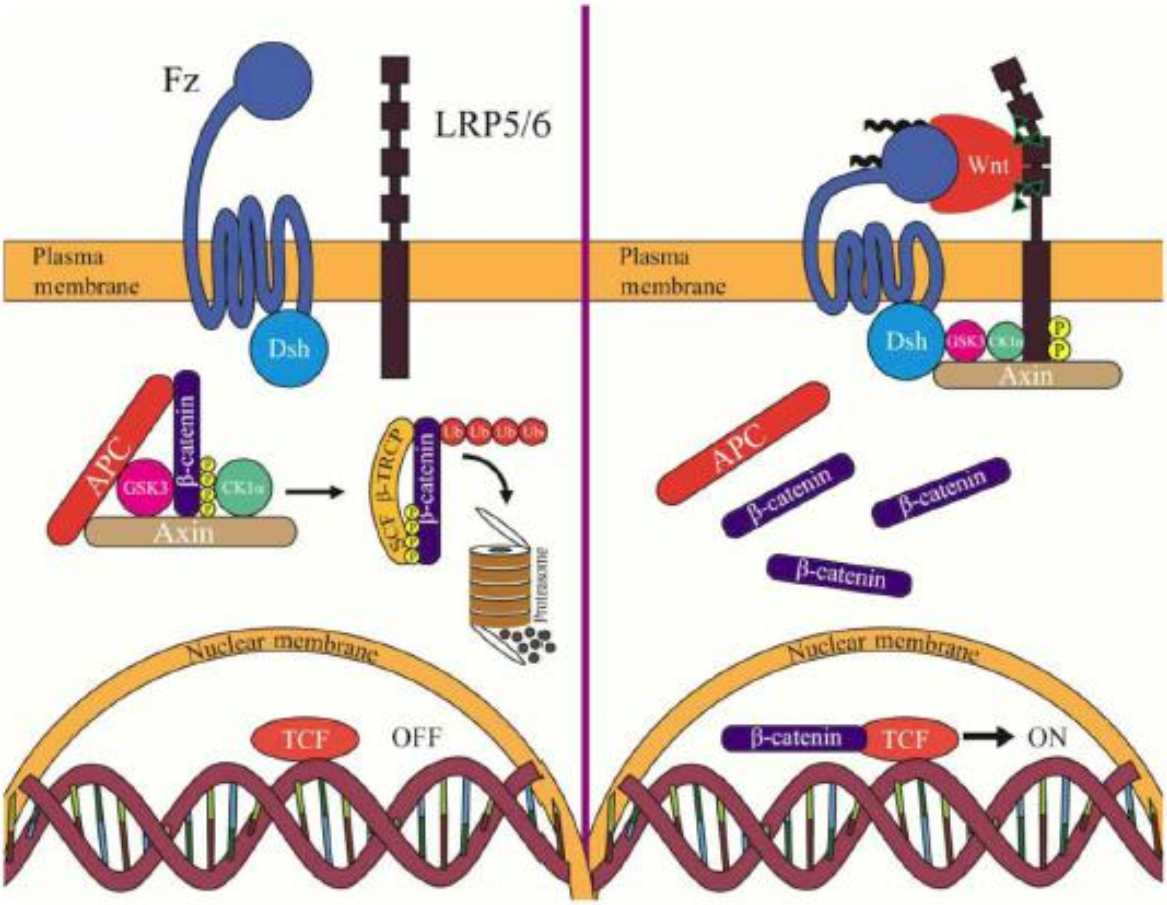

Figure 1.1. The current model of Wnt/β-catenin signaling. (Left panel) In the absence of Wnt, cytoplasmic β-catenin forms a complex with APC, Axin, GSK3, and CK1α. β-Catenin is phosphorylated by CK1α and subsequently phosphorylated by GSK3. The phosphorylated form of β-catenin is recognized by the E3 ubiquitin ligase SCFβ-TRCP, which targets β-catenin for proteasomal degradation. In the absence of nuclear β-catenin, Wnt target genes are repressed. APC, adenomatous polyposis coli; GSK3, glycogen synthase kinase 3; CK1α, casein kinase 1 alpha. (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 CK1α 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. Fz, Frizzled; Dsh, Dishevelled; TCF, T-cell factor [Figure from (Saito-Diaz et al., 2013)].

Activation of Wnt/β-catenin signaling removes APC from the complex and relocalizes the other components to the plasma membrane via the adaptor Dsh, thus stabilizing β-catenin which enters the nucleus to mediate transcription (Figure 1). Thus, Wnt/β-catenin signaling can be divided into three general molecular events: (1) surface receptor activation, (2) inhibition of the β-catenin destruction complex, and (3) activation of a Wnt-specific nuclear transcriptional complex. The next sections of this document consider each of these steps more closely.

Wnt/β-catenin Signaling: Surface Receptor Activation

The secreted Wnt proteins are cysteine-rich morphogens between approximately 350-400 amino acids which can act in both short-range and long-range signaling. There are at least 19 vertebrate Wnt proteins and are capable of activating the pathway. Wnt ligands bind to their receptor Frizzled (Fz). The structural basis of Wnt-receptor interactions has been characterized (Janda et al., 2012). All of the Wnt ligands contain an N-terminal signal peptide for secretion and are N-linked glycosylated (Smolich et al., 1993; Takada et al., 2006; Willert et al., 2003). The N-glycosylation of the *Drosophila* Wnt homolog Wg is stimulated by lipid modifications (Tanaka et al., 2002). Early studies suggested that glycosylation of Wnt was dispensable for Wnt activity (Mason et al., 1992), but more recent studies have demonstrated the requirement of glycosylation for Wnt secretion (Komekado et al., 2007; Kurayoshi et al., 2007).

The Wnt proteins contain multiple charged amino acids and undergo lipid modifications which are required for activity (Bradley and Brown, 1990). Wnt3a protein (and by extension Wnts in general) is acylated with a palmitate at Cys77 and a palmitoleate at Ser209 (Takada et al., 2006; Willert et al., 2003). Interestingly, the

crystal structure of the Wnt/receptor complex shows Cys77 engaged in disulfide bonding and the palmitoleate at Ser209 docked inside a hydrophobic groove on a cysteine-rich domain (CRD) of the receptor, playing a direct role in Wnt-receptor interaction (Janda et al., 2012).

These lipid modifications of Wnt are mediated by an endoplasmic reticulum (ER)-embedded, multi-pass transmembrane *O*-acetyl transferase known as Porcupine (Porc) [reviewed in (MacDonald et al., 2009; Port and Basler, 2010; Saito-Diaz et al., 2013)]. *Porc* was initially identified in *Drosophila* as a segment polarity gene was the first gene shown to be required in Wnt-secreting cells. Loss-of-function of *Porc* leads to accumulation of Wnt in the ER (Kadowaki et al., 1996; van den Heuvel et al., 1993) and overexpression of *Porc* results in a high percentage of Wnts that are lipid-modified (Galli et al., 2007). The p24 family of proteins is required for modified Wnts to get transported from the ER to the Golgi (Buechling et al., 2011; Port et al., 2011). Once in the Golgi, the *trans*-Golgi seven-pass transmembrane protein Wntless (Wls transports Wnt from the Golgi to the plasma membrane. Wls binds to the palmitoylated Ser209 which is mediated by Porc (Herr and Basler, 2011). WIs is recycled back to the plasma membrane via a protein complex known as the retromer. The retromer complex routes WIs back from endosomes into *trans*-Golgi in a retrograde manner (Coudreuse et al., 2006; Port and Basler, 2010). WIs gets degraded in the endosome in the absence of the retromer complex (Yang et al., 2008). The addition of exogenous WIs bypasses the requirement of the retromer (Franch-Marro et al., 2008; Port et al., 2008) Together, Porc, Wls, and indirectly, the retromer complex, form a pathway critical for secretion of Wnt ligands (Figure 1.2).

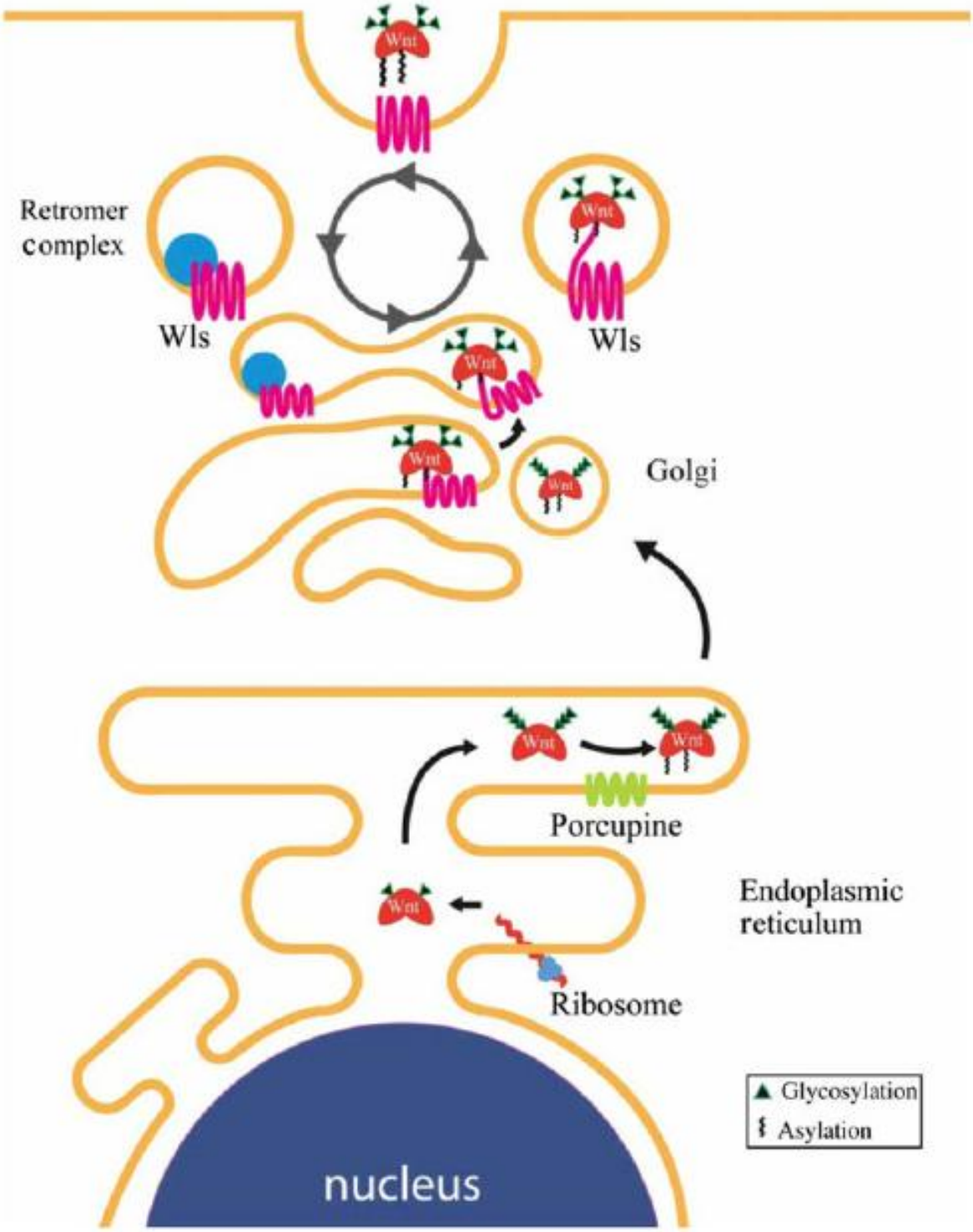

Figure 1.2. Synthesis and export of Wnt ligand. Wnt ligand undergoes multiple posttranslational modifications in the ER. Glycosylation and palmitoylation of Wnt ligand (the latter mediated by the transmembrane protein Porc) are required for its translocation to the Golgi apparatus. Palmitoylation of Wnt allows it to bind Wls, which provides a mechanism for transportation to the plasma membrane. The retromer complex recycles Wls from the plasma membrane back to the Golgi [Figure from (Saito-Diaz et al., 2013)].

The soluble Wnt ligands bind to the Frizzled (Fz) family of seven transmembrane domain receptors, which share structural features with G-protein coupled receptors (GPCRs). Biochemical experiments showed that Wnt binds to the CRD domain of Fz with a binding affinity in the low nanomolar range (Bhanot et al., 1996; Hsieh et al., 1999). Because of Fz's topological similarity to classical GPCRs, heterotrimeric G-protein signaling has been hypothesized as critical in transducing Wnt signaling. A link between G proteins and Wnt signaling has been suggested in several studies. First, in *Drosophila*, studies suggest that $G_{\alpha o}$ transduces signaling through Fz and interacts with the scaffold protein Axin to promote its localization to the plasma membrane (Egger-Adam and Katanaev, 2009; Katanaev et al., 2005). In mammalian cell culture, depletion of $G_{\alpha o}$ and $G_{\alpha q}$ inhibited Wnt/β-catenin (Liu et al., 2005). Reconstitution experiments in *Xenopus* egg extract show that $G_{\alpha o}$, $G_{\alpha q}$. $G_{\alpha i2}$, and $G_{\beta\gamma}$ can inhibit β-catenin phosphorylation and turnover. $G_{\beta\gamma}$ was proposed to promote GSK3 recruitment to the membrane that enhanced low-density lipoprotein receptor-related protein 6 (LRP6) phosphorylation and activation (Jernigan et al., 2010).

LRP5 and LRP6 are functionally redundant single pass transmembrane receptors which serve as co-receptors of Wnt/β-catenin signaling (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000). In *Drosophila* wingless signaling, the lone LRP family member is known as Arrow. Although there have been some differences in potency, LRP5 and LRP6 were shown to be mechanistically nearly identical in the Wnt/β-catenin signaling pathway, despite some differences during development (He et al., 2004; Mi and Johnson, 2005). Biochemical and structural studies have shown that

different Wnt ligands bind to different extracellular domains of LRP5/6 (Ahn et al., 2011; Bourhis et al., 2010; Chen et al., 2011; Cheng et al., 2011). Wnt binding to Fz and LRP5/6 leads to the production of phosphatidylinositol 4,5-biphosphate (PIP_2) (Pan et al., 2008). The production of PIP_2 has been hypothesized to promote the oligomerization and clustering of Fz and LRP6 into "signalosomes" upon activation of Wnt/β-catenin signaling. The *in vivo* physiological significance of signalosome formation is still being investigated (Bilić et al., 2007; Cong et al., 2004b). PIP_2 production also promotes recruitment of destruction complex components to LRP5/6 on the plasma membrane, possibly through Amer1/WTX (APC membrane recruitment 1 or Wilms tumor gene on the X chromosome), a tumor suppressor in Wilms' tumor which binds to Axin and GSK3. Amer1/WTX's recruitment to the plasma membrane is PIP_2- dependent (Major et al., 2007; Tanneberger et al., 2011). The recruitment of the destruction complex to the plasma membrane upon Wnt binding leads to the phosphorylation of LRP5/6 in an event known as the "initiation step" of Wnt/β-catenin signaling (Baig-Lewis et al., 2007). LRP5/6 is phosphorylated by the destruction complex kinases GSK3 and CK1 at PPPSPxS motifs on LRP5/6 which are both necessary and sufficient to activate Wnt/β-catenin signaling (Davidson et al., 2005; MacDonald et al., 2009; MacDonald et al., 2008; Tamai et al., 2004; Wolf et al., 2008; Zeng et al., 2005). The recruitment of the concentration-limiting scaffold protein Axin (Lee et al., 2003) brings additional GSK3 and CK1 molecules to the plasma membrane during the "amplification step" (Baig-Lewis et al., 2007). Subsequently, the activated and phosphorylated LRP6 intracellular domain inhibits further GSK3 activity by directly binding to it (Cselenyi et al., 2008; Piao et al., 2008; Wu et al., 2009). This GSK3 inhibition by phosphorylated LRP6 frees up β-catenin

from getting phosphorylated by GSK3 and targeted for ubiquitin-mediated degradation, thus transducing the signal further downstream. The mechanistic relationship between LRP6 and GSK3 amplification and then inhibition requires further study.

Other molecules linked to agonizing or antagonizing Wnt/β-catenin signaling have been identified. There are two classes of secreted Wnt/β-catenin antagonists. One class, consisting of secreted Fz-related proteins (sFRPs) and Wnt inhibitory factors (WIFs), bind and sequester Wnt ligands and prevent their interaction with Wnt receptors (Bovolenta et al., 2008). The other class, made up of Dkk1 and Wise/SOST members, binds to LRP5/6 and blocks its interaction with Wnt ligands (Mao et al., 2002; Semenov et al., 2001). Other Wnt/β-catenin agonists include Norrin and R-Spondin (Kazanskaya et al., 2004; Kim et al., 2006; Nam et al., 2006; Wei et al., 2007; Xu et al., 2004). R-spondin may be a driver of colorectal cancer (Seshagiri et al., 2012) and has been shown to bind to leucine-rich repeat-containing GPCRs 4,5 and 6 (LGR4/5/6), which are intestinal stem cell markers , but how this binding agonizes Wnt/β-catenin signaling is still unclear (Barker et al., 2007; Carmon et al., 2011; de Lau et al., 2011; Glinka et al., 2011; Snippert et al., 2010). Recent studies suggest that R-spondin stabilizes the Wnt/β-catenin receptors Fz and LRP5/6 by inhibiting the activity of two E3 Ubiquitin Ligases, *RNF43* and *ZNRF3*, which target Fz and LRP6 for degradation (Hao et al., 2012; Koo et al., 2012). This stabilization of Fz and LRP5/6 potentiates Wnt/β-catenin signaling. Very recently, the type 1 transmembrane protein Tiki was identified in an expression cloning screen that perturbed axis formation in *X. laevis* embryos (Zhang et al., 2012). Tiki was identified as a novel metalloprotease that cleaves the N-terminal 8 amino acids of mature Wnt proteins which results in the formation of large, soluble

oligomeric Wnt complexes due to oxidation and the formation of disulfide bonds *in vitro*. Whether the formation of these inactive Wnt complexes is how Tiki affects the Wnt/β-catenin pathway *in vivo* is still unclear.

Another critical component of the Wnt/β-catenin pathway is the cytoplasmic effector protein Dishevelled (Dsh). Dsh is required genetically in *Drosophila* wingless signaling (Klingensmith et al., 1994) and there are 3 vertebrate paralogs encoded by 3 distinct genes (*Dvl1-3*) (Semenov and Snyder, 1997; Sussman et al., 1994; Yang et al., 1996). Dsh gets phosphorylated and recruited to the cytoplasmic portion of the receptor upon Wnt-receptor binding (Rothbacher et al., 2000; Semenov and Snyder, 1997; Yanagawa et al., 1995). This Dsh phosphorylation is independent of LRP6 activation (Gonzalez-Sancho et al., 2004). Dsh contains 3 known structural domains, the DEP, the PDZ, and the DIX domains. The PDZ and DIX domains have been shown to be important in Dsh binding to Fz (Tauriello et al., 2012; Wong et al., 2003; Wong et al., 2000). The DIX domain is thought to polymerize and promote receptor clustering (Schwarz-Romond et al., 2007). Though Dsh is thought to be upstream of LRP6 in the Wnt/β-catenin pathway (Tolwinski et al., 2003) and can stimulate PIP_2 production (Pan et al., 2008), in *Drosophila* and *Xenopus* egg extracts Dsh activates Wnt/β-catenin independently of Arrow/LRP6 (Salic et al., 2000b; Wehrli et al., 2000). In another invertebrate species, *Caenorhabditis elegans*, there is a Dsh homolog but no LRP5/6 homolog, suggesting that Dsh might play a more critical role in different phyla (Phillips and Kimble, 2009). Dsh is likely regulated by ubiquitin-mediated proteasomal degradation through at least 3 known E3 ubiquitin ligases, the HECT-type ligases NEDL1 and ITCH and the SCF-type ligase KLHL12 (Angers et al., 2006; Miyazaki et al.,

2004; Wei et al., 2012). This degradation is also mediated by the Naked2 protein as a co-factor (Hu et al., 2010). Additionally, the deubiquitinase CYLD (encoded by the familial cylindromatosis tumor suppressor gene) negatively regulated Wnt/β-catenin signaling (Tauriello et al., 2010).

These Wnt/β-catenin pathway components (Wnt, Fz, LRP5/6, and Dsh) and their regulators (Porc, WIs, sFRPs, WIFs, PIP_2, ITCH, NEDL1, KLHL12, and others) combine to form a highly regulated network of plasma membrane surface proteins that are critical for Wnt/β-catenin signal transduction. After surface receptor activation and transduction of the signal, the cytoplasmic β-catenin destruction complex comes into play.

Wnt/β-catenin Signaling: The β-catenin destruction complex

The β-catenin destruction complex is a macromolecular machine that efficiently phosphorylates β-catenin and targets it for degradation. I will first describe the molecules involved in the formation of the β-catenin destruction complex (Figure 1.1) and follow with the current model of Wnt/β-catenin signaling pathway upon receptor activation [reviewed in (Chen et al., 2014b; Saito-Diaz et al., 2013)].

The transcriptional regulator β-catenin, as mentioned earlier, is the primary effector of the Wnt/β-catenin signaling pathway. In the absence of Wnt/β-catenin signaling, the destruction complex targets β-catenin for degradation by $SCF^{\beta\text{-TRCP}}$, a Skp1-Cullin-Fbox (SCF) E3 Ubiquitin Ligase complex family member. When Wnt/β-catenin signaling is active, β-catenin degradation is inhibited and translocates from the cytoplasm to the nucleus to activate Wnt/β-catenin signaling. There are other substrates of the destruction complex but their physiological relevance is still unclear. β-catenin was originally identified in *Drosophila* as the segment polarity *armadillo* as a component

of the adherens junction in *Xenopus* (McCrea et al., 1991; Nusslein-Volhard and Wieschaus, 1980). Structurally, β-catenin contains a central core consisting of 12 helical 42 amino acid armadillo repeats which form a superhelix (Huber et al., 1997). The unstructured N-terminal and C-terminal ends of β-catenin form dynamic interactions with the armadillo repeats (Xing et al., 2008). These armadillo repeats form a positively charged groove which regulates β-catenin's interaction with other Wnt/β-catenin pathway components (i.e. APC, Axin, TCF/Lef) as well as E-cadherin (Graham et al., 2000; Huber et al., 1997; Huber and Weis, 2001; Xing et al., 2003; Xing et al., 2004). The cellular signals that regulate whether newly synthesized β-catenin mediates gene transcription or maintains the adherens junction are not well-understood. There is substantial evidence that overexpression of cadherins inhibits Wnt/β-catenin gene transcription and promotes localization of β-catenin to the membrane (Gottardi et al., 2001; Heasman et al., 1994; Sadot et al., 1998; Sanson et al., 1996; Shtutman et al., 1999; Stockinger et al., 2001). Further evidence of the interplay between cadherins and Wnt/β-catenin signaling occurs when the proteolytic cleavage of cadherins by ADAM1- and presinilin-1 (a subunit of γ-secretase) activates Wnt/β-catenin target gene expression (Marambaud et al., 2002; Maretzky et al., 2005; Reiss et al., 2005; Uemura et al., 2006). Evidence for direct crosstalk between cadherins and Wnt/β-catenin signaling has been elusive, as E-cadherin knockdowns did not activate Wnt/β-catenin signaling (Herzig et al., 2007; Kuphal and Behrens, 2006). These results combine to suggest that there are two distinct pools of β-catenin, which is further supported by a study demonstrating that β-catenin can exist as a monomer and a dimer bound to α-catenin (Gottardi and Gumbiner, 2004). The monomeric form preferentially activates

Wnt/β-catenin signaling and the dimeric form preferentially binds cadherins. Surprisingly, β-catenin's mechanism of nuclear translocation is still unclear.

The scaffold protein Axin is a critical, concentration-limiting negative regulator of Wnt/β-catenin signaling (Lee et al., 2003). Axin is encoded by the *fused* gene locus in mice (Zeng et al., 1997). Its primary function is to serve as a scaffold for the destruction complex by binding to the other components and bringing them into close proximity with each other (Figure 1.1). Structural analysis has visualized the interactions between Axin and APC (Spink et al., 2000), Axin and β-catenin (Xing et al., 2003), and Axin and GSK3β (Dajani et al., 2003). Studies in *Drosophila* embryos suggest that Axin forms oligomers *in vivo*, and can potentially act as a cytoplasmic anchor of Armadillo/β-catenin and prevent nuclear translocation, thus inhibiting Wnt/β-catenin signaling (Peterson-Nedry et al., 2008; Tolwinski and Wieschaus, 2001). Axin is found at low concentrations and serves as the concentration-limiting component of destruction complex formation in *Xenopus* (Lee et al., 2003)**.** The concentration of Axin plays a critical role in creating specificity for Wnt/β-catenin signaling as many component of the destruction complex play roles in other signaling pathway (i.e. GSK3) (Forde and Dale, 2007; Lee et al., 2003). Due to the critical nature of Axin concentration on Wnt/β-catenin signaling, Axin protein levels are very highly regulated. GSK3 phosphorylation inhibits Axin degradation (Yamamoto et al., 1999), and studies in *Xenopus* egg extract and in *Drosophila* show that APC is required for Axin turnover, likely due to compensatory regulation due to fluctuation in APC protein levels (Lee et al., 2003). Axin stability is regulated by the E3 Smad ubiquitin regulatory factor 2 (Smurf2) (Kim and Jho, 2010) and the poly(ADP-Ribose) Polymerase (PARP) Tankyrase, which poly(ADP-ribosy)lates

(PARsylates) Axin through the addition of poly(ADP-Ribose) moieties to promote the ubiquitination and degradation of Axin through its poly(ADP-Ribose) moieties (Huang et al., 2009a). The discovery of two distinct Tankyrase inhibitors, IWR-1 and XAV939, which stabilize Axin and inhibit Wnt/β-catenin signaling further confirms the importance of Axin protein levels (Chen et al., 2009; Huang et al., 2009a). These tankyrase inhibitors act by inhibiting Axin PARsylation and thus inhibiting Axin turnover. Recently, two separate groups have identified RNF146 as the poly(ADP-Ribose)-directed E3 ubiquitin ligase that ubiquitinates and targets Axin for degradation (Callow et al., 2011; Zhang et al., 2011). RNF146 directly binds to poly(ADP-Ribose) and maintains low steady-state levels of Axin. In addition, the deubiquitinase ubiquitin-specific protease 34 (USP34), catalyzed the deubiquitination of Axin and increases its steady-state levels in cells (Lui et al., 2011). Axin can also be stabilized by SUMOylation at its C-terminus which inhibits ubiquitination (Kim et al., 2008). Very recently, quantitative measurements of Axin protein levels in a large panel of mammalian cells suggest that Axin protein levels dynamically regulate the dynamics of Wnt/β-catenin signaling (Tan et al., 2012). These results combine to strongly suggest that regulating Axin protein levels is likely a major mechanism for regulating Wnt/β-catenin signaling.

The serine/threonine kinase GSK3 is a critical regulator of β-catenin degradation. GSK3 is widely expressed and plays a role in many different cellular processes (Forde and Dale, 2007) and inhibition of GSK3 activity is critical for activation of Wnt/β-catenin signaling in all paradigms. The *Drosophila* homolog of GSK3 is called *shaggy,* or *zeste white 3* (Siegfried et al., 1992). Mammals have two distinct GSK3 genes, α and β, which are functionally redundant in Wnt/β-catenin signaling (Doble et al., 2007). GSK3 gets its

name from its initial discovery in glucose metabolism as a kinase for glycogen synthase (Embi et al., 1980). GSK3 usually requires its substrates to be phosphorylated (or primed), and thus often acts in concert with other kinases. GSK3 phosphorylates β-catenin at Ser33, Ser37, and Thr41 and this phosphorylation is required for β-catenin degradation (Peifer et al., 1994; Yost et al., 1996). The structure of GSK3β contains an activation loop which gives it its priming mechanism and a bilobed topology including a β-sheet domain linked to a C-terminal α-helix domain (Dajani et al., 2001; Haar et al., 2001). GSK3 activity is also regulated by an auto-inhibitory phosphorylation at Ser9 which blocks access to the catalytic site (Cross et al., 1995; Dajani et al., 2001). GSK3 phosphorylates other components of the Wnt/β-catenin pathway in addition to β-catenin (Rubinfeld et al., 1996; Willert et al., 1999; Zeng et al., 2005).

The priming kinase that acts in concert with GSK3 to regulate Wnt/β-catenin signaling is CK1α. The CK1 family of kinases has seven different paralogs encoded by 7 distinct genes (α, β, γ1, γ2, γ3, δ, and ε) (Knippschild et al., 2005; Price, 2006). Similar to GSK3, CK1 is widely expressed and plays important roles in multiple cellular processes. All the CK1 family members have highly similar catalytic domains, but the length and sequence of their C-terminal non-catalytic domains differ significantly. CK1α, which contains a short (~24 amino acid) C-terminal domain, appears to be an outlier compared with the other family members, which contain longer C-terminal tails (~200 amino acids). CK1α, γ, δ, and ε are thought to be positive regulators of the Wnt/β-catenin pathway through phosphorylation of pathway components (Cong et al., 2004a; Gao et al., 2002; Kishida et al., 2001; Lee et al., 2001; Peters et al., 1999; Sakanaka et al., 1999; Swiatek et al., 2004; Yanagawa et al., 1995; Zeng et al., 2005; Zhang et al.,

2006). Some CK1 paralogs are also thought to negatively regulate the Wnt/β-catenin pathway (Gao et al., 2002; Hammerlein et al., 2005; Kishida et al., 2001; Liu et al., 2002; Rubinfeld et al., 2001). CK1α phosphorylates β-catenin at Ser45 and serves as the priming kinase for GSK3 at the destruction complex (Liu et al., 2002). Two separate genome-wide S2 *Drosophila* RNAi screens identified CKIα as critical to suppress Wnt/β-catenin signaling, which is consistent with the dual kinase (priming kinase followed by processive kinase) model at the destruction complex (DasGupta et al., 2005; Lum et al., 2003). Consistent with this model, CK1α activation by the antihelminthic drug pyrvinium strongly inhibited Wnt/β-catenin signaling by enhancing β-catenin phosphorylation and subsequent degradation (Thorne et al., 2010).

The 2843 amino acid scaffold protein APC, which is 310 kDa, acts as a negative regulator of Wnt/β-catenin signaling. The gene was first identified as a mutation site in FAP, a familial form of colon cancer (Kinzler et al., 1991). Like β-catenin, APC plays many cellular roles and which likely occur due to different subpopulations of protein (Faux et al., 2008). The C-terminal region of APC regulates microtubule dynamics in mitosis and cell migration through binding to EB1 and Discs large (Matsumine et al., 1996; Su et al., 1995), though this function is independent of Wnt/β-catenin signaling (Nathke, 2006). APC binds to β-catenin and mutations in APC increased β-catenin protein levels in cancer cells (Rubinfeld et al., 1993; Su et al., 1993). APC also binds to GSK3 and Axin (Fagotto et al., 1999; Ikeda et al., 1998; Itoh et al., 1998; Rubinfeld et al., 1996). In fact, overexpression of the concentration-limiting protein Axin can compensate for the loss of APC (Lee et al., 2003). Additionally, a mutant form of Axin which can’t bind to APC can still inhibit Wnt/β-catenin signaling similarly to wild-type

Axin, suggesting that APC isn't strictly required for Wnt/β-catenin inhibition in the presence of Axin (Hart et al., 1998). Unfortunately, APC's precise mechanistic role in regulating Wnt/β-catenin signaling is still unclear and several different models have been proposed [reviewed in (Cadigan and Peifer, 2009; Chen et al., 2014b; MacDonald et al., 2009; Saito-Diaz et al., 2013)]. None of the proposed models are mutually exclusive and the strongest evidence supports APC's role in regulating the steady state levels of cytoplasmic β-catenin, but it is very likely that APC plays multiple roles in the Wnt/β-catenin signaling pathway similar to several other components of the pathway (Chen et al., 2014b; Saito-Diaz et al., 2013). APC is regulated by post-translational modifications such as phosphorylation (Morin et al., 1997; Rubinfeld et al., 1996; Salic et al., 2000b) and ubiquitination. The E3 ubiquitin ligase that targets APC for degradation is still unknown. APC has, however, been linked to two deubiquitinases: the COP9 signalosome-associated deubiquitinase, USP15, which stabilizes APC and binds the destruction complex(Huang et al., 2009b), and Trabid, which removes K63-linked ubiquitin chains from APC and acts as a positive regulator of Wnt/β-catenin signaling (consistent with APC being a negative regulator of Wnt/β-catenin signaling) (Tran et al., 2008). The mechanism of K63-linked ubiquitin chains regulating APC is still unknown.

All the previous destruction complex components mentioned have been reconstituted biochemically and are considered "core" components of the destruction complex. Some molecular studies have identified other components which may also be a part of the destruction complex which have not been confirmed biochemically. One of these is the heterotrimeric phosphatase PP2A. Multiple studies in multiple systems have implicated PP2A as both an activator of Wnt/β-catenin signaling (Hsu et al., 1999;

Ratcliffe et al., 2000; Willert et al., 1999) but also as an inhibitor of Wnt/β-catenin signaling (Gao et al., 2002; Li et al., 2001; Seeling et al., 1999). It is likely that PP2A, similar to GSK3 and CK1, can both activate and inhibit the Wnt/β-catenin in a context-dependent manner. Presenilin 1 (PS1), the catalytic subunit of γ-secretase, a protease critical in both Notch signaling and Alzheimer's disease, has been shown to inhibit Wnt/β-catenin signaling (Kang et al., 2002; Killick et al., 2001). PS1 appears to function as an alternative scaffold to Axin to promote GSK3 phosphorylation of β-catenin and uses Protein Kinase A (PKA) as a priming kinase instead of CK1 (Kang et al., 2002). Interestingly, PS1's ability to promote β-catenin degradation is dependent on E-cadherin, possibly linking the hypothesized two pools of β-catenin described earlier (Serban et al., 2005). Other proteins that have been implicated in regulating Wnt/β-catenin signaling through the destruction complex include PP2C, PP1, Amer1/WTX, and the ankyrin protein Diversin (Itoh et al., 2009; Luo et al., 2007; Major et al., 2007; Schwarz-Romond et al., 2002; Strovel et al., 2000).

The β-catenin destruction complex is evolutionarily conserved from metazoans to humans. Even though it is traditionally considered a cytoplasmic complex, it has also been found functional in the nucleus (Bienz, 2002; Cong and Varmus, 2004; Sierra et al., 2006; Wiechens et al., 2004). The complex is constitutively active, with cells constantly cycling between synthesis and degradation of β-catenin. On the surface, this appears to be a futile cycle of synthesis and degradation. However, the existence of these futile cycles in signaling is thought to be critical for more diverse modulation of these signals, allowing for complex behaviors such as stochastic bistability (Samoilov et al., 2005). Axin nucleates the formation of the complex by binding to GSK3, CK1, and

APC. These interactions have already been mapped (Dajani et al., 2003; Sobrado et al., 2005; Spink et al., 2000). β-catenin then binds to APC and Axin and enters the assembled complex. The kinetics of complex formation and whether it is stochastic or ordered are still unclear (Lee et al., 2003). The phosphorylation of Axin by GSK3 and of APC by CK1 and GSK3 increases their respective affinities for β-catenin (Ha et al., 2004; Willert et al., 1999). The N-terminal region of β-catenin, upon Axin binding, becomes positioned for phosphorylation by CK1 at Ser45. This priming phosphorylation leads to subsequence successive phosphorylation at Thr41, Ser37, and Ser33 (Amit et al., 2002; Liu et al., 2002). Phosphorylated APC competes β-catenin off of Axin and thus allows for a new β-catenin molecule to bind Axin, continuing the cycle (Kimelman and Xu, 2006). APC phosphorylation may also prevent the action of PP2A on β-catenin (Su et al., 2008). GSK3 phosphorylation of β-catenin causes recognition of β-catenin by β-TRCP, a recognition subunit of SCF complex E3 ubiquitin ligases (Jiang and Struhl, 1998; Kitagawa et al., 1999; Lagna et al., 1999; Liu et al., 1999; Marikawa and Elinson, 1998). $SCF^{\beta\text{-}TRCP}$ directly catalyzes the polyubiquitination of β-catenin (via K48 linkages) and its subsequent proteasome-mediated degradation. This degradation ensures low steady state levels of β-catenin are maintained to prevent aberrant signal transduction. One recent study shows that the HECT domain E3 Ligase EDD ubiquitinates β-catenin and prevents its degradation (Hay-Koren et al., 2010). The full physiological significance of EDD ubiquitination still needs to be elucidated.

The constitutively active β-catenin destruction complex becomes inhibited upon Wnt binding and receptor activation and β-catenin protein levels increase. The actual mechanistic details are still being investigated but the central unifying principle in all

proposed models is the inhibition of GSK3 enzymatic activity. These models are as follows: 1) Dissociation of the destruction complex upon Wnt activation, 2) Inhibitory phosphorylation of GSK3 at Ser9, 3) LRP6 Binding and direct inhibition of GSK3, 4) Axin degradation upon Wnt activation which prevents formation of the complex and 5) global inhibition of GSK3 through sequestration into multi-vesicular bodies (MVBs). The details of each individual model are reviewed in [(Saito-Diaz et al., 2013) and (Chen et al., 2014b)]. The inhibition of GSK3 and thus the β-catenin destruction complex allows for the accumulation of β-catenin in the cytoplasm where it eventually translocates to the nucleus and activates a Wnt/β-catenin specific transcriptional response. As mentioned earlier, β-catenin serves as the main effector of the pathway and transduces the signal into the nucleus.

Wnt/β-catenin signaling: Transcriptional activation

β-catenin accumulation in the cytoplasm, and subsequently the nucleus, was widely considered the driving force of Wnt/βcatenin signaling. Contrary to the prevailing model, recent studies have shown that the fold change, rather than the absolute concentration increase, of β-catenin activates Wnt/β-catenin signaling (Goentoro and Kirschner, 2009). The same group also showed that an approximately 2 fold change in β-catenin levels is sufficient to activate Wnt/β-catenin signaling (Goentoro et al., 2009). β-catenin does not contain any classical nuclear localization signals (NLS) or nuclear export signals (NES) and how its localization is regulated is still under intense investigation. Once it gets into the nucleus, β-catenin interacts and acts as a co-factor with the TCF/LEF family of transcription factors which are critical for Wnt/β-catenin signal transduction (Behrens et al., 1996; Molenaar et al., 1996). TCF, in the absence of

β-catenin, interacts with the co-repressor Groucho/transduction-like enhancer (Gro/TLE1-3) to repress gene transcription. TCF binds to the DNA at a Wnt-responsive element (WRE), of which there are over 6000 in a colon cancer cell line that regulate the transcription of 300-400 genes (Hatzis et al., 2008). These TCF proteins are regulated by post-translational modifications such as phosphorylation (Hammerlein et al., 2005; Hikasa et al., 2010; Hikasa and Sokol, 2011; Ishitani et al., 2003; Ishitani et al., 1999; Lee et al., 2001; Lo et al., 2004; Smit et al., 2004) and ubiquitination (Yamada et al., 2006). There is also evidence of a deubiquitinase USP4 regulating TCF4 (Zhao et al., 2009). In the classical model of Wnt/β-catenin signaling, the displacement of Gro/TLE by β-catenin causes TCF/LEF to switch from a repressor to a transcriptional activator. This was originally thought to be due to direct displacement (Daniels and Weis, 2005), but recent studies show that the X-linked inhibitor of apoptosis (XIAP) monoubiquitinates Gro/TLE and decreases its affinity for TCF/LEF, thus allowing β-catenin to bind TCF/LEF (Hanson et al., 2012).

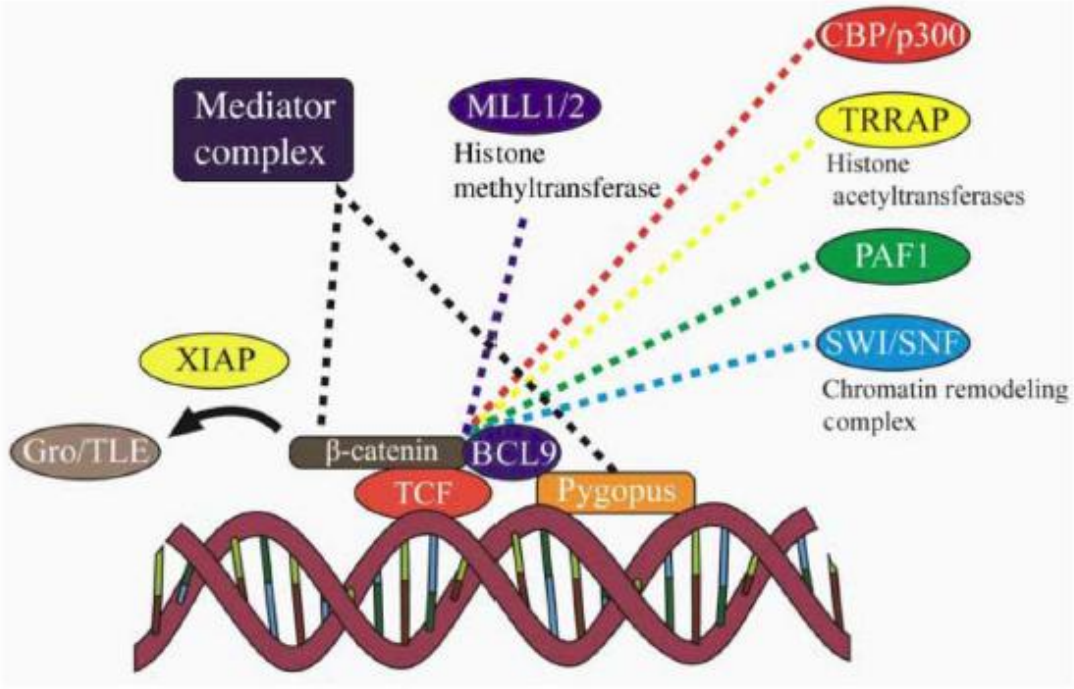

Figure 1.3. Nuclear TCF/β-catenin transcriptional complexes. Upon Wnt/β-catenin signaling, DNA-bound TCF/β-catenin recruits many other transcriptional complexes to Wnt target genes. Dotted lines represent interactions between the transcriptional complexes and β-catenin. During active Wnt target gene transcription, the co-repressor Gro/TLE cycles on and off of β-catenin in an XIAP-dependent manner with the other transcriptional complexes. Gro/TLE, Groucho/transducin-like enhancer of split [Figure from (Saito-Diaz et al., 2013)].

β-catenin binds to the nuclear transcriptional co-factors BCL9 and Pygopus (Pygo) to mediate Wnt/β-catenin pathway-specific transcription (Figure 1.3) (Belenkaya et al., 2002; Parker et al., 2002; Thompson et al., 2002). Pygo, BCL9, TCF, and β-catenin represent a core transcriptional complex required for Wnt/β-catenin transcription (Fiedler et al., 2008; Schwab et al., 2007; Sustmann et al., 2008). In addition, β-catenin also interacts with multiple proteins involved in chromatin remodeling (Mosimann et al., 2009; Willert and Jones, 2006). Wnt/β-catenin signaling requires responses at the plasma membrane, in the cytoplasm via the destruction complex, and in the nucleus via the β-catenin transcriptional complex. The Notch signaling pathway, the other pathway I will be describing, shares many features in common with the Wnt/β-catenin pathway, including a role for ligand-receptor interactions at the membrane, cytoplasmic regulatory events on the primary effector of the pathway, and required transcriptional complex formation in the nucleus leading to transcriptional activation. In fact, the Wnt/β-catenin and the Notch pathway have extensive cross-talk and there is evidence that activation of one pathway can regulate the activation of the other pathway. These will be discussed later on in this chapter.

Historical Significance: Notch Signaling in Development and Disease

The canonical Notch signaling pathway is a highly conserved developmental signaling pathway critical in cell fate determination through lateral inhibition, differentiation, proliferation, cell death, and neuronal development in developing embryos and stem cell and tissue maintenance in adults. The Notch pathway is named for its family of single transmembrane Notch receptors. The *Notch* gene was first identified by John Dexter in the lab of Thomas Hunt Morgan who noticed a notched wing phenotype in *Drosophila melanogaster* (Dexter, 1914). A few short years later, Morgan identified the mutant alleles (Mohr, 1919; Morgan, 1917; Morgan and Bridges, 1916). Details about the Notch pathway can be found in several excellent reviews [reviewed in (Fortini, 2012; Kopan, 2010)].

The following decades yielded genetic data indicating that the Notch locus was X-linked and had extremely complex allelic interactions [reviewed in (Artavanis-Tsakonas and Muskavitch, 2010)] leading to multiple speculative hypotheses on its biochemical nature (Foster, 1973; Thorig et al., 1981a; Thorig et al., 1981b). The *Notch* gene was identified as a “neurogenic” mutation in *Drosophila* in the 1980s, linking the mutation to developmental phenotypes (Lehmann et al., 1983). Further confirming the importance of Notch signaling in development, Nusslein-Volhard and Weischaus conducted a series of *Drosophila* mutagenesis screens for embryonic phenotypes yielded six loci that were later identified as core components in the Notch signaling pathway (Nüsslein-Volhard et al., 1984; Nusslein-Volhard and Wieschaus, 1980). In the mid-1980s, Spyros Artavanis-Tsakanos and Michael Young independently cloned the *Notch* receptor and identified it as a single-pass transmembrane receptor and attributed

its wing-notching phenotype to gene haploinsufficiency (Artavanis-Tsakonas et al., 1983; Kidd et al., 1986; Kidd et al., 1983; Wharton et al., 1985). Notch was subsequently cloned in other organisms, including *C. elegans* and *Xenopus* (Austin and Kimble, 1987; Coffman et al., 1990; Greenwald et al., 1987). These initial studies provided insight into the role of Notch signaling in multiple fields of biology, including developmental and stem cell biology, neuroscience, and cancer biology (Fortini et al., 1993).

The *Notch* locus in *Drosophila* and was shown to be both pleiotropic and haploinsufficient. *Notch* loss-of-function mutations lead to a change in cell fate from dermoblasts to neuroblasts in *Drosophila* embryos. One of the major insights from the experiments in *Drosophila* embryos was the apparent necessity for the Notch-sending and Notch-receiving cells to be adjacent to each other (Doe and Goodman, 1985; Greenspan, 1990). Further studies in other tissues and other animals confirmed Notch's broad pleiotropic effect and its requirement in signaling between neighboring cells. Proper regulation of Notch pathway is critical in nearly all cell fate decisions made between neighboring cells and this pleiotropic effect can be extended to multiple developmental processes including differentiation, proliferation, and apoptosis. The particular developmental process affected by Notch affects in a specific tissue is likely context-dependent, but it is clear that very tight regulation of Notch activity is critical for determining cell fates in adjacent cells. The Notch pathway is very sensitive to dosage effects, as loss-of-function and gain-of function mutations in Notch can often lead to the "same phenotype". Notch signaling has also been associated with stem cell maintenance and proliferation (Austin and Kimble, 1987). Stem cell maintenance and

differentiation is dependent on cell-cell communication between stem cells and their surrounding environment, or niche, and as mentioned above, Notch is critical for processes that require cell-cell communication. The list of tissue-specific stem cells regulated by proper Notch signaling is expanding rapidly (Liu et al., 2010). In fact, many Notch reviewers have termed Notch a "stem cell pathway" because of its extensive involvement in stem cell biology (Brack et al., 2008; Casali and Batlle, 2009; Dreesen and Brivanlou, 2007; Farnie and Clarke, 2006).

Developmental pathways are often misregulated in cancers due to their critical roles in cellular growth, differentiation, proliferation, and cell-cell signaling. The Notch pathway, similar to the Wnt pathway, is misregulated in many types of cancers. Perhaps the most well-characterized link between the Notch signaling pathway and tumorigenesis is from studies on the molecular mechanisms underlying T-cell acute lymphoblastic leukemia (T-ALL). Gain-of-function mutations in the Notch pathway were first identified in cancer in the early 1990s (Ellisen et al., 1991; Gallahan and Callahan, 1997; Gallahan et al., 1987; Jhappan et al., 1992; Reynolds et al., 1987). These were the first human homologs of the *Drosophila Notch* gene and they were identified as a chromosomal translocation within T-ALL patients (Ellisen et al., 1991; Reynolds et al., 1987). In the original study, four out of the 40 T-ALL patients had this mutation, that results in a dominant active, ligand-independent NOTCH1 receptor, which was termed TAN1 for translocation-associated Notch homolog. This discovery was the first direct link between Notch signaling and human cancer. A few years later, experiments using murine bone marrow (BM) reconstitution showed that TAN1 was causative for disease development. Mice transplanted with TAN1-expressing BM progenitors developed T cell

neoplasms two weeks after BM transplantation (Pear et al., 1996). This evidence was supported by *in vitro* studies and *in vivo* studies (Capobianco et al., 1997; Girard et al., 1996). It wasn't until the early 2000s, however, when Aster and colleagues identified activating mutations in NOTCH1 were present in over 50% of all T-ALL patient cases (Weng et al., 2004). NOTCH1 mutations were later identified in many other types of hematopoietic tumors as well as solid tumors [reviewed in (Ntziachristos et al., 2014; South et al., 2012). Interestingly, the Notch pathway has both oncogenic and tumor suppressive roles in human cancers in a context-dependent manner , including breast cancer, lung cancer, skin cancer, liver cancer, colorectal cancer, glioblastoma, AML, CLL, and others (Balint et al., 2005; Fabbri et al., 2011; Klinakis et al., 2011; Licciulli et al., 2013; Qi et al., 2003; Sun et al., 2014; Villanueva et al., 2012; Wang et al., 2011; Weng et al., 2004). Unsurprisingly, Notch signaling is also often misregulated in congenital developmental diseases consistent with its role in progenitor cell regulation (Eldadah et al., 2001; Garg et al., 2005; Joutel et al., 1996; Li et al., 1997; McDaniell et al., 2006; Oda et al., 1997; Simpson et al., 2011; Sparrow et al., 2006). Due to the Notch pathway's critical roles in both development and disease, understanding the molecular mechanisms governing Notch signaling is critical for our understanding of the pathophysiological effects of Notch pathway misregulation and for designing therapeutics against the Notch signaling pathway. Unfortunately, many of the molecular mechanisms of the Notch pathway have not been fully elucidated biochemically and many of the present therapeutics for the Notch pathway have proven unsuccessful when taken to clinical trials due to non-therapeutic Notch-mediated effects in the GI tract and the formation of skin cancers.

The Current Model of the Notch Signaling pathway

The most widely characterized pathway initiated by the classical Notch-ligand interaction is generally referred to as “canonical” Notch signaling. Other Notch-dependent signaling pathways can occur independently of the processes and molecules required for the classical Notch pathway or through cross-talk with other pathways (such as the Wnt/β-catenin pathway). These other Notch pathways are referred to as “non-canonical” Notch signaling. Details on non-canonical Notch signaling has been reviewed elsewhere [reviewed in (D'Souza et al., 2010; Heitzler, 2010)] and is outside the scope of this document. Unless specifically referred to as non-canonical, all references to Notch signaling refer to canonical Notch signaling.

The Notch signaling pathway, at its core, results in the generation and translocation of the Notch Intracellular Domain (NICD) into the nucleus to activate a Notch-specific transcriptional program. In the core Notch signaling pathway, the Notch transmembrane receptor (existing as a heterodimer) on a signal-receiving cell interacts extracellularly with the canonical Notch pathway ligands Delta/Serrate/Lag-2 (DSL) on a neighboring signal-sending cell. This ligand-receptor interaction initiates an ADAM 10 metalloprotease proteolysis (S2) which allows the remaining Notch receptor to be proteolyzed by the ubiquitously expressed protease γ-secretase (S3). This γ-secretase proteolysis generates the release of NICD. The S3 proteolysis can occur at the plasma membrane or in the early endosome, as γ-secretase is present at both cellular compartments. Recent studies suggest that γ-secretase is more active at the low PH of the early endosome. The stability of the NICD is regulated but very little is known about how NICD stability is regulated. Finally, the NICD translocates to the nucleus and binds

to the transcriptional co-activator C-promoter binding factor1 [CBF1 (also known as recombination signal binding protein for immunoglobulin kappa J region (RBPJ-κ)]. In *Drosophila,* CBF1 is known as Suppressor of Hairless (Su(H)) and in *C. elegans,* Longevity-assurance gene-1 (LAG-1). Collectively, this transcriptional co-factor is called CSL (for CBF1/Su(H)/LAG-1). The transcriptional complex, consisting of NICD, MAM, and CSL is thought to activate a canonical Notch-mediated transcriptional program [Figure 1.4, adapted from (Andersson et al., 2011)].

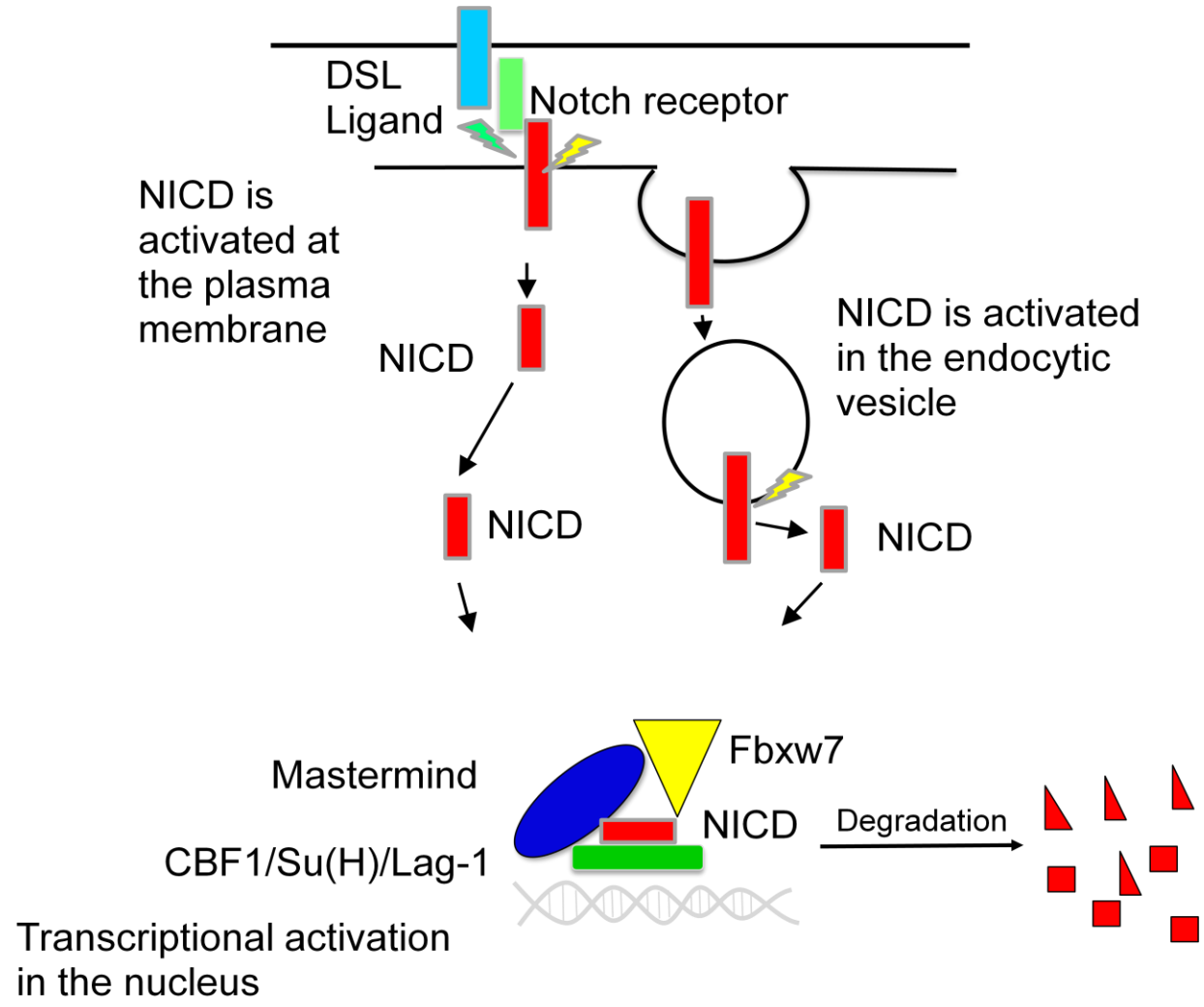

Figure 1.4. The core Notch pathway contains a limited set of components that form the signal transmitting chain in the pathway: a ligand (light blue), a Notch receptor (green and red) and the transcription factor CSL (green). In addition, some components (Furin (not shown), ADAM 10 secretase (green lightning bolt), γ-secretase (yellow lightning bolt) and MAML (blue oval)) are not part of conveying the signal but are nevertheless crucial for allowing the signal to be transmitted from one step to the next in the pathway. Briefly, the Notch receptor is synthesized as a single transmembrane receptor that is Furin cleaved to yield a bipartite heterodimeric Notch receptor, which is presented on the cell surface of a 'receptor-expressing' cell. This receptor can be activated at the plasma membrane by binding to Notch ligands on 'ligand-expressing' cells. This leads to the removal of the extracellular domain of Notch, which is then targeted for lysosomal degradation. The remaining portion of the receptor, termed the Notch extracellular truncated (NEXT) domain, undergoes sequential cleavage by ADAM secretases and γ-secretase as it becomes endocytosed, yielding the Notch intracellular domain (NICD). NICD then translocates to the nucleus where it binds the DNA-binding protein CSL (CBF1/Suppressor of Hairless/LAG-1) and activates the transcription of Notch target genes [adapted from (Andersson et al., 2011)].

The Notch pathway is very unusual among signal transduction pathways because there is no evidence of an amplification step in between receptor activation and transcriptional activation. The Notch receptor also serves as the transcriptional co-activator rather than acting through another effector protein. Interestingly, each activated Notch receptor generates one NICD and is consumed during the signal transduction process, making the Notch pathway particularly sensitive to gene dosage. The core Notch signal transduction pathway can be broken down into three distinct molecular events: 1) Notch surface receptor activation, 2) Regulation of the NICD, and 3) Activation of a Notch-specific nuclear transcriptional complex by NICD. The next sections of this document will describe each of these steps in greater detail as well as how the non-core components regulate the Notch pathway.

Notch Signaling: Surface Receptor Activation

The Notch receptor is a family of single pass trans-membrane receptors that is critical in cell-cell signaling. In mammals, there are 4 Notch receptors (called Notch1-4) [reviewed in (D'Souza et al., 2010)]. All of the mammalian Notch receptors share structural similarities (Figure 1.5). These receptors contain many of the same structural motifs and are likely to be regulated in a similar fashion. These Notch receptors bind to the cell surface expressed Delta-Serrate-LAG-2 ligands (Jagged1, Jagged 2, Delta-like 1 (Dll1), Delta-like 3 (Dll3), and Delta-like 4 (Dll4)) (Figure 1.6).

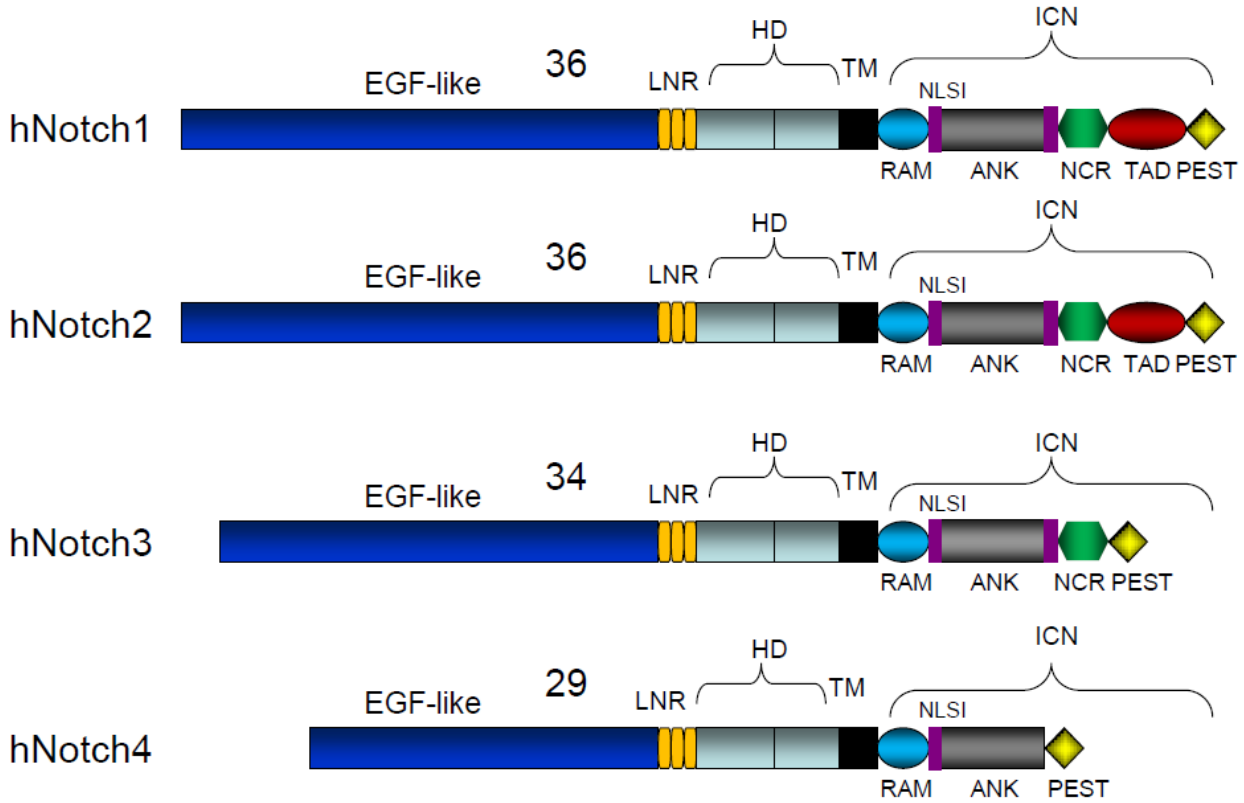

Figure 1.5. The structural conservation of mammalian Notch receptors. Diagrammatic representation of the four known mammalian receptors. EGF: epidermal growth factor; HD: heterodimerization domain; ICN: intracellular domain; LNR: cysteine-rich LNR repeats; TM: transmembrane domain; RAM: RAM domain; NLS: nuclear localizing signals; ANK: ankyrin repeat domain; NCR: cysteine response region; TAD: transactivation domain; PEST: region rich in proline (P), glutamine (E), serine (S) and threonine (T) residues [adapted from (Pancewicz and Nicot, 2011)].

The Notch receptors are translated as pre-proproteins and are cleaved by a Furin-like protease (S1) in the trans-Golgi network and then trafficked to the plasma membrane. This cleaved heterodimer is prsented on the cell surface composed of a large extracellular domain non-covalently linked to the intracellular domain (Blaumueller et al., 1997; Logeat et al., 1998). The extracellular domain of all Notch receptors contains different numbers of epidermal growth-factor-like repeats (EGF-like) (36 for Notch1 and Notch2, 34 for Notch3, and 29 for Notch4) which are critical in ligand-receptor binding (Fig 1.5). A subset of the EGF-like repeats are calcium-binding EGF-like repeats (cbEGF), that are required for ligand binding. Genetic experiments in *Drosophila* and cell aggregation assays have identified EGF-like repeats 11 and 12 on Notch as the major ligand-binding site. This region binds in a calcium-dependent manner but does not have full functionality, suggesting that other EGF-like repeats likely also contribute to ligand binding (Rebay et al., 1993; Rebay et al., 1991). Structural studies later indicated that EGF-like 12, via an interaction between an aromatic residue (Y/F/W) and a both a hydrophobic residue (I/L/V/P) with a glycine is the critical binding pocket for the Notch ligand in a calcium-dependent manner (Cordle et al., 2008; Hambleton et al., 2004). Additionally, the relative strength of receptor-ligand binding can be modulated by post-translational modifications of these EGF-like repeats. O-glycosylation of the EGF-like repeats, including the addition of O-fucose and O-glucose on EGF-like repeat 12, primes the Notch receptor for further modification (Stanley and Okajima, 2010). The addition of O-fucose is mediated by O-fucosyltransferase 1 (Pofut1) and is not directly required for Notch signaling (Okajima et al., 2008) but is required for subsequent glycosylation of Notch receptors by the glycosyltransferase

Fringe proteins (Lunatic Fringe, Manic Fringe, and Radical Fringe in mammals). The Fringe proteins add N-acetylglucosamine (GlcNAc) sugars to the O-fucose moiety and modulate ligand-receptor binding by increasing the affinity for Delta-like and decreasing affinity for Serrate-like ligands (Hicks et al., 2000; Kato et al., 2010; Okajima et al., 2003). The expression domains of Fringe genes often coincides with either Delta or Jagged but not both and this expression often leads to active Notch signaling only at the margins in between the Delta and Jagged expressed regions (Irvine and Wieschaus, 1994; Marklund et al., 2010; Wu and Rao, 1999). A substitution in the O-fucose site on EGF-like repeat 12 led to decreased Notch signaling (Ge and Stanley, 2008; Lei et al., 2003). Notch can also be glycosylated by the glycosyltransferase Rumi (Poglut1) and this glycosylation is required for Notch signaling (Acar et al., 2008; Fernandez-Valdivia et al., 2011). Two enzymes of the glycosyltransferase 8 family also glycosylate the Notch receptor (Sethi et al., 2010). Recently, a secreted Fringe protein, chondroitin sulfate synthase 1(CHSY1) was identified as a negative regulator of Notch signaling (Tian et al., 2010). It is clear that glycosylation plays a critical role in regulating Notch signaling through regulation of receptor-ligand binding, but further structural, molecular, and biochemical studies are still needed to identify the individual contributions of each glycosylation event.

The extracellular domain of the Notch receptor also contains three LIN-12-Notch (LNR) repeats and a hydrophobic region known to mediate heterodimerization (HD). Those two regions, along with the S1 cleavage site (Furin-mediated) and the S2 cleavage site (ADAM 10/17 metalloprotease-mediated) comprise the negative regulatory region (NRR) of the Notch receptor. The NRR normally autoinhibits the S2

cleavage by sterically blocking access to the S2 cleavage site through a hydrophobic core (Gordon et al., 2009). Mutations in the NRR which mutate the hydrophobic core and cause ligand-independent Notch receptor activation are the most common mutation in T-ALL, a Notch-mediated cancer (Malecki et al., 2006; Weng et al., 2004). The autoinhibitory conformation of the NRR is calcium-dependent, as depletion of calcium activates the receptor (Rand et al., 2000). The autoinhibitory effect of the NRR is relieved by a conformational change in the Notch heterodimer, through ligand endocytosis in response to ligand-receptor binding.

The 5 canonical mammalian Notch ligands (Jagged1, Jagged2, Dll1, Dll3, Dll4) can be divided into two general classes, a Delta/Delta-like family and a Serrate/Jagged family. All of the ligands have a Notch-binding site within a DSL domain as well as N-terminal domain and EGF-like repeats. The Jagged family of ligands also contains a cysteine rich domain (CRD) and Jagged1, Jagged2, and Dll1 all contain two Delta and OSM-11-like proteins (DOS) domains (Figure 1.6).

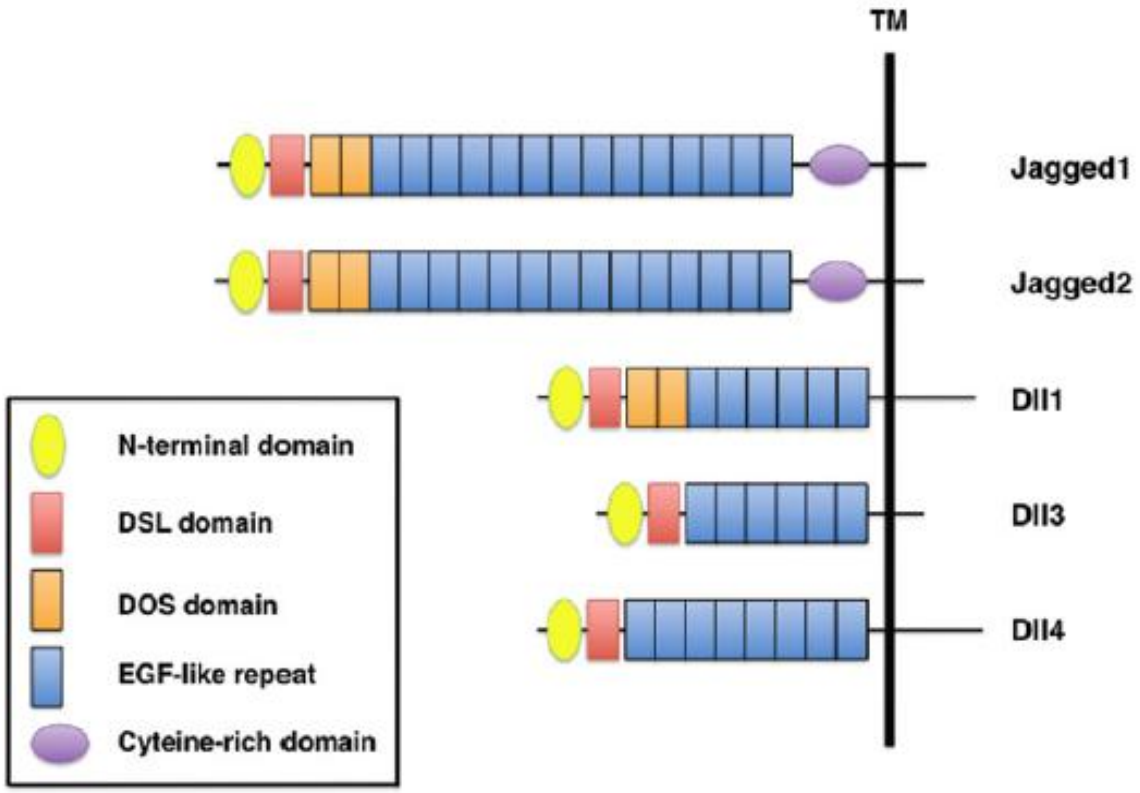

Figure 1.6. Domain organization of mammalian Notch ligands. Five mammalian ligands are classified into two categories, Delta-like (Dll1, Dll3, Dll4) and Serrate-like (Jagged1, Jagged2), based on structural homology to the two Drosophila ligands, Delta and Serrate. All Notch ligands have an N-terminal domain, a DSL (Delta/Serrate/LAG-2) domain and EGF-like repeats. Jagged1 and Jagged2 contain a cysteine-rich domain, whereas Jagged1, Jagged2 and Dll1 have two DOS (Delta and OSM-11-like proteins) domains located immediately following the DSL domain. DSL ligands are transmembrane proteins of which the extracellular domain contains a characteristic number of EGF-like repeats and a cysteine rich N-terminal DSL domain. The DSL domain is a conserved motif found in all DSL ligands and required for their interaction with Notch [adapted from (Kume, 2012)].

To date, there is little evidence of differences in signaling output between different receptor-ligand combinations with the exception of Dll3, which lacks a DOS domain and is the most structurally divergent of the Notch ligands (Dunwoodie et al., 1997). Dll3 is incapable of activating Notch receptors from a neighboring cell (Ladi et al., 2005) and is rarely present on the cell surface (Chapman et al., 2011; Geffers et al., 2007). Because there are differences in signaling output between different receptor-ligand combinations, there must be some mechanism for establishing the specificity of Notch signaling. One way that Notch provides specificity is through restricting the distribution of specific ligands and receptors to specific intracellular compartments. An example of this phenomenon is in *Drosophila* sensory organ development, in which Delta is specifically recycled during asymmetric cell division and sorted exclusively into cells adjacent to Notch-expressing cells (Emery et al., 2005; Jafar-Nejad et al., 2005). Notch ligands can also be specifically localized to cellular extensions such as filopodia, which can activate signaling at distances larger than typical cell-cell distances (Cohen et al., 2010b; De Joussineau et al., 2003). There is also some evidence that cell motility dynamics can affect the specificity of Notch signaling (Del Bene et al., 2008).

Because most cells express both canonical Notch pathway ligands and Notch receptors at their cell surface, the proper directionality of Notch signaling must be firmly established. One way that directionality of Notch signaling is established is through *cis*-inhibition, in which ligands that activate receptors on neighboring cells (*trans*-activation), inhibit receptors expressed on the same cell surface (de Celis and Bray, 1997; del Alamo et al., 2011; Micchelli et al., 1997; Miller et al., 2009; Sprinzak et al., 2010). *Cis*-inhibition downregulates the Notch receptor at the cell surface (Matsuda and Chitnis,

2009) but this does not always occur (Fiuza et al., 2010), and *cis*- inhibition also cell-autonomously downregulates Notch target genes. As mentioned earlier, Dll3 likely serves exclusively as a *cis*-inhibiting ligand and cannot activate receptors in *trans* (Ladi et al., 2005). Some recent reports have started to unravel how the individual ligands can affect *trans*-activation and *cis*-inhibition. The extracellular DSL-EGF-like repeat 3 domain of Serrate is critical in both *trans*-activation and *cis*-inhibition (Cordle et al., 2008) and mutations in the intracellular domain of Serrate affect *trans*-activation but not *cis*-inhibition (Glittenberg et al., 2006). Additionally, reports have shown that Notch ligand and receptor intracellular domains (ICDs) display competitive interactions. NICD can suppress the antiproliferative effect of Delta ICD in endothelial cells (Kolev et al., 2005). Conversely, Jag1 ICD suppresses NICD-induced transcription in COS cells (LaVoie and Selkoe, 2003). Interestingly, the signal-sending cell can also undergo *cis*-inhibition in which the Notch receptor inhibits the ligand on the same cell surface (Becam et al., 2010). Also, many of the non-canonical ligands of Notch signaling are capable of *cis*-inhibition. One specific example is Delta-like homolog 1/2 (Dlk1/2), which competes with *trans*-presented canonical ligands to bind Notch receptors (Baladron et al., 2005). A model for *trans*-activation vs. *cis*-inhibition was proposed in which *trans*-activation occurs in a graded manner in response to increasing concentrations of ligand, while *cis*-inactivation occurs with a sharp threshold of Notch ligand co-expression, potentially leading to a bistable switch which generates mutually exclusive sending and receiving states (Sprinzak et al., 2010) .This model still needs to be further tested *in vivo*.

Upon ligand-receptor binding, the ADAM metalloproteases cleave the Notch receptor (whose autoinhibitory NRR has been removed) in the extracellular space between the two cells at the S2 cleavage site. Different ADAM proteases have been implicated in Notch S2 cleavage (Brou et al., 2000; Canault et al., 2010; Tian et al., 2008; Tousseyn et al., 2009; van Tetering et al., 2009), and one recent study claims that specific ADAM proteases cleave Notch in a ligand-dependent or –independent manner (Bozkulak and Weinmaster, 2009). The structural aspects of S2 cleavage were reviewed recently [reviewed in (Kovall and Blacklow, 2010)]. The S2 cleavage is often considered the limiting regulatory step in Notch receptor activation.

The S2 cleavage creates the membrane-tethered Notch extracellular truncation (NEXT) region. The NEXT immediately becomes a substrate for regulated transmembrane cleavage by the γ-secretase complex at the S3 cleavage site. γ-secretase is a multi-subunit protease complex containing presenilin, nicastrin, presenilin enhancer 2 (Pen2) and anterior pharynx-defective 1 (Aph1) (Jorissen and De Strooper, 2010). γ-secretase is ubiquitously expressed and cleaves transmembrane proteins at residues within the transmembrane domain. Although the original model for S3 cleavage suggested that it follows constitutively after the S2 cleavage, recent studies suggest that the activity of γ-secretase is regulated, both with regard to cleavage efficacy and the position of the cleavage site in the receptor. γ-secretase complexes containing different presenilin subunits (PS1 or PS2) have different cleavage preferences for amyloid precursor protein (APP), although how this difference affects Notch signaling is yet to be determined (Jorissen and De Strooper, 2010). One report suggests that nicastrin is not required for γ-secretase-mediated processing of Notch, but

important for the stability of the γ-secretase complex (Zhao et al., 2010). Other proteins that regulate the function of the γ-secretase complex include CD147 (also known as BSG), transmembrane protein 21 (Tm21, also known as Tmed10) and γ-secretase activating protein (GSAP also known as Pion) (Chen et al., 2006; He et al., 2010; Zhou et al., 2006). The actual effect of these regulatory proteins on Notch receptor processing still needs to be investigated. The S3 cleavage has been shown heterogeneous in terms of the cleavage site. NICD fragments generated by S3 cleavage can have either an N-terminal valine (Val) or an N-terminal serine/leucine (Ser/Leu) and the Ser/Leu-NICD fragments are less stable than Val-NICD fragments (Tagami et al., 2008). Notch processing is also regulated by the estrogen receptor (ER), and inhibition of ER activity by tamoxifen increases Notch activity (Rizzo et al., 2008). Neuronal activity can also enhance Notch processing through the protein activity-regulated cytoskeleton-associated protein (Arc)/activity-regulated gene 3.1 protein homolog (Arg3.1) (Alberi et al., 2011), giving another way to modulate Notch signaling.

Notch Signaling: Regulation of the NICD

It is not clear whether the S3 cleavage occurs at the cell surface or in the early endosome upon endocytosis of the receptor. Endocytosis of the Notch receptor is thought to be a critical step in transduction of the Notch signal. Notch initially binds to the γ-secretase complex at the cell surface (Hansson et al., 2005), but there is evidence that the majority of cleavage occurs after internalization of the receptor by endocytosis (Vaccari et al., 2008) as well as evidence of cleavage at the membrane (Kaether et al., 2006; Sorensen and Conner, 2010; Tarassishin et al., 2004). It is likely that the localization of Notch cleavage is context-dependent and serves as another method of

modulating Notch signaling (Tagami et al., 2008). Notch receptor endocytosis requires monoubiquitination of the receptor at lysine 1749 (Gupta-Rossi et al., 2004). This monoubiquitination is followed by deubiquitination by eIF3f, a subunit of translation initiation factor E74-like factor 3 (Elf3), which is required for Notch to be processed by γ-secretase (Moretti et al., 2010). The Notch receptor is regulated by the putative E3 ubiquitin ligase Deltex, which has been implicated in the regulation of Notch processing and internalization (Diederich et al., 1994; Hori et al., 2004; Matsuno et al., 1995; Wilkin et al., 2008; Yamada et al., 2011). Deltex also may serve as a bridge between eIF3f and Notch in early endosomes (Moretti et al., 2010). The exact role of Deltex in the Notch pathway is still in question. It has mostly been described as a positive regulator of Notch signaling (Fuwa et al., 2006; Matsuno et al., 1995; Matsuno et al., 2002; Wilkin et al., 2008), but several reports have also described it as a negative regulator of Notch signaling (Mukherjee et al., 2005; Sestan et al., 1999). Additionally, Deltex may not be required for Notch signaling in all developmental contexts (Fuwa et al., 2006). Loss of Deltex function does not seem to severely affect T-cell development, a Notch-dependent process, in the mouse (Lehar and Bevan, 2006). Recently, it has been hypothesized that canonical Notch signaling and Deltex-activated Notch signaling are two distinct events activated in different endocytic compartments (Yamada et al., 2011).

Another regulator of Notch signaling is the endocytic adaptor protein Numb. Numb, which is found in both *Drosophila* and vertebrates, and Numb-like, the mammalian homolog of Numb, act as suppressors of Notch signaling (Rhyu et al., 1994; Uemura et al., 1989; Zhong et al., 1997). Numb acts mechanistically by recruiting the E3 ubiquitin ligase itchy (Itch), the mammalian homolog of *Drosophila* Suppressor of

deltex [Su(dx)], to promote degradation of the Notch receptor (Beres et al., 2011) and to regulate post-endocytic sorting for Notch1 (McGill et al., 2009). Numb differentially regulates the different Notch receptors and a recent report shows that Numb can negatively regulate Notch1 and Notch2 but not Notch3 during myogenic differentiation (Beres et al., 2011). There are 6 alternatively spliced *NUMB* isoforms in humans. The two most recently identified ones, NUMB5 and NUMB6, are less potent Notch antagonists than the others (Karaczyn et al., 2010), though it is possible that the observed difference is due to Numb's interaction with other signaling pathway components such as p53 and Gli1, a Hedgehog pathway effector (Colaluca et al., 2008; Di Marcotullio et al., 2006). Sanpodo, a *Drosophila* transmembrane protein with no known vertebrate homolog, also regulates Notch signaling by associating with Notch and Numb during asymmetric cell division (O'Connor-Giles and Skeath, 2003). Sanpodo agonizes Notch signaling in the absence of Numb but inhibits Notch signaling in the presence of Numb (Babaoglan et al., 2009). These data suggest that the relationship between Notch and Numb may not be unidirectional as Notch may regulate Numb as well. For example, high levels of Notch reduce Numb and Numb-like protein levels in the developing chick CNs and in cultured cells (Chapman et al., 2006). In addition, Notch controls the expression of Numb by upregulating it in cells that not did inherit Numb during cell division but require Numb for Notch repression (Rebeiz et al., 2011).

Intracellular trafficking has been shown to regulate Notch signaling after its internalization. Ectopic ligand-independent Notch signaling is activated when the sorting of Notch from early endocytic vesicle to multivesicular bodies (MVBs) or lysosomal compartments is defective. This is shown in endosomal sorting complex required for

transport (ESCRT) and *lethal giant discs* [*lgd*; also known as *l(2)gdl*] mutants (Childress et al., 2006; Jaekel and Klein, 2006; Vaccari et al., 2008). *Drosophila* sensory organ precursors (SOPs) traffic Notch via a specialized endocytic route which generates differential Notch signaling in the resulting daughter cells. This trafficking is mediated by Smad anchor for receptor activation (SARA) endosomes, which segregate into one of the two daughter cells during asymmetric SOP cell division. Delta and Notch are both internalized into SARA endosomes and asymmetrically localized to one of the cells during SOP mitosis, resulting in the ligand-dependent appearance of NICD in only that cell (Coumailleau et al., 2009). SARA itself is not required for this process.

The Notch intracellular domain serves as the major effector of the canonical Notch signaling pathway. All Notch receptors contain the RAM23 domain (which mediate interactions with CSL) and seven Ankyrin/CDC10 repeats (ANK), necessary for protein-protein interactions. In addition, Notch receptors 1-3 contain two nuclear localization signals (NLS) compared to one NLS in Notch4. The NLS is necessary to target the intracellular domain to the nucleus where the transcriptional activation domain (TAD) activates downstream events. Notch3 and Notch4 contain no identifiable TAD domain and weakly activate transcription. All four Notch receptors contain a C-terminal Pro-Glu-Ser-Thr rich domain (PEST) for degradation (Kovall and Blacklow, 2010). It has become increasingly clear in recent years that the NICD is subject to a large number of post-translational modifications which have the potential to modulate Notch signaling.

NICD undergoes multiple post-translational modifications, including phosphorylation, ubiquitination, hydroxylation, and acetylation [Figure 1.7; adapted from

(Andersson et al., 2011)]. Below, I will describe, separately, the regulation of each type of post-translational modification on NICD and their impact on Notch signaling.

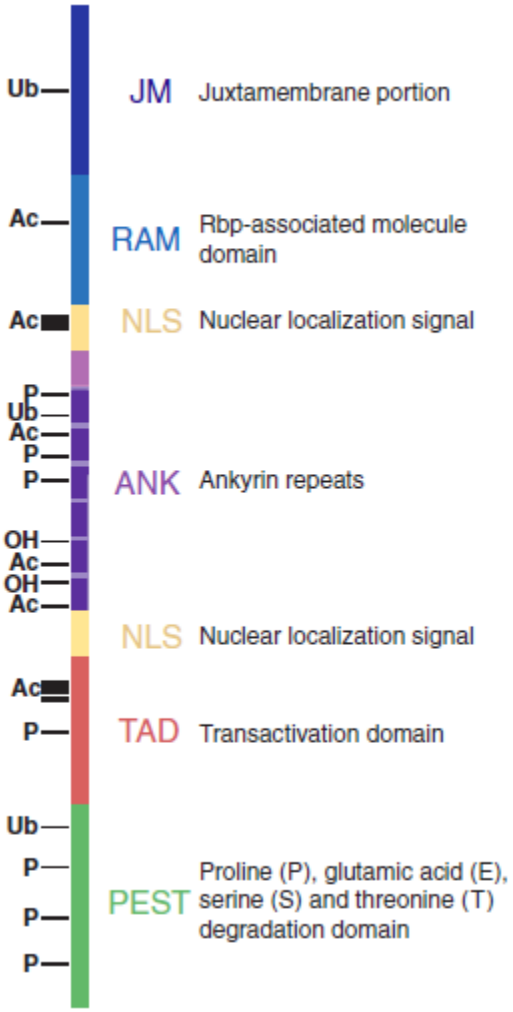

Figure 1.7. The NICD undergoes multiple post-translational modifications. The NICD is composed of several domains (JM, RAM, ANK, TAD and PEST), two nuclear localization signals and several ankyrin repeats. These various domains and motifs can be modified by phosphorylation, hydroxylation, ubiquitination or acetylation to alter signaling through NICD. The specific proteins that mediate these modifications are described in the text [from (Andersson et al., 2011)].

Phosphorylation: The NICD is modified extensively by phosphorylation. One of the key kinases in developmental signaling pathways is GSK3β. The literature is conflicted about GSK3's role in Notch signaling. One report suggests that GSK3β phosphorylates NICD C-terminally to the ANK repeats and inhibits NICD2-mediated induction of Notch target genes such as hairy and enhancer of split 1 (*Hes1*) (Espinosa et al., 2003) but another suggests that GSK3 stabilizes NICD1 (Foltz et al., 2002). Other reports have been published which suggest GSK3 as a positive regulator of Notch signaling through stability or localization (Guha et al., 2011; Han et al., 2012). Other reports suggest that GSK3 is in fact a negative regulator of Notch signaling (Jin et al., 2009b; Kim et al., 2009). Cyclin C/CDK8 phosphorylates NICD and has been shown to be critical in regulating the activity and stability of the NICD (Fryer et al., 2002; Fryer et al., 2004). Granulocyte colony stimulating factor (Csf) also phosphorylates NICD2 at Ser 2078, leading to its transcriptional activation (Ingles-Esteve et al., 2001). Notch has also been shown to be phosphorylated by Akt (Song et al., 2008), Calcium/Calmodulin-dependent kinase IV (CaMKIV) (Choi et al., 2013), CK2 (Ranganathan et al., 2011a), Nemo-like kinase (NLK) (Ishitani et al., 2010), Down syndrome associated kinase DYRK1A (Fernandez-Martinez et al., 2009), Abelson tyrosine kinase (Abl) (Xiong et al., 2013), CaMKII (Ann et al., 2012; Mamaeva et al., 2009), Disabled-1 (Dab-1) tyrosine kinase (Keilani et al., 2012), Protein Kinase Cδ (PKCδ) (Kim et al., 2012), Adaptor-Associated Kinase 1 (AAK1) (Gupta-Rossi et al., 2011), and DDR1 tyrosine kinase (Kim et al., 2011).

Ubiquitination: The NICD can also be modified by ubiquitination. The ubiquitination of NICD has been shown to modulate its half-life [reviewed in (Le Bras et

al., 2011)]. As mentioned earlier, the putative E3 ubiquitin ligase Deltex likely serves as a positive regulator of Notch signaling, possibly through a ligand-independent pathway distinct from canonical Notch signaling (Yamada et al., 2011). The most well-characterized E3 ubiquitin ligase for NICD is F-box and WD-40 domain protein 7 (Fbxw7; also known as Cdc4 and SEL-10), which ubiquitinates NICD within the PEST domain and promotes its rapid degradation (Fryer et al., 2004; Gupta-Rossi et al., 2001; Oberg et al., 2001; Wu et al., 2001). Functionally, Fbxw7 is critical in controlling stemness and neuronal fate versus glial differentiation in the developing brain (Matsumoto et al., 2011). The transcriptional activity of NICD1, but not NICD4, was enhanced in by a dominant negative form of Fbxw7 (Wu et al., 2001). In contrast to those results, *Fbxw7*$^{-/-}$ mice showed that Notch4 ICD, but not Notch1, 2, and 3 ICDs was elevated following *Fbxw7* knockout (Tsunematsu et al., 2004). These results also strongly suggest that the individual Notch receptors are regulated differently. Recently, it has been shown serum- and glucocorticoid-inducible kinase (SGK1) forms a trimeric complex with NICD and Fbxw7 and enhances Fbxw7-mediated NICD degradation (Mo et al., 2011). The importance of NICD stability is supported by the fact that *NOTCH1* and *FBXW7* mutations are found in T-cell acute lymphoblastic leukemia (T-ALL) (Erbilgin et al., 2010; Malyukova et al., 2007). Gain-of-function mutations in *NOTCH1* are found in over 50% of T-ALL patients (Weng et al., 2004) and loss-of-function mutations in *FBXW7* have also been found (Malyukova et al., 2007; Mansour et al., 2009; O'Neil et al., 2007). Interestingly, there is some evidence that NICD1 stability is still modulated even in the absence of FBXW7/PEST domain-mediated ubiquitination (O'Neil et al., 2007; Tsunematsu et al., 2004). These results suggest that there is

another region of NICD1 that regulates its stability. These *NOTCH1* mutations are concentrated at the HD domain and the PEST domain; the HD domain mutants promote ligand-independent activation and the PEST domain mutants confers resistance to ubiquitination and subsequent degradation (Weng et al., 2004). Additionally, T-ALL cell lines lacking functional FBXW7 have extended NICD1 half-lives (Malyukova et al., 2007; Mansour et al., 2009; O'Neil et al., 2007). Mutations in the PEST domain of *NOTCH1* have also been found in non-small-cell lung cancer (Westhoff et al., 2009), suggesting that NICD stability, and consequently Notch signaling, can lead to cancer. One other E3 ubiquitin ligase that ubiquitinates Notch is Itch (Cornell et al., 1999; Qiu et al., 2000) which is required for Notch1 degradation in the absence of ligand (Chastagner et al., 2008). An extensive list of NICD interacting proteins can be found in (Andersson et al., 2011). Much is still not known about many of these interactions, which have only been observed in overexpression studies. Whether they interact with NICD under physiological conditions in cultured cells or *in* vivo, with free NICD in the cytoplasm or nucleoplasm of the signal-receiving cell, or with the NICD-CSL-MAML transactivating complex inside the nucleus could inform future studies on NICD regulation.

Hydroxylation and Acetylation: NICD is also affected by two other types of post-translational modification, hydroxylation and acetylation. NICD is hydroxylated by the asparagines hydroxylase factor-inhibiting HIF1α (FIH1l also known as HIF1AN), which hydroxylates NICD at N1945 and N2012 (Coleman et al., 2007; Zheng et al., 2008). This might contribute to signaling specificity because Notch2 and Notch3 are hydroxylated whereas Notch4 is not. The *in vitro* studies suggest that HIF1α a negative regulator of Notch, but HIF1α targeted mice do not display a Notch gain-of-function

phenotype (Zhang et al., 2010). Another recent study has recently identified the deacetylase sirtuin 1 (Sirt1) as critical to the acetylation/deacetylation of NICD in endothelial cells, affecting NICD half-life (Guarani et al., 2011).

These results clearly indicate that the NICD is extensively modified by post-translational modifications, which serve to regulate the NICD. Most of these modifications affect the stability or half-life of NICD, implicating NICD protein turnover as a critical step in regulating the canonical Notch signaling pathway. Further studies on the regulation of NICD protein turnover would be extremely critical for understanding the mechanisms of the pathway between receptor activation and transcriptional activation. Not much is known about how these mechanisms that affect NICD turnover alter canonical Notch signaling.

Notch Signaling: Activation of a NICD-CSL-MAML transcriptional complex

Upon Notch activation, the liberated NICD translocates to the nucleus where it binds to CSL and the scaffold protein MAML; this NICD-CSL-MAML complex subsequently activates downstream target genes. This transcriptional activation is central to canonical Notch signaling (Kovall and Blacklow, 2010). The Notch-mediated transcriptome appears to be very diverse in different cell types, contributing to the specificity of Notch signaling. Genome-wide Notch transcriptome studies in healthy or mutated T-cells (Chadwick et al., 2009; Dohda et al., 2007; Palomero et al., 2006; Weerkamp et al., 2006), mouse embryonic stem (ES) cells (Main et al., 2010; Meier-Stiegen et al., 2010), alveolar epithelial cells (Aoyagi-Ikeda et al., 2011), endometrial stromal cells (Mikhailik et al., 2009), C2C12 mouse myoblast cells (Buas et al., 2009), and *Drosophila* myogenic cells (Krejci et al., 2009) yield a vast array of diversity in their

target gene transcriptomes. In addition to the diversity in target gene activation, Notch signaling is also affected by cell cycle stage [reviewed in (Kageyama et al., 2009)], and through cell lineage progression(e.g. in T-cell development [reviewed in (Radtke et al., 2010)]), and during neural differentiation of ES cells *in vitro*, when cyclin D1 is activated in a specific temporal window during ES cell neural differentiation (Das et al., 2010).

The canonical Notch signaling pathway directly activates the downstream hairy and enhancer of split-related (HESR) genes, a family of basic helix-loop-helix (bHLH) transcriptional repressors. The HESR genes have been shown to be activated by Notch during tumor progression (Sethi et al., 2011; Wendorff et al., 2010). Surprisingly, in the five cell types listed above which have done genome-wide transcriptome analysis, hairy/enhancer-of-split related with YRPQ motif 1 (*Hey1)* was upregulated in four of the five. Additionally, *Hes5* was upregulated in only the ES cells. These data suggest that even though there are some common upregulated target genes, there is no universal target gene upregulated in all cases of Notch signaling. The list of immediate Notch target genes which are upregulated in parallel with the HESR genes is quite extensive; including *c-Myc* (Rao and Kadesch, 2003; Satoh et al., 2004; Weng et al., 2006), cyclin D1 (Cohen et al., 2010a; Ronchini and Capobianco, 2001; Satoh et al., 2004), cyclin D3 (Joshi et al., 2009), cyclin-dependent kinase 5 (*CDK5*) (Palomero et al., 2006), *p21* (Rangarajan et al., 2001), *Snail* (Sahlgren et al., 2008), and platelet-derived growth factor receptor beta (*PDGFRβ*) (Jin et al., 2008; Morimoto et al., 2010). The differences in Notch-mediated transcriptome response after receptor activation is only partially understood. The prevailing model is that CSL binds DNA via conserved CGTGGGAA motifs to target promoters and in the absence of NICD represses transcription. Upon

Notch pathway activation, NICD, along with MAML, is thought to displace co-repressors and recruit co-activators to the NICD-CSL complex, leading to transcriptional activation of target genes. Certain genes, at least in *Drosophila*, appear repressed in the absence of Notch signaling (Bardin et al., 2010; Castro et al., 2005; Koelzer and Klein, 2006). However, other studies in *Drosophila* suggest that Su(H), the *Drosophila* homolog of CSL, is actively recruited to its binding sites by NICD rather than being placed there during the "Notch-off" state (Krejci and Bray, 2007). The binding coefficient between CSL and DNA is weaker than previously thought (Friedmann and Kovall, 2010), and the affinity of CSL for the RAM domain of NICD is unchanged by DNA binding (Friedmann et al., 2008), suggesting that the interaction between CSL and DNA is likely to be dynamic.

Each different Notch receptor has distinct tissue-specific expression patterns, and this distinction between the Notch receptors can lead to transcriptional specificity. The configuration of CSL-binding sites influences the likelihood of recruiting NICD1 or NICD3 (Ong et al., 2006). NICD1 is highly active on paired CSL binding sites while NICD3 is highly active when binding to CSL motifs adjacent to zinc-finger transcription factor binding sites (Ong et al., 2006). The dimerization potential of NICD can also influence the target gene repertoire by restricting the response to dimeric CSL binding sites (Cave et al., 2005). Structural analysis of the NICD dimeric complex suggests flexibility in spacer length is tolerable (Arnett et al., 2010). There is evidence that NICD multimerization is an initial step in forming the active transcriptional complex (Vasquez-Del Carpio et al., 2011). Additionally, because there is some difference in target gene expression, it is likely that there might be distinct biological functions for each Notch

receptor. NICD2, but not NICD1, promotes tumor growth in xenografts in a medulloblastoma model (Fan et al., 2004). NICD1 and NICD3 signaling generate distinct phenotypes in the pancreas (Apelqvist et al., 1999; Hald et al., 2003) but have similar phenotypes in adult CNS progenitor cells (Tanigaki et al., 2001). NICD3, but not NICD1 or NICD2, can drive the formation of invasive gliomas during embryonic CNS development (Pierfelice et al., 2011). One recent study suggests that the difference in signaling between the Notch receptors comes primarily from its extracellular domain (Liu et al., 2013).

Another way to generate signaling specificity is through feedback loops with downstream target genes. One example is c-Myc, which activates genes in concert with NICD-CSL that NICD-CSL does not activate alone (Palomero et al., 2006). In smooth muscle cells, Hey1 and Hey2 are activated by Notch and dampen Notch-mediated transcription by blocking NICD-CSL binding to DNA (Tang et al., 2008) that may affect the duration of the Notch signal. Another example of Notch feedback is the Notch target gene Notch-regulated ankyrin repeat protein (*Nrarp*), which feeds back to modulate Notch signaling while also potentiating Wnt signaling (Ishitani et al., 2005; Phng et al., 2009).

The cooperation of NICD-CSL and other transcription factors may also play a role in generating signal specificity. Proneural bHLH proteins cooperate with NICD-CSL to regulate HESR gene expression (Holmberg et al., 2008) and synergistic transcriptional responses between NICD-CSL and GATA factors (Neves et al., 2007), NF-κB (Vilimas et al., 2007), and Twist (Bernard et al., 2010) has also been demonstrated. In addition, the spacing between the binding sites has been shown to be

important (Swanson et al., 2010). The binding of CSL to the N-terminal region of NICD likely also affects the regulation of the NICD itself, as described earlier. As mentioned previously, SCF^{Fbxw7} then serves as an “off” switch to turn off Notch transcription by binding and degrading the NICD. This degradation also serves as a mechanism to continually refresh the Notch signal by degrading a post-transcriptionally active NICD and freeing up CSL and MAML to bind another NICD. Much remains unknown about why CSL is sometimes bound to the DNA in the absence of NICD but in other cases is recruited to the DNA by NICD.

The Notch signaling pathway has many key features in common with other developmental signaling pathways, including the Wnt/β-catenin pathway. However, very little is known about the cytoplasmic mechanism of the Notch pathway relative to other pathways. Notch is unique in that an obvious amplification step in the cytoplasm has yet to be identified, and the receptor itself serves as the downstream effector and transcriptional co-activator. Due to the importance of the NICD and the critical role that the downstream effector plays in other pathways, it is very likely that the NICD is very highly regulated at the protein level, possibly through cross-talk with other pathways but also through distinct mechanisms that affect only the NICD. The next section of the introduction focuses on regulation of Notch signaling through crosstalk with other developmental pathways, specifically the Wnt/β-catenin pathway.

Notch and Wnt Signaling: Evidence for Wntch Signaling?

Due to the small number of developmentally critical signaling pathways and the diverse functions required for embryonic development, it has become increasingly clear

that many of these pathways operate cooperatively to form a signaling network. Interestingly, Notch cross-talk has been shown to occur at all three levels of pathway interaction; epistasis of one pathway to another, convergence of two pathways to the same target genes, and direct interaction between components of each pathway.. The most well-characterized pathway that has been shown to cross-talk with the Notch pathway is the Wnt/β-catenin pathway and in this document I will focus on the cross-talk between canonical Notch signaling and canonical Wnt/β-catenin signaling.

Wnt/β-catenin signaling and Notch signaling are oftentimes required for similar developmental processes (Arias and Hayward, 2006) and multiple instances of Wnt-Notch crosstalk have been observed. Wnt/β-catenin signaling upregulates *Jag1* as a target gene of β-catenin in the hair follicle (Estrach et al., 2006), upregulates *Dll4* during vascular remodeling (Corada et al., 2010) and induced Notch2 expression in colorectal cancer cells (Ungerback et al., 2011). These studies suggest that Wnt/β-catenin signaling promotes Notch signaling. These two pathways can also converge downstream of the receptor; β-catenin can bind to NICD in neural precursor cells (Shimizu et al., 2008) and form complexes with NICD-CSL in arterial cells but not venous endothelial cells (Yamamizu et al., 2010). Intriguingly, the Dll1 ICD induces Wnt reporter activity and upregulates the expression of connective tissue growth factor (CTGF) (Bordonaro et al., 2011). Additionally, the scaffold protein MAML, which is critical for Notch transcriptional activation, binds to both GSK3β (Saint Just Ribeiro et al., 2009) and β-catenin (Alves-Guerra et al., 2007). MAML binding to GSK3β (which is inhibited by Wnt/β-catenin signaling) decreases MAML-mediated transcriptional activity (Saint Just Ribeiro et al., 2009) and MAML can act as a transcriptional co-activator for

β-catenin to increase expression of *cyclin D1* and *c-myc*, target genes of Wnt/β-catenin signaling (Alves-Guerra et al., 2007). These data further supports that Notch signaling and Wnt/β-catenin signaling can positively regulate each other. Another unexpected level of crosstalk occurs at the receptor level, where the soluble Frizzled-related proteins (sFRPs), Wnt/β-catenin antagonists, bind to ADAM10 metalloprotease and downregulate its activity, inhibiting Notch signaling. This regulation affects the Notch-dependent process of retinal neurogenesis, which is also Wnt-independent (Esteve et al., 2011). All of these suggest that Notch signaling and Wnt/β-catenin signaling affect each other positively. However, other studies have actually identified another method of crosstalk in which Notch signaling inhibits Wnt/β-catenin signaling (Hayward et al., 2008; Munoz-Descalzo et al., 2012).

Notch loss of function has been shown to result in ligand independent activation of Wnt/β-catenin signaling (Brennan et al., 1999a; Demehri and Kopan, 2009; Hanlon et al., 2010; Kwon et al., 2011; Kwon et al., 2009; Lawrence et al., 2001; Lin et al., 2008; Nicolas et al., 2003; Pan et al., 2004). This regulation is likely a post-translational effect of Notch on Wnt signaling. In some systems, gain of function of Notch downregulates the activity of β-catenin (Acosta et al., 2011; Deregowski et al., 2006; Hayward et al., 2005; Kwon et al., 2011; Langdon et al., 2006; Nicolas et al., 2003; Sanders et al., 2009). Additionally, Notch's regulation of Wnt/β-catenin signaling is independent of its transcriptional activity as it does not depend on CSL binding or generation of the NICD (Acosta et al., 2011; Hayward et al., 2005; Kwon et al., 2011; Sanders et al., 2009). Genetic experiments in *Drosophila* have also identified alleles of Notch that affect interactions with Wnt/β-catenin signaling (Brennan et al., 1997; Brennan et al., 1999c;

Langdon et al., 2006; Ramain et al., 2001; Ruel et al., 1993). These data all strongly suggest a role for Notch in inhibiting Wnt/β-catenin signaling *in vivo* and *in vitro*.

Structure function analysis has identified functionally distinct domains in Notch receptors for Wnt/β-catenin signaling and for canonical Notch signaling (Arias, 2002; Brennan et al., 1999b; Brennan et al., 1999c; Heitzler, 2010; Langdon et al., 2006). This suggests that Notch may interact with components of Wnt/β-catenin signaling directly. In support of this possibility, multiple reports have shown that Notch interacts either genetically or molecularly with key components of the Wnt/β-catenin signaling pathway: Dishevelled, APC, Axin, TCF/LEF, GSK3 and β-catenin itself (Axelrod et al., 1996; Espinosa et al., 2003; Foltz et al., 2002; Hayward et al., 2006; Hayward et al., 2005; Herranz et al., 2008; Jin et al., 2009a; Kwon et al., 2011; Langdon et al., 2006; Lee et al., 2009; Munoz-Descalzo et al., 2010; Munoz-Descalzo et al., 2011; Sanders et al., 2009; Shimizu et al., 2008; Strutt et al., 2002). Furthermore, NICD and β-catenin can be found in the same endocytic vesicles.

There is strong evidence that Notch signaling and Wnt/β-catenin signaling can be described as two arms of a signaling network rather than as two distinct, discrete signaling pathways. In cell culture studies, the Wnt/β-catenin and Notch pathways cooperate to maintain adult stem cells in the skin (Blanpain and Fuchs, 2009; Blanpain et al., 2006; Lowry et al., 2005), the intestine (Robine et al., 2005; Sancho et al., 2004; van Es et al., 2005), and skeletal muscle (Brack et al., 2008). In the intestine, treatment with gamma secretase inhibitors (GSIs) to inhibit Notch signaling also inhibits Wnt/β-catenin signaling (van Es et al., 2005) but loss of function of CSL does not suppress Wnt/β-catenin signaling (Peignon et al., 2011). These results strongly favor a model in

which Notch and Wnt signaling are functionally interconnected and can be integrated into a single functional signaling unit which some have termed “Wntch” signaling (Hayward et al., 2008; Munoz-Descalzo et al., 2010; Munoz-Descalzo et al., 2011; Munoz Descalzo and Martinez Arias, 2012; Sanders et al., 2009). These interactions can be summarized as one interconnected molecular network in Figure 1.8 [Figure 1.8, from (Munoz Descalzo and Martinez Arias, 2012)].

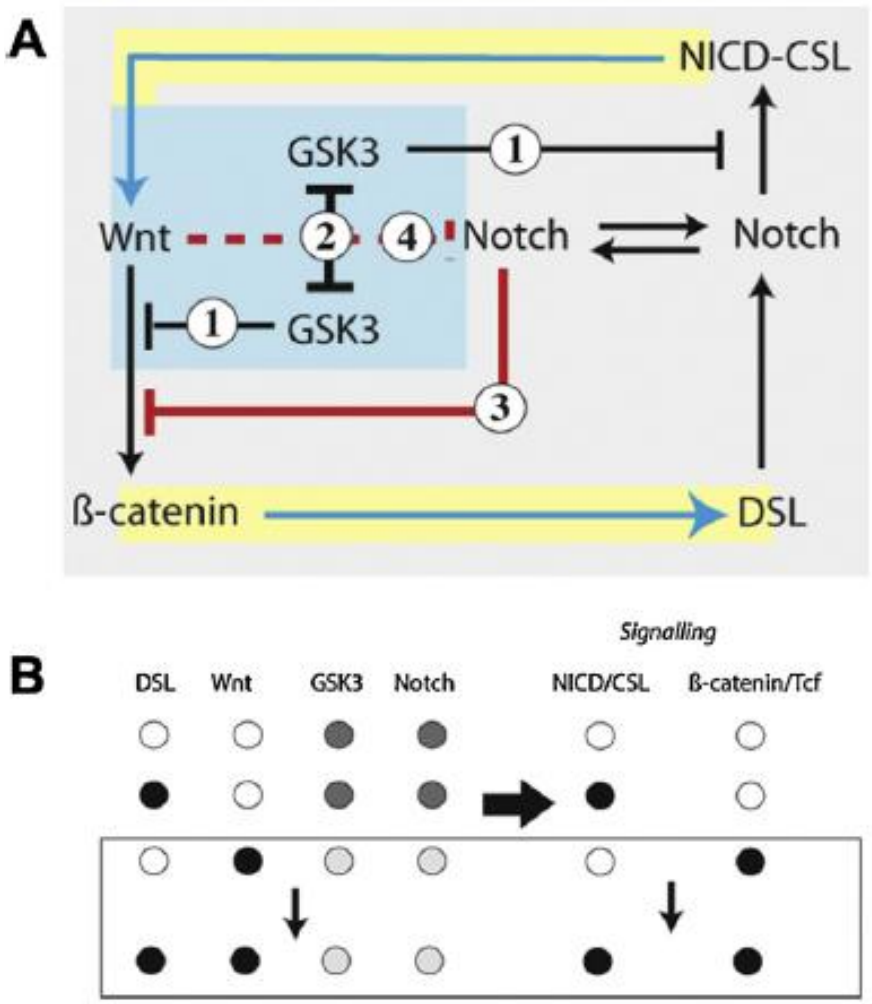

Figure 1.8. Structure and function of Wntch. (A) Summary of the interactions between elements of Wnt and Notch signalling and outline of the network that configures Wntch signalling. For details see text. The transcriptional interactions are labelled in yellow. (1) Effects of GSK3 activity that destabilized β-catenin and NICD. (2) Wnt signalling inhibits GSK3 and thus stimulates β-catenin and CSL/NICD function. These effects are likely to be cell type specific and depend on basal levels of GSK3 activity. (3) Notch, in a CSL independent manner, inactivates the transcriptional activity of β-catenin. (4) Wnt signalling inhibits the CSL independent activity of Notch. (B) Activity of the network outlined in A in different conditions. Notice that activation of Wnt signalling can lead to DSL-Notch-CSL signalling [from (Munoz Descalzo and Martinez Arias, 2012)].

The simplest and most obvious explanation for “Wntch” signaling is that the transcriptional effectors somehow converge to then regulate both pathways. However, the results have that been observed cannot be easily explained by that hypothesis alone. One key observation is that ligand independent Notch trafficking inhibits Wnt/β-catenin signaling. Experiments in *Drosophila* imaginal discs, mammalian cells, and embryos show that Notch inhibits β-catenin activity even when the Notch receptors cannot interact with DSL ligands nor bind to CSL for transcriptional activation (Hayward et al., 2005; Kwon et al., 2011; Sanders et al., 2009). Even though these Notch receptors cannot activate canonical Notch signaling, they still get endocytosed and trafficked, which allows them to specifically target the transcriptionally competent form of Arm/β-catenin but does not significantly affect the overall cytosolic pool of β-catenin (Hayward et al., 2005; Kwon et al., 2011; Sanders et al., 2009). There is evidence that the dephosphorylated transcriptionally competent form of β-catenin is recruited to the plasma membrane upon Wnt activation and might have different regulators than the cytosolic pool (Hendriksen et al., 2008). Notch’s regulatory effect on β-catenin might be through Axin, which is thought to be membrane-anchored and has been shown to interact with Notch and APC to regulate Armadillo in *Drosophila* (Hayward et al., 2006; Munoz-Descalzo et al., 2011; Tolwinski et al., 2003; Tolwinski and Wieschaus, 2001). It is still unclear whether Axin’s interactions with Notch are related to its function as a membrane anchor.

The effect of Notch on dephosphorylated β-catenin likely occurs through other adaptor proteins. In *Drosophila*, ligand independent trafficking of Notch requires Dsh, a positive regulator of Wnt/β-catenin signaling (Munoz-Descalzo et al., 2010; Munoz-

Descalzo et al., 2011). In mammalian cells, the Notch regulation of β-catenin is likely through the adaptor protein Numb (Cheng et al., 2008; Kwon et al., 2011). In both *Drosophila* and mammalian cells, the putative E3 and adaptor protein Deltex participates in CSL independent Notch signaling to regulate β-catenin (Langdon et al., 2006; Ordentlich et al., 1998; Ramain et al., 2001). Taken together, a model for Notch-mediated inhibition of Wnt/β-catenin signaling emerges where Notch interacts with dephosphorylated β-catenin at the membrane and degrades or sequesters β-catenin. This likely requires some combination of Dsh, Numb, and Deltex (Gupta-Rossi et al., 2004). Validation of such a model will require further biochemical studies.

There is also evidence that Wnt signaling can modulate Notch signaling. Wingless, the *Drosophila* homolog of Wnt, has been shown to regulate Notch signaling (Munoz-Descalzo et al., 2010; Wesley, 1999). Multiple reports have also shown that the classical DSL ligand *Jagged1* is a target gene of β-catenin-mediated transcription (Amoyel et al., 2005; Estrach et al., 2006; Galceran et al., 2004; Rodilla et al., 2009). Interestingly, MAML is also a transcriptional co-activator for β-catenin (Alves-Guerra et al., 2007; Kankel et al., 2007).

The idea of a single functional Wntch signaling module is a compelling one, as evidence shows that each of these individual pathways can influence the output of the other one. This strongly suggests that these two pathways are functionally interconnected, forming a signaling network to regulate cell growth and proliferation and cell fate decisions. This is likely the first step towards integrating signal transduction into one large network where the activation of one pathway regulates the activation of other pathways. More studies to elucidate the mechanistic underpinnings of “Wntch” signaling

will critical towards an integration of Notch and Wnt signaling into a single functional paradigm.

CHAPTER II

MATERIALS AND METHODS: PROTEIN DEGRADATION IN *XENOPUS* EGG EXTRACT

One critical aspect of studying developmental signaling networks is the use of proper systems to study them in. I used *Xenopus* egg extract as one of the primary systems to study degradation of Notch. Details about the use of *Xenopus* egg extract can be found in this chapter and in Chen et al. 2014a (Chen et al., 2014a). This system can be used to study both Wnt signaling and Notch signaling.

Reconstitution of β-catenin degradation in *Xenopus* egg extract

Introduction

Xenopus laevis egg extract has been used extensively to study many cell biological processes including cytoskeletal dynamics, nuclear assembly and import, apoptosis, ubiquitin metabolism, cell cycle progression, signal transduction, and protein turnover(Blow and Laskey, 1986; Chan and Forbes, 2006; Dabauvalle and Scheer, 1991; Forbes et al., 1983; Glotzer et al., 1991; Kornbluth et al., 2006; Lohka and Masui, 1983; Maresca and Heald, 2006; Masui and Markert, 1971; Mitchison and Kirschner, 1984; Murray, 1991; Newport and Kirschner, 1984; Salic et al., 2000b; Shennan, 2006; Theriot et al., 1994; Tutter and Walter, 2006; Verma et al., 2004). The *Xenopus* egg extract system is amenable to the biochemical analysis of a legion of cellular processes because egg extract represents essentially undiluted cytoplasm that contains all the essential cytoplasmic components necessary to execute these processes and enable

investigation. Large quantities of egg extract can be prepared at one time for biochemical manipulations that require large amounts of material (e.g. protein purification or high-throughput screening)(Thorne et al., 2010; Thorne et al., 2011; Yu et al., 1996). Another advantage is that the concentration of specific proteins in *Xenopus* egg extract can be precisely adjusted by addition of recombinant protein and/or immunodepletion of endogenous proteins in contrast to transfection of plasmid DNA where expression of the protein of interest is difficult to control. In addition, the lack of available recombinant proteins can be overcome by the addition of transcripts encoding the protein of interest, taking advantage of the freshly prepared *Xenopus* egg extract's high capacity to translate exogenously added mRNA.

The regulation of protein degradation is critical for the control of many cellular pathways and processes(Hinkson and Elias, 2011). *Xenopus* egg extract has been used extensively to study protein degradation as the system allows for multiple ways to monitor protein turnover without confounding influences of transcription and translation. The Wnt signaling pathway is a highly conserved signaling pathway that plays critical roles in development and disease. The turnover of β-catenin, the major effector of the Wnt pathway, is highly regulated, and an increased steady-state level of β-catenin is critical for the activation of Wnt target genes. The importance of β-catenin degradation is highlighted by the fact that mutations in the Wnt pathway that inhibit β-catenin degradation found in ~90% of all sporadic cases of colorectal cancer(Kinzler and Vogelstein, 1996). β-catenin degradation by components of the Wnt pathway can be faithfully recapitulated in *Xenopus* egg extract to study the mechanism of its turnover as well as to identify novel small molecule modulators of its degradation (Cselenyi et al.,

2008; Guger and Gumbiner, 1995; Jernigan et al., 2010; Lee et al., 2001; Lee et al., 2003; Major et al., 2007; Salic et al., 2000b; Seeling et al., 1999; Thorne et al., 2010; Thorne et al., 2011).

Methods for the preparation of *Xenopus* egg extract for studying the cell cycle have been described in previous *JoVE* publications (Cross and Powers, 2008a; Cross and Powers, 2008b; Willis et al., 2012). The current protocol describes a modification of these methods and is optimized for the degradation of [^{35}S]-radiolabeled β-catenin and luciferase-tagged β-catenin in *Xenopus* egg extract. The radiolabeled degradation assay allows for direct visualization of protein levels via autoradiography. [^{35}S]methionine is incorporated into the protein of interest using an *in vitro* translation reaction that can then be directly added to a degradation reaction. In addition, the radiolabeled protein turnover assay does not require an antibody against the protein of interest or an epitope tag, which can influence protein stability. Because even small changes in protein levels, as reflected in changes in the intensity of the radiolabeled protein band, are readily visualized by autoradiography, the [^{35}S]-radiolabeled degradation assay represents a very useful method for visualization of protein turnover(Salic et al., 2000b).

Fusion of β-catenin to firefly luciferase (hereafter referred to as simply”luciferase”) allows for more precise and quantitative measurements of protein level, and the capacity to more readily determine the kinetic properties of β-catenin turnover(Thorne et al., 2010; Thorne et al., 2011). A major advantage of the luciferase assay is that it provides a strong quantitative system that is easily scaled up. The following protocol provides simple methods for assaying β-catenin degradation and a

robust, efficient, and effective method for high-throughput screening of novel β-catenin modulators.

Protocol

1. Preparation of *Xenopus* egg extract

Each frog yields approximately 1 mL of usable egg extract. Extracts from 10 frogs are typically prepared at one time, and the volume of buffer described below is for performing a 10-frog Xenopus egg extract prep. The buffer volume can be adjusted accordingly for larger or smaller preparations of egg extract. The process of collecting eggs and processing them into extract is most efficient when conducted by two people.

1.1) Egg collection

1.1.1) To prime the frogs, inject each female frog with 100 U of Pregnant Mare Serum Gonadotropin (PMSG) from a freshly made 250 U/mL stock. Use a 3 mL tuberculin syringe with a 27g needle to inject subcutaneously, with the bevel of the needle up, into the dorsal lymph sac. This is approximately 1 cm towards the midline from the notched discolorations along the length of the legs of the frog.

1.1.2) Store primed frogs in water at 18°C for 5-10 days. For standing water tank systems, the animal density is approximately 4 liters of water per female frog. The minimum time required for priming to take effect is 5 days, and the effects of priming wear off after 10 days.

1.1.3) Prepare 0.5 x Marc's Modified Ringers (MMR) solution from a 20 x MMR stock. 20 x MMR consists of 2 M sodium chloride, 40 mM potassium chloride, 40 mM calcium

chloride, 20 mM magnesium chloride, and 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4.

1.1.4) Set up buckets for all injected frogs (one frog per 4 L bucket). Although more than one frog can be placed in the same bucket for egg collection, if one of the frogs lays predominantly poor quality eggs, a substantial amount of effort will be required to separate the poor quality eggs from those that are suitable for making extract. Thus, maximizing the number of frogs in the same tank to minimize the amount of buffer used for egg collect is not worth the risk.

1.1.5) Inject 750 U Human Chorionic Gonadotropin (HCG) into the dorsal lymph sac of each frog using a 27g needle as described in 1.1.1.

1.1.6) Place each of the HCG-injected frogs into individual 4 L buckets containing 0.5 x MMR cooled to 16°C.

1.1.7) Place the containers with the frogs in a 16°C incubator to collect eggs overnight (15-16 hours). Maintaining the proper temperature is critical for the entire procedure, from collecting eggs to preparing egg extract.

1.2 Dejellying eggs

Eggs are covered with a jelly coat that must be removed prior to making extract. The likelihood of spontaneous lysis of the eggs increases as the time between egg laying and extract preparation increases. Thus, it is important to proceed through the following steps as rapidly as possible.

1.2.1) Prepare 4 L of 1 x MMR, 50 mL of 0.1 x MMR, and 400 mL of 2% cysteine, pH 7.7, made in distilled water. All solutions should be maintained at 16°C.

1.2.2) To expel additional eggs, one can gently squeeze the lower back and abdomen of the frog.

1.2.3) Remove the frogs and the bulk of the MMR, to leave the eggs in approximately 1-200 mL of MMR in each bucket.

1.2.4) Remove debris with a transfer pipet, and assess the quality of the eggs. High quality eggs are generally marked by a clear separation between the darkly pigmented animal hemisphere and the lightly colored vegetal hemisphere and have the highest dark-to-light contrast. Discard with a transfer pipet any eggs that appear stringy, mottled, or lysed (white and puffy) as they will decrease the overall quality of the extract. If >10% of the eggs are of poor quality, the entire batch should be discarded.

1.2.5) Combine eggs into a 500 mL glass beaker and pour out as much MMR as possible while keeping eggs submerged.

1.2.6) Rinse eggs by gentle swirling with twice the egg volume of MMR. Repeat 2X, and remove any debris or obviously poor quality eggs.

1.2.7) Add approximately 100 mL of 2% cysteine to the glass beaker, swirl gently to mix, and allow eggs to settle for 5 minutes at 16°C. Pour off the cysteine. Dejellying is marked by the gradual appearance of jelly coats floating above the eggs and the more compact packing of the eggs as they now occupy a smaller volume without the jelly coat.

1.2.8) Add another 100 mL of 2% cysteine, gently swirl, wait 5 minutes, and then slowly pour off the cysteine. Repeat until eggs have become tightly compacted (usually by the third cysteine treatment). Note that if eggs are left too long in cysteine, they are prone to lysis. Similarly, dejellied eggs are fragile and prone to mechanical lysis if they are

swirled too vigorously or if they are exposed to air. Once the eggs have been dejellied, it is important to rapidly proceed to the centrifugation steps.

1.2.9) Pour off cysteine, and rinse away the jelly coat and other debris by gently washing eggs in 1 x MMR. Pour off buffer carefully along the side of the beaker. Repeat 2X or until MMR solution is no longer cloudy. While rinsing with 1 x MMR continue to remove the bad eggs using a transfer pipet.

1.2.10) Perform a final gentle rinse with 30 mL 0.1 x MMR, and gently pour off as much of the buffer as possible. Again, remove any obviously bad eggs.

1.3) Packing and crushing eggs by centrifugation

The extract described below that is used for β-catenin degradation is a variant of the cytostatic factor extract (metaphase II-arrested). In contrast to the low-speed and high-speed extract used for cell cycle studies, intermediate speed extract works best for β-catenin degradation. Interphase extract similarly promote robust β-catenin degradation although is more labor intensive to prepare.

1.3.1) Add Leupeptin, Pepstatin, Aprotinin mixture (LPA, a protease inhibitor) at 10 µg/mL (diluted from a 10 mg/mL stock solution in DMSO) and Cytochalasin D at 20 µg/mL (diluted from a 10 mg/mL stock solution in DMSO) into the remaining 20 mL of 0.1 x MMR.

1.3.2) Add 0.1 x MMR containing LPA and Cytochalasin D to the washed eggs, swirl gently, and incubate for 5 minutes at 16°C.

1.3.3) Transfer eggs into 16°C pre-chilled 50 mL centrifuge tubes, allow the eggs to settle, and remove residual buffer from the top. To prevent exposure to air, withdraw a small amount of buffer into the transfer pipet prior to withdrawing eggs for transfer.

Continue to transfer additional eggs into the centrifuge tubes and remove residual buffer from the top until the eggs fill the centrifuge tube to the top (maximize the yield of extract).

1.3.4) To pack the eggs, spin centrifuge tubes at 400 x *g* for 60 seconds at 4°C. Remove residual buffer from the top of the centrifuge tubes.

1.3.5) For the crushing spin, spin tubes at 15,000 x *g* for 5 minutes at 4°C.

1.4) Collecting cytoplasmic layer of extract

(At this point extract should be kept cold during throughout the process, and all steps should be performed at 4°C.)

1.4.1) Clear a hole in the lipid layer using P1000 pipet tip.

1.4.2) Collect the cytoplasmic layer (between the dark pigmented layer and the light lipid layer) using a new P1000 pipet tip (See Figure 1) into clean pre-chilled centrifuge tubes. For high-quality extract that robustly degrades β-catenin, the amount of pigmented and lipid layer that is withdrawn with the cytoplasmic layer should be minimized.

1.4.3) Spin extracted cytoplasmic layer at 15,000 x *g* for 10 minutes at 4°C and again collect the cytoplasmic layer. Repeat the spin and extraction 1X. The extract should be "straw colored." If there is substantial contamination with the pigmented and lipid layers at this point, one can repeat the spin one more time (although excessive spins will decrease the capacity of the extract to degrade β-catenin).

1.4.4) Add LPA and Cytochalasin D to the extract at final concentrations of 10 µg/mL each.

1.4.5) (optional) Freshly prepared extract has a high capacity to translated exogenously added mRNA. Unfortunately, this capacity is lost once the extract is frozen. For

translation, add capped mRNA (0.1 mg/ml), RNAsin (1.5 U/μL), and energy regeneration mix (2.1.1) and incubate the reaction at RT for 2 hr. Use translated extract immediately for β-catenin degradation assays or snap-freeze in liquid nitrogen for later use. Capped mRNA can be readily prepared using commercially available kits.

1.4.6) Snap-freeze extract in liquid nitrogen. Extracts are stored in small (200 μL) aliquots for single use because they rapidly lose their capacity to degrade β-catenin if refrozen. For long-term storage, extract can be stored in liquid nitrogen. For short-term storage, extract can be stored at -80°C, although the capacity of extract to degrade β-catenin can be dramatically reduced with extended storage at -80°C (longer than 2 months).

2. Preparing extract for β-catenin degradation assay

2.1) Depletion from *Xenopus* extract

A major advantage of *Xenopus* extract is the capacity to readily deplete components of a pathway and precisely add back a defined amount of a protein in order to determine its dose-dependent effects.

2.1.1) Use freshly prepared *Xenopus* egg extract or quickly thaw frozen extract and place on ice. All manipulations should be performed in the cold.

2.1.2) Add extract to 1/10 the volume of pelleted antibody or affinity beads (e.g. 20 μL pelleted beads to 200 μL extract). In order to minimize dilution of the extract, care should be taken to withdraw as much liquid from the beads as possible before addition of extract. Gel loading tips with long, tapered tips used for gel loading work well for this purpose.

2.1.3) Rotate extract-bead mix at 4°C for 1 hour.

2.1.4) Spin extract-bead mix at 12,600 x *g* in microfuge at 4 °C for 30 seconds. Alternatively, if magnetic beads are used, apply magnetic field to collect beads.

2.1.5) Transfer depleted extract to a fresh microfuge tube on ice. Be careful not to transfer any beads with the extract.

2.1.6) Prepare extract for β-catenin degradation assay as described in 2.2.

2.1.7) Confirm efficiency of depletion by immunoblotting both depleted extract and beads.

2.2) Optimizing *Xenopus* extract for β-catenin degradation

β-catenin degradation in *Xenopus* egg extract is an energy-dependent process that quickly depletes the endogenous ATP stores. Consequently, an energy regeneration system is required to maintain robust β-catenin degradation.

2.2.1) Prepare a 20 x energy regeneration (ER) mix consisting of 150 mM creatine phosphate, 20 mM ATP, 600 µg/mL creatine phosphokinase, and 20 mM $MgCl_2$. ER should be aliquoted and stored at -80°C. Repeated freeze/thaw cycles should be avoided, so small frozen aliquots are preferable.

2.2.2) Quickly thaw *Xenopus* egg extract by rubbing the frozen tube between your hands. Place the tube on ice just before all of the extract has melted.

2.2.3) Add 10 µL of energy regeneration mix (20 x ER) into an aliquot (200 uL) of *Xenopus* egg extract. Mix thoroughly by quickly flicking the tube and vortexing. Pulse-spin and immediately place on ice.

2.2.4) (optional) The turnover of β-catenin can be slightly enhanced in *Xenopus* egg extract by addition of ubiquitin (1.25 mg/ml final). Cycloheximide (0.1 mg/ml final) can also be added to minimize translation of endogenous transcripts.

2.2.5) Aliquot the appropriate volumes for degradation assay into pre-chilled microfuge tubes on ice. For radiolabeled β-catenin degradation assays, withdraw 2-5 μl extract for each time point.

3. Radiolabeled β-catenin degradation assay in *Xenopus* egg extract

(All steps should be performed on ice unless otherwise indicated).

3.1) Preparing radiolabeled β-catenin

3.1.1) Prepare freshly *in vitro*-synthesized [^{35}S]methionine-radiolabeled protein using commercially available kits. Generating ^{35}S-labeled proteins are easily and efficiently produced using commercially available *in vitro*-coupled transcription-translation kits. It is important that the translated protein is sufficiently labeled such that changes in protein turnover can be readily visualized.

3.1.2) To confirm successful radiolabeling, perform SDS-PAGE/autoradiography with 0.5 μL of the translated protein. The radiolabeled β-catenin protein band should be clearly visible on film within a few hours (4-6 hours). The intensity of the radiolabeled β-catenin band can be quantified using ImageJ, ImageQuant, or an alternative quantitative software program.

3.1.3) Snap-freeze the radiolabeled protein in liquid nitrogen for storage until use. Prolonged storage (>2 months) and multiple freeze/thaw (greater than 2) can severely impact the capacity of the radiolabeled β-catenin to degrade robustly in *Xenopus* egg extract.

3.2) Performing β-catenin degradation assay

3.2.1) Add 1-3 μL (depending on the strength of the radiolabeled band signal) of *in vitro*-translated β-catenin (and other proteins, small molecules, etc. that are being tested) into

20 µL of *Xenopus* reaction mix on ice. Mix thoroughly by quickly flicking the tube and vortexing. This is an important step as *Xenopus* egg extract is very viscous, and incomplete mixing will affect the consistency of the results. Pulse spin and place on ice.

3.2.2) Start the β-catenin degradation reaction by shifting the tubes to room temperature.

3.2.3) At the designated time point, remove 1-5 µL of the sample and mix immediately with SDS sample buffer (5 x volume) to stop the reaction. To make sure the degradation reaction is completely terminated, flick tube several times and vortex vigorously.

3.2.4) Perform SDS-PAGE/autoradiography. Run 1 µL equivalents (~ 50 µg of protein) of the extract for each time point/lane. Degradation of β-catenin in *Xenopus* egg extract should be evidenced by the time-dependent decrease in intensity of the radiolabeled β-catenin band (see Figure 2). Results can be quantified using ImageJ, ImageQuant, or other preferred imaging software if necessary.

3.2.5) (optional) Soak SDS-polyacrylamide gel in fixing solution (10% acetic acid and 30% methanol in distilled water) prior to drying to decrease background radioactivity and increase the quality of the image.

4. β-catenin-luciferase degradation assay in *Xenopus* egg extract

Perform all steps on ice unless otherwise indicated.

4.1) Preparing β-catenin-luciferase

4.1.1) Non-radiolabeled, luciferase-tagged β-catenin can be synthesized using the transcription-translation coupled system with complete amino acid mix.

4.1.2) Confirm production of the luciferase-tagged β-catenin by measuring luciferase activity from 0.5-1 µL of the reaction. Background luminescence can be assessed by

measuring luminescence from an untranslated reaction mix. Multiple commercial kits are available for measuring luciferase activity. Long-lived luminescence, however, works particularly well for the degradation assay.

4.2) Performing β-catenin-luciferase degradation assay

4.2.1) Thaw and prepare and *Xenopus* egg extract as in 2.2.

4.2.2) Add *in vitro*-translated β-catenin-luciferase fusion (from 4.1) into prepared *Xenopus* reaction mix (from 2.2) on ice and mix well as in 3.2.1. The activity of the β-catenin luciferase that is added to the extract is typically between 20-50,000 relative luminescence units (RLU)/μL of extract (based on measurements obtained from 4.1.2). Starting signal should be approximately 100,000 RLU (2-5 μL of the *in vitro*-translated β-catenin-luciferase fusion).

4.2.3) Shift the extract to room temperature to start the degradation reaction.

4.2.4) Remove an aliquot of the reaction at the indicated time and snap-freeze in liquid nitrogen. Triplicate samples are typically removed for analysis for each time point. Frozen extract can be stored at -80°C until they are ready to be analyzed.

4.2.5) Thaw samples ice, transfer samples to standard white 96 well plates on ice, and process for luciferase activity.

Representative Results

Xenopus egg extract is a robust biochemical system for investigating β-catenin turnover. The concentration of β-catenin in *Xenopus* egg extract is ~25 nM (Salic et al., 2000b). Under optimal conditions, the egg extract is capable of degrading β-catenin at a rate of 50-100 nM/hr and is half-maximal at 200 nM (Lee et al., 2003). There are several critical steps for successful reconstitution of β-catenin degradation using *Xenopus* egg extract. These include 1) generating high quality *Xenopus* egg extract and the manner by which egg extract is prepared, 2) generating quality radiolabeled β-catenin and β-catenin-luciferase protein, 3) optimizing reaction conditions to support β-catenin degradation, and 4) proper processing of reaction time points.

A high-quality preparation of *Xenopus* egg extract in which β-catenin is robustly degraded depends on both the quality of the eggs and how the eggs are handled prior to the crushing step as well how the extract is subsequently centrifuged to obtain the final extract. As mentioned in the protocol, it is important to ensure that only high quality eggs (evidenced by sharp contrast between the dark animal hemisphere and light vegetal hemisphere) are used, that poor quality eggs (stringy, mottled, or white puffs) are removed throughout the process, that eggs are maintained at a cool temperature (16°C) throughout the procedure, and that the procedure is carried out as quickly as possible. It is important not to sacrifice quality for quantity of extract. Additionally, as previously mentioned, eggs from a frog that lays poor quality eggs (>10%) should be entirely discarded.

The extract described above is a modification of meiosis II-arrested CSF extract (Masui and Markert, 1971). The preparation of *Xenopus* egg extract for β-catenin

degradation (intermediate speed extract) differs from classic extracts prepared for studying cell cycle (Cross and Powers, 2008a; Cross and Powers, 2008b; Willis et al., 2012), which are low-speed or high-speed extracts (Salic and King, 2005). Adapting *Xenopus* egg extract for optimal degradation of other proteins may require altering the centrifugation speed and number of spins. Low-speed extract contains intact organelles and other large cellular components. Thus, higher speed spins will alter the composition of the extract and potentially remove inhibitory and/or essential components that will affect degradation of the protein of interest. The preparation of intermediate speed extract herein described is optimized for degradation of β-catenin by components of the Wnt pathway. Spinning the extract greater than 4X can significantly decrease the capacity of the extract to degrade β-catenin (although it has minimal effect on the degradation of another Wnt component, Axin). β-catenin is susceptible to caspase-mediated proteolysis in low-speed spin extract and does not noticeably degrade in high-speed spin extract. Thus, it is likely that different speed extracts will be optimal for degradation of components of other signaling pathways.

The generation of ^{35}S-β-catenin and β-catenin-luciferase can be performed using a number of commercially available kits. Protein production using transcription-translation coupled systems is highly dependent on the quality of the plasmid: highly pure midi-preparations of plasmids work better and are more reliable compared to DNA mini-preparations. For radiolabeling β-catenin, freshly obtained [^{35}S]methionine or translabel ([^{35}S]methionine plus [^{35}S]cysteine) is preferred. Note that methionine and cysteine both have a tendency to oxidize with prolonged storage thereby decreasing their incorporation into β-catenin in the *in vitro* transcription-translation coupled reaction.

Because prolonged storage and repeated freeze-thawing cycles can inhibit degradation, small amounts of the radiolabeled β-catenin are prepared at a time and used soon after.

The amount of protein translated and the number of methionines in the protein determine the strength of the radiolabeled signal. For β-catenin, there should be no problems obtaining strong radioactive signals for degradation assays. For proteins with few methionines and/or that do not translate well, a [^{35}S]methionine and [^{35}S]cysteine mix can be used instead of [^{35}S]methionine and/or added an epitope tag (Myc) that contains multiple methionines. Although success with various expression plasmids containing the appropriate phage promoters (T7, T3, and SP6) can be obtained for *in vitro* transcription-translation reactions, pCS2-based plasmid generally gives the best results. In addition, the signal of the translated protein can sometimes be dramatically increased by deleting the endogenous 5' UTR. Finally, for large-scale experiments (e.g. high-throughput screening), a large quantity of recombinant β-catenin-luciferase may be required. β-catenin-luciferase from the *Sf9*/baculovirus system degrades with similar kinetics as the wild-type β-catenin in *Xenopus* extract and embryos (Salic et al., 2000b). Alternatively, a high-yield *in vitro* expression system (e.g. wheat germ-based) has been successfully used for high-throughput screening (Thorne et al., 2010; Thorne et al., 2011).

In order to reliably measure the degradation of β-catenin in the extract system, it is important that the initial signal from either the radiolabeled β-catenin or the luciferase β-catenin fusion is sufficiently robust. Thus, some amount of optimization by the experimenter on the size of the degradation assay, the number of time points needed, and the efficiency of the *in vitro* translation reaction will be required. When assembling

the degradation reaction, there are several steps one can take to enhance the chance of obtaining robust degradation. Firstly, all reagents should be thawed quickly and placed on ice prior to their complete thaw. Because the degradation of β-catenin is highly energy dependent, it is important that the ER is relatively fresh. Secondly, *Xenopus* egg extract is viscous, and it is critical that the reaction is thoroughly mixed after addition of radiolabeled β-catenin/β-catenin-luciferase fusion, ER, protein, small molecule modulators, etc. Thirdly, pre-incubating the reaction mix for 20-30 minutes on ice gives more reproducible results when testing effects of small molecule modulators.

The capacity to deplete proteins from *Xenopus* egg extract represents a powerful tool to assess protein function and their concentration-dependent effects. As an example, we demonstrate that depleting GSK3 from *Xenopus* egg extract blocks the degradation of β-catenin, indicating the important role of GSK3 in β-catenin turnover. Different depletion conditions will need to be empirically determined for different proteins. For example, abundant proteins will require an increase in the amount of antibody or affinity ligand beads used to achieve full depletion. A good starting point is to use packed beads at 1/10 the volume of the extract in order to minimize extract dilution. In addition to the strength of binding of the antibody/affinity ligand to the target protein, the type of beads (e.g. sepharose, agarose, or magnetic) used may impact the efficiency of protein depletion from *Xenopus* egg extract(Trinkle-Mulcahy et al., 2008). Finally, it would be ideal to analyze the extent of depletion (typically by immunoblotting). Once the β-catenin reaction is completed, the manner by which the samples are processed can greatly affect the results of the experiment. The concentration of *Xenopus* egg extract preps prepared in the manner described is 52.41 mg/mL ± 5.40

mg/mL, and it is important not to overload the capacity of the SDS-PAGE gel; this is not a problem with the luciferase-β-catenin assay. Thus, for each time point, no more than 1 μL equivalent of extract should be loaded into each lane of an SDS-PAGE gel. To further enhance the quality of the results, the gel fixation step (optional) will decrease background radioactivity and increase the signal-to-noise ratio. For the luciferase assay, a decrease from an initial signal of 100,000 RLU in *Xenopus* egg extract faithfully reflects the change in the protein levels of the β-catenin-luciferase fusion protein.

The *Xenopus* egg extract system represents an attractive system to study the regulation of β-catenin degradation. The extract system allows for the precise control of the concentration of individual proteins via depletion and reconstitution. The capacity to monitor their effects on the kinetics of β-catenin turnover over time allows for a better understanding the biochemical mechanism of this crucial step in Wnt signal transduction. Such manipulations have provided deep insight into the complex molecular interactions between components of the Wnt pathway and were critical for the development of the first mathematical model of the Wnt pathway (Lee et al., 2003). One caveat is that the concentrations of Wnt pathway components in *Xenopus* egg extract and mammalian cell lysates have been found to differ, possibly reflecting differences in the way the Wnt pathway is regulated during embryogenesis versus the adult situation (Tan et al., 2012). The capacity to perform both radioactive and enzymatic, luciferase-based assays using *Xenopus* egg extract provides added powerful complementary qualitative and qualitative tools for studying the biochemical regulation of β-catenin degradation.

In addition to monitoring the turnover of β-catenin, degradation of the negative Wnt regulator, Axin, can be monitored in *Xenopus* egg extract either as a radiolabeled protein or fusion to luciferase. Using a variant of luciferase, Renilla, fused to Axin, was previously adapted for the luciferase degradation assay into a high-throughput format to simultaneously screen for modulators of two Wnt pathway proteins, β-catenin and Axin (Thorne et al., 2010; Thorne et al., 2011). This biochemical screen identified the FDA-approved drug, pyrvinium that increased and decreased the degradation rate of β-catenin and Axin, respectively, in *Xenopus* egg extract (Thorne et al., 2010). Pyrvinium was subsequently validated in cultured human cells and in various model organisms (e.g. *Xenopus* and *C. elegans*) as a small molecule inhibitor of the canonical Wnt pathway. In summary, the *Xenopus* egg extract system is a versatile biochemical system that can be exploited in a multitude of ways to study the mechanism of Wnt signaling as well as for identification of small molecule modulators of the Wnt pathway. The biochemical method described herein can be applied to other signaling pathways in which protein degradation may play a critical role.

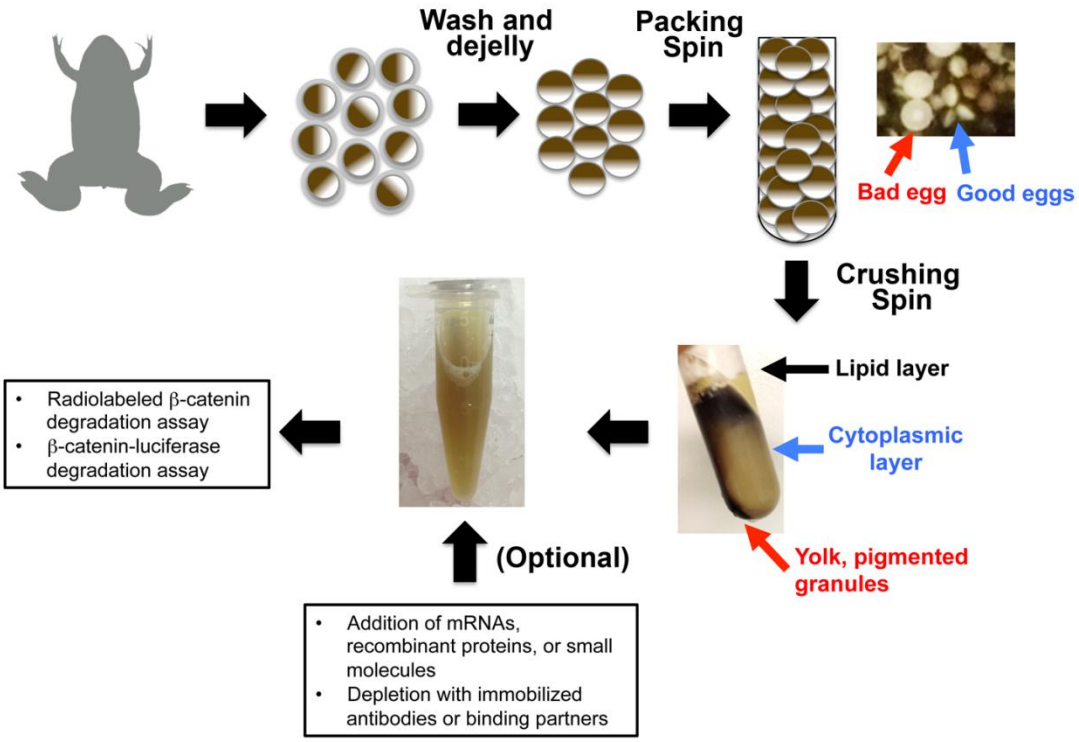

Figure 2.1. A schematic representation of the preparation of concentrated *Xenopus* egg extract. Eggs are collected from HCG-injected *Xenopus laevis* females, compacted with a low-speed (400 x *g*) spin, and crushed and separated with a medium-speed (15,000 x *g*) spin. Take care to inject subcutaneously into the dorsal lymph sac, remove the bad eggs carefully, and carefully separate the high-quality cytoplasmic extract from the lower-quality darker extract [Figure from (Chen et al., 2014a)].

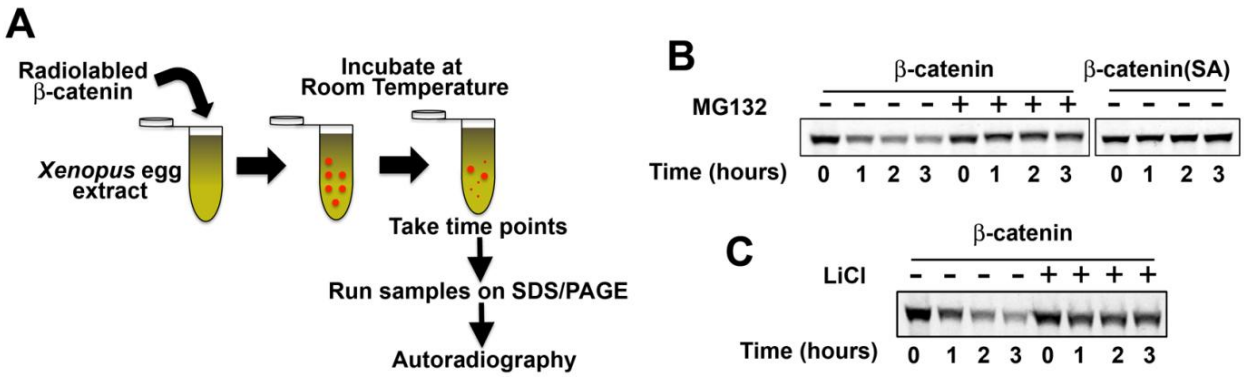

Figure 2.2. β-catenin degrades robustly when incubated in *Xenopus* egg extract. (A) A schematic of a degradation assay. (B) [^{35}S]-labeled β-catenin prepared by an IVT reaction degrades robustly when incubated in *Xenopus* egg extract. Addition of MG132 (200 μM) to *Xenopus* egg extract inhibits β-catenin degradation. Mutation to alanines of the GSK3 phosphosites within β-catenin (β-catenin SA) or addition of LiCl (25 mM) (C) inhibits β-catenin degradation. [Figure adapted from (Chen et al., 2014a)].

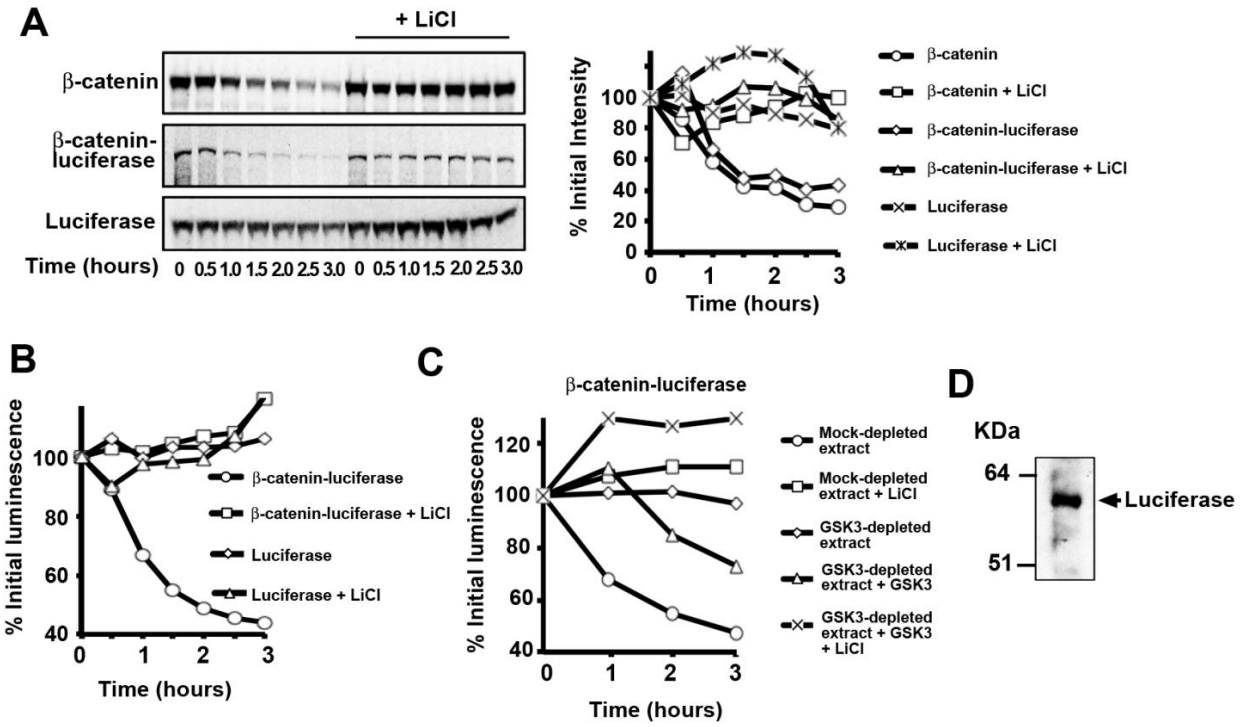

Figure 2.3. Luciferase-tagged β-catenin degrades when incubated in *Xenopus* egg extract. (A) Radiolabeled β-catenin-luciferase degrades at a rate similar to that of untagged β-catenin protein. As with the wild-type protein, addition of LiCl (25 mM) inhibits β-catenin-luciferase degradation. Luciferase alone does not noticeably turn over in *Xenopus* egg extract. Changes in luciferase activity of the β-catenin-luciferase fusion parallel the changes in its protein levels. (B) Addition of LiCl (25 mM) or depletion of GSK3 (C) inhibits β-catenin-luciferase turnover as assessed by measuring luciferase activity. (D) Immunoblotting for *in vitro* translated β-catenin-luciferase (using an anti-luciferase antibody) revealed significant amounts of free luciferase protein in certain preps that likely contributes to the higher background when compared to the radiolabeled degradation assay. [Figure from (Chen et al., 2014a)].

Representative degradation plate

	1	2	3	4	5	6	7	8
A	126920	147920	122176	148270	123269	148403	125468	149957
B	150481	124617	145384	120010	143542	119177	146333	126939
C	121823	144963	121436	143189	120606	143137	118852	145645
D	148711	121535	149984	118229	143885	120014	146080	126763
E	127243	145848	120879	144259	117748	144723	120644	149164
F	150088	119839	147795	121746	148981	121215	147723	131853

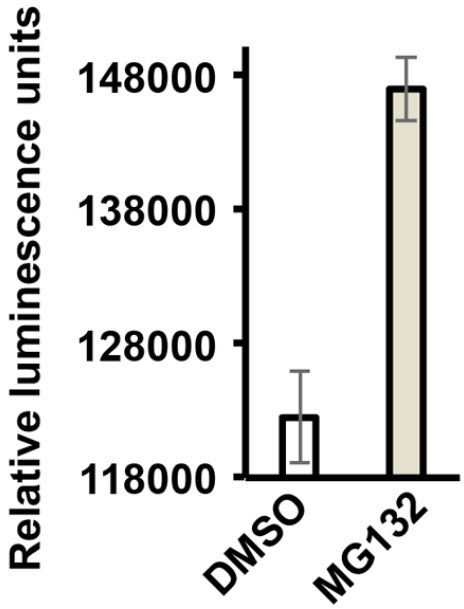

Figure 2.4. Regulated degradation of β-catenin-luciferase in *Xenopus* extract can be adapted to a high-throughput format. A representative checkerboard analysis for β-catenin-luciferase using MG132 (450 μM) as an inhibitor of β-catenin degradation. Shaded columns indicate MG132-treated wells, and lighter columns represent vehicle (DMSO)-treated wells. Quantification and Z-factor calculation result in a score of 0.3 indicating an acceptable assay for potential use in high-throughput screening. [Figure from (Chen et al., 2014a)].

CHAPTER III

IDENTIFICATION OF A NOTCH1 INTRACELLULAR DOMAIN DEGRON THAT REGULATES SIGNALING IN A PARALOG-SPECIFIC MANNER

Materials and Methods

Preparation of *Xenopus* egg extract

Meiosis II-arrested cytostatic factor (CSF) *Xenopus* egg extract was prepared as described in Chen et al. 2014 (Chen et al., 2014a). The *Xenopus* egg extract generated was used for all [S^{35}]-methionine radiolabeled degradation assays and luciferase-tagged fusion protein degradation assays. A schematic showing the general process of preparing *Xenopus* egg extract is shown in Figure 2.1 (Figure 2.1).

[S^{35}]-radiolabeled degradation assay in *Xenopus* egg extract

Xenopus egg extract was collected as previously described (Chen et al., 2014a). Either [S^{35}] radiolabeled protein or Luciferase-tagged protein was *in vitro* transcribed and translated using rabbit reticulocyte lysate using the Promega protocol (Rabbit reticulocyte lysate, nuclease treated, Promega). *In vitro* transcribed and translated protein was incubated in *Xenopus* egg extract and samples were taken at specific time points and placed into SDS-PAGE sample buffer. The [S^{35}] radiolabeled samples at each time point were subjected to SDS-PAGE and were assessed by autoradiography as previously described (Chen et al., 2014a). The difference in radioactivity over time directly corresponds to the protein levels at those specific time points. A decrease in

radioactivity is directly proportional to a decrease in protein levels also. An example of a [S^{35}]-radiolabeled degradation assay in *Xenopus* egg extract is shown in Figure 2.2 [Figure 2.2 (Chen et al., 2014a)].

Luciferase-tagged fusion protein degradation assay in *Xenopus* egg extract

Additionally, Luciferase-tagged proteins were also used to assess protein degradation. Luciferase-tagged proteins are valid tools for assessing protein degradation because Luciferase-tagged proteins still exhibits the same kinetics as untagged proteins [Figure 2.3; (Chen et al., 2014a)]. Because SDS Sample Buffer quenches the enzymatic activity of Luciferase, the Luciferase-tagged *in vitro* transcribed and translated proteins were snap-frozen in liquid nitrogen immediately for each time point. After all time points have been taken, the Luciferase-tagged samples for each time point were placed into a 96-well plate and assessed by luminescence readings taken by a luminometer as previously described (Chen et al., 2014a). Just as for the [S^{35}]-radiolabeled samples, a change in the luminescence over time is directly proportional to a change in protein levels. An example of this is shown in Figure 2.3 [Figure 2.3; (Chen et al., 2014a)]. This change can be quantified because the luminescence readout gives specific numbers for each time point. Since NICD1 has similar degradation kinetics with [S^{35}]-radiolabeling and Luciferase fusions, both of these readouts were used to assess NICD degradation. His-CSL-Flag was purified from SF21 cells as previously described (Vasquez-Del Carpio et al., 2011). GST-β-catenin (gift from Wenqing Xu) was expressed and purified from bacterial cell lysates with glutathione beads (Merck Millipore) according to the manufacturer's suggestions.

High-throughput degradation assay in *Xenopus* egg extract

One advantage that studying protein degradation in *Xenopus* egg extract has over cell culture is the ability to adapt the egg extract system to high-throughput assays, such as screening for modulators of Notch signaling (Chen et al., 2014a; Thorne et al., 2011). An example of data from the high-throughput luciferase assay is shown in figure 2.4 (Figure 2.4). We used the high-throughput assay to perform a preliminary small molecule screen for kinase inhibitors that modulate NICD turnover. Please see future directions for more about this screen.

Plasmids/Cloning

The *AIP4* and *AIP4 C830A* plasmids were generous gifts from Dr. Adriano Marchese at Loyola University-Chicago. The full-length mNotch1 and the Hes-1 reporter plasmid were gifts from Stacey Huppert. The *Fbxw7* clone came from DNASU (HsCD00404060, DNASU).

The hNICD(NT1-CT2), (NT1-CT3), (NT1-CT4), (CT2-NT1), (CT3-NT1), and (CT4-NT1) fusion plasmids were generated by NdeI digestion at an internal NdeI site at position 1016 of NICD1 and position 1034 of NICD2 (NdeI, NEB). NdeI sites were created using single site directed mutagenesis inside NICD3 and NICD4 at the conserved residues that corresponded to the NdeI sites in NICD1 and NICD2. These mutagenesis reactions did not change amino acid sequences and only changed the nucleotide sequence. The NdeI sites were near regions conserved in all NICD paralogs. The two distinct regions of each individual NICD paralog were then ligated together

using traditional ligation protocols (T4 DNA Ligase, NEB) and sequenced to confirm correct insertions.

Other DNA plasmids were generated by traditional PCR-based subcloning methods. Standard PCR amplification of template DNA containing the 8 base-cutting restriction endonuclease FseI at the 5’ end and the 8 base-cutting restriction endonuclease AscI at the 3’ end (FseI, AscI, both from NEB) was performed to generate PCR amplicons. These PCR amplicons were then ligated into their respective vectors using standard ligation protocols (T4 DNA Ligase, NEB). The complete list of primers used for this subcloning is listed in Table 2.1.

Primer Name	Primer sequence
hNICD1 FseI Start extra A Fwd	CGCGGGCCGGCCAATGGTGCTGCTGTCCCGCAAGCGC
hNICD1 Stop AscI Rev	CAATGGCGCGCCTTACTTGAAGGCCTCCGGAATGCGGGC
hNICD1 no stop AscI R	CAATGGCGCGCCCTTGAAGGCCTCCGGAATGCG
hNICD2 FseI extra A F	CGGCCGGCCAATGGTAATCATGGCAAAACGAAAGCGT
hNICD2 AscI R	CAATGGCGCGCCTCACGCATAAACCTGCATGTT
hNICD2 no stop R	CAATGGCGCGCCCGCATAAACCTGCATGTTGTT
hNICD3 FseI extra A F	CGGCCGGCCAATGGTCATGGTGGCCCGGCGCAAG
hNICD3 AscI R	CAATGGCGCGCCTCAGGCCAACACTTGCCTCTT
hNICD3 no stop R	CAATGGCGCGCCGGCCAACACTTGCCTCTTGGG
hNICD4 FseI extra A F	CGGCCGGCCAATGGTCCTCCAGCTCATCCGGCGT
hNICD4 AscI R	CAATGGCGCGCCCTATTTTTTACCCTCTCCTCCTTGGTT
hNICD4 no stop R	CAATGGCGCGCCTTTTTTACCCTCTCCTCCTTGGTT
hNICD1 ΔPEST R	CAATGGCGCGCCCTAGGAGCTGTCCAGCAGGCAGCC
hNICD1 ΔPEST no stop R	CAATGGCGCGCCGGAGCTGTCCAGCAGGCAGCC
hNICD1 2342* AscI R	CAATGGCGCGCCTCAGGGGGCCTGTGTGCTCAG
hNICD1 2342* no stop R	CAATGGCGCGCCGGGGGCCTGTGTGCTCAGGGG
hNICD1 2399* AscI R	CAATGGCGCGCCTCACAGGTTCTGCTGCTGCATCTGTAA
hNICD1 2399* no stop R	CAATGGCGCGCCCAGGTTCTGCTGCTGCATCTGTAACAG
hNICD1 2493* AscI R	CAATGGCGCGCCTCAGGAGTAGCTGTGCTGCGAGGGGGGCGTCAG
hNICD1 2493* no stop R	CAATGGCGCGCCGGAGTAGCTGTGCTGCGAGGGGGGCGTCAGGAA
hNICD1 2518* AscI R	CAATGGCGCGCCTCAAGGAACACGGGACGGGGTGAGGAAGGG
hNICD1 2518* no stop R	CAATGGCGCGCCAGGAACACGGGACGGGGTGAGGAAGGGGTG
hNICD1- NICD2 1 Fwd	CGGCGGGAGCCCCTCGGCCAGGATGCTGTGGGGCTG
hNICD1- NICD2 1 Rev	CAGCCCCACAGCATCCTGGCCGAGGGGCTCCCGCCG
hNICD1- NICD2 2 Fwd	GACGTCAATGTCCGCGGGCCAGATGGCTGCACCCCA
hNICD1- NICD2 2 Rev	TGGGGTGCAGCCATCTGGCCCGCGGACATTGACGTC
hNICD1- NICD2 3 Fwd	ACAGACCGCACGGGCGAGATGGCCCTGCACCTTGCA
hNICD1- NICD2 3 Rev	TGCAAGGTGCAGGGCCATCTCGCCCGTGCGGTCTGT
hNICD1 NT-10aa Fwd	GAATGGCCGGCCAATGCATGGCCAGCTCTGGTTCCCT
hNICD1 NT50 Rev	ATTGGGCGCGCCACCGTCTGAAGCGTTCTTCAG
hNICD2 NT51 Rev	ATTGGGCGCGCCAGCTTCTGAGACTTGCAC
hNICD3 NT50 Rev	ATTGGGCGCGCCCTCACCCTTGGCCATGTT
hNICD4 NT50 Rev	ATTGGGCGCGCCTGCCTTTGGCTTCAGTGC
mNICD1 FseI F	CGGCCGGCCGATGGTGCTGCTGTCCCGCAAGCGC

mNICD1 AscI R	CGGCGCGCCCTAGTTTATTTTCTTGGAACAG
mNICD4 FseI F	GAATGGCCGGCCAATGGTCCTCCAGCTCATTCGGCGA
mNICD4 AscI R	CAATGGCGCGCCCTAGTTCAGATTTCTTACAAC
dNICD FseI Fwd	TATAGGCCGGCCAATGGTGAAATACGTAATTACT
dNICD AscI Rev	ATATGGCGCGCCTCAAATGTAGATGGCCTC
Fbxw7 FseI extra A F	CGGCCGGCCAATGAATCAGGAACTGCTCTCT
Fbxw7 ΔFbox extra A	CGGCCGGCCAATGAACAAATTACGGCAAAGT
Fbxw7 AscI R	CAATGGCGCGCCTCACTTCATGTCCACATCAAA
NICD1 CT NdeI F	GGATCATATGGACCGCCTGCCGCGCGACATC
NICD2 CT NdeI F	AGACCATATGGATCGTCTTCCCCGGGATGTG
NICD3 CT NdeI F	AGACCATATGGACAGGCTGCCGCGGGACGTA
NICD4 CT NdeI F	AGACCATATGGGGCTAGCGCCGGCGGACGTC
NICD3 Add NdeI F	AACCGTGAGATCACCGACCATATGGACAGGCTGCCGC GGGAC
NICD3 Add NdeI R	GTCCCGCGGCAGCCTGTCCATATGGTCGGTGATCTCAC GGTT
NICD4 Add NdeI F	GCCCGAGAGCTGCGGGACCATATGGGGCTAGCGCCGG CGGACGT
NICD4 Add NdeI R	ACGTCCGCCGGCGCTAGCCCCATATGGTCCCGCAGCT CTCGGGC

Table 2.1. List of all primers used in this thesis. All sequences are read from left to right and 5' to 3'.

Site-directed Mutagenesis

Another large portion of the DNA plasmids generated in this study were generated through either single site directed mutagenesis or multi site directed mutagenesis. Multi site directed mutagenesis was performed according to original Agilent Technologies protocol (QuikChange Lightning Multi Site Directed Mutagenesis Kit, Agilent Technologies) and using the Agilent Technologies PCR program. Single site directed mutagenesis was performed using the following protocol adapted from Agilent Technologies:

Step 1

PCR reaction containing:

35.4 µL H_20

5.0 µL 10X PFU Turbo Polymerase Buffer

5.0 µL 2.5 mM dNTPs

1.0 µL template DNA (30 ng/µL)

1.3 µL Forward primer (100 ng/µL)

1.3 µL Reverse primer (100 ng/µL)

1.0 µL PFU turbo (Agilent Technologies)

for a total volume of 50 µL

Step 2

PCR the reaction mix with the following program:

Lid 95°C

1) 95°C 50 seconds (can use 98°C if needed)

2) 95°C 50 seconds (can use 98°C if needed)

3) 55°C 50 seconds

4) 68°C 24 minutes

Repeat Steps 2-4 17 times

5) 68°C 24 minutes

6) Hold at 12°C indefinitely

Step 3

Add 1 µL of DpnI (DpnI, NEB) into each PCR reaction mix and incubate at 37°C for 1 hour. DpnI digests methylated DNA so should digest template DNA but not amplified DNA.

Step 3

Transform into bacteria (10 µL of DpnI-digested PCR product into 100 µL of DH5α competent cells) and plate onto agar plates containing antibiotic for plasmid.

Step 4

Pick bacterial colonies and miniprep to isolate plasmid DNA.

Step 5

Sequence colonies to identify the correctly mutagenized clones.

The complete list of mutagenesis primers used in this study is provided in Table 2.2.

Primer Name	Primer Sequence
hNICD1 S2538A F	ACTGGTCCGAGGGCGTCTCCGCCCCTCCCACCAGCATGCAGTC
hNICD1 S2538A R	GACTGCATGCTGGTGGGAGGGGCGGAGACGCCCTCGGACCAGT
hNICD1 S2514A/S2517A F	AGCACCCCTTCCTCACCCCGGCCCCTGAGGCCCCTGACCAGTGGTCCAG CTC
hNICD1 S2514A/S2517A R	GAGCTGGACCACTGGTCAGGGGCCTCAGGGGCCGGGGTGAGGAAGGG GTGCT
hNICD1 L2X F	CATCGATGGCCGGCCAATGGTGCTGTCCCGCAAGCGCCGGCGGC
hNICD1 L2X R	GCCGCCGGCGCTTGCGGGACAGCACCATTGGCCGGCCATCGATG
hNICD1 S4M F	GGCCGGCCAATGGTGCTGCTGATGCGCAAGCGCCGGCGGCAGCATG
hNICD1 S4M R	CATGCTGCCGCCGGCGCTTGCGCATCAGCAGCACCATTGGCCGGCC
hNICD1 R5A F	CGGCCAATGGTGCTGCTGTCCGCCAAGCGCCGGCGGCAGCATGGCC
hNICD1 R5A R	GGCCATGCTGCCGCCGGCGCTTGGCGGACAGCAGCACCATTGGCCG
hNICD1 Q10K F	GCTGTCCCGCAAGCGCCGGCGGAAGCATGGCCAGCTCTGGTTCCCTG
hNICD1 Q10K R	CAGGGAACCAGAGCTGGCCATGCTTCCGCCGGCGCTTGCGGGACAGC
hNICD1 Q13S F	CAAGCGCCGGCGGCAGCATGGCTCGCTCTGGTTCCCTGAGGGCTTC
hNICD1 Q13S R	GAAGCCCTCAGGGAACCAGAGCGAGCCATGCTGCCGCCGGCGCTTG
hNICD1 Bas Reg 1 F	GCTCTGGTTCCCTGAGGGCTTCACTCTTCGCCGAGAGGCCAGCAAGAAG AAGCGG
hNICD1 Bas Reg 1 R	CCGCTTCTTCTTGCTGGCCTCTCGGCGAAGAGTGAAGCCCTCAGGGAAC CAGAGC
hNICD1 Bas Reg 2 F	GAGGGCTTCACTCTTCGCCGAGATGCCAGCAATCACAAGCGGCGGGAG CCCCTCGGC
hNICD1 Bas Reg 2 R	GCCGAGGGGCTCCCGCCGCTTGTGATTGCTGGCATCTCGGCGAAGAGT GAAGCCCTC
hNICD1 L2X/S4M F	CATCGATGGCCGGCCAATGGTGCTGATGCGCAAGCGCCGGCGGCAGC
hNICD1 L2X/S4M R	GCTGCCGCCGGCGCTTGCGCATCAGCACCATTGGCCGGCCATCGATG
hNICD1 L2X/S4M R5A F	CATCGATGGCCGGCCAATGGTGCTGATGGCCAAGCGCCGGCGGCAGCA TG
hNICD1 L2X/S4M R5A R	CATGCTGCCGCCGGCGCTTGGCCATCAGCACCATTGGCCGGCCATCGAT G
hNICD1 L2X/S4M Q10K F	CTGATGCGCAAGCGCCGGCGGAAGCATGGCCAGCTCTGGTTCCC
hNICD1 L2X/S4M Q10K R	GGGAACCAGAGCTGGCCATGCTTCCGCCGGCGCTTGCGCATCAG
hNICD1 R1758S F	CGGCCAATGGTGCTGCTGTCCTCCAAGCGCCGGCGGCAGCATGGCC
hNICD1 R1758S R	GGCCATGCTGCCGCCGGCGCTTGGAGGACAGCAGCACCATTGGCCG
hNICD1 R1761W F	GTGCTGCTGTCCCGCAAGCGCTGGCGGCAGCATGGCCAGCTCTGC
hNICD1 R1761W R	GCAGAGCTGGCCATGCTGCCGCCAGCGCTTGCGGGACAGCAGCAC
hNICD1 S1776C F	GTTCCCTGAGGGCTTCAAAGTGTGTGAGGCCAGCAAGAAGAAGCGG
hNICD1 S1776C R	CCGCTTCTTCTTGCTGGCCTCACACACTTTGAAGCCCTCAGGGAAC
hNICD1 R1783W F	GTCTGAGGCCAGCAAGAAGAAGTGGCGGGAGCCCCTCGGCGAGGAC
hNICD1 R1783W R	GTCCTCGCCGAGGGGCTCCCGCCACTTCTTCTTGCTGGCCTCAGAC

hNICD1 R1784L F	GAGGCCAGCAAGAAGAAGCGGCTGGAGCCCCTCGGCGAGGACTCC
hNICD1 R1784L R	GGAGTCCTCGCCGAGGGGCTCCAGCCGCTTCTTCTTGCTGGCCTC
hNICD1 P1796H F	GAGGACTCCGTGGGCCTCAAGCACCTGAAGAACGCTTCAGACGGT
hNICD1 P1796H R	ACCGTCTGAAGCGTTCTTCAGGTGCTTGAGGCCCACGGAGTCCTC
hNICD1 WAAAAP F	CCTGAGTCCCCTGACCAGTGGGCCGCCGCGGCCCCGCATTCCAACGTCTCCGAC
hNICD1 WAAAAP R	GTCGGAGACGTTGGAATGCGGGGCCGCGGCGGCCCACTGGTCAGGGGACTCAGG
hNICD1 1771-1774A F	CAGCATGGCCAGCTCTGGTTCGCTGCGGCCGCCAAAGTGTCTGAGGCCAGCAAG
hNICD1 1771-1774A R	CTTGCTGGCCTCAGACACTTTGGCGGCCGCAGCGAACCAGAGCTGGCCATGCTG
hNICD1 D1839N/R1841Q F	CCTGACCTGGACGACCAGACAAACCACCAGCAGTGGACTCAGCAGCACCTG
hNICD1 D1839N/R1841Q R	CAGGTGCTGCTGAGTCCACTGCTGGTGGTTTGTCTGGTCGTCCAGGTCAGG
hNICD2 NT2-5 F	CATCGATGGCCGGCCAATGGTACTGCTGTCCCGCAAACGAAAGCGTAAGCATGGC
hNICD2 NT2-5 R	GCCATGCTTACGCTTTCGTTTGCGGGACAGCAGTACCATTGGCCGGCCATCGATG
hNICD2 NT2-5 K9Q F	CTGTCCCGCAAACGAAAGCGTCAGCATGGCTCTCTCTGGCTGCCTG
hNICD2 NT2-5 K9Q R	CAGGCAGCCAGAGAGAGCCATGCTGACGCTTTCGTTTGCGGGACAG
hNICD2 N1 Bas Reg 1 F	CTCTGGCTGCCTGAAGGTTTCAAAGTGTCTGAGGATGCAAGCAATCACAAGCGT
hNICD2 N1 Bas Reg 1 R	ACGCTTGTGATTGCTTGCATCCTCAGACACTTTGAAACCTTCAGGCAGCCAGAG
hNICD2 N1 Bas Reg 2 F	GAAGGTTTCAAAGTGTCTGAGGCCAGCAAGAAGAAGCGTCGTGAGCCAGTGGG
hNICD2 N1 Bas Reg 2 R	CCCACTGGCTCACGACGCTTCTTCTTGCTGGCCTCAGACACTTTGAAACCTTC
mN1 FL L1744X/S1747M F	CTTTGTGGGCTGTGGGGTGCTGATGCGCAAGCGCCGGCGGCAGCA
mN1 FL L1744X/S1747M R	TGCTGCCGCCGGCGCTTGCGCATCAGCACCCCACAGCCCACAAAG
mN1 FL L1744X/S1747M/Q1753K F	CTGATGCGCAAGCGCCGGCGGAAGCATGGCCAGCTCTGGTTCCCT
mN1 FL L1744X/S1747M/Q1753K R	AGGGAACCAGAGCTGGCCATGCTTCCGCCGGCGCTTGCGCATCAG
mN1 FL 2467* F	CCTTCCCAGCACAGTTACTCCTAGTCCCCTGTGGACAACACCCCC
mN1 FL 2467* R	GGGGGTGTTGTCCACAGGGGACTAGGAGTAACTGTGCTGGGAAGG

Table 2.2. List of all primers used for mutagenesis. Sequences are read left to right and 5' to 3'.

Gene	Vector	Collection #
hNICD1	CS2	EL1868
hNICD1-Luciferase	CS2	EL1869
hNICD1-MYC	CS2	EL1870
hNICD1-GFP	CS2	EL1871
hNICD2	CS2	EL1872
hNICD2-Luciferase	CS2	EL1873
hNICD2-MYC	CS2	EL1874
hNICD3	CS2	EL1875
hNICD3-Luciferase	CS2	EL1876
hNICD3-MYC	CS2	EL1877
hNICD4	CS2	EL1878
hNICD4-Luciferase	CS2	EL1879
hNICD4-MYC	CS2	EL1880
$hNICD1^{S3A}$	CS2	EL1881
$hNICD1^{S3A}$-Luciferase	CS2	EL1882
$hNICD1^{S3A}$-MYC	CS2	EL1883
$hNICD1^{S2493\Delta}$	CS2	EL1891
$hNICD1^{S2493\Delta}$ -Luciferase	CS2	EL1892
$hNICD1^{S2493\Delta}$ -MYC	CS2	EL1893
hNICD1/2(NT1-CT2)	CS2	EL1896
hNICD1/2(NT1-CT2)-Luciferase	CS2	EL1897
hNICD1/3(NT1-CT3)	CS2	EL1898
hNICD1/3(NT1-CT3)-Luciferase	CS2	EL1899
hNICD1/4(NT1-CT4)	CS2	EL1900

hNICD1/4(NT1-CT4)-Luciferase	CS2	EL1901
hNICD2/1(NT2-CT1)	CS2	EL1902
hNICD2/1(NT2-CT1)-Luciferase	CS2	EL1903
hNICD3/1(NT3-CT1)	CS2	EL1904
hNICD3/1(NT3-CT1)-Luciferase	CS2	EL1905
hNICD4/1(NT4-CT1)	CS2	EL1906
hNICD4/1(NT4-CT1)-Luciferase	CS2	EL1907
hNICD1/2(NT35)	CS2	EL1908
hNICD1/2(NT35)-Luciferase	CS2	EL1909
hNICD1/2(NT125)	CS2	EL1910
hNICD1/2(NT125)-Luciferase	CS2	EL1911
hNICD1/2(NT176)	CS2	EL1912
hNICD1/2(NT176)-Luciferase	CS2	EL1913
$hNICD1^{L1755\Delta}$	CS2	EL1914
$hNICD1^{L1755\Delta}$-Luciferase	CS2	EL1915
$hNICD1^{L1755\Delta}$-MYC	CS2	EL1916
$hNICD1^{S1757M}$	CS2	EL1917
$hNICD1^{S1757M}$-MYC	CS2	EL1918
$hNICD1^{Q1763K}$	CS2	EL1920
$hNICD1^{Q1763S}$	CS2	EL1921
$hNICD1^{KRR}$	CS2	EL1922
$hNICD1^{NT\Delta10}$	CS2	EL1851
$hNICD1^{NT\Delta10}$-Luciferase	CS2	EL1852
$hNICD1^{NT\Delta10}$-MYC	CS2	EL1923
$hNICD1^{L1755\Delta}$-GFP	CS2	EL1925

hNICD1^{S1757M}-GFP	CS2	EL1926
hNICD1$^{L1755\Delta / S1757M}$-GFP	CS2	EL1927
hNICD1^{LSQ}	CS2	EL1931
hNICD1^{LSQ}-MYC	CS2	EL1932
hNICD1(1-50)-Luciferase	CS2	EL1933
hNICD1(1-50)-GFP	CS2	EL1934
hNICD1(1-50)$^{L1755\Delta}$-Luciferase	CS2	EL1935
hNICD1(1-50)^{S1757M}-Luciferase	CS2	EL1937
hNICD1(1-50)^{S1757M}-GFP	CS2	EL1938
hNICD1(1-50)$^{L1755\Delta / S1757M}$-Luciferase	CS2	EL1947
hNICD1(1-50)$^{L1755\Delta / S1757M}$-GFP	CS2	EL1948
hNICD1(1-50)^{LS}-Luciferase	CS2	EL1950
hNICD1(1-50)^{LS}-GFP	CS2	EL1951
hNICD2(1-50)-GFP	CS2	EL1952
hNICD2(1-50)-Luciferase	CS2	EL1953
hNICD3(1-50)-GFP	CS2	EL1954
hNICD3(1-50)-Luciferase	CS2	EL1955
hNICD4(1-50)-GFP	CS2	EL1956
hNICD4(1-50)-Luciferase	CS2	EL1957
hNICD1$^{NT\Delta10 / S2493\Delta}$-MYC	CS2	EL1958
hNICD1$^{NT\Delta10 / S2493\Delta}$	CS2	EL1959
hNICD1$^{LSQ / S2493\Delta}$	CS2	EL1962
hNICD1$^{LSQ / S2493\Delta}$-MYC	CS2	EL1963
hNICD1^{R1758S}	CS2	EL1964
hNICD1^{R1758S}-MYC	CS2	EL1965

hNICD1^{R1761W}	CS2	EL1966
hNICD1^{R1761W}-MYC	CS2	EL1967
hNICD1^{S1776C}	CS2	EL1968
hNICD1^{S1776C}-MYC	CS2	EL1969
hNICD1^{R1783W}	CS2	EL1970
hNICD1^{R1783W}-MYC	CS2	EL1971
hNICD1^{R1784L}	CS2	EL1972
hNICD1^{R1784L}-MYC	CS2	EL1973
hNICD1$^{LSQ / S2A}$	CS2	EL1976
hNICD1$^{LSQ / S2A}$	CS2	EL1977
hNICD1$^{NTΔ10 / S2A}$	CS2	EL1978
hNICD1$^{NTΔ10 / S2A}$-MYC	CS2	EL1979
hNICD1^{W4AP}	CS2	EL1980
hNICD1^{W4AP}-MYC	CS2	EL1981
hNICD1$^{S2A / W4AP}$	CS2	EL1982
hNICD1$^{S2A / W4AP}$-MYC	CS2	EL1983
hNICD1$^{NTΔ10 / W4AP}$	CS2	EL1984
hNICD1$^{NTΔ10 / W4AP}$-MYC	CS2	EL1985
hNICD1$^{NTΔ10 / S2A / W4AP}$	CS2	EL1986
hNICD1$^{NTΔ10 / S2A / W4AP}$-MYC	CS2	EL1987
hNICD1$^{1771-74A}$	CS2	EL1988
hNICD1$^{1771-74A}$-MYC	CS2	EL1989
hNICD1/2^{KRR}-Luciferase	CS2	EL1994
hNICD1/2$^{NT10 / KRR}$-Luciferase	CS2	EL1996
hNICD1$^{NTΔ10 / 1771-74A}$-MYC	CS2	EL1997

hNICD1$^{1771\text{-}74A\ /\ S2493\Delta}$-MYC	CS2	EL1998
hNICD$^{NT\Delta10\ /\ 1771\text{-}74A\ /\ S2493\Delta}$-MYC	CS2	EL1999
mNotch1$^{S2467\Delta}$	pcDNA3.1	EL2001
mNotch1^{LSQ}	pcDNA3.1	EL2003
mNotch1$^{LSQ\ /\ S2467\Delta}$	pcDNA3.1	EL2004
mNICD1	CS2	EL2007
mNICD4	CS2	EL2008
dNICD1	CS2	EL2009
Fbxw7DN	CS2	EL2013
Fbxw7	CS2	EL2014
mNotch1	pcDNA3.1	Gift from Stacey Huppert
FLAG-Itch	pCMV-10	Gift from Adriano Marchese
FLAG-Itch^{C380A}	pCMV-10	Gift from Adriano Marchese
Renilla luciferase	CS2	EL986
Hes1-Luciferase	pGL2-Basic	Gift from Stacey Huppert
GST-β-catenin	pGEX	Gift from Wenqing Xu
GST	pGEX	GE Healthcare Life Sciences

Table 2.3 List of all plasmids generated in this thesis

siRNA constructs

Two distinct siRNA sequences for RBPj knockdown (Dharmacon) were transfected into HEK293 cells. The sequences are as follows:
RBPj 1 sense 5'-GGAAAUAGUGACCAAGAAAUGUU-3'
RBPj 1 antisense 5'-CAUUUCUUGGUCACUAUUUCCUU-3'
RBPj 2 sense 5'-GGUGAGUGCUUCAGUUAUAGUUU-3'
RBPj 2 antisense 5'-ACUAUAACUGAAGCACUCACCUU-3'

Mammalian Cell Culture

HEK293 cells were maintained in Dulbecco's Modified Eagle Medium (Corning) supplemented with 1% L-Glutamine, 10% (v/v) FBS, 100 µg/ml streptomycin, and 100 U/ml penicillin at 37^{o}C with 5% CO_2. Transient transfections were performed using Fugene HD (Promega) according to the manufacturer's suggestions. For cycloheximide (Sigma-Aldrich) chase experiments, media supplemented with 100 µg/ml cyclohexamide was added to cells at the 0 minute time point. Cell were then incubated in the presence of cyclohexamide for the duration of the experiment. For microscopy, cell were plated on glass bottom MatTek dishes (MatTek Corporation) for imaging.

Hes-1 Reporter Assays

HEK293 cells were transiently transfected with 1 µg Hes1-Luciferase, 0.5 µg Renilla luciferase, and 1 µg of the indicated Notch construct. Luciferase and Renilla luciferase activities were assessed after 24 hrs using the Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's instructions. Experiments were performed in triplicate and replicated at least two times, and significance was determined using a two-tailed student t-test. The luciferase levels are read on a luminometer (Optima Fluostar, as described by Promega (Dual-Glo Luciferase Assay

System, Promega). Renilla is used as a control for background luciferase and for cell death. The readings are normalized to wild-type NICD or full-length Notch1.

Zebrafish Somite Formation Assay

mRNAs were synthesized using the SP6 or T7 mRNA Message Machine (Ambion). Zygotes were either injected with 25 pg/nl or 100 pg/nl mRNA mixed with phenol red using the MPPI-2 injection system (Applied Scientific Instruments). Injected embryos were incubated at 28^{o}C until the 10-13 somite stage and scored for proper somitogenesis using a Zeiss stemi 2000-CS scope. Live embryos were embedded in 0.8% agarose and images taken with an Olympus DP72 camera at 4X magnification. For immunostaining, embryos were permeabilized with 10 µg/ml Proteinase K and stained with α-EphA4 (Tyr-602) (ECM Biosciences) overnight at 4^{0}C. Primary antibodies were then detected with Alexa-488 secondary antibodies (Life Technologies) and imaged on a Nikon Eclipse 80I equipped with a photometrics CoolSNAP ES camera. Significance was assessed using Fisher's exact test.

Immunoblot analysis

Cells were incubated in non-denaturing lysis buffer (50 mM Tris-HCl pH7.4, 300 mM NaCl, 5 mM EDTA, 1% (w/v) Trition X-100, 1 mM PMSF) for 30 min on ice. Lysates were rigorously vortexed once at 15 min during the incubation. At the end of the incubation, lysates were cleared by centrifugation. Total protein was assessed using the Bio-Rad protein assay dye reagent (Bio-Rad). SDS-PAGE and immunoblotting were performed using standard techinques. To assesses changes in

steady-state protein levels, 1 ug of each DNA construct was transfected into an equivalent number of HEK293 cells. 48 hr post-transfection, cells were lysed and 50 µg of total protein processed for SDS-PAGE and immunoblottting. The following antibodies were used: α-Fbxw7 (Bethyl Laboratories, Cat# A301-720A), α-MYC (9E10), α-Flag (Sigma-Aldrich), α-β-Tubulin (Clone E7, Developmental Studies Hybridoma Bank, University of Iowa, IA). Secondary antibodies conjugated to horseradish peroxidase were purchased from Bethyl Laboratories. Blots were analyzed using ImageJ software.

Live-Cell Imaging

MatTek dishes were maintained at 37°C by heated stage (Warner Instruments). Single-plane confocal videos were taken using a Yokogawa QLC-100/CSU-10 spinning disk head (Visitec assembled by Vashaw) attached to a Nikon TE2000E microscope using a CFI PLAN APO VC 100× oil lens, NA 1.4, with or without 1.5× intermediate magnification, and a back-illuminated EM-CCD camera Cascade 512B (Photometrics) driven by IPLab software (Scanalytics). A krypton-argon laser (75 mW 488; Melles Griot) with AOTF was used for color excitation. Custom double dichroic mirror and filters (Chroma) in a filter wheel (Ludl) were used in the emission light path. Intensity in live-cells expressing various GFP constructs was measured using ImageJ software. Cells of interest were outlined with a selection tool, and measurements were set (area, integrated density, and mean gray value). Data points are plotted as the percent of initial intensity.

Introduction

The Notch pathway is a highly conserved, metazoan signaling pathway critical for organismal development (Koch et al., 2013; Kopan and Ilagan, 2009). Improper regulation of the Notch pathway has been shown to contribute to numerous human diseases including cancer (Koch and Radtke, 2010; Louvi and Artavanis-Tsakonas, 2012; Ntziachristos et al., 2014; Ranganathan et al., 2011b; South et al., 2012). The Notch pathway communicates transcriptional decisions between adjacent cells through the direct interaction of a Delta/Serrate/Lag-2 (DSL) type-1 transmembrane ligand on the signaling cell and a Notch type-1 transmembrane receptor on a receiving cell (D'Souza et al., 2010). The interaction between ligand and receptor promotes a series of proteolytic events resulting in liberation of the Notch intracellular domain (NICD) from its membrane tether. Once NICD is liberated into the cytoplasm, it enters the nucleus where it forms a complex consisting of CSL (CBF1/RBPjk/Su(H)/Lag-1), MAML (Mastermind-like), and CoA (coactivators) (Kovall and Blacklow, 2010). Formation of this multimeric complex drives transcription of Notch target genes to promote differentiation, stem cell maintenance, proliferation, or apoptosis. In the prevailing model, transcriptional termination is mediated, in part, by the E3 ubiquitin ligase complex, SCF^{Fbxw7}, which promotes its ubiquitin-mediated degradation (Moretti and Brou, 2013).

Much of our understanding of Notch pathway regulation comes from studies of Notch mutations in human leukemias. Notch1 is a major driver of T-Cell Acute Lymphoblastic Leukemia (T-ALL), and mutations of Notch1 in T-ALL have been studied in detail (Ellisen et al., 1991; South et al., 2012; Sulis et al., 2008; Weng et al., 2004).

Most mutations occur in the heterodimerization domain (HD) that lead to constitutive cleavage and liberation of NICD1 from the holoprotein as well as mutations that truncate the C-terminal PEST domain, thereby leading to increased NICD1 stability. Enhanced stability of these truncated forms of NICD1 is due to loss of the Fbxw7 recognition sequence, LTPSPE, and a poorly characterized phosphoregulated domain (WSSSSP) (Chiang et al., 2006; Ellisen et al., 1991; Sulis et al., 2008; Weng et al., 2004). The importance of NICD1 turnover in limiting Notch signaling is further highlighted by findings, which implicate loss-of-function mutations in Fbxw7 to drive T-ALL (Malyukova et al., 2007; O'Neil et al., 2007; Thompson et al., 2007).

Multiple studies indicate that SCF^{Fbxw7}-mediated turnover of NICD1 is not the sole mechanism for regulating its steady-state levels, and, thus, its transcriptional activity. Mutants of NICD1 that disrupt its interaction with SCF^{Fbxw7} are still ubiquitinated and degraded (O'Neil et al., 2007; Thompson et al., 2007). Similarly, NICD1 is degraded in *Fbxw7*$^{-/-}$ mouse embryonic fibroblasts (Tsunematsu et al., 2004). Non-SCF^{Fbxw7}-mediated degradation has been shown to occur via the HECT type E3 ligase, Itch, although this mechanism remains unclear (Moretti and Brou, 2013).

We have previously used the *Xenopus* egg extract system to study the cytoplasmic regulation of β-catenin turnover (Chen et al., 2014a; Lee et al., 2001; Salic et al., 2000a). We now apply the *Xenopus* egg extract system to study cytoplasmic human NICD1 (hNICD1) protein turnover and have identified a novel hNICD1-specific degron at its N-terminal end distinct from its C-terminal PEST domain degradation elements. We show that mutations in this degron stabilize hNICD1 and potentiate hNICD1 activity *in vitro* and *in vivo*. Degradation mediated by the N-terminal degron is

inhibited by hNICD1 binding to CSL. Finally, we present evidence that mutations within the N-terminal degron may function as a driver of Notch1 signaling in human cancers.

Results

NICD1 degrades robustly in *Xenopus* egg extract

To recapitulate cytoplasmic NICD turnover, we utilized the *Xenopus* egg extract system that has been previously shown to support β-catenin degradation via components of the Wnt pathway (Chen et al., 2014a; Lee et al., 2003; Salic et al., 2000b). We found that radiolabeled *in vitro*-translated (IVT) hNICD1 degrades robustly in *Xenopus* egg extract. The addition of MG132, a proteasome inhibitor, inhibited the degradation of both hNICD1 and β-catenin (Figure 3.1A). Recombinant GST-β-catenin, however, potently inhibited the turnover of radiolabeled IVT β-catenin but had no effect on the turnover of hNICD1 (Figure 3.1B). β-catenin degradation by Wnt pathway components requires its phosphorylation by Glycogen synthase kinase 3 (GSK3), and addition of inhibitors of GSK3 (e.g. LiCl or BIO) blocks β-catenin turnover in *Xenopus* egg extract (Figure 3.2; (Chen et al., 2014a; Salic et al., 2000b)); in contrast, addition of LiCl or BIO to extract does not observably decrease the half-life of hNICD1 (Figure 3.2). These results show that hNICD1 degradation in *Xenopus* egg extract occurs in a proteasome-dependent manner distinct from that of β-catenin.

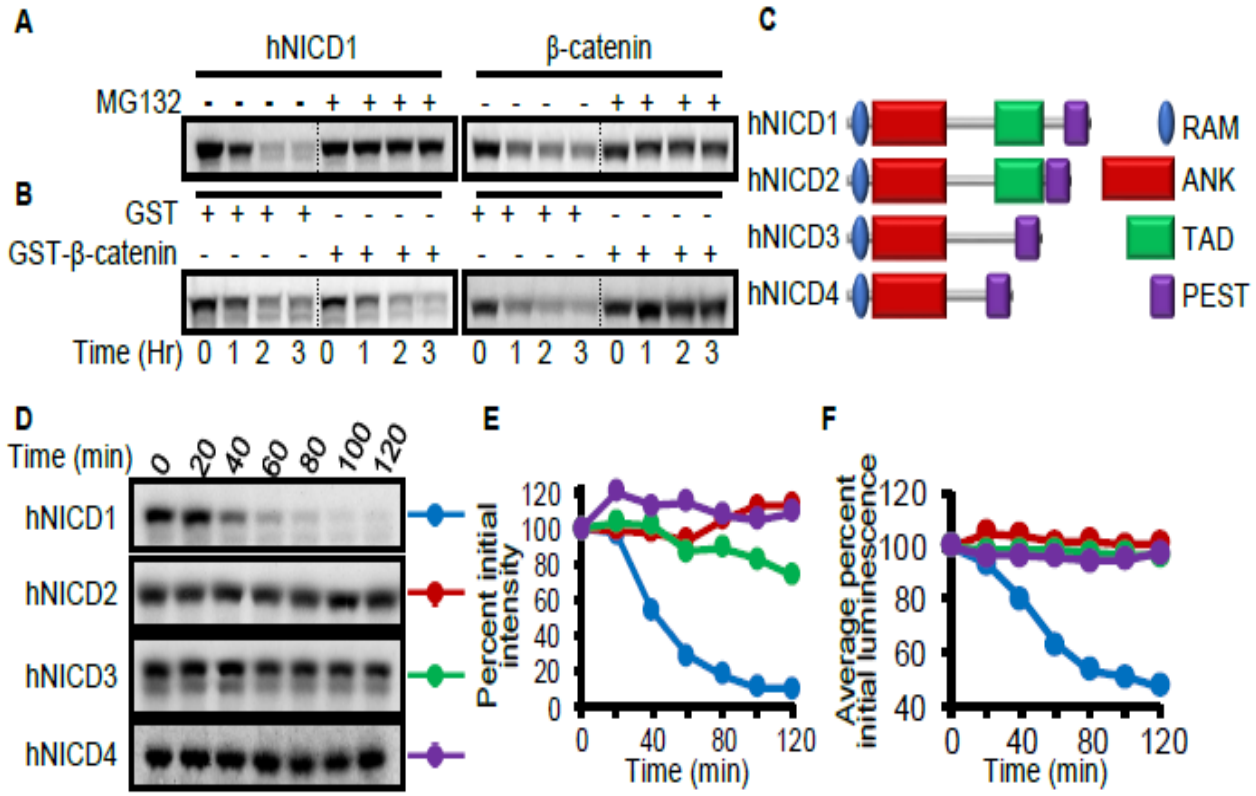

Figure 3.1. hNICD1 is degraded in *Xenopus* egg extract. (A) *In vitro*-translated [^{35}S]hNICD1 and [^{35}S]β-catenin were incubated in *Xenopus* egg extract in the presence of DMSO (-) or MG132 (+). Samples were removed at indicated times and analyzed by SDS-PAGE/autoradiography. (B) Same as in (A) except extract was supplemented with MG132 and recombinant GST or GST-β-catenin. (C) Schematic of the four human NICD Notch paralogs. RAM is RBP-Jκ Associated Module domain, ANK is Ankyrin repeats domain, TAD is Transcriptional Activation Domain, and PEST is Proline, Glutamic acid, Serine, Threonine rich region. (D) Radiolabeled hNICD paralogs were incubated in extract. Samples were removed at the indicated times for analysis by SDS-PAGE/autoradiography. In contrast to hNICD1, hNICD2, 3, and 4 did not noticeably degrade in *Xenopus* egg extract. (E) Graph of densitometry measurements from (D). (F) Degradation of hNICD Luciferase fusions (hNICD1, 2, 3, and 4-Luc) parallels degradation of their radiolabeled, untagged versions in *Xenopus* egg extract. *In-vitro* translated NICD Luciferase fusions were incubated in extract and samples removed at the indicated times for luminescence measurement. Graph represents mean Luciferase signal for two independent experiments (performed in triplicate) normalized to the value of the initial time point (100%).

NICD degradation within *Xenopus* egg extract is restricted to the NICD1 paralog

To determine whether turnover of NICD is paralog-specific, we tested the capacity of extract to degrade human NICD2, NICD3, and NICD4 in *Xenopus* egg extract (Figure 3.1C). In contrast to hNICD1, we find that hNICD2, hNICD3 and hNICD4 are stable throughout the time course of the experiment (Figure 3.1D-E). In order to more readily quantify the degradation of NICD proteins in *Xenopus* egg extract, we generated hNICD paralogs fused at their C-terminal ends to firefly Luciferase (Luciferase), normally a stable protein in extract (Chen et al., 2014a). We find that the hNICD1 Luciferase fusion has a similar half-life in extract as radiolabeled hNICD1 (Fig 3.1F and Fig 3.3B). The half-lives of hNICD2, 3, and 4 Luciferase fusions were also similar to the half-lives of the radiolabeled non-fusion proteins (Figure 3.1F). Residual Luciferase activities of the fusion proteins likely reflect the presence of background Luciferase protein produced by internal translational start sites in the IVT reaction (Chen et al., 2014a). The differential degradation of NICD paralogs is evolutionarily conserved: both mouse NICD1 and mouse NICD4 degraded with similar kinetics as their human orthologs (Figure 3.3B). Interestingly, we find that the single *Drosophila* NICD ortholog is stable in *Xenopus* egg extract (Figure 3.3B).

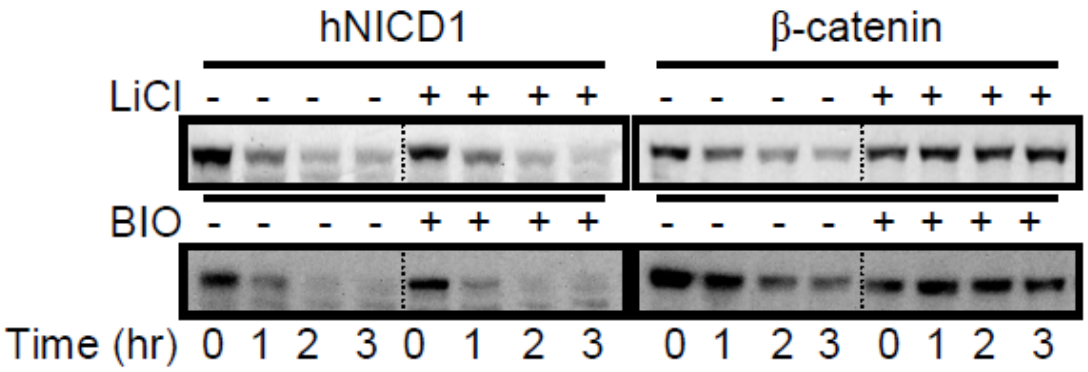

Figure 3.2. hNICD1 degradation in *Xenopus* egg extract occurs independent of GSK3. Radiolabeled *in vitro*-translated [^{35}S]β-catenin or [^{35}S]hNICD1 were incubated in the presence or absence of the GSK3 inhibitors LiCl (25 mM) or BIO (375 μM). Samples were removed at the indicated times and processed for SDS-PAGE/autoradiography. Water and DMSO were controls for LiCl and BIO, respectively.

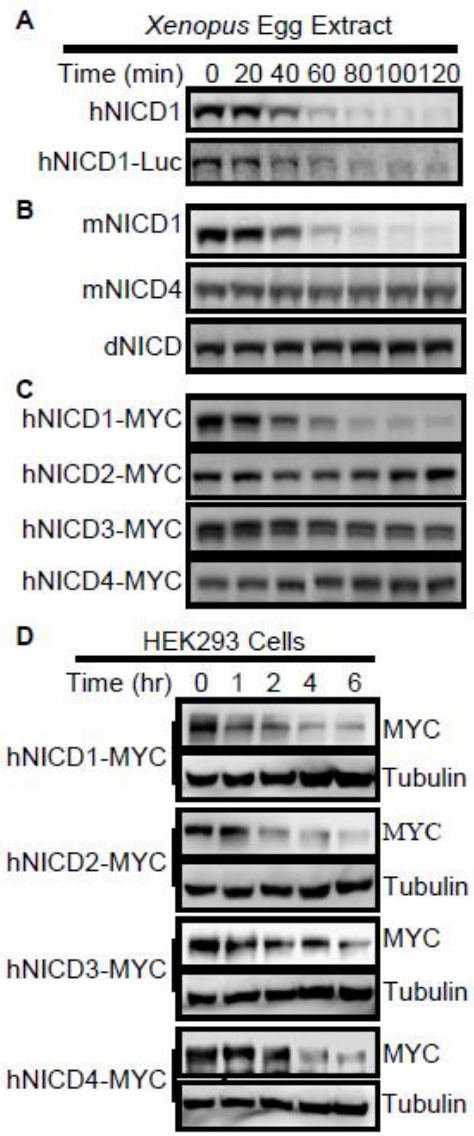

Figure 3.3. NICD1 is degraded in *Xenopus* egg extract, in contrast to other NICD paralogs, and degradation is not affected by C-terminal fusions. (A) [^{35}S]Met-labeled hNICD1 and hNICD1-Luc (Luciferase) were incubated in *Xenopus* egg extract, and samples were removed at the indicated times for analysis by SDS-PAGE/autoradiography. (B) Mouse NICD1 and NICD4 paralogs (mNICD1 and mNICD4) degrade similarly to their human counterparts in *Xenopus* egg extract. *Drosophila* dNICD does not degrade in extract. Mouse NICD1 (mNICD1), mouse NICD4 (mNICD4), and *Drosophila* NICD (dNICD1) were incubated in *Xenopus* egg extract and analyzed as in (A). (C) Degradation rates of radiolabeled hNICD paralogs fused at the C-terminal end to a MYC epitope are indistinguishable from their non-tagged versions. Radiolabeled hNICD paralogs fused at their C-terminal ends with the MYC epitope were incubated in *Xenopus* egg extract and analyzed by SDS-PAGE/autoradiography. (D) In contrast to *Xenopus* egg extract, C-terminal MYC fusions of all hNICD paralogs degrade when expressed in HEK293 cells. Cells expressing hNICD-MYC paralogs were collected at the indicated times after addition of cycloheximide (100 µg/ml) and immunoblotting was performed. Tubulin is loading control.

NICD1 degradation in *Xenopus* egg extract does not require its PEST domain or Fbxw7

We next sought to assess differences between the turnover of NICD proteins in *Xenopus* egg extract and in cultured human cells. To facilitate detection by immunoblotting, NICD paralogs were fused at their C-terminal ends with a MYC epitope. When NICD MYC fusions were added to *Xenopus* egg extract, we observed essentially identical turnover rates as their non-tagged versions (Figure 3.4C). We next assessed protein turnover in HEK293 cells by cycloheximide chase. In contrast to our *Xenopus* egg extract experiments, we find that all NICD paralogs degrade with similar half-lives comparable to those that have been previously reported in cultured human cells (Figure 3.4D) (Choi et al., 2013; Fryer et al., 2004; Malyukova et al., 2007; Mo et al., 2007; Palermo et al., 2012; Tsunematsu et al., 2004).

All NICD paralogs contain a conserved C-terminal PEST domain that regulates its turnover and is recognized, in part, by the SCF^{Fbxw7} ubiquitin ligase complex (Gupta-Rossi et al., 2001; Moretti and Brou, 2013; Oberg et al., 2001; Wu et al., 2001). Because our *Xenopus* egg extract preparation is predominantly cytoplasmic in character, whereas the Fbxw7 E3 ligase that mediates NICD turnover is predominantly nuclear (O'Neil et al., 2007), it is possible the inability of extract to support degradation of NICD2, 3, and 4 reflects the absence of a component of the SCF^{Fbxw7} complex. Consistent with this possibility, we found that, in contrast to HEK293 cell lysates, we were unable to detect Fbxw7 in our extract using an antibody that recognizes a highly conserved region of Fbxw7 present in the *Xenopus* protein (Figure 3.4A).

If Fbxw7 is absent or present at a very low concentration in *Xenopus* egg extract, we hypothesize that hNICD1 degradation is occurring independently of the SCF^{Fbxw7} complex. $hNICD1^{S3A}$ is a mutant in which three phosphorylated serine residues at positions 2514, 2517, and 2539 necessary for recognition by Fbxw7 have been changed to alanines (Figure 3.4B) (Fryer et al., 2004; O'Neil et al., 2007; Thompson et al., 2007). The $hNICD^{S2493\Delta}$ mutant (found in T-ALL patients) encodes a truncation at residue S2493 that removes its Fbxw7 recognition site (Figure 2B) (Weng et al., 2004). We found that both mutants degrade at rates indistinguishable from that of wild-type hNICD1 in *Xenopus* egg extract (Figure 3.4C-E). Essentially identical results were obtained with other mutants encoding truncations within the PEST domain found in T-ALL patients and with a truncation that completely abolishes the C-terminal PEST domain (data not shown).

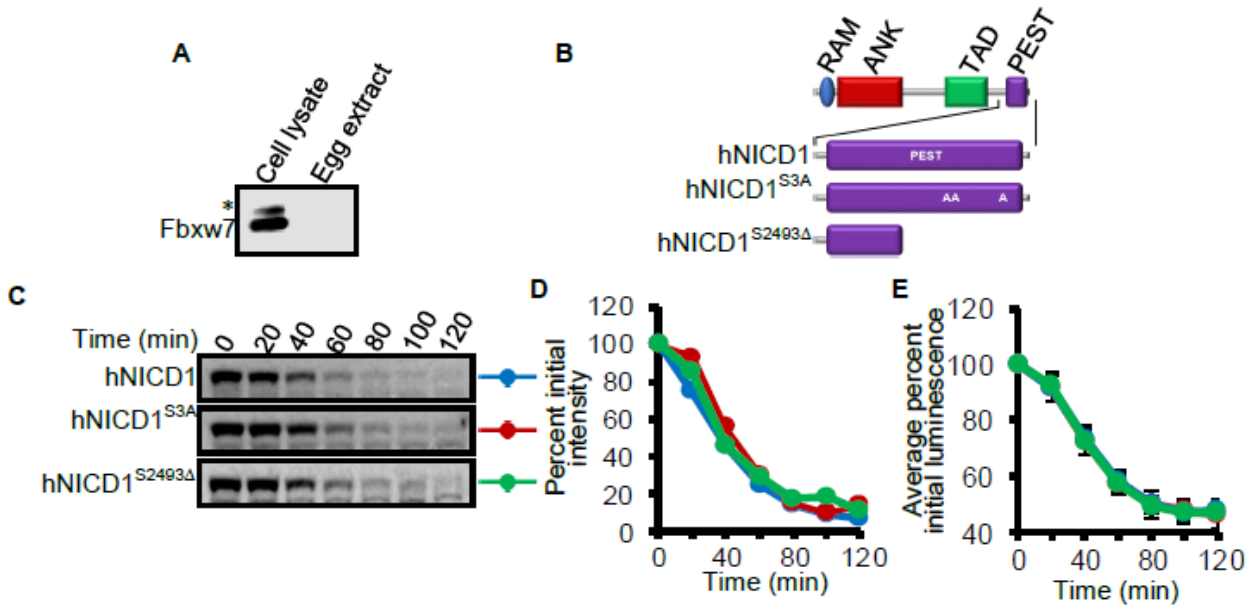

Figure 3.4. PEST domain mutants of hNICD1 degrade in *Xenopus* egg extract. (A) Fbxw7 is not detectable in *Xenopus* egg extract. Equivalent amounts of HEK293 cell lysates and *Xenopus* egg extract (30 ug each) were processed for immunoblotting. Fbxw7 was detected using an antibody that recognizes a conserved region of Fbxw7 present in the *Xenopus* and human proteins. Asterisks indicates non-specific band. (B) Schematic of hNICD1 PEST domain mutants. (C) The Fbwx7 binding mutant, hNICD^{S3A}, and the PEST domain truncation mutant hNICD$^{S2493\Delta}$ degrade at similar rates as wild-type hNICD1. (D) Graph of densitometry measurements in (C). (E) Turnover rates of hNICD1 PEST mutants fused to Luciferase are similar to their untagged, radiolabeled forms in *Xenopus* extract. *In-vitro* translated hNICD1^{S3A} and hNICD1$^{S2493\Delta}$ Luciferase fusions were added to *Xenopus* extract and sample removed at the indicated times for Luciferase activity measurements. Graph represents mean of two independent experiments (performed in triplicate) and normalized to value of the initial time point (100%).

The N-terminal end of hNICD1 encodes a degron required for degradation in *Xenopus* egg extract

To identify the PEST-independent degron of hNICD1, we generated chimeric proteins in which the N- and C-terminal portions of hNICD1 (just upstream of the ANK repeats) were swapped for the corresponding regions of other hNICD paralogs (Figure 3.5A and E). We found that all swaps containing the N-terminal ends of hNICD1 degraded at a similar rate as wild-type hNICD1 (Figure 3.5B-D). In contrast, constructs with C-terminal fragments of hNICD1 were stable throughout the time course of the experiment (Figure 3.5F-H). These data suggest that the regulatory region controlling hNICD1 degradation in *Xenopus* egg extract is located within its N-terminal end. We performed further swapping analysis to delineate the N-terminal region of hNICD1 responsible for promoting its degradation in *Xenopus* egg extract (Figure 3.6A). We found that the N-terminal 35 amino acid fragment of hNICD1 is sufficient to confer robust degradation of hNICD2 (Figure 3.6B). Collectively, these results demonstrate that the amino terminus of hNICD1 contains a Notch1-specific degron (N1-Box) that is both necessary and sufficient to degrade hNICD1 in *Xenopus* egg extract.

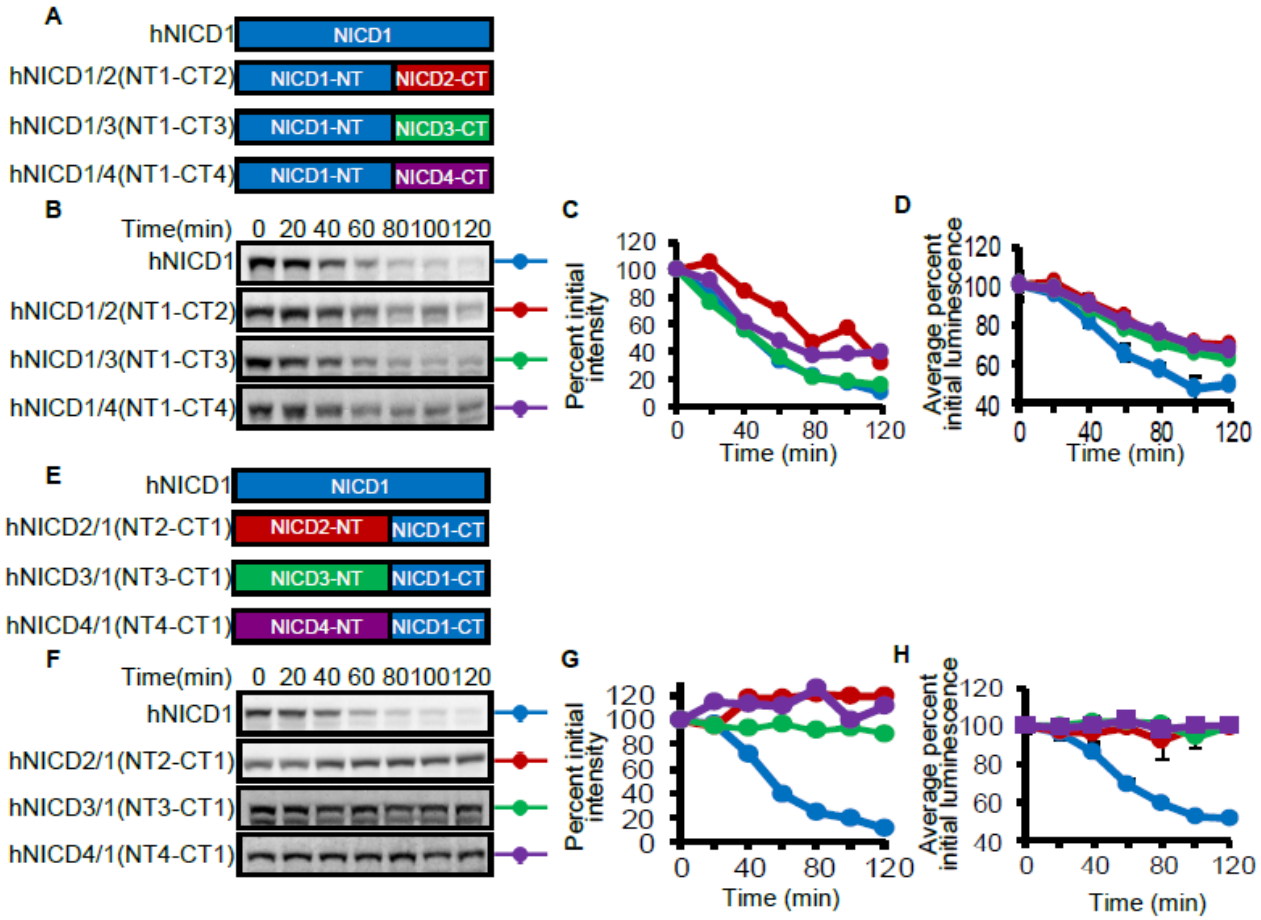

Figure 3.5. The N-terminal half of hNICD1 promotes hNICD1 degradation in *Xenopus* egg extract. (A) Schematic of hNICD chimeras containing N-terminal hNICD1 fusions. (B) Chimeric mutants of NICD paralogs containing the N-terminal half of hNICD1 all degrade in *Xenopus* egg extract. Radiolabeled chimeric mutants were incubated in extract, and samples removed at the indicated time points for analysis by SDS-PAGE/autoradiography. (C) Graph of densitometry measurements in (B). (D) N-terminal hNICD1 chimeras fused to Luciferase degrade at a similar rate as the non-tagged proteins. *In vitro*-translated proteins were incubated in *Xenopus* egg extract, samples were removed at the indicated times, and Luciferase activity determined. Experiments were performed in triplicate. Graphs show mean SD of Luciferase signal normalized to the value of the initial time point. (E) Schematic of hNICD chimeras encoding C-terminal hNICD1 fusions. (F) Chimeric mutants of NICD paralogs containing the C-terminal half of hNICD1 are stable in *Xenopus* egg extract. (G) Graph of densitometry measurements in (F). (H) Similar to their radiolabeled, untagged versions, C-terminal hNICD1 chimeras fused to Luciferase are stable in *Xenopus* egg extract.

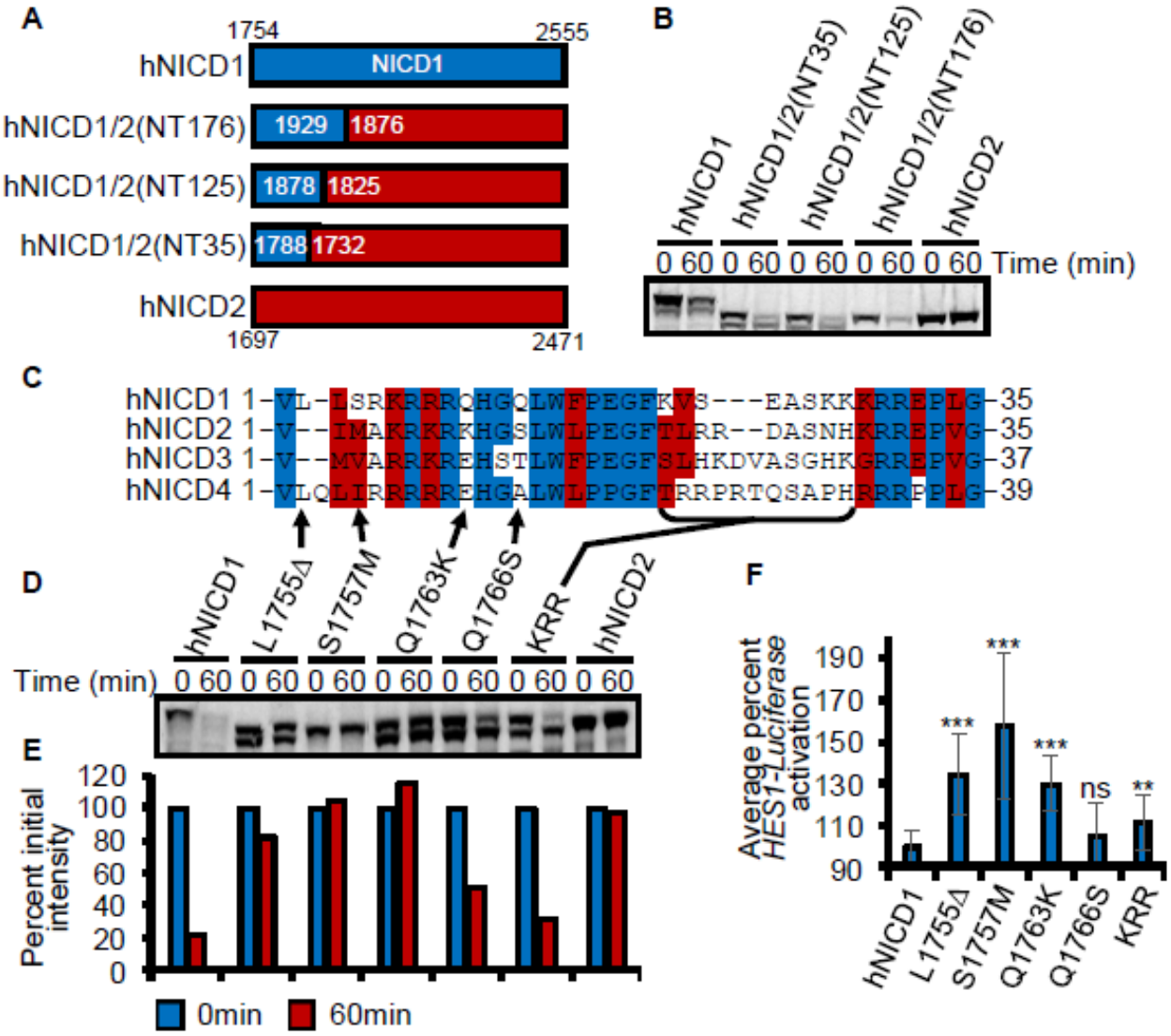

Figure 3.6 Mutations within the 35 amino acid N-terminal region of hNICD1 inhibit its degradation in *Xenopus* egg extract. (A) Schematic of hNICD1 and hNICD2 chimeras. Parenthesis indicates the number of N-terminal amino acids of hNICD1 present in each chimera. (B) Radiolabeled hNICD1/2 chimeras containing at least the N-terminal 35 amino acids of hNICD1 degrade robustly in *Xenopus* extract. (C) Alignment of the N-terminal regions of the human NICD paralogs. (D) Mutation of hNICD1 at position L1755, S1757, or Q1763 potently inhibits degradation of hNICD1. Radiolabeled hNICD1 point mutants of non-conserved residues within the N-terminal 35 amino acids were incubated in extract and their stability analyzed by SDS-PAGE/autoradiography. Δ indicates amino acid deletion. KRR is Lysine rich region. (E) Quantification of densitometry from (D). (F) N-terminal mutants of hNICD1 are transcriptionally active. hNICD1 point mutants were co-transfected with a HES1-Luciferase Notch reporter into HEK293 cells and Luciferase activity assessed after 24 hr. Graph represents ± S.D. of Luciferase signal normalized to *Renilla* luciferase (transfection control) of at least 3 independent experiments (performed in triplicate).. ***p-value<0.0001, **p-value<0.05 relative to wild-type hNCID1. ns is not significant.

We next sought to identify the essential N1-Box residues that facilitate hNICD1 turnover in *Xenopus* egg extract. Alignment of the N-terminal end of all four human NICD paralogs (Figure 3.6C) revealed non-conserved residues within the first 35 amino acids of hNICD1 (corresponding to amino acids 1754-1788 of full-length Notch1) that may contribute to hNICD1-specific degradation in extract. To test this possibility, non-conserved residues within this region of hNICD1 were mutated to their corresponding hNICD2 residues, and their stability in extract was assessed (Figure 3.6D-E). We find that, in contrast to wild-type hNICD1, the L1755Δ (deletion of residue 2), S1757M, and Q1763K mutants of hNICD1 are all stable in *Xenopus* egg extract as is the triple mutant ($hNICD1^{LSQ}$; Figure 3.7A). Further confirmation of the importance of the first 10 amino acids in hNICD1 comes from our demonstration that the $hNICD1^{NT\Delta10}$ mutant (lacking the N-terminal 10 amino acids) is stable in extract (Figure 3.7A).

To confirm that the increased stability of point mutants is not due to protein misfolding, we performed Notch transcription assays (Figure 3.6F). All five mutants tested retained the capacity to stimulate HES1-Luciferase reporter activity, indicating that the proteins were not grossly misfolded. Furthermore, the three stabilized mutants demonstrate increased reporter activity (Figure 3.6F). Based on these results, we tested whether replacing the N-terminal 10 amino acids of hNICD2 with those of hNICD1 was sufficient to promote degradation of the predominantly hNICD2 chimera (Figure 3.7B-C). Whereas hNICD1/2 chimeras that exchanged the first 10 residues ($hNICD1/2^{NT10}$) or the Lysine rich region ($hNICD1/2^{KRR}$) did not promote degradation, a combination of these swaps ($hNICD1/2^{NT10/KRR}$) showed enhanced turnover (Figure 3.7B). Substantial degradation was observed for the hNICD1/2 chimera that replaced the N-terminal 35

amino acids of hNICD2 with those of hNICD1 (Figure 3.7B). These results suggest that, although the N-terminal 10 amino acids of hNICD1 are critical for mediating its degradation, the N-terminal 35 amino acids of hNICD1 represents the minimal unit that is required to impart hNICD2 with the capacity to degrade in extract (the N1-Box).

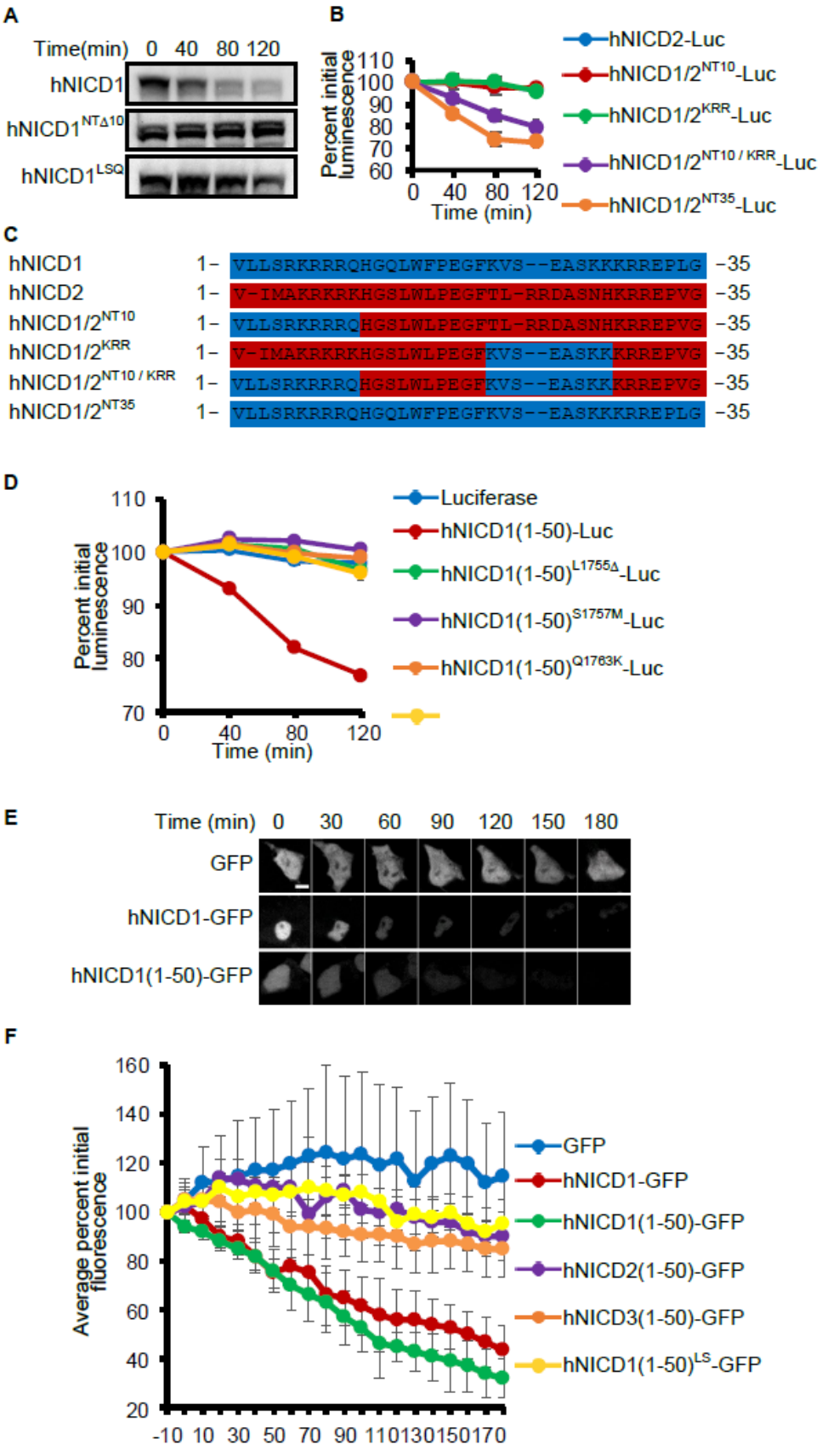
A
Time(min) 0 40 80 120
hNICD1
hNICD1NTΔ10
hNICD1LSQ
B
Percent initial luminescence
110
100
90
80
70
60
0 40 80 120
Time (min)
hNICD2-Luc
hNICD1/2NT10-Luc
hNICD1/2KRR-Luc
hNICD1/2NT10 / KRR-Luc
hNICD1/2NT35-Luc
C
hNICD1 1- VLLSRKRRRQHGQLWFPEGFKVS--EASKKKRREPLG -35
hNICD2 1- V-IMAKRKRKHGSLWLPEGFTL-RRDASNHKRREPVG -35
hNICD1/2NT10 1- VLLSRKRRRQHGSLWLPEGFTL-RRDASNHKRREPVG -35
hNICD1/2KRR 1- V-IMAKRKRKHGSLWLPEGFKVS--EASKKKRREPVG -35
hNICD1/2NT10 / KRR 1- VLLSRKRRRQHGSLWLPEGFKVS--EASKKKRREPVG -35
hNICD1/2NT35 1- VLLSRKRRRQHGQLWFPEGFKVS--EASKKKRREPLG -35
D
Percent initial luminescence
110
100
90
80
70
0 40 80 120
Time (min)
Luciferase
hNICD1(1-50)-Luc
hNICD1(1-50)L1755Δ-Luc
hNICD1(1-50)S1757M-Luc
hNICD1(1-50)Q1763K-Luc
E
Time (min) 0 30 60 90 120 150 180
GFP
hNICD1-GFP
hNICD1(1-50)-GFP
F
Average percent initial fluorescence
160
140
120
100
80
60
40
20
-10 10 30 50 70 90 110 130 150 170
GFP
hNICD1-GFP
hNICD1(1-50)-GFP
hNICD2(1-50)-GFP
hNICD3(1-50)-GFP
hNICD1(1-50)LS-GFP

Figure 3.7. The first 10 amino acids of hNICD1 are critical for N1-Box-mediated degradation, whereas the first 50 amino acids of hNICD1 are required to promote degradation of heterologous proteins. (A) The N-terminal ten amino acids of hNICD1 contain amino acids critical for its degradation. Radiolabeled hNICD1$^{NT\Delta10}$ and hNICD1^{LSQ} were incubated in *Xenopus* egg extract and samples removed at the indicated times for analysis by SDS-PAGE/autoradiography. (B) Degradation of hNICD1/2 chimera-Luciferase fusions in *Xenopus* egg extract parallels degradation of the radiolabeled, untagged versions in extract. *In vitro*-translated hNICD1/2 chimeras fused to Luciferase were incubated in extract, samples removed at the indicated times, and Luciferase activity determined. Graph represents mean ± s.e.m. from three independent experiments (performed in triplicate). (C) Schematic showing the first 35 residues of the hNICD1/2 chimeras used in (B). (D) The N-terminal 50 amino acids of hNICD1 and their mutants were fused to Luciferase and incubated in *Xenopus* egg extract. Samples were removed at the indicated times, and Luciferase activity measured. Graph represents mean ± s.e.m. of two independent experiments (performed in triplicate) and normalized to the value of the initial time point. (E) Fusion of the N-terminal 50 amino acids of hNICD1 to GFP promotes its degradation in human cultured cells. GFP, hNICD1-GFP and hNICD1(1-50)-GFP were expressed in HEK293 cells. At time 0 min, cycloheximide (100 µg/ml) was added and live cell imaging performed. Representative images are shown. (F) In contrast to hNICD1, the N-terminal 50 amino acids of hNICD2 and 3 fused to GFP were stable in HEK293 cells. Graph represents mean ± s.e.m. of GFP fluorescence normalized to value of the initial time point. Experiments were performed at least twice with >10 cells quantified for each construct.

To determine whether the N-terminal region of hNICD1 can act in an autonomous fashion to promote turnover of a stable heterologous protein, we fused the N-terminal 50 residues of hNICD1 to Luciferase. We chose 50 amino acids because, based on secondary structure predictions, it represents a discrete structural unit, and we sought to minimize protein misfolding. We found that hNICD1(1-50)-Luc degraded in extract in contrast to Luciferase alone (Figure 3.7D). To further demonstrate that mutants identified in our hNICD1/2 chimera studies represent critical amino acids of the N1-Box (rather than altering accessibility or conformation of a degradation signal elsewhere on the protein), we generated the analogous L1755Δ, S1757M, and Q1763K mutants in the hNICD1(1-50)-Luc fusion (Figure 3.7D). As expected, all the mutants were stable in *Xenopus* extract, indicating that residues L1755Δ, S1757M, and Q1763K are critical for N1-Box activity.

We tested if the hNICD1 N1-Box was functional in human cells by fusing the N1-Box to GFP and monitoring its turnover by live-cell imaging upon cycloheximide treatment. In contrast to GFP alone, hNICD1(1-50)-GFP showed a loss of fluorescence similar to the rate for the full-length hNICD1-GFP fusion (Figure 3.7E,F). As controls, we showed that GFP fusions of the first 50 amino acids of hNICD2 and 3, and the hNICD1(1-50) triple mutant (hNICD1(1-50)^{LSQ}) did not exhbibit substantial GFP turnover over the time course of our measurements (Figure 3.7F). These results indicate that the degradation machinery that recognizes the N1-Box of hNICD1 is evolutionarily conserved in both *Xenopus* egg extract and cultured human cells.

The N1-Box controls hNICD1 stability and activity *in vitro* and *in vivo*

Our initial studies with the L1755Δ, S1757M, and Q1763K mutants of hNICD1, which were all more stable than the wild-type protein in *Xenopus* egg extract, indicate that they stimulated higher levels of Notch reporter activity (Figure 3.6F). We next examined whether N1-Box-regulated degradation of hNICD1 correlates with Notch transcriptional activity. The N1-Box mutants, hNICD1$^{NT\Delta10}$ and hNICD1^{LSQ}, were tagged at their C-terminal end with the MYC epitope to facilitate their detection, equivalent amounts of DNA were transfected into HEK293 cells, and protein levels were assessed by immunoblotting (Figure 3.8A). As expected, both N1-Box mutants display higher steady-state levels of protein compared to wild-type hNICD1, consistent with a decreased rate of degradation of the mutants (Figure 3.8A and 3.9). Consistent with their increased stability, both hNICD1$^{NT\Delta10}$ and hNICD1^{LSQ} have greater transcriptional activity than wild-type hNICD1 (Figure 3.8B). To ensure that the increased transcription we observe is not an artifact of transiently expressing the intracellular domain fragment, and to demonstrate evolutionary conservation, we generated the analogous N1-Box mutant in the full-length mouse Notch1 receptor (mNotch1^{LSQ}). Consistent with our hNICD1 studies, full-length mNotch1^{LSQ} demonstrated elevated Notch transcriptional activity compared to full-length wild-type mNotch1 (Figure 3.8C). A mutation in the HD domain of human Notch1 (L1601P) identified in T-ALL patients has been shown to result in a constitutively activated “leaky” Notch1 receptor due to its constitutive cleavage from the plasma membrane (Chiang et al., 2006; Thompson et al., 2007; Weng et al., 2004). When the L1601P mutant is combined with mNotch1^{LSQ}, an additional increase in transcriptional activation is observed (Figure 3.8C). These studies

indicate that regulation of steady-state levels of NICD1 by N1-Box alters Notch1 transcriptional activation.

In order to demonstrate that the N1-Box regulates Notch1 function in an *in vivo* context, we assessed its effects on somite formation in the developing embryo of *Danio rerio*. The Notch1 receptor has an established role in somitogenesis in mice and in *D. rerio* (Harima and Kageyama, 2013; Lewis et al., 2009), and misregulation of Notch signaling during development results in readily observable disruption of the symmetric, bilaterally formed somites (Figure 3.8D). Conveniently, this biological readout is highly dose-sensitive: injecting 100 pg *mNotch1* mRNA results in defects in somitogenesis in 90% of the embryos, whereas only 25% of embryos are affected with injection of 25 pg *mNotch1* mRNA (Figure 3.8D). Consistent with a role for N1-Box *in vivo*, injecting embryos with 25 pg mRNA of the mouse Notch1 N1-Box mutant, *mNotch1*^{LSQ}*,* resulted in 47% of embryos with defective somite formation, nearly double that of wild-type *mNotch1* mRNA (Figure 3.8E). A similar effect on somitogenesis is observed in the background of the constitutively activated L1601P mutation (Figure 3.8E). These results support a role for N1-Box in regulating Notch1 signaling *in vivo*.

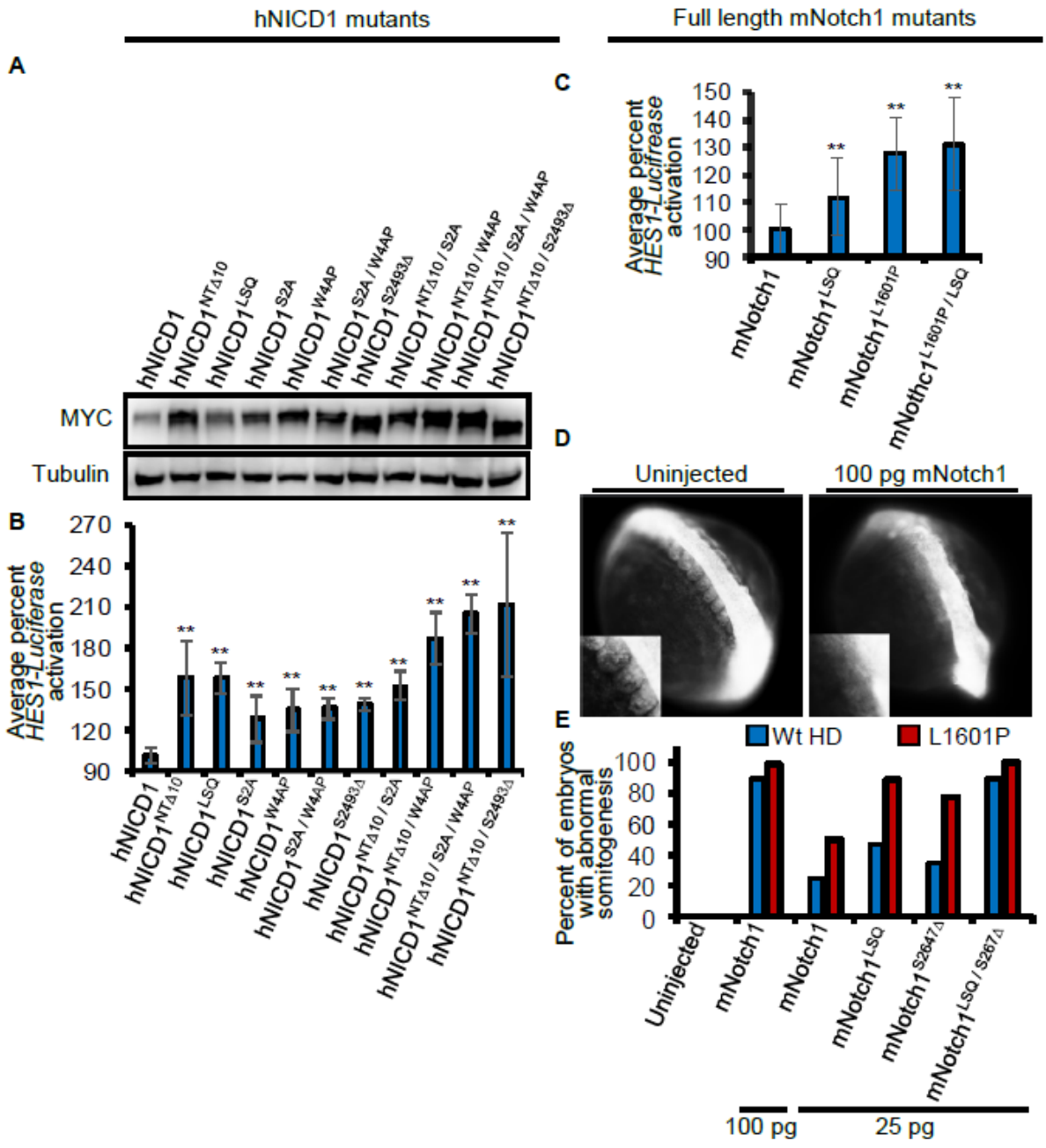
hNICD1 mutants
Full length mNotch1 mutants
A
MYC
Tubulin
hNICD1
hNICD1 NTΔ10
hNICD1 LSQ
hNICD1 S2A
hNICD1 W4AP
hNICD1 S2A / W4AP
hNICD1 S2493Δ
hNICD1 NTΔ10 / S2A
hNICD1 NTΔ10 / W4AP
hNICD1 NTΔ10 / S2A / W4AP
hNICD1 NTΔ10 / S2493Δ
B
Average percent HES1-Luciferase activation
270
240
210
180
150
120
90
C
Average percent HES1-Luciferase activation
150
140
130
120
110
100
90
mNotch1
mNotch1 LSQ
mNotch1 L1601P
mNothc1 L1601P / LSQ
D
Uninjected
100 pg mNotch1
E
Wt HD
L1601P
Percent of embryos with abnormal somitogenesis
100
80
60
40
20
0
Uninjected
mNotch1
mNotch1 LSQ
mNotch1 S2647Δ
mNotch1 LSQ / S2671Δ
100 pg
25 pg

Figure 3.8. hNICD1 N1-Box mutants have elevated steady-state levels and increased activity in cultured human cells and zebrafish embryos. (A) N1-Box mutants ($hNICD^{NT\Delta 10}$ and $hNICD^{LSQ}$), the Fbwx7 binding mutant ($hNICD1^{S2A}$), the WSSSSP mutant ($hNICD1^{W4AP}$), or combinations of these mutants were expressed in HEK293 cells and immunoblotting of lysates performed. Tubulin is control. (B) Stabilizing hNICD1 mutants of the N1-Box, Fbwx7, and WSSSP exhibit higher transcriptional activity compared to wild-type hNICD1. hNICD1 mutants alone or in combination were transfected with a HES1-Luciferase Notch reporter into HEK293 cells and luciferase activity assessed after 24 hr. Graphs show mean ± S.D.. of Luciferase signal normalized to *Renilla* Luciferase (transfection control) of at least 2 independent experiments (performed in triplicate). p-value is <0.002 relative to wild-type hNICD1 for all mutants. (C) The full-length Notch1 N1-Box mutant exhibits increased Notch transcriptional activity. HEK293 cells were transfected with plasmids encoding full-length mNotch1 receptor, $mNotch1^{LSQ}$, $mNotch1^{L1601P}$, or $mNICD1^{L1601P/LSQ}$ plus the HES1-Luciferase Notch reporter. $mNICD1^{L1601p}$ is an HD mutant that is constitutively cleaved from the holoreceptor to release the intracellular domain. Graphs shows the mean ± S.D. Luciferase activity normalized to *Renilla* luciferase (transfection control) and is representative of four independent experiments performed in triplicate. *p-value<0.007 relative to wild-type. (D) The N1-Box Notch1 mutant exhibit enhanced capacity in zebrafish embryos to disrupt somitogenesis. (Top) Representative images taken at the 10-13 somite stage of an uninjected *D. rerio* embryo or injected with 100 pg of *mNotch1* mRNA. (Bottom). Graph is percentage of *D. rerio* embryos at the 10-13 somite stage with defective somites. All data is from 3 clutches collected from 3 unique breeding pairs per clutch. n=78-200 embryos per injection. Compared to 25 pg *Notch1* mRNA injection: $P \leq 0.05$. Compared to 25 pg injection of $hNICD1^{LSQ;\ S2467\Delta}$ mRNA injection: $P \leq 0.005$, except for comparison to the *Notch1* 100 pg injection which is insignificant.

Two *cis* elements have been identified within the PEST domain that facilitate NICD turnover - the conserved LTPSPE sequence recognized by the SCF^{Fbxw7} complex (Fryer et al., 2002; Fryer et al., 2004; O'Neil et al., 2007; Thompson et al., 2007) and the WSSSSP sequence (Chiang et al., 2006). In order to determine the contributions of these two degradation signals and the N1-Box on NICD1 protein stability, we made mutants that disrupted their functions both singly and in combination. Consistent with previous studies, we find that mutation within the LTPSPE (S2514A/S2517A; $hNICD1^{S2A}$), WSSSSP ($hNICD1^{W4AP}$), both LTPSPE and WSSSSP ($hNICD1^{S2A/W4AP}$), or truncation of the PEST region ($hNICD1^{S2493\Delta}$), results in increased protein levels when expressed in cultured cells (Figure 3.8A) (Chiang et al., 2006; Fryer et al., 2004; O'Neil et al., 2007; Thompson et al., 2007; Weng et al., 2004). Increased steady-state levels of $hNICD1^{S2A}$ and $hNICD1^{W4AP}$ mutants are also observed in combination with the NTΔ10 mutation when compared to wild-type hNICD1. Interestingly, there is an observable enhancement in protein levels when the $hNICD1^{NT\Delta10}$ mutation is combined with W4AP and/or S2493Δ, but not with the S2A mutation. All stabilizing mutants activate the HES1 reporter to a greater extent than wild-type hNICD1, and differences in protein levels of individual hNICD1 mutants roughly correlate with the capacity of the mutant to activate Notch transcription in reporter assays (Figure 3.8B). Consistent with their enhanced stability, $hNotch1^{W4AP}$ and $hNotch1^{S2493\Delta}$, when combined with NTΔ10, exhibit increased transcriptional activation (in contrast to $hNICD1^{S2A}$) (Figure 3.8B). These results suggest the WSSSSP sequence and the N1-Box act independently of each other. The absence of further activation by $hNICD1^{NT\Delta10}$ or $hNICD1^{W4AP}$ upon mutation in the Fbwx7 binding

site may indicate that stabilization by NTΔ10 or WSSSSP fully saturates the SCF^{Fbxw7} complex, which may be limiting.

This pattern of interaction between the NTΔ10 and S2493Δ mutations in hNotch1 was also observed in zebrafish embryos in our somitogenesis assay. The number of embryos with somitogenesis defects following injection of *mNotch1*$^{S2467\Delta}$ mRNA was statistically greater when compared to injection of wild-type *mNotch1* mRNA (Figure 3.8E). Similarly, the N1-Box mutant ($mNotch^{LSQ}$) was more potent than the PEST truncation mutant, and the effect of the N1-Box and PEST truncation double mutant on somitogenesis was greater than either one alone (Figure 3.8E). A similar pattern was observed in zebrafish somitogenesis in the L1601P mutant background.

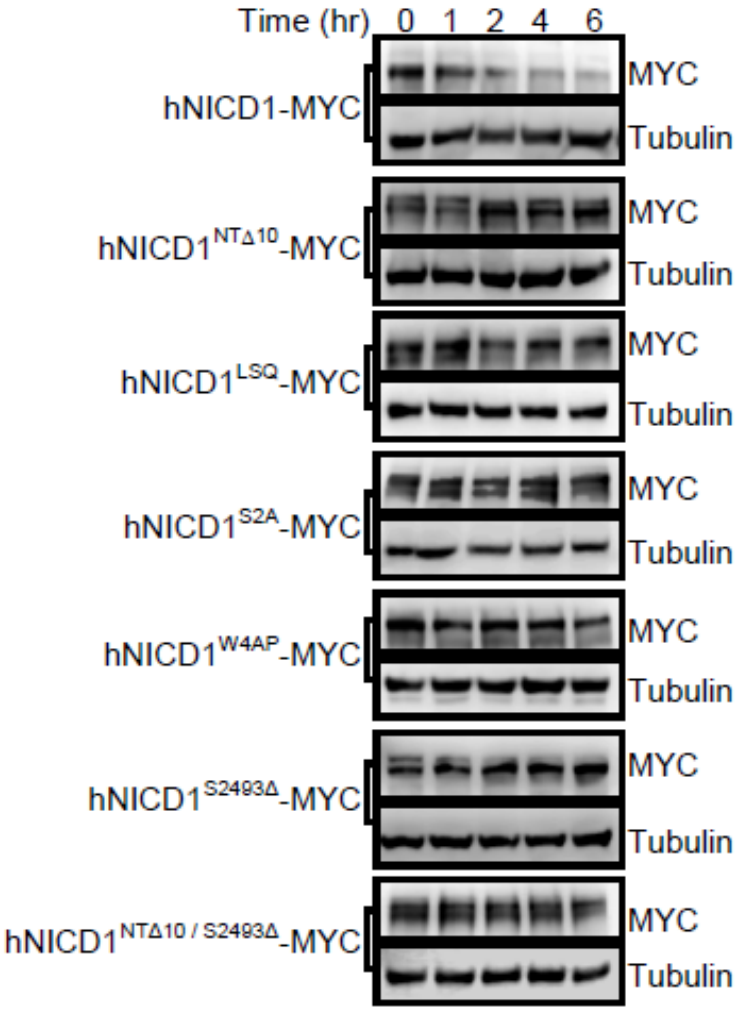

Figure 3.9. hNICD1 degron mutants have decreased rates of degradation in cultured human cells. MYC-tagged hNICD1, N1-Box mutants (hNICD$^{NT\Delta10}$ and hNICD^{LSQ}), PEST mutants (hNotch1^{S2A}, hNotch1^{W4AP}, and hNICD1$^{S2493\Delta}$) and a combination mutant (hNICD1$^{NT\Delta10/S2493\Delta}$) were expressed in HEK293 cells. Cycloheximide (100 µg/ml) was added at 0 min and samples collected at the indicated times for immunoblotting. Tubulin is loading control.

The N1-Box is not regulated by Fbxw7 or Itch

It is possible that there are Fbxw7 sites in hNICD1 other than its PEST region. To more carefully assess whether Fbxw7 regulates the N1-Box, we overexpressed Fbxw7 and a dominant-negative form (Fbxw7DN) that lacks the Fbox domain necessary for interaction within the SCF complex (Skaar et al., 2013; Wu et al., 2001). As previously reported, overexpression of Fbxw7 decreases, whereas overexpression of Fbxw7DN increases, steady-state levels of hNICD1 in HEK293 cells (Figure 3.10A) (Gupta-Rossi et al., 2001; Wu et al., 2001). In contrast, overexpressing Fbxw7 or Fbxw7DN did not affect levels of the Fbxw7 binding site mutant, hNICD1^{S2A} (Figure 3.10A). The effects of overexpressing Fbxw7 and Fbxw7DN on wild-type hNICD1 were similarly observed for hNICD1^{W4AP} (Figure 3.10A), consistent with a previous study suggesting that the WSSSSP site acts independently of the SCF^{Fbxw7} complex (Chiang et al., 2006). A similar effect was observed with NICD1$^{NT\Delta10}$, indicating that the N1-Box-mediated degradation of NICD1 is independent of the SCF^{Fbxw7} complex.

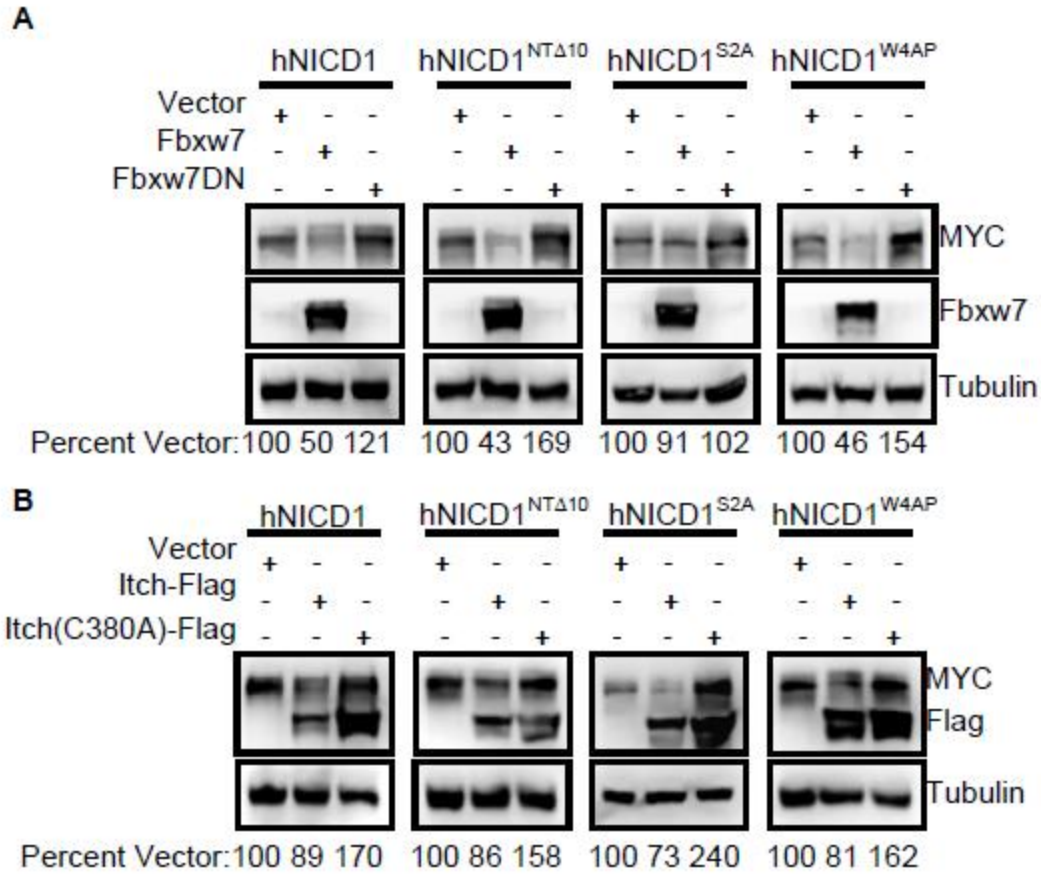

Figure 3.10. N1-Box mutants do not affect the capacities of the E3 ligases, Fbxw7 and Itch, to regulate hNICD1 levels in cultured human cells. (A) Overexpression of Fbxw7 and Fbxw7DN decreases and increases, respectively, steady-state levels of hNICD1, hNICD$^{NT\Delta10}$, and hNotch1^{W4AP}. In contrast, overexpression of Fbxw7 and Fbxw7DN had no effect on levels of the Fbxw7 binding mutant, hNICD^{S2A}. (B) Overexpression of ITCH and its dominant-negative mutant, ITCH^{C380A}, decreases and increases, respectively, steady-state levels of all hNICD1 constructs. Expression studies were performed in HEK293 cells. hNICD1 constructs were tagged at their C-terminal ends with MYC, and Itch and Itch^{C380A} were tagged at their C-terminal ends with FLAG to facilitate their detection by immunoblotting. Tubulin is loading control.

The E3 ubiquitin ligase, ITCH, has also been shown to promote PEST domain-independent NICD1 degradation, although its binding site has not been well characterized (Mazaleyrat et al., 2003; McGill and McGlade, 2003; Qiu et al., 2000). As previously shown, overexpression of ITCH decreases, whereas overexpression of a dominant-negative form of ITCH, ITCH^{C380A}, increases, the steady-state levels of hNICD1 in HEK293 cells (Figure 3.10B). We find that similar results are obtained for all the mutants, indicating that ITCH does not mediate NICD1 degradation through the N1-Box, WSSSSP, or LTPSPE.

Binding of CSL to hNICD1 regulates its stability

Because of the sequence overlap between the N1-Box and the RAM domain (the major *cis* factor involved in CSL binding (Nam et al., 2003; Tamura et al., 1995)) (Figure 3.11A), we tested whether the binding of CSL to hNICD1 could influence hNICD1 stability. To examine this possibility, we incubated recombinant CSL (expressed and purified from the *Sf21*/baculovirus system) with *Xenopus* egg extract and assessed its effect on hNICD1 turnover. We find that CSL inhibits degradation of hNICD1 in extract in a dose-dependent manner (Figure 3.11B-C). If the regulation of NICD1 stability by CSL occurs via direct binding, mutants of NICD1 that prevent it from binding to CSL should no longer be inhibited by CSL. This appears to be the case as the degradation of the hNICD1$^{1771-74A}$ mutant, which lacks the capacity to interact with CSL (Chu and Bresnick, 2004; Vasquez-Del Carpio et al., 2011), is not inhibited by recombinant CSL (Figure 3.11B-C). We speculate that the predominantly cytoplasmic character of *Xenopus* egg extract contains low levels of CSL in contrast to human cells; thus, hNICD1$^{1771-74A}$ should turn over at a faster rate than wild-type hNICD1. Consistent with

this, we observe lower steady-state level of hNICD1$^{1771\text{-}74A}$ than wild-type hNICD1 when expressed in HEK293 cells (Figure 3.11D-E).

Because degradation of the NICD via the SCF^{Fbxw7}/PEST domain is dependent on assembly of a transcriptional complex involving CSL (Fryer et al., 2002; Fryer et al., 2004), we predict that turnover of the hNICD1$^{1771\text{-}74A}$ mutant is primarily mediated by the N1-Box. If this is the case, an N1-Box/CSL-binding double mutant should be more stable than the N1-Box mutant. This increased stability is due to incapacity of the CSL-binding mutant to assemble into a transcriptional complex (in contrast to the N1-Box mutant) and, thus, would not be degraded via the SCF^{Fbxw7} complex. Consistent with this idea, we find that the steady-state level of hNICD1$^{NT\Delta10/1771\text{-}74A}$ is greater than that of hNICD1$^{NT\Delta10}$ (Figure 3.11D-E).

Conversely, in an hNICD1 PEST mutant, the inability to bind CSL should lead to decreased protein stability due to enhanced N1-Box-mediated degradation. Consistent with this model, we find that the steady-state level of hNICD1$^{1771\text{-}74A\ /\ S2493\Delta}$ is lower than that of hNICD1$^{S2493\Delta}$ (Figure 3.11D-E). These results provide strong evidence that CSL binding to NICD1 inhibits its turnover by blocking N1-Box-mediated degradation.

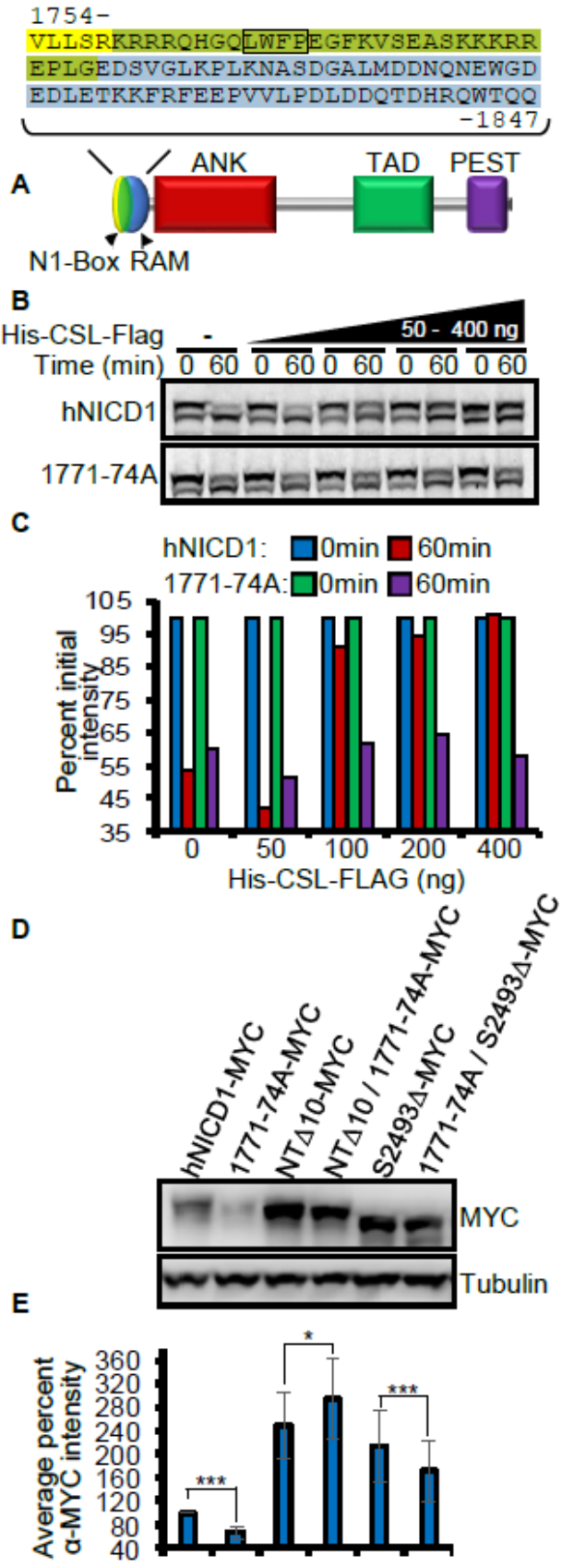
1754-
VLLSRKRRRQHGQLWFPEGFKVSEASKKKRR
EPLGEDSVGLKPLKNASDGALMDDNQNEWGD
EDLETKKFRFEEPVVLPDLDDQTDHRQWTQQ
-1847
A
ANK
TAD
PEST
N1-Box
RAM
B
His-CSL-Flag
-
50 - 400 ng
Time (min)
0 60 0 60 0 60 0 60 0 60
hNICD1
1771-74A
C
hNICD1: 0min 60min
1771-74A: 0min 60min
Percent initial intensity
105
95
85
75
65
55
45
35
0
50
100
200
400
His-CSL-FLAG (ng)
D
hNICD1-MYC
1771-74A-MYC
NTΔ10-MYC
NTΔ10 / 1771-74A-MYC
S2493Δ-MYC
1771-74A / S2493Δ-MYC
MYC
Tubulin
E
Average percent α-MYC intensity
360
320
280
240
200
160
120
80
40
*
***
***

Figure 3.11. N1-Box-mediated hNICD1 degradation in *Xenopus* egg extract and cultured human cells is inhibited by its binding to CSL. (A) Schematic showing overlap of the N1-Box (yellow) and RAM domains (blue). Overlapping sequence is displayed in green. The ΦWΦP motif is outlined by a black box. (B) Recombinant CSL inhibits degradation of hNICD1 (but not a CSL binding mutant of hNICD) in a dose-dependent manner in *Xenopus* egg extract. Radiolabeled hNICD1 or hNICD$^{11771\text{-}74A}$ were incubated in *Xenopus* egg extract in the presence of increasing amounts of recombinant CSL. Samples were removed after 1 hr and subjected to SDS-PAGE/autoradiography. (C) Graph of densitometry measurements in (B) normalized to Tubulin. (D) Steady-state levels of the CSL binding mutant, hNICD1$^{11771\text{-}74A}$, are lower than the wild-type protein in cultured human cells. Plasmids encoding the indicated N1-Box and CSL binding mutants tagged with the MYC epitope were transfected into HEK293 cells and immunoblotting performed. Tubulin is loading control. (E) Graph of densitometry measurements in (D). Graph shows mean ± SD of MYC intensities normalized to Tubulin for 4 independent experiments. ***p-value<0.01, *p-value<0.1.

Mutations within the N1-Box are potential drivers of Notch-mediated tumorigenesis

Multiple studies have identified mutations in the Notch1 receptor as a major cancer driver (Koch et al., 2013; Ntziachristos et al., 2014; South et al., 2012). Because our studies indicate that loss of N1-Box mutation promotes Notch signaling to an equal or greater extent than mutations found in T-ALL patients, we sought to determine whether mutations within the N1-Box also occur in human cancers. Utilizing the NIH Catalogue of Somatic Mutations in Cancer (COSMIC) database, we identified two gain-of-function mutations in patient tumors that are located within the first 35 residues of hNICD1 (Figure 3.12A). We find that both mutants (hNICD1^{R1758S} and hNICD1^{S1776C}) exhibit greater Notch transcriptional activity than wild-type hNICD1 and have elevated steady state protein levels when compared to wild-type hNICD1 (Figure 3.12B-C). Furthermore, we demonstrate that these mutants have enhanced activity *in vivo*, and injection of *D. rerio* embryos with mRNA from either mutant show significantly increased defects in somitogenesis compared to injection of wild-type hNICD1 mRNA (Figure 3.12D). These studies are consistent with mutations within the N1-Box as potential cancer drivers that act by stabilizing NICD1.

A

COSMIC ID	Mutation (CDS)	Mutation (AA)	Cancer Type
COSM 422441	5272 C>A	R1758S	Bladder/ Urinary tract
COSM 1554560	5327 C>G	S1776C	Lung

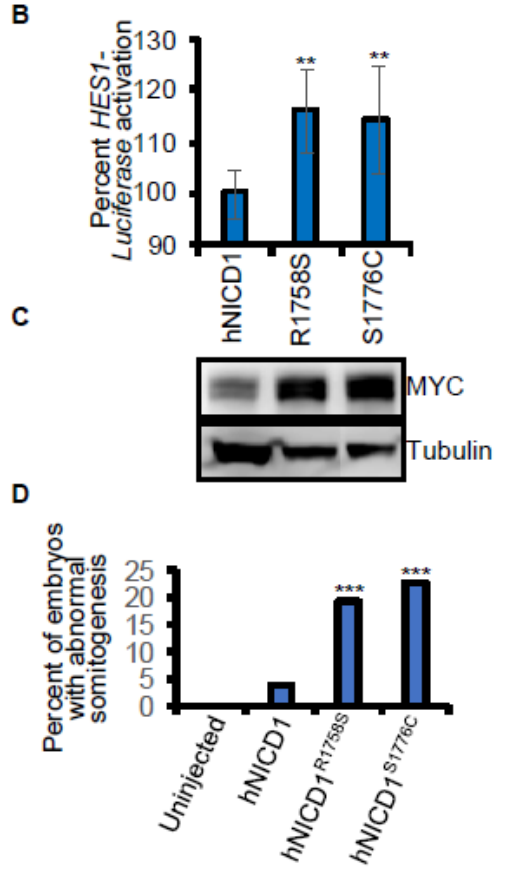

Figure 3.12. Mutations within the N1-Box of hNotch1 in human cancers have enhanced signaling activity. (A) Table of somatic mutations found within residues 1754-1788 of hNotch1 (residues 1-35 of hNICD1) from the COSMIC database. (B) Both mutants in the N1-Box demonstrate elevated Notch reporter activity. HEK293 cells were transfected with plasmids encoding hNICD1 mutants found in human cancers in (A) and the HES1-Luciferase Notch reporter. Graph is representative of at least three independent experiments performed in triplicate and show ± S.D. of Luciferase signal normalized to *Renilla* luciferase (transfection control). **p-value≤0.007 for all mutants versus hNICD1. (C) The R1758S and S1776C mutants of hNotch1 have elevated steady-state protein levels when expressed in cultured cells. HEK293 cells were transfected with MYC epitope-tagged hNICD1 encoding the human N1-Box cancer mutants and immunoblotting performed. Tubulin is loading control. Intervening lanes were removed. (D) Graph is percentage of *D. rerio* embryos at the 10-13 somite stage with defective somites injected with 50 pg of the indicated hNICD1 mRNA. All data is from at least 3 clutches collected from 3 different breeding pairs. n=30-320 embryos per injection. ***p-value<0.0005 versus hNICD1.

Discussion

In T-ALL, mutations of *Notch1* that truncate the C-terminal PEST domain remove key *cis* factors necessary for its proteasomal-mediated degradation, highlighting the importance of regulating NICD1 protein levels upon its liberation from the plasma membrane (Ntziachristos et al., 2014; South et al., 2012). Prior studies indicated that degradation of the intracellular domain of Notch1 (the most ubiquitously expressed of the four Notch paralogs) differs from those of the other three Notch paralogs (Chiang et al., 2006; Tsunematsu et al., 2004; Wu et al., 2001). Herein, we identify the N1-Box, a novel degron of NICD1 that we identified using *Xenopus* egg extract. We show that the N1-Box is transferable as an autonomous degron and that its activity is inhibited by NICD1 binding to CSL. We show that the N1-Box is conserved in vertebrates and that mutations in the N1-Box lead to increased Notch1 activity *in vivo* in zebrafish. Finally, we demonstrate that mutations in the N1-box of Notch1 found in human cancers have increased steady-state levels and enhanced activity. Further evidence for an *in vivo* role for the N1-Box comes from a previous report demonstrating that a chimeric receptor encoding the Notch2 extracellular domain and the Notch1 intracellular domain (Notch21) is two-fold more active compared to wild-type Notch1 when expressed at similar levels in mice (Liu et al., 2013). Interestingly, the Notch21 chimeric fusion deleted the N1-Box of NICD1. Thus, it is possible that the increased activity of this chimera is due to increased stability of its intracellular domain. Based on our results, we propose a model (Figure 3.13) in which the liberated NICD1 (upon cleavage from the Notch1 receptor by γ-secretase) has two fates: (1) rapid degradation and inactivation via its N1-Box or (2) binding to CSL and Notch transcriptional complex components to drive

transcription of Notch target genes (Figure 3.13). Termination of Notch1 signaling occurs upon ubiquitin-mediated degradation by the SCF^{Fbxw7}/proteasome.

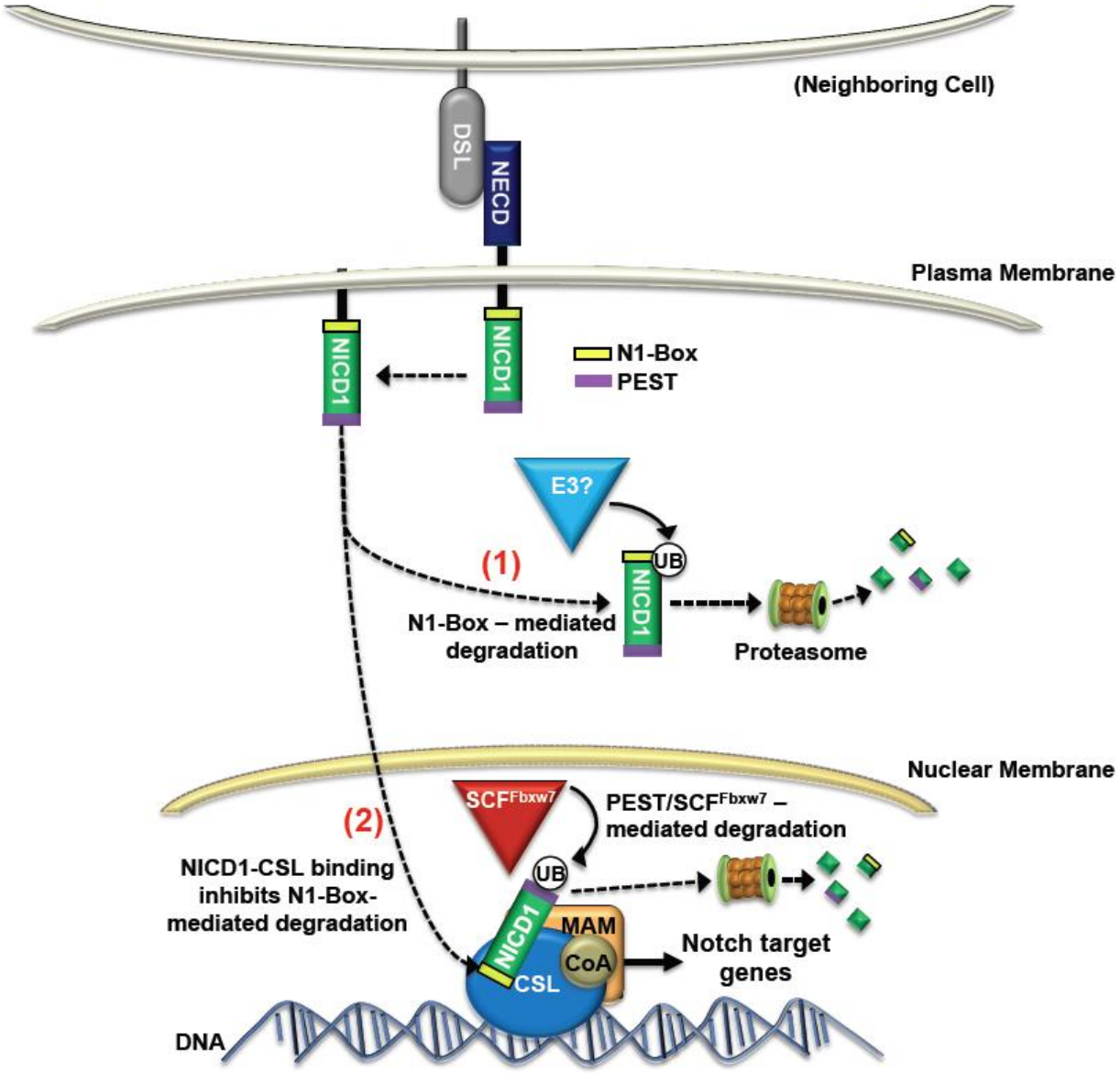

Figure 3.13. Model of N1-Box-mediated regulation of hNotch1 signaling. See text for details.

Previous studies have shown that the NICD has the capacity to assemble into higher order (intramolecular or intermolecular) forms, and disruption of these higher order forms are required for NICD binding to CSL (Kelly et al., 2007; Vasquez-Del Carpio et al., 2011). It is possible that these higher order complexes may promote N1-box-mediated degradation by blocking NICD1 binding to CSL. Alternatively, these higher order structures may inhibit NICD1 turnover as well as transcriptional activation by blocking the activity of the N1-box and its overlapping RAM domain. Further studies are needed to resolve these two possibilities.

Although it is obvious that there are advantages for a mechanism to terminate transcription by proteolysis, it is not as clear why a cell would have a mechanism for degrading cytoplasmic NICD1. Why is the cytoplasmic level of NICD1 not limited exclusively by its release from the holoprotein upon receptor activation? One possibility is that this system dampens the stochastic flux in the system, thereby minimizing noise. Thus, a threshold level of Notch receptor activation needs to occur in order for transcription to be activated. This may explain why the *Drosophila* NICD does not encode an N1-Box as stochastic flux in Notch signaling plays an important role during neuroblast differentiation via the mechanism of lateral inhibition (Artavanis-Tsakonas et al., 1999). Conversely, once the transcriptional complex is fully saturated (i.e. all CSL is occupied by NICD1), unbound NICD1 could be degraded to limit the activation window. This model is consistent with the digital response model proposed for Notch1 pathway activation (Ilagan et al., 2011). Regardless, the importance of NICD1-mediated degradation in maintaining normal Notch1 signaling in humans is evidenced by our

findings that mutations of the N1-Box that lead to hNICD1 stabilization are found in human cancers.

Of the four Notch paralogs, why is the N1-Box only present in the Notch1 paralog? It is possible that differences in regulation between the four Notch paralogs may also simply reflect differences in their transcriptional behavior. One example is the requirement for Notch2 over Notch1 in liver development (Sparks et al., 2010), in which decreased levels of the Notch2 receptor, but not Notch1, leads to Alagille syndrome (McDaniell et al., 2006). Also consistent with differences in the transcriptional behavior between different Notch paralogs is the observation that certain Notch-driven cancers are associated with particular Notch paralogs (Bellavia et al., 2000; Callahan and Raafat, 2001; Capobianco et al., 1997; Kiaris et al., 2004; Pancewicz and Nicot, 2011).

Currently, γ-secretase inhibitors (GSIs) are the best characterized Notch pathway therapeutics. Unfortunately, because of their pan-Notch inhibitory activities, there are many associated adverse effects that limit their long-term use (Espinoza and Miele, 2013; Extance, 2010; Milano et al., 2004; Morell and Strazzabosco, 2013; van Es et al., 2005; Wong et al., 2004). Thus, one long-term goal for treating Notch-driven cancers is to develop paralog-specific drugs in order to minimize adverse effects associated with pan-Notch inhibition. Our identification of the NICD1-specific N1-Box provides a potential path to develop Notch1-selective inhibitors. Compounds that disrupt NICD1-CSL interaction may be particularly potent as they would not only block NICD1-mediated transcription, but also promote NICD1 degradation. The development of such compounds would be greatly facilitated by the identification of the E3 ubiquitin ligase for the N1-Box.

CHAPTER IV

DISCUSSION AND FUTURE DIRECTIONS

Introduction

The work presented in this dissertation represents mostly biochemical, cell-based, and *in vivo* experiments identifying a novel regulatory region of NICD1 which stabilizes the NICD and hyperactivates Notch-mediated transcription which elucidates a novel mechanism that modulates the Notch signaling pathway. In the current Chapter, I discuss and provide future directions for the results described in the previous two Chapters. I have divided this chapter into parts I and II. Part I focuses exclusively on the regulation of the NICD via its novel N1-Box and the implications for Notch signaling. Part II focuses on the future of the Notch-Wnt signaling network and the implications for "Wntch" signaling.

Part I

Discussion

Utilizing *Xenopus* egg extract, cell-based assays, and zebrafish embryos we identified and characterized the N1-Box region of NICD1 as a regulatory domain of Notch signaling, which has not been previously characterized. This novel region is very likely a driver of Notch-mediated cancers in which Notch acts as an oncogene. Additionally, we have identified *cis*-acting factors of NICD1 that regulate the capacity of the N1-Box to promote degradation.

Our initial observation was that human NICD1 degrades robustly in *Xenopus* egg extract, and NICD2, 3, and 4 do not (Figure 3.1). NICD1 degradation is proteasome-dependent, as the addition of MG132 inhibits its turnover (Figure 3.1). NICD1's degradation occurs distinctly from the previously characterized β-catenin degradation in *Xenopus* egg extract (Figure 3.1). Additionally, degradation of NICD1 is GSK3-independent, contradicting previous reports about Notch1 degradation (Espinosa et al., 2003; Foltz et al., 2002; Jin et al., 2009b) (Figure 3.2). These results were validated using mouse NICD1 and NICD4 and tagged versions of the human NICDs, which also degrade differentially (Figure 3.2). Interestingly, all 4 Myc-tagged NICD constructs degrade in HEK293 cells as assessed by cycloheximide chase assay, even though only NICD1-Myc degrades in *Xenopus* egg extract (Figure 3.3). The *Xenopus* egg extract system allows us to separate distinct dynamic degradation pathways, which is much more difficult in cell culture. The degradation of NICD1 in *Xenopus* egg extract is independent of previously-identified PEST/Fbwx7-dependent degradation of NICD1 in cell culture (Figure 3.4). So which domain regulates NICD1 turnover in *Xenopus* egg extract?

We generated N-terminal and C-terminal fragments of NICD1 fused with the respective N-terminal and C-terminal fragments of the other NICD paralogs to address that question. We were able to identify that NICD1 degradation is N-terminal dependent (Figure 3.5). We narrowed the N-terminal regulatory region of NICD1 down to the first 35 amino acids of NICD1 (Figure 3.6). The NICD1 and the Notch 2, 3, 4 ICDS have conserved N-terminal domains within those 35 amino acids but the very N-terminus is not conserved among the Notch paralogs. We found that three key residues at the very

N-terminal region of NICD1 (L2, S4, and Q10 in human NICD1) stabilize the NICD1 when they are individually mutated to resemble NICD2 in *Xenopus* egg extract (Figure 3.6). These stable mutated forms of NICD1 also hyperactivate Notch signaling in HEK293 cells (Figure 3.6). When these three residues are collectively mutated to resemble NICD2, the NICD1 is stable in HEK293 cells (Figure 3.6). These results indicate that the N-terminal region of NICD1 is necessary for its degradation. But is this N-terminal region sufficient to promote degradation?

The heterologous addition of the N-terminal region of N1ICD onto the stable proteins NICD2, GFP, and Luciferase was sufficient to promote their degradation in *Xenopus* egg extract and in HEK293 cells (Figure 3.6; Figure 3.7). Our results show that the N-terminal region of NICD1 is both necessary and sufficient to promote degradation of proteins, fully validating it as a novel degron. We have designated this new regulatory region of NICD1 as the N1-Box. Our results show that the N1-Box is both necessary and sufficient to promote degradation of proteins in *Xenopus* egg extract and HEK293 cells.

Next, we investigated the potential cooperation between the newly identified N1-Box and previously established regulatory domains of NICD1, particularly the PEST domain and the WSSSSP domains (Chiang et al., 2006; Fryer et al., 2004). The N1-Box hyperactivates Notch signaling to a higher extent than the other previously established domains and this hyperactivation is additive with the WSSSSP domain but none of the others (Figure 3.8), strongly suggesting that the WSSSSP domain acts through a parallel pathway with the N1-Box or acts upon the N1-Box in some way. Up until this point, all of our experiments were performed with the NICD1 rather than the full-length

canonical Notch receptor. Do these stabilizing N1-Box mutations affect canonical Notch signaling in the context of full-length Notch receptors?

The stabilized N1-Box mutant isoform of full-length mouse Notch1 hyperactivates Notch signaling in HEK293 cells and in zebrafish embryos (Figure 3.8). These results were validated using a constitutively active form of mouse Notch1 (Figure 3.8). Interestingly, the N1-Box mutant isoform of Notch1 hyperactivates Notch signaling to a larger extent than the PEST domain mutant of Notch1, supporting our cell culture transcriptional reporter assay results (Figure 3.8). These results, taken collectively, show that the N1-Box of Notch1 both stabilizes the NICD1 and hyperactivates canonical Notch signaling in mammalian cell culture and *in vivo* in zebrafish embryos. This transcriptional hyperactivation is higher for the N1-Box mutants than for the previously characterized PEST domain mutants. We next ask whether mutations in the N1-Box are found in human cancers and whether these cancers have elevated Notch signaling.

We found that mutations in the N1-Box identified from the COSMIC database in Notch-driven cancers also confer stability to NICD1 in cell-based assays, which strongly suggests that the N1-Box mutations are correlated with Notch-driven cancers (Figure 3.12). These results show that mutations in the N1-Box of Notch1 are associated with human diseases, marking the novel N1-Box regulatory region as a potential therapeutic for Notch-mediated cancers. Curiously, there are not a large number of mutations in the N1-Box that are linked to cancer. Our hypothesis is that *cis*-binding factors on Notch1 affect the N1-Box's ability to promote degradation.

Next, we investigated the role of known *cis*-binding factors on regulation of the N1-Box-dependent degradation. Based on structural analysis, the CSL/Su(H)/Lag-1

binding site is located in the RAM domain, adjacent to the N1-Box. The addition of recombinant CSL inhibits NICD1 degradation in a dose-dependent manner in *Xenopus* egg extract and this effect is reversed in a CSL-binding mutant that cannot bind to CSL (1771-1774A) (Figure 3.11). These results are validated in HEK293 cells, where the steady state level of the CSL-binding mutant is significantly lower than wild-type NICD1 because it can no longer be shielded from degradation by CSL (Figure 3.11). Interestingly, an N1-Box and CSL-binding double mutant appears to be even more stable than the N1-Box mutant alone (based on the quantification), further supporting the idea that CSL binding affects the N1-Box. These results partially elucidate a mechanism for regulation of the N1-Box which is dependent on CSL binding and subsequent CSL-mediated transcription.

Based on our results, a model emerges in which the N1-Box is actively degrading NICD1 upon cleavage by δ-secretase. This degradation is independent of ligand activation. Then, upon recruitment of CSL to the N1ICD, the N1-Box domain no longer promotes degradation as it is sterically inhibited by CSL binding to the RAM domain (Figure 3.13). But why does Notch1 specifically have this extra level of regulation that is not present in the other paralogs? Notch1 is the most widely expressed paralog of Notch and there is some evidence of ligand-independent signaling through Deltex (Fuwa et al., 2006; Yamada et al., 2011). It is likely that this mechanism of regulating protein levels is used to modulate the natural variability of signaling between cells as Notch signaling requires a very precise amount to undergo proper cell-cell signaling.

One emerging idea about signal transduction pathways is the idea of bistability within the system, where a threshold of signal must be reached within each individual

cell which then acts in a binary fashion to either activate signaling maximally or not activate signaling. Our data suggest that the N1-Box may be the switch that imparts Notch1 signaling bistability in an organismal setting. Because Notch1 is so highly expressed in comparison to other Notch paralogs, it is likely that Notch1 requires an additional level of signal modulation in order to prevent ectopic signaling, and the N1-Box is likely at least a partial contributor to Notch1's potential bistability. So how does our model for the regulation of the Notch1 intracellular domain fit into the prevailing models of how Notch signaling is regulated?

The previous prevailing model for the regulation of NICD stability was that it was regulated by the N-end rule of protein stability. In the N-end rule, the N-terminal amino acid of a protein determines its half-life (Bachmair et al., 1986). NICDs which start with valine or methioninie were thought to escape the N-end rule which made them stable enough to activate transcription (Tagami et al., 2008). Our results showed that the N-end rule is not a likely explanation for the stability of the Notch1 mutants, as the wild-type NICD1 starts with a valine but is significantly less stable than the NICD1 (NTΔ10) that starts with an histidine and should be subject to the N-end rule. In addition, point mutations in the N1-Box which do nto affect the initial valine can also greatly affect stability of the NICD1. Our results show that NICD1's stability is regulated by additional factors other than the traditional N-end rule.

One other recently proposed model is that the Notch extracellular domain is the only domain critical for Notch signaling. One recent report showed that a chimeric Notch receptor containing the Notch2 extracellular domain and the Notch1 intracellular domain (Notch21) is two-fold more active than wild-type Notch1 when expressed at similar

levels in mice. Their conclusion was that the Notch extracellular domain was the critical region in the regulation of Notch signaling and that the intracellular domains are actually interchangeable to transduce the signal (Liu et al., 2013). Ostensibly, this conflicts with our conclusions that the N1-Box of the intracellular domain is likely the most important region for regulation of Notch signaling. Interestingly, the Notch21 chimeric fusion deletes the N1-Box of NICD1, which according to our results leads to a more stable intracellular domain. Thus, it is possible that the increased activity of this chimera is due to increased stability of its intracellular domain. A series of experiments to fully combine these two models would start by comparing Notch signaling from the Notch21 chimeric receptor to the Notch1 receptor in which the N1-Box is mutated. Our data suggests that the Notch21 chimera and either the N1(LSQ) or the N1(NTΔ10) would have similar levels of Notch signaling, due to an increase in the stability of the NICD1.

Future Directions

Identifying the complete regulatory mechanism of the N1-Box

This dissertation identifies a novel regulatory region of Notch1 termed the N1-Box which serves as a major regulator of Notch1 protein stability. It also elucidates a mechanism for how the N1-Box can be regulated *in vivo* upon CSL binding to inhibit N1-Box-mediated degradation. However, the full mechanism of N1-Box regulation still needs to be elucidated. Proteins are often regulated by post-translational modifications. The degradation of NICD1 is proteasome-dependent in *Xenopus* egg extract. Proteasome-mediated degradation requires the involvement of an E3 ubiquitin ligase which adds ubiquitin moieties to lysine residues and targets the protein to the

proteasome. The candidate E3s which have been previously linked to Notch1 stability (ITCH, SCF^{Fbxw7}) degraded NICD1 in HEK293 cells in the N1-Box stabilized mutant similarly to wild-type NICD1 (Figure 3.10). In addition, there is a basic region in NICD1 which contains a string of lysines that is not found in the other NICD paralogs, strongly suggesting that NICD1 is being regulated by an E3 distinct from those we tested. These residues are very likely to be targets of ubiquitination and degradation.

One way to identify the E3 ubiquitin ligase for the N1-Box of NICD1 is to perform a screen for mammalian E3s that increase NICD1 turnover. The *Xenopus* egg extract system would be very amenable to this type of screen and using luciferase-tagged proteins we could do a high throughput screen with a quantifiable readout. Validation of any hits from the screen would then be performed in cell culture using a transcriptional reporter assay also using luciferase levels as the readout for protein levels. Any hits identified can be validated in cultured cells and *Xenopus* egg extract in a low throughput manner. The effect of any E3s that alter NICD1 turnover via the N1-Box of NICD1 needs to be investigated thoroughly. If it is directly affecting the N1-Box, then the degradation of NICD1 would be inhibited in the N1-Box mutant. In Chapter I, I discussed that NICD1 is modulated by other post-translational modifications, and these can affect the stability of NICD1 and are worth investigating. Oftentimes, ubiquitination is regulated by phosphorylation at nearby residues.

The other major post-translational modification that could potentially regulate the stability of the NICD1 is phosphorylation. Previous literature has suggested that phosphoregulation of NICD1 can regulate its stability (Choi et al., 2013; Foltz et al., 2002; Fryer et al., 2004). Because the regulation of Notch is so tightly regulated *in vivo*,

modulation of Notch1 in either direction could be critical in affecting canonical Notch signaling. We have performed a small molecule kinase inhibitor screen for modulators of NICD1 turnover in *Xenopus* egg extract using luciferase-tagged NICD1 and assessing for changes in NICD1 degradation via luciferase signal. The library we used was the TOCRIS kinase inhibitor toolbox [used in (Jester et al., 2010)] (http://www.tocris.com/dispprod.php?ItemId=224690#.U-L8TM90zwo).

Our screen identified Ataxia telangiectasia mutated (ATM) and Ataxia telangiectasia and Rad3-related protein (ATR), two well-characterized DNA damage responding serine/threonine kinases that phosphorylate key tumor suppressors such as p53 and CHK2, as modulators of NICD1 turnover in *Xenopus* egg extract. Further characterization of the role of ATM and ATR on NICD1's stability should be pursued. These studies should include structure-function analysis, functional assays in cell culture, and then potentially even *in vivo* studies. Follow up validation on any other hits from the screen should also be investigated. If validated, the screen can begin to identify other novel targets for therapeutics for treatment on Notch-mediated cancers. Additionally, it would be interesting to identify whether any of these kinases affects the capacity of the N1-Box to promote degradation. The N1-Box contains multiple serines and threonines, making it highly likely to be phosphorylated.

If an E3 and a kinase that act on the N1-Box can both be identified, the next step would be to identify the relationship between these two post-translational modifications. Phosphorylation can both promote and inhibit ubiquitination and subsequent proteasome-mediated degradation in other signaling pathways (Orford et al., 1997; Yamamoto et al., 1999). Would the phosphorylation of the N1-Box promote or inhibit its

ubiquitination? Is it context-dependent or tissue-specific? These are the key questions that still need to be answered about the regulation of the N1-Box.

We have also already shown that CSL binding to the N1-Box affects its ability to get degraded. Previous literature showed that CSL binds to the RAM and Ankyrin domains of NICD with high affinity (Hsieh et al., 1996). But is CSL binding to NICD1 regulated? Previous literature has shown that in the nucleus, the scaffold protein MAML binds to the CSL/NICD complex and is required to activate transcription of the CSL/NICD transcriptional complex (Fryer et al., 2002). Potentially the post-translational modifications are actually affecting CSL binding to the RAM domain in the N1-Box, which would then promote degradation of NICD1. The role of ubiquitination and/or phosphorylation on CSL binding is still unclear, and this would be another interesting question to address.

How about the role of the prevailing models in the regulation of Notch signaling? One key experiment would be to assess the stability of the Notch21 chimera vs. wild-type Notch1 and vs. stabilized Notch1. It is likely that the differential signaling levels for the Notch21 chimera vs wild-type Notch1 is due to a difference in the stability of each respective ICD, as we have shown that the stable mutants of NICD1 lead to hyperactivation of Notch signaling in mammalian cell culture and strongly suggests Notch hyperactivation in *D. rerio* embryos.

In vivo **disease models of the N1-Box**

Although we have identified a novel degron, called the N1-Box, in NICD1, and shown that it is functional in canonical Notch signaling in cell culture and in zebrafish

embryos, there are still many unanswered questions about the *in vivo* importance of the N1-Box in Notch-mediated disease. The first step is to do tumorigenic assays in cancer cell lines using our N1-Box mutants, such as colony formation assays to assess for tumorigenesis. If N1-Box mutants can induce tumorigenesis in cell culture, one very obvious next step is to make a mouse model of the stabilized N1-Box mutant of NICD1. Because T-ALL and other leukemias are primarily Notch-driven in humans, we propose to express an inducible N1-Box mutant Notch1 under the control of a leukocyte specific promoter in an inducible Cre-LoxP background mouse. Upon tamoxifen induction, N1-Box-mutant NICD1 expression will potentially induce tumorigenesis in the adult mouse, which would demonstrate that the N1-Box can in fact induce Notch-mediated tumors *in vivo*. Alternatively, we could generate tissue-specific mouse models for the other tissues that are susceptible to Notch-driven cancers, such as mammary glands, liver, skin, lungs and others. In addition, we could make a stabilized N1-Box mouse that is not inducible which express mutant N1 throughout development and look for other Notch-driven phenotypes. Generating tissue-specific mutant lines for the specific mutations that stabilized human NICD1 (Figure 3.5) and assessing their ability to induce tumorigenesis would also be quite interesting and important. Are these stable mutants identified from a human database actually able to induce tumorigenesis, or is the Notch1 mutation a secondary mutation? The mouse model is an ideal model to test many of these hypotheses.

The generation of a mouse model will allow us to assess whether the differential signaling observed in the Notch21 chimeric mice can be explained by the increased stability of the N1-Box. Based on our results in *Xenopus* egg extract, mammalian cell

culture, and zebrafish embryos we would expect the N1-Box mutant mice to have an increased level of Notch signaling, similar to the Notch21 chimeric mice. Further experiments should also be performed to fully elucidate the relationship between the chimeric receptors and the stabilized N1-Box mutant receptors.

Additionally, Notch mutations have been linked to multiple developmental defects in humans, including Alagille syndrome (McDaniell et al., 2006), aortic valve disease (Garg, 2006; Garg et al., 2005), Hajdu-Cheney syndrome (Simpson et al., 2011), CADASIL (Joutel et al., 1996), and T-ALL (Weng et al., 2004). The N1-Box mutant mouse models in which the mutant form of NICD is expressed at birth would be very useful in studies involving developmental defects caused by misregulated Notch signaling. In summary, a mouse model of the N1-Box mutant would be very beneficial to helping fully elucidate the mechanisms regulating Notch signaling and also the mechanisms regulating the N1-Box.

Part II

Discussion

As discussed in Chapter I, there appears to be extensive crosstalk between the Notch signaling pathway and the Wnt signaling pathway (Hayward et al., 2008; Munoz Descalzo and Martinez Arias, 2012), and some refer to the Wnt/Notch signaling network as the “Wntch” signaling network. Many aspects of these two pathways converge and studying how one pathway affects the other would be quite interesting and the identification of a novel regulatory region of NICD1 opens up new avenues for exploring the interplay between Wnt and Notch signaling.

One example of convergence into the “Wntch” pathway is the observation that Presenilin 1, a core subunit of the δ-secretase complex which cleaves Notch, negatively regulates β-catenin stability and transcriptional activity and can promote phosphorylation of β-catenin independent of Axin (Kang et al., 2002; Killick et al., 2001). Other examples of Wnt/Notch signaling convergence have been described extensively in Chapter I and include convergence on Dsh (Ramain et al., 2001; Sokol, 1996), TCF/LEF (Galceran et al., 2004; Ross and Kadesch, 2001; Shimizu et al., 2008), and even the core components themselves (Corada et al., 2010; Hayward et al., 2006; Hayward et al., 2005; Jin et al., 2009a). Based on previous literature, regulating one of these two pathways can also regulate the other one, allowing for combinatorial treatment for both Wnt and Notch-mediated diseases.

Identifying Novel Therapeutic Targets of Notch Signaling

Due to the importance of Notch signaling in many types of diseases, both developmental defects and cancer, studying how this signaling network is properly regulated would be critical to addressing Notch-mediated disease. How would the discovery of the novel N1-Box of Notch1 help address that question? This allows researchers to identify novel targets for development of therapeutics against Notch-mediated cancers. Most of the current treatments for Notch focus on δ-secretase inhibitors (GSIs), most of which are unable to pass clinical trials due to toxicity issues and off-target side effects due to δ-secretase’s promiscuity and effect on multiple cell types and tissues. Our research has uncovered both a novel regulatory region of the

NICD1, the N1-Box, as well as a novel regulatory mechanism involving the *cis*-binding factors CSL and the WSSSSP motif of NICD1.

There are two novel approaches to take for development of therapeutics based on our research. The first is to target the N1-Box directly by activating the E3 responsible for promoting the degradation of NICD1. E3 activation would decrease NICD1 levels and reduce Notch signaling, which would be critical in treating Notch-dependent cancers which have ectopic Notch signaling. Identifying small molecules which target NICD1 degradation via the N1-Box would be one way to specifically target Notch1 as opposed to the other Notch paralogs and other proteins that are dependent on δ-secretase. This would reduce potential off-target effects and likely reduce toxicity to the patients. One caveat with targeting an E3 is that most E3s have multiple substrates and it is possible that there will be other off-target effects distinct from the GSIs. Regardless, our studies open up a significant opportunity for the development of anti-cancer therapeutics against Notch1-driven cancers by increasing the specificity of the therapeutic and decreasing off-target effects.

In Chapter III, we showed that CSL binding can inhibit N1-Box-mediated degradation and stabilize the NICD1 in *Xenopus* egg extract and HEK293 cells. A second novel approach for therapeutics would be to target CSL binding to NICD1. Previous literature has suggested that CSL is a nuclear specific protein (Chiang et al., 2006; Ong et al., 2006) which binds to DNA and activates transcription in a complex with NICD and MAML inside the nucleus. Inhibiting CSL binding would clearly affect CSL-dependent Notch signaling. Previous literature provides us evidence that CSL-independent Notch signaling is mediated by Deltex (Hori et al., 2004). However, in

Xenopus egg extract (a cell-free and nucleus-free system), CSL binding disrupts NICD1 degradation through the N1-Box. Interestingly, this inhibition is independent of the transcriptional inhibition due to loss of CSL binding in cell culture as a CSL-binding mutant further changes the stability of N1-Box mutants in *Xenopus* egg extract (which does not undergo CSL-mediated transcriptional activity). Identifying small molecules which disrupt CSL binding to NICD1 would allow for constitutive activation of N1-Box-mediated degradation and modulation of Notch signaling. Although CSL binds to all the Notch paralogs to activate signaling, NICD2, 3, and 4 do not contain the N1-Box, and, thus, their stability is not likely to be affected by CSL binding.

In addition, the disruption of the WSSSSP motif likely affects N1-Box-mediated degradation. The WSSSSP motif is heavily phosphorylated and is shown to regulate NICD stability. Identifying small molecules which could affect the phosphorylation of the WSSSSP motif could also serve as a potential new target for therapeutics. We showed that mutation of the WSSSSP motif cooperates with the N1-Box to hyperactivate Notch signaling, which strongly suggests that the WSSSSP motif is actually inhibiting Notch signaling in some fashion, likely through promoting its degradation. Being able to stabilize the phosphorylation of the WSSSSP motif through a potential therapeutic would also help be useful in the treatment of Notch-mediated cancers.

Collectively, our data identifies novel regulators of Notch signaling which have not been previously reported. WE show that NICD1 degrades in a cell-free system giving researchers additional tools for identifying modulators of the Notch signaling network (specifically the Notch pathway) that were not available previously. Because the N1-Box is specific to Notch1, inhibiting the N1-Box should not lead to global Notch

phenotypes but only Notch1-specific phenotypes, allowing researchers to parse out the involvement of Notch1 specifically vs. the other Notch paralogs *in vivo*. This work will help us take a small step forward towards identifying effective and usable new therapeutics for Notch signaling. Any potential therapeutics for Notch signaling may also have the potential to potentiate or dampen Wnt/β-catenin signaling because of the extensive crosstalk between the Notch and Wnt/β-catenin signaling pathways.

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