Ubiquitin Specific Protease 34 (USP34), a new positive regulator of Canonical Wnt/β-catenin signalling

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

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Abstract

The Wnt pathway is a fundamental signalling pathway conserved in all animals, regulating growth, differentiation, embryonic development, and tissue homeostasis in adults. Wnt signalling is kept quiescent by ubiquitin-mediated degradation of the transcription factor β-catenin, orchestrated by a group of proteins called the Destruction Complex. Aberrant Destruction Complex activity is a common theme in many cancers, and is the primary cause of colon cancer. Through mass spectrometry analysis of Axin protein complexes (a key Destruction Complex component) we identified the deubiquitinating enzyme USP34 as an Axin-interacting protein. Functional studies showed USP34 functions to positively regulate Wnt signalling, acting downstream of β-catenin stabilization. While characterizing USP34 we also discovered a new positive regulatory role for Axin in promoting signalling that is dependent on its nuclear localization. Our results suggest that USP34 stabilizes the nuclear pool of Axin through regulating its ubiquitination and offers a potential strategy to target pathological Wnt signalling.
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# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title</td>
<td>i</td>
</tr>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>iii</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>iv</td>
</tr>
<tr>
<td>List of Figures and Tables</td>
<td>vii</td>
</tr>
<tr>
<td>1.1: Introduction: Wnt (Wingless/Int-1)</td>
<td>1</td>
</tr>
<tr>
<td>Evolutionary origins</td>
<td>2</td>
</tr>
<tr>
<td>Wnt pathway functions</td>
<td>2</td>
</tr>
<tr>
<td>Canonical signalling</td>
<td>3</td>
</tr>
<tr>
<td>Non-Canonical signalling</td>
<td>5</td>
</tr>
<tr>
<td>Disease related to Wnt signalling</td>
<td>7</td>
</tr>
<tr>
<td>1.2: Canonical Wnt Signalling Pathway Details</td>
<td>9</td>
</tr>
<tr>
<td>Wnt production and secretion</td>
<td>9</td>
</tr>
<tr>
<td>The inactive Wnt pathway at steady state</td>
<td>10</td>
</tr>
<tr>
<td>The Destruction Complex</td>
<td>10</td>
</tr>
<tr>
<td>Wnt activation</td>
<td>11</td>
</tr>
<tr>
<td>Nuclear signalling events</td>
<td>14</td>
</tr>
</tbody>
</table>
1.3: Ubiquitin, Ubiquitin-Proteasome System (UPS)

Ubiquitination

Mono- and poly-ubiquitination, branching

Proteasome mediated degradation

De-ubiquitination

Disease caused by UPS disruption

UPS and Wnt signalling

1.4: Project Hypothesis

2.0: Materials and Methods

Plasmid Constructs

Tissue Culture and Transfection

Wnt3A Conditioned Media

Western Blotting/Antibodies

Tandem-affinity Purification and Mass Spectrometry

TOPFlash Luciferase Reporter Assays

Co-immunoprecipitation

K48 Chain Cleavage

UBL-PLA2Assay

Immunofluorescence

β-catenin stabilization assay

Axin Ubiquitination
3.0: Results

3.1: Ubiquitin Specific Protease 34 (USP34) found within Axin protein complex by mass spectrometry proteomic analysis

3.2: USP34 confers ubiquitin specific protease activity to the Axin complex

3.3: USP34 positively regulates β-catenin dependent transcription downstream of the Destruction Complex

3.4: A new positive regulatory role for Axin in the Wnt pathway, downstream of the Destruction Complex

3.5: USP34 controls the nuclear-cytoplasmic shuttling of Axin

3.6: Axin ubiquitination is dynamic

3.7: USP34 stabilizes Axin, likely via deubiquitination

4.0: Discussion

References

Abbreviations
List of Figures and Tables

Table 1: Wnt pathway major components 65
Table 2: Mass Spectrometry: interactors within Axin1/2 protein complex 66
Figure 1. Regulation of β-catenin by the Destruction Complex. 67
Figure 2. Canonical Wnt Signalling. 68
Figure 3. The Ubiquitin Cycle. 69
Figure 4. Identification of the Ubiquitin Specific Protease 34 (USP34) as an Axin interacting protein 70
Figure 5. USP34 confers ubiquitin protease activity to the Axin protein complex. 71
Figure 6. USP34 has a positive regulatory function in Wnt signalling. 72
Figure 7. Uncovering a positive regulatory role for Axin in pathological Wnt signalling. 74
Figure 8. USP34 controls the nuclear-cytoplasmic shuttling of Axin. 76
Figure 9. Ubiquitinated Axin is sensitive to ubiquitin specific protease activity (USP). 77
Figure 10. USP34 knockdown destabilizes Axin. 78
1.0: Introduction: Wnt (Wingless/Int-1)

In 1982, Roel Nusse and Harold Varmus discovered a preferential insertion site of the mouse mammary tumor virus (MMTV) within the *Mus musculus* genome at a locus they named Int-1\(^1\). Sequencing and alignment later revealed that the *Drosophila melanogaster* morphogen Wingless (Wg) was orthologous to Int-1, linking Int-1 to the Wg signalling pathway studied at the time and hence the name Wnt (Wg+Int). There are 19 Wnt genes in vertebrates, 7 in arthropods, and 5 in *Caenorhabditis elegans*\(^2\).

In *D. melanogaster* embryogenesis, Wg acts as a morphogen and is responsible for establishing body patterning, with high levels specifying development of naked cuticle, while low levels instructing denticle formation. During larval development Wingless is also needed to promote wing growth and sensory bristle specification\(^3\). These easily observable morphological changes have made the fruit fly a powerful research model, with subsequent genetic screens and epistasis analysis helping to establish the main components and their chain of action within the Wnt signalling pathway. Analogously, the importance of Wnt proteins for vertebrate development became clear with observations that injection of *Xenopus laevis* embryos with mouse Wnt1 mRNA resulted in embryos with two heads\(^4\). This experiment established that Wnts are important for dorsal axis specification and provided the framework to study the signalling pathways controlled by Wnt proteins during animal development. The discovery that the main Wg signalling components such as Frizzled (Fz), Arrow (LRP), Zeste-white (GSK3), Dishevelled (Dvl) and Armadillo (β-catenin) have vertebrate homologs (Table 1)\(^5\) further highlighted that this pathway is evolutionary conserved.
1.1: Evolutionary origins

Wnt signalling developed very early in the evolutionary history of multi-cellular animals; the sea anemone *Nematostella vectensis* (Cnidaria) shares 18 of the 19 vertebrate Wnt genes\(^2\), while the sponge *Oscarella carmela* (Porifera) also shares many pathway components including Wnt, Frizzled, Dickkopf, β-catenin, and Dishevelled\(^6\). Cnidaria and Porifera are sister branches to Bilataria (vertebrates and all other animals), which diverged over 550 million years ago, before the Cambrian explosion\(^7,8\). The basic functions of these conserved proteins have also been retained, with β-catenin being responsible for specifying oral-aboral axis during gastrulation in *N. vectensis*\(^9\), much as it does for *D. melanogaster* and for vertebrates. This evolutionary conservation suggests a primordial and important function for the Wnt pathway in all multi-cellular animals.

1.2: Wnt pathway functions

One common interpretation of our knowledge of Wnt signalling has been to divide the pathway into at least two molecularly distinct branches that produce different cellular responses. The more studied of the two has been termed the Wnt/β-catenin or Canonical pathway and is involved in specifying cell fate and regulating cellular proliferation\(^10\). As its name implies, the activity of the Wnt/β-catenin pathway requires the function of the transcriptional co-activator β-catenin.
The second branch is the less well defined Non-Canonical pathway, responsible for establishing cell polarity and is also implicated in some instances of cell motility, such as the migration of cells during convergence and extension movements during gastrulation\textsuperscript{11}. This distinction between Canonical and Non-canonical signalling is merely a simplification, as there is increasing recognition that Wnt signalling is more complex and depends on spatial and temporal contexts, such as the presence of unique regulatory factors or the differential expression of ligands and receptors\textsuperscript{12}.

1.2.1: Canonical Wnt signalling

Canonical Wnt signalling is well known for establishing the dorsal-ventral axis in vertebrates, body segmentation in \textit{D. melanogaster}, development of the gut in \textit{C. elegans}\textsuperscript{13}, and also acts in tandem with Notch signalling in somite patterning in mice\textsuperscript{10}. Dorsal-ventral axis formation is a critical developmental process that is evolutionarily conserved and easily observable in common vertebrate model organisms such as zebrafish and Xenopus, which have become invaluable tools for studying this pathway. In this context, β-catenin signalling is important for the specification of the dorsal axis of the embryo. Furthermore, recent \textit{in vivo} studies using these models have established a fundamental role for maternally deposited Wnt11 ligand as a dorsalizing factor in the embryo acting as early as the 4 cell stage\textsuperscript{14}. Canonical signalling is also implicated in generating the neural crest\textsuperscript{15}, and establishing posterior structures such as the hindbrain and spinal cord during central nervous system development\textsuperscript{16}. In addition to its importance in early development, the pathway is also critical in adults for tissue homeostasis. Indeed, the maintenance of several adult stem cell populations has been demonstrated to be
under the influence of Wnt proteins\textsuperscript{17}. One particularly well studied example is the control of intestinal stem cells that supports the rapid turnover of the intestinal epithelium\textsuperscript{17}. Bulge cells in the hair follicle responsible for hair growth and hematopoietic stem cells (HSCs) of the bone marrow also require canonical Wnt signalling to achieve homeostasis\textsuperscript{18}.

At the molecular level, canonical Wnt signalling revolves around the post-translational regulation of the transcription factor \(\beta\)-catenin. Under resting conditions, \(\beta\)-catenin is constantly targeted for ubiquitin-mediated degradation by a cytoplasmic protein complex named the Destruction Complex, an assembly of proteins that include Axin, Adenomatous Polyposis Coli (APC), as well as the protein kinases Casein Kinase 1\(\alpha\) (CK1\(\alpha\)) and Glycogen Synthase Kinase 3\(\beta\) (GSK3). The role of the Destruction Complex is to orchestrate the phosphorylation and presentation of \(\beta\)-catenin to \(\beta\)-TrCP (an E3 ubiquitin ligase, see chapter 3.1: Ubiquitination) for ubiquitination and subsequent degradation by the proteasome\textsuperscript{19}. Thus, under resting conditions, the cellular levels of \(\beta\)-catenin are kept low by the activity of the Destruction Complex.

A subgroup of Wnt proteins such as Wnt1, Wnt3a, and Wnt8\textsuperscript{13} have been shown to interact and activate the Frizzled and LRP6 receptors at the cell surface and to stimulate the Wnt/\(\beta\)-catenin pathway. Once activated, Frizzled can engage the cytoplasmic protein Dishevelled (Dvl) which disrupts the formation and function of the Destruction Complex. This causes an accumulation of newly synthesized \(\beta\)-catenin (no longer ubiquitinated and degraded) that can then enter the
nucleus where it acts as a transcriptional co-activator to initiate transcription of Wnt target genes.

1.2.2: Non-canonical signalling

In addition to the canonical/β-catenin pathway, Wnt-activated Frizzled (Fz) receptors can also stimulate at least two other molecularly distinct signalling pathways that do not involve β-catenin: the Planar Cell Polarity (PCP) pathway and the Wnt Ca2+ pathway. These pathways are alternatively classified as β-catenin-independent or Non-Canonical signalling pathways.

The PCP pathway provides a means for the cell to sense polarity within its environment. In addition to the well characterized apical basal polarity, cells also exhibit polarity with respect to the plane of the epithelium. PCP signalling plays a crucial role in D. melanogaster development where it is important in establishing the chiral/asymmetric arrangement and orientation of the photoreceptor cells within each ommatidia in the eye and in coordinating the hairs of the wings. Each Drosophila wing cell produces a single hair at the distal tip of the cell with all the hairs pointing in the same direction in wild-type flies. When PCP proteins are mutated, defects in hair polarity and ommatidia orientation are observed. These easily observable outcomes of PCP signalling have established Drosophila as an important model organism for studying the non-canonical Wnt pathway and have allowed the identification of several pathway members.

PCP signalling is also critical for tissue organization during vertebrate development. One well characterized role of PCP signalling in vertebrates is the polarized organization of auditory hair
cells of the organ of Corti, within the inner ear\textsuperscript{21}. Another context where PCP signalling plays important roles is during convergence and extension cell movements during embryonic gastrulation. These polarized cell movements allow mesodermal cells to migrate towards the midline (convergence), intercalate together and allow the extension of the developing embryos into the anterior-posterior axis.\textsuperscript{22} Wnt proteins such as Wnt5a and Wnt11 that are not typically activating the β-catenin pathway have been found to regulate these processes\textsuperscript{23}. Several of the Drosophila PCP protein homologues have also been described to participate in these polarized cell movements in vertebrates\textsuperscript{24}.

Although Non-canonical and Canonical pathways share several components, namely Wnt proteins binding to Fz receptors and downstream activation of the protein Dishevelled (canonical pathway reviewed in depth in Chapter 2.4), the PCP pathway utilizes a different set of molecules including Flamingo, Prickle, Vangl, and Diego, while the Wnt/Ca\textsuperscript{2+} pathway involves activation of Calcium/Calmodulin-dependent kinase II (CamKII) and PKC. Both pathways are thought to lead to reorganization of the cytoskeleton as a means of generating polarity and modulating cell adhesion for mobility\textsuperscript{22}. However, these β-catenin independent pathways are not completely functionally detached from canonical signalling as stimulation of non-canonical signalling can inhibit canonical pathway activity\textsuperscript{25}.

Activation of these β-catenin independent pathways does not directly result in cellular proliferation and mutations and these pathways have been less frequently associated with
human cancers and other diseases. Since my thesis is focused on the canonical Wnt/β-catenin signalling, I will not review these non-canonical pathways in detail here.

1.3: Diseases related to Wnt signalling

Because of its fundamental role in development and homeostasis, dysregulation of Wnt signalling has serious consequences and has been found to be the cause of several human diseases. One clear example is colon cancer, the second leading cause of cancer-related deaths in Canada, with an estimated 22,000 new cases and 9,100 deaths in 200926.

Wnt signalling was found to be constitutively activated in colon cancers from patients with inherited Familial Adenomatous Polyposis (FAP)27, where the tumour suppressor gene APC is mutated28. Patients with FAP develop several hundred benign colon adenomas (or polyps) that eventually progress to malignant carcinomas following additional cancer promoting mutations. Furthermore, almost 90% of sporadic colon cancers exhibit aberrant Wnt signalling30, of which a majority (80%) stems from mutations in APC29, and to a lesser degree, from mutations in β-catenin and Axin31. Regulation of Wnt/β-catenin signalling is thus critical to maintaining normal colonic homeostasis and perturbation of this pathway leads to colon cancer.

Our understanding of the etiology of colon cancer has increased the recognition of Wnt involvement in other cancers due to pathway dysregulation18. In chronic myelogenous leukemia (CML) the fusion protein BCR-ABL can only transform hematopoietic stem cells (HSCs) capable of self-renewal, a process controlled by Wnts, into leukemic cancer stem cells32. Wnt1 and
Wnt2 appear to be upregulated in non-small-cell lung cancers (NSCLCs) along with loss of the Wnt antagonists WIF-1 and sFRP1\textsuperscript{33}. Many mouse models of breast cancer exhibit a high incidence and early onset of mammary tumours, primarily due to congenital transmission of the Mouse Mammary Tumour Virus (MMTV)\textsuperscript{34}, which induces tumourigenesis by causing Wnt-1 over-expression\textsuperscript{1}. In hepatocellular carcinomas (HCCs) alteration of Wnt signalling due to mutations in β-catenin account for 18% of cases\textsuperscript{35}, with mutations in other components such as Axin also found in 5% of HCCs\textsuperscript{36}.

Disruption of canonical signalling is also increasingly recognized in other diseases. Evidence has been mounting that neurodevelopmental defects significantly contribute to Schizophrenia\textsuperscript{37}, with Disrupted in Schizophrenia 1 gene (DISC1) (first gene found highly associated with this disease) shown to be required for maintaining neuro-progenitor cells through promoting canonical Wnt signalling\textsuperscript{38}. One hallmark of Alzheimer’s Disease (AD) is the accumulation of β-amyloid and hyperphosphorylated tau proteins that lead to neurotoxicity, both leading to increased GSK3 activity and reduced β-catenin levels\textsuperscript{39,40}. Indeed Wnt pathway stimulation is neuroprotective and can ameliorate some behavioral deficiencies of AD in animal models\textsuperscript{41}. Familial Exudative Vitreoretinopathy (FEVR) is an example of canonical pathway malfunction affecting the Wnt receptors. Pediatric FEVR patients suffer vision loss due to progressive retina deterioration (abnormal vascularization), ultimately leading to retinal detachment. It is now known that FEVR is caused by mutations in Frizzled4 and Norrin (a Wnt-like ligand)\textsuperscript{42} and less frequently in LRP5/6.
2.0: Canonical Wnt Signalling Pathway

2.1: Wnt production and secretion

Wnts are a set of small hydrophobic proteins (38-42 kDa) characterized by a unique pattern of cysteine residues, glycosylation sites, and an N-terminal signal sequence. The signature alignment of these conserved cysteines is often used to identify Wnt proteins in different organisms \(^4^3\).

Newly synthesized Wnt protein is lipid-modified with palmitic acid by the acyltransferase Porcupine (Porc) within the Endoplasmic Reticulum (ER). This lipid modification is required for subsequent glycosylation and lipoprotein binding, which help target it for exocytosis and extracellular distribution \(^4^4\) (lipoproteins may also help the interaction with heparin sulfate proteoglycans (HSPGs) in the extracellular matrix (ECM) and aid Wnt diffusion \(^4^5\)).

Palmitoylated and glycosylated Wnt is then transported to the Golgi network, where it is bound to the transmembrane protein Wntless (Wls) \(^4^6\). Wntless carries Wnt from the Trans-Golgi-Network (TGN) to the cell surface via vesicles where it is released in the extracellular environment \(^4^7\). Wls is then retrieved from the plasma membrane by endocytosis and degraded, or recycled for further use by the retromer complex; a mechanism for recycling endosomal components that are otherwise destined for lysosome degradation (e.g. surface receptors) \(^4^3\). Retromer mediated recycling of Wls seems to be necessary for releasing sufficient Wnt for long range signalling \(^4^8\).
2.2: The Wnt pathway at steady state

Canonical Wnt signalling is peculiar in many aspects, starting with the dual nature of the transcription factor/junctional protein β-catenin. β-catenin is an essential subunit of adherens junctions, working in tandem with α-catenin to anchor actin cytoskeleton filaments to plasma membrane cadherins\(^4\) and essential for cell-cell adhesion. β-catenin can be targeted to adherens junctions via serine/threonine phosphorylation by Casein Kinase II (CK2)\(^5\) while junctional β-catenin can be released via tyrosine phosphorylation by receptor tyrosine kinases (RTKs)\(^6\). β-catenin can also exist in the cytoplasm and in the nucleus, and it is this non-membrane bound pool that is responsible for Wnt signalling. Whether β-catenin can shift between these distinct pools is still controversial\(^7\) and in *C. elegans* the junctional and transcriptional roles are fulfilled by two distinct β-catenin homologs (HMR-1 and BAR-1 respectively)\(^8\).

At resting state, the small fraction of β-catenin not localized to the adherens junctions is actively phosphorylated by the Destruction Complex, targeted for ubiquitination and rapidly degraded via the proteasome. Although this unique regulation may seem futile as cells constantly produce and destroy β-catenin to keep the pathway inactive, it offers a very tight control of pathway activity by controlling the cellular levels of β-catenin and allowing the cell to sensitively and rapidly respond to circulating levels of Wnt proteins.

2.3: The Destruction Complex
The Destruction Complex is a cytoplasmic assembly of several proteins that coordinate the phosphorylation and ubiquitination of β-catenin, targeting it to the proteasome for destruction. It is composed of the kinases Casein Kinase 1α (CK1α) and Glycogen Synthase Kinase 3 (GSK3β), both anchored to the scaffold protein Axin, which is also bound to the tumour suppressor Adenomatous Polyposis Coli (APC)\textsuperscript{19}.

β-catenin binding to Axin initiates a ‘priming’ phosphorylation of β-catenin at residue S45 by CK1α\textsuperscript{53}, subsequently allowing GSK3β to sequentially phosphorylate residues T41, S37, and S33\textsuperscript{54}. Phosphorylated β-catenin is very vulnerable to dephosphorylation by Protein Phosphatase 2A (PP2A), and must be protected by APC\textsuperscript{55} in order to be recognized as a substrate for ubiquitination by β-Transducin repeat-Containing Protein (β-TrCP), a component of the SCF\textsuperscript{β-TrCP} ubiquitin ligase machinery (see Chapter 3.0: Ubiquitin for details). Following K48 poly-ubiquitination, β-catenin is consigned to destruction via proteasome-mediated proteolysis\textsuperscript{56}. Although β-catenin regulation (and pathway inhibition at steady state) involves many steps, APC is especially important within this complex since several mutations in APC that result in the inactivation of Destruction Complex function and hyperactive Wnt/β-catenin signalling have been identified in human cancers (Figure 1).

2.4: Wnt pathway activation

Various receptors for the Wnt proteins can be found at the cell surface. The Frizzled receptor (Fz), which belongs to the seven-pass transmembrane G-protein coupled receptor family, were the first Wnt receptors discovered and are the best characterized\textsuperscript{57,58}, with 4 \textit{D. melanogaster}
and 10 vertebrate isoforms. Fz receptors bind their ligands (primarily Wnts) through an extracellular N-terminal Cysteine Rich Domain (CRD), and are involved in signal transduction for both the canonical and non-canonical Wnt pathways\textsuperscript{59}. Different Fz receptors also exhibit overlapping specificity towards Wnt ligands\textsuperscript{57} but it is commonly thought that specific Wnts interact preferentially with given Fz receptors to allow for signalling specificity. Unique to canonical signalling, Fz also associates with the single-transmembrane domain Low density lipoprotein Receptor-related Protein 5 and 6 (LRP5/6) that act as co-receptors to interact with Wnt proteins and initiate the activation of the pathway\textsuperscript{60}.

Aside from Frizzled, the tyrosine kinase receptors Ryk and Ror have recently been identified as atypical Wnt receptors. Ror binds Wnt via a similar CRD, while Ryk interacts with Wnts via a WIF (Wnt Inhibitory Factor) domain. In \textit{D. melanogaster} the Ryk homolog Derailed binds Wnt5a and guides neuron guidance\textsuperscript{61} while vertebrate Ryk has recently been shown to be important for neurogenesis\textsuperscript{62}. Ror2 on the other hand has been shown to help Wnt5a-mediated convergent extension in Xenopus\textsuperscript{63} and to be required for non-canonical Wnts to inhibit the Wnt/β-catenin pathway\textsuperscript{64}.

In addition to Wnt proteins, other endogenous pathway agonists and antagonists have been found. The protein Norrin, mutated in Norrie disease (congenital blindness), can activate canonical signalling through Fz4 binding, and is important for vascular development of the eye and ear\textsuperscript{65}. The Wnt inhibitor Dickkopf (Dkk) blocks signalling by binding to LRP6 and causing its
internalization\textsuperscript{66}, while Wnt Inhibitory Factors (WIFs) and Soluble Frizzled-Related Proteins (SFRPs) are secreted proteins that sequester Wnt\textsuperscript{44} in the ECM.

The Fz-LRP6 receptor complex recognizes Wnt proteins and is responsible for canonical signalling. Wnt binding to Fz promotes the formation of the Fz-LRP6-Wnt complex, leading to activation of the cytoplasmic protein Dvl. Activated Dvl then disrupts the Destruction Complex by promoting Axin binding to LRP6 at the plasma membrane. GSK3 (previously recognized for phosphorylating β-catenin for ubiquitination/degradation) and CK1γ phosphorylate LRP6, thereby producing more high affinity docking sites for Axin. Because Axin is the least abundant component of the canonical signalling pathway the net result of Wnt-induced Axin sequestration to LRP6 is a disruption of the cytoplasmic Destruction Complex\textsuperscript{67,68}. This effectively releases β-catenin from ubiquitination and degradation and allows it to accumulate in the cytoplasm and enter the nucleus to initiate signalling (Figure 2). It is interesting to note the dual and opposite roles GSK3 plays in this system, first as a negative regulator by phosphorylating β-catenin for ubiquitination, then as a positive regulator by promoting Axin recruitment to LRP6. Although we now understand how the major components act together to initiate and propagate Wnt signalling, many questions remain: how is Wnt stimulation quantified/translated into proportionate levels of β-catenin for signalling? Are there mechanisms in place to deactivate signalling, perhaps through dephosphorylation of Axin-LRP6 complexes? What regulates Axin dissociation from the Destruction Complex, and how is that controlled? Answering these questions will provide new insights into the mechanism of
canonical signalling and potentially allow us to derive new means of controlling the aberrant signalling that is the source of many diseases.

2.5: Nuclear signalling events

The mechanism of β-catenin entry into the nucleus is yet to be fully elucidated, but it is known that β-catenin lacks defined nuclear localization sequences (NLS), and that its translocation is independent of importins, the main machinery regulating nuclear import\textsuperscript{69}. Recent work has found that the phosphorylation of the β-catenin C-terminus at S191 and S605 by c-Jun N-terminal Kinase 2 (JNK2) appears to be required for nuclear import\textsuperscript{70}, while glycosylation may also play a role in blocking nuclear entry\textsuperscript{71}. Axin and APC, two critical members of the Destruction Complex, also contain active nuclear localization and nuclear export signals (NLS, NES respectively) allowing them to shuttle between the cytoplasm and the nucleus\textsuperscript{72,73}, presenting one possible mechanism of β-catenin nuclear accumulation/export. However, another study examining the kinetics of β-catenin transport between the cytoplasm and the nucleus concluded that Axin and APC merely act passively in anchoring β-catenin to a particular subcellular compartment\textsuperscript{74}.

Although the means by which β-catenin can enter the nucleus is debated, it has been shown that only N-terminally dephosphorylated β-catenin (sites of GSK3β phosphorylation\textsuperscript{75}) can promote canonical Wnt signalling. Despite proteasome inhibitor (ALLN, MG132) induced accumulation of β-catenin, only N-terminally dephosphorylated β-catenin appears competent at triggering Wnt target gene expression both \textit{in vitro}\textsuperscript{76} and \textit{in vivo}\textsuperscript{77}.  

14
Upon entering the nucleus, β-catenin acts as a co-activator by displacing the transcriptional repressor Groucho/TLE from the T-Cell Factor (TCF)/Lymphoid Enhancer-binding Factor (LEF) DNA binding transcription factor\textsuperscript{78} and initiate the transcription of Wnt target genes, such as Axin2, Naked1, and Troy\textsuperscript{79} (Figure 2).

Interestingly, many of the components involved in canonical signalling also have regulatory functions within the nucleus, but their effects may not necessarily coincide with their cytoplasmic functions. In \textit{D. melanogaster}, in addition to its negative role in the Destruction Complex, cytoplasmic APC can also positively regulate signalling through down-regulating Axin\textsuperscript{80}, while nuclear APC can also repress Wnt signalling by promoting histone H3K4 deacetylation via recruitment of CtBP\textsuperscript{81}. In addition to its role in the cytoplasm to inhibit the Destruction Complex, it appears that Dvl also localizes to the nucleus where it has been found to promote signalling via stabilizing the interaction between β-catenin and TCF\textsuperscript{82}. Nuclear β-TrCP interacts with and enhances transcription by β-catenin-TCF (a positive regulatory role), in contrast to its negative role within the SCF\textsuperscript{β-TrCP} E3 ligase responsible for ubiquitinating β-catenin\textsuperscript{83}. Axin has also been found to shuttle between the cytoplasm and the nucleus, but at this point the functional relevance of this behaviour is unknown\textsuperscript{72}.

3.0: Ubiquitin, Ubiquitin-Proteasome System (UPS)

Cellular protein homeostasis is achieved through a balance of synthesis and turnover by dynamic regulation of transcription and protein degradation. Ever since Rudolf Schoenheimer
demonstrated the *in vivo* incorporation of radiolabeled dietary amino acids into cellular proteins almost 70 years ago, protein degradation has been recognized as a fundamental and critical cellular process. Eukaryotes employ two major pathways for protein turnover: lysosome mediated general proteolysis by acidic proteases and the precisely tuned ubiquitin-proteasome degradation of targeted proteins that is responsible for destroying most endogenous proteins. 80-90% of protein breakdown is mediated by the ubiquitin-proteasome system.

Ubiquitin is a small (76aa, 8.5kDa) heat stable protein that is strongly conserved in all eukaryotes. It was first isolated from mice thymus extracts in 1966 as a putative stimulator of T-cell differentiation, but was refuted as an artifact of endotoxin contamination. A decade later in 1977, Ubiquitin was found covalently linked to histones H2A/H2B, but its function was not elucidated until recently as a regulator of histone methylation. At the same time the Ubiquitin-histone association was being characterized, Ciechanover, Hershko and Rose found ‘APF-1’ (ATP-dependent Proteolysis Factor 1) which they believed to be responsible for the non-lysosomal degradation of intracellular proteins. This led to the realization that APF-1 and Ubiquitin were the same protein and to the discovery that it functioned as a molecular tag for selective and controlled protein degradation.

3.1: Ubiquitination

Ubiquitin and its homologous molecules Nedd8, Sumo, and ISG15 (Ubls, ubiquitin-like protein modifiers) all share a characteristic ‘ubiquitin fold’ structure, along with a C-terminal di-glycine that forms an isopeptide bond with an amino group of the target protein. The ε-amino group of
lysine is the most common ubiquitin acceptor, while N-terminal binding is also possible. Protein ubiquitination is a three step process catalyzed by distinct enzymes at each stage. Free ubiquitin must first be activated by an ATP-dependent mechanism by the E1 enzyme, followed by transfer to an E2 conjugating enzyme that transfer ubiquitin to a target substrate in coordination with the E3 ubiquitin ligase which provide the specificity for substrate recognition\textsuperscript{90} (Figure 3).

The human genome has 11 genes coding for Ubiquitin and Ubls, along with 13 E1 enzymes that process the Ubls, with two E1 genes specific for Ubiquitin. E2 conjugating enzymes are more varied with 49 types, conferring selectivity towards different E3 ligases\textsuperscript{91} and may also affect the type of ubiquitin chain that is ligated to a given target protein (see below). E3 ligases are the most diverse with 617 genes predicted in the human genome, facilitating precise discrimination of target proteins and insuring the specificity of the three-step conjugation process\textsuperscript{92}. E3 ligases are classified into three types: Homologous to E6AP Carboxy Terminus (HECT), Really Interesting New Gene (RING), and UFD2 homology (U-box)\textsuperscript{90}.

In canonical Wnt signalling, phosphorylated $\beta$-catenin is recognized by $\beta$-TrCP, the F-box component of the SCF complex (RING-type E3 ligase). The SCF complex has four units: an F-box protein that binds the phosphorylated substrate, an E2 binding protein Rbx1 (the ring finger protein), the scaffold Cullin (Cul1), and the Skp1 adaptor that joins F-box to Cullin (Figure 1), this complex is responsible for ubiquitinating $\beta$-catenin\textsuperscript{93}. 
3.2: Mono- and poly-ubiquitination, ubiquitin chains

Ubiquitin itself contains seven lysines (K6, 11, 27, 29, 33, 48, 63), making it susceptible to further conjugation by ubiquitin units thereby forming polyubiquitin chains with unique branching structures depending on which lysine residues are utilized. Unique types of poly-ubiquitination and branching of such chains add an extra layer of complexity thereby increasing the possibilities for functional roles. K48-linked ubiquitin chains longer than 4 units, where each unit is linked to lysine 48 of the preceding ubiquitin, is the most well known poly-ubiquitin tag and constitutes a signal that targets the modified protein for degradation by the proteasome\textsuperscript{94}. K63-linked ubiquitination is another common signal with roles reported to regulate kinase activation, vesicle trafficking, and DNA repair\textsuperscript{95}.

Proteins can also be modified with only one ubiquitin residue in a process termed monoubiquitination. Mono-ubiquitin signals are involved in many different cellular processes, such as endocytosis of plasma membrane proteins, viral vesicle budding/release, post-translational protein sorting by the Trans-Golgi Network\textsuperscript{96} and nuclear-cytoplasmic shuttling of proteins. Mono-ubiquitination also plays an important role in chromosomal remodelling. Indeed, Histone H2B monoubiquitination (residue K123) is required for H3 methylation (residue K4) and subsequent transcriptional activation\textsuperscript{88}.

Moreover, varying the type of ubiquitin-like molecules conjugated at a single amino acid locus on a protein can achieve different functional effects. For example, the behaviour of the Proliferating Cell Nuclear Antigen (PCNA) protein depends on whether it is conjugated to SUMO
or Ubiquitin. Under normal circumstances, PCNA is tagged by SUMO at K164, driving PCNA activity towards promoting DNA replication\(^97\). DNA damage leads to replacement of SUMO by monoubiquitin at K164 and promotes DNA repair by trans-lesion polymerases (error-prone), while K63 poly-ubiquitination at the same site leads to repair using a template of the sister duplex (error-free)\(^98\). NMR studies of K48 and K63 ubiquitin linkages suggest the unique properties of each chain is due to conformational differences, with K48 ubiquitin chains exhibiting a compact structure while K63 having a more open and extended conformation\(^99\).

### 3.3: Proteasome mediated degradation

The 26S Proteasome holoenzyme is a large protein complex of 2.5MDa in size, consisting of the 19S Regulatory Particle and the 20S Core Particle. The 20S subunit is a hollow cylindrical complex containing proteolytic active sites facing the inner surface. Proteins are blocked from random entry by the 19S Regulatory unit, which serves as a cap on the open sides of the 20S Core and can selectively bind, de-ubiquitinate, unfold, and translocate K-48 ubiquitinated proteins into the 20S core unit in an ATP-dependent manner. The compact, hydrophobic surface of K48 chains seems to be responsible for affinity towards the 19S Regulatory unit\(^89\). Once the substrate is bound to the proteasome, protein unfolding is initiated in unstructured regions of the substrate and the protein begins entry into the 20S core unit for proteolysis\(^100,101\).

Proteasome mediated protein degradation occurs primarily in the cytoplasm, where it participates in protein quality control, cellular signalling, foreign antigen processing, and
general protein turnover. An estimated 30% of newly synthesized proteins do not pass quality control checkpoints for proper folding and assembly and are quickly degraded by the proteasome. Internalization of some membrane proteins for degradation or recycling can also be mediated by ubiquitination/neddylation. Proteins destined for extracellular export must unfold to enter the endoplasmic reticulum for trafficking, and are also subject to quality control by Endoplasmic Reticulum Associated Degradation (ERAD) where defective proteins undergo retrograde transport back to the cytoplasm for destruction. Misfolded and otherwise defective proteins can be harmful if they aggregate and precipitate (in the case of Creutzfeldt-Jakob, Alzheimer’s, and Parkinson’s diseases). The processing of foreign proteins (e.g. viral proteins) into short peptides by the proteasome is also a critical step in the generation of antigens for initiating immune responses. The proteasome also functions within the nucleus, facilitating nuclear protein turnover and also in modulating transcription through the non-proteolytic function of the 19S regulatory unit that acts by recruiting chromatin remodelling factors.

3.4: De-ubiquitination

Analogous to other covalent protein modifications (phosphorylation, methylation), ubiquitination is dynamic and reversible. The removal or cleavage of ubiquitin is mediated by deubiquitinating enzymes (DUBs). Currently there are 79 predicted functional DUBs encoded by the human genome. DUBs are classified into two major groups based on their enzymatic mechanism: cysteine proteases (USP, UCH, OTU, and MJD domain Dubs), and metalloproteases (JAMM domain). JAMM domain DUBs employ Zn²⁺ to catalyze ubiquitin cleavage, while the
Cysteine proteases utilize a catalytic triad of cysteine-histidine-aspartate residues in their active site. Cysteine protease DUBs are further divided based on the structure and sequence homology of their catalytic domains: Ubiquitin C-terminal Hydrolases (UCH), Machado-Joseph Disease Protein Domain Proteases (MJD), Ovarian Tumor Proteases (OTU), and Ubiquitin Specific Proteases (USP), with USPs being the largest subgroup\textsuperscript{107}. Interestingly, some viruses have also evolved their own unique deubiquitinases (no homology to human DUBs) to counteract the proteasomal processing of ubiquitinated foreign material to generate antigens in order to benefit infection\textsuperscript{108}.

DUBs have been described to serve four important roles within the cell: 1. Cleave \textit{de novo} synthesized ubiquitin into monomer units (Ubls are coded as inactive multimers or fusion proteins\textsuperscript{89}); 2. Editing or rescue of ubiquitinated proteins (both proteolysis dependent and independent roles); 3. Removal of ubiquitin conjugates from proteins to allow entry into the proteasome for degradation; and 4. Recycling of free polyubiquitin chains (from #3) into monomers (reviewed in \textsuperscript{4}). Through these functions DUBs maintain a steady supply of free ubiquitin and regulate the stability and/or localization of targeted proteins.

Out of the 79 putative DUBs, only three DUBs (POH1, UCH-L5, USP14) are known to operate at the proteasome to deubiquitinate proteins and facilitate their unfolding and entry into the proteolytic 20s core\textsuperscript{89}. DUBs have been implicated in a wide range of critical cellular processes, regulating transcription\textsuperscript{109}, endocytosis\textsuperscript{110}, protein stability\textsuperscript{111} and protein localization\textsuperscript{112}. For example, many histones are ubiquitinated, and deubiquitination of H2B by Ubp8 is required for
efficient transcription\textsuperscript{109}. Following activation, many receptors are ubiquitinated, which acts as a signal for internalization. Upon activation, the T-cell Receptor (TCR) is ubiquitinated by the Cbl E3 ligase, leading to endocytosis of the receptor and entry into the endosomal sorting complex. If TCR remains mono-ubiquitinated, it is targeted to the lysosome for degradation, while deubiquitinated TCR (by the deubiquitinase DUB-2\textsuperscript{113}) can be recycled back to the surface for reuse. Disruption of this endocytic process can attenuate ligand-dependent down-regulation of the receptor and cause hypersensitivity\textsuperscript{110}.

DUBs also play a key role in the regulation of some transcription factors. For example the proto-oncogene MYC is ubiquitinated/degraded in the nucleus by FBW7 (a F-box E3 ligase), but is stabilized by USP28 (which is also required for tumour proliferation)\textsuperscript{111}. DUBs can also indirectly control protein levels by regulating the stability of E3 ligases. Under normal conditions the stress sensor/tumour suppressor p53 is constitutively degraded by the E3 ligase MDM2, which is stabilized by USP7/HAUSP (HAUSP has affinity for both p53 and Mdm2). Upon exposure to stress (hypoxia, DNA damage, oncogene activation) HAUSP preferentially associates with and deubiquitinates p53 rather than Mdm2, protecting p53 from degradation\textsuperscript{114}.

HAUSP also acts on the FOXO family of transcription factors, but in a degradation independent fashion. The mono-ubiquitination of FOXO serves as a nuclear localization signal promoting FOXO shuttling from the cytoplasm into the nucleus, which can be antagonized by HAUSP mediated deubiquitination\textsuperscript{112}. In the Transforming Growth Factor β (TGFβ) pathway, monoubiquitination of the transcription factor Smad4 by the ubiquitin ligase Ectoderin (Ecto)
inhibits Smad4 interaction with Smad2, preventing formation of an active transcription complex in the nucleus\textsuperscript{115}. The DUB FAM/USP9x specifically targets Smad4, opposing Ecto and acts as a positive regulator of TGF\(\beta\) signalling\textsuperscript{116}.

Our growing awareness of the balance between E3 ligases and DUBs in regulating ubiquitin modifications has helped redefine the biological role of ubiquitination from a simple one-way signal for degradation into a dynamic regulatory system akin to phosphorylation in modulating protein stability, activity and localization.

### 3.5: Disease caused by UPS malfunction

Reflecting the fundamental importance of the UPS system in so many different cellular processes for maintaining homeostasis, aberrant function of the UPS can be associated to a broad range of diseases including cancers, neurological disorders, and autoimmune diseases. Cancer is one large area of research; many oncogenes are transcription factors rendered hyperactive due to loss of UPS regulation. The tumour suppressor p53 (pro-apoptotic, initiates DNA repair, induces growth arrest) is normally down-regulated by the E3 ligase MDM2\textsuperscript{117}. Activating mutations in MDM2 (20% soft tissue cancers, 7% of all cancers\textsuperscript{118}) leads to further loss of p53, removing one major means of tumour suppression. The autosomal dominant disorder Von Hippel–Lindau (VHL) disease predisposes affected individuals to a variety of tumors. The VHL gene codes for an E3 ligase that regulates Hypoxia Inducible Factor 1 (HIF1), the dysregulation of which leads to increased survival in hypoxic conditions and promotes tumour survival\textsuperscript{119}. FBW7, an F-box substrate adaptor of the SCF E3 ligase complex, is a strong
tumour suppressor responsible for UPS regulation of several proto-oncogenes (c-Myc, Cyclin E, Jun, Notch), and is mutated in about 6% of primary tumours\textsuperscript{120}. FBW7 ubiquitin ligase activity is also countered by USP28, which deubiquitinates and stabilizes c-MYC, and is often highly expressed in colon and breast carcinomas\textsuperscript{111}. Within the canonical Wnt pathway, disruption of UPS function by mutations in APC, Axin, and/or β-catenin can all lead to cancer, especially when it occurs in stem cell populations like those found in the colon Crypts of Lieberkuhn\textsuperscript{121}.

As mentioned briefly in previous sections, several prominent neurological diseases such as Schizophrenia and Alzheimer’s Disease are associated with altered Wnt signalling. Disrupted in Schizophrenia 1 (DISC1) was the first gene found highly associated with Schizophrenia, which normally acts by inhibiting GSK3-mediated β-catenin degradation. Loss of DISC1 function leads to increased β-catenin phosphorylation by GSK3 and subsequent degradation, damping canonical Wnt signalling and loss of neuro-progenitor cells\textsuperscript{38}. In Parkinson’s disease (PD), a build-up of α-synuclein forms pathogenic fibrils that lead to cell death in the brain. α-synuclein is normally degraded via ubiquitination by Parkin, an E3 ligase\textsuperscript{122}. Interestingly, the DUB UCH-L1 has been associated with susceptibility to PD. UCH-L1 is abundant in the brain (1-2% of total protein)\textsuperscript{123}, and exhibits both DUB and ligase activity depending on its dimerization\textsuperscript{124}. However, Parkin has been associated with a multitude of other non-proteolytic ubiquitination targets\textsuperscript{125}, and there is conflicting evidence regarding whether fibrillized α-synuclein (deposited in Lewy Bodies commonly found in distressed neurons of PD patients) is protective\textsuperscript{126}, so the precise etiology of PD remains controversial.
The ubiquitin mediated endocytosis of receptors is also another area of active research, where dysregulated ubiquitination/deubiquitination can lead to increased receptor recycling back to the plasma membrane for signalling and may induce activation of auto-reactive T-cells that can lead to autoimmune disease\textsuperscript{110}. The activity of the UPS system is thus often associated with human diseases. It is therefore important to further our knowledge of the normal cellular processes regulated by the UPS systems to better understand when it is malfunctioning during diseases.

3.6: UPS and Wnt signalling

Aside from regulating β-catenin stability, recent developments have revealed that other critical members of the canonical pathway are also subjected to regulation by ubiquitination, and not only through affecting protein stability. The cytoplasmic protein Dvl is ubiquitinated for degradation by the KLHL12-Cullin-3 E3 ligase\textsuperscript{127}, dampening both canonical and non-canonical Wnt signalling. In the absence of signalling, APC can also be degraded via ubiquitination in a process involving Axin. In this case, Wnt pathway activation is thought to inhibit this process,\textsuperscript{129}. Additionally, APC is also a substrate for K63-linked ubiquitination that is regulated by the DUB Trabid, which acts as a positive regulator of signalling\textsuperscript{130}. It is speculated that K63-ubiquitin may alter APC repressor activity within the nucleus in a degradation-independent manner. The Destruction Complex scaffold Axin is also down-regulated upon Wnt signalling\textsuperscript{131}, presumably in a ubiquitin dependent manner since Axin C-terminal sumoylation protects it from degradation by preventing Axin ubiquitination\textsuperscript{132}. Interestingly, the β-TrCP E3 ligase responsible for ubiquitinating β-catenin also serves an opposite positive role for signalling in the nucleus as a
co-activator (along with p300) to promote β-catenin signalling. The UPS thus plays critical roles in repressing and promoting Wnt signalling, yet the seemingly opposing roles these proteins play depending on context suggests that our current understanding of the molecular mechanisms regulating the canonical pathway is incomplete.

4.0: Project Goal

The scaffold protein Axin is the central component of the β-catenin Destruction Complex. How Axin functions, beyond scaffolding the Destruction Complex kinases and APC to catalyze the destruction of β-catenin is incompletely understood. Using a proteomic approach employing tandem affinity purification followed by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS), we identified Ubiquitin Specific Protease USP34 as a novel Axin-interacting protein. Given the central role of the Ubiquitin Proteasome System in regulating Wnt signalling, the goal of this thesis was to pursue the molecular and functional characterization of the Axin-USP34 interaction and study its potential role in the regulation of canonical Wnt signalling.
5.0: Materials and Methods

5.1: Plasmid Constructs

Human Axin1 and Axin2 cDNA were cloned into the pGLUE tandem-affinity purification plasmid\textsuperscript{127} (pGlue-hAxin1, pGlue-hAxin2) by PCR from a human brain cDNA library. Human point mutant β-catenin (pGlue-pt.mutant-hβcatenin) and human Dishevelled 2 (pGlue-hDvl2) are described previously\textsuperscript{133}. All PCR amplified regions were sequenced and validated.

5.2: Tissue Culture and Transfection

Human HEK293T, RKO colon carcinoma (ATCC: CRL-2577), SW480 colorectal adenocarcinoma (CCL-228), HCT116 colorectal carcinoma (CCL-247), and Mouse L cells (CRL-2647/CRL-2648) were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10\% fetal bovine serum (FBS) and penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO) in a 37°C humidified incubator with 5% CO\textsubscript{2}. HEK293T stable cell lines were generated by transfection with calcium phosphate followed by puromycin selection (2μg/ml) and maintained in culture with media supplemented with puromycin. Transient cDNA transfections were performed following the manufacturer’s recommendations using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

For siRNA experiments, cells were transfected with 25nM of siRNA with recommended amounts of Lipofectamine RNAiMax (Invitrogen). Previously validated siRNAs against β-Catenin, Axin1, Axin2\textsuperscript{134}, and Control-Non targeting (Dharmacon, Lafayette, CO) were used, while a set of 4 siRNAs targeting USP34 was tested by western blot and the siRNA exhibiting the most
efficient knockdown (USP34 siRNA A: 5’-GCAGGGAAGUUCGACGAAUU-3’) was used for all other experiments.

For the epistasis experiments involving expression of pt.mutant.β-Catenin or Dvl2 with a given siRNA, siRNA were first reverse transfected using RNAiMax at the time of seeding cells, followed by replacement of media 24h after seeding and cDNA transfection using Lipofectamine 2000. Cells were then assayed 36h after cDNA transfection using the TOPFlash reporter assay.

5.3: Wnt3A Conditioned Media

Mouse L Wnt-3A cells (CRL-2647) were cultured until reaching 90% confluence, upon which media was collected and refreshed every two days for a total of 6 days. Collected media containing Wnt3A was aliquoted and stored at 4°C. Media from different days was assayed using TOPFlash assays to determine fractions with maximal activity and subsequently used for Wnt stimulation experiments. Parental Mouse L cells not producing Wnt3A (CRL-2648) were also cultured and media similarly collected for use as negative control media.

5.4: Western Blotting/Antibodies

Proteins lysates were resolved with SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Blots were stained with antibodies indicated in the figure legends, then stained with horseradish peroxidase-conjugated secondary antibody and detected by chemiluminescence. Antibodies: α-β-Catenin (#9587, Cell Signalling Technologies); p44/42 MAP
Kinase (ERK) (#9102, Cell Signalling Technologies); α-Axin (C95H11)(#2074, Cell Signalling Technologies); α-Acive-β-Catenin (05-665, Millipore); α-USP34 (A300-824A, Bethyl Labs); α-Lamin B (sc-6217, Santa Cruz Biotechnology); α-HA (MMS-101P, Covance); α-Flag (F1804, Sigma); peroxidase-conjugated secondary anti-goat/rabbit/mouse (705-035-147, 711-035-152, 715-035-150 Jackson ImmunoResearch Laboratories).

5.5: Tandem-affinity Purification and Mass Spectrometry

HEK293T cells (2×10^8 cells) expressing SBP-HA-CBP-tagged Axin construct were used for the tandem-affinity purification procedure as previously described127. Briefly, cells were lysed with tandem affinity purification lysis buffer (TAP lysis: 10% glycerol, 50mM Hapes-KOH pH 8.0, 100mM KCl, 2mM EDTA, 0.1% NP40, 2mM DTT, 10mM NaF, 0.25mM Na3VO₄, protease inhibitors (Sigma)), lysates were incubated at 4 °C with 100μl packed streptavidin resin (GE Healthcare). Resin beads were washed and protein complexes were then eluted from the streptavidin resin in calmodulin binding buffer supplemented with 2mM biotin. The second round of affinity purification was performed using 100μl of calmodulin resin (GE Healthcare). Following washes, the protein complexes were eluted with two 100μl elutions with Calmodulin Elution Buffer (50mM Ammonium Bicarbonate pH 8.0, 10mM EGTA) and the proteins in the complex were reduced in 25mM dithiothreitol and alkylated using 100M iodoacetimide (Sigma), and brought to 1mM CaCl2, then directly digested with sequenced-grade trypsin (Promega) overnight at 37°C. Following digestion, the sample was lyophilized and then resuspended in reversed-phase HPLC buffer A (0.1% formic acid, 5% acetonitrile, 95% H₂O). Microcapillary reversed-phase columns (76-um inner diameter, 361-um outer diameter;
Polymicro Technology) were cut to a final length of 15–20 cm, and spray tips were pulled in-house by hand. Columns were packed in-house (12 cm) with Jupiter proteo 90-Å, 4-um silica particles (Phenomenex) using a pressure bomb. Prior to loading the sample, columns were subjected to a 30 mins equilibration in HPLC buffer B (95% Acetonitrile, 5% H2O, 0.1% Formic Acid) and 30 mins in HPLC buffer A. Half of the trypsin-digested sample was then applied to the column using a pressure bomb. The LC column was then placed in front of a LTQ-XL mass spectrometer (Thermo) programmed for data dependent MS/MS acquisition (one survey scan, five MS/MS of the most abundant ions). After sequencing the same m/z species (+/- 3 Da) three times, it was placed on an exclusion list for 3 min. Peptides were eluted from the reversed-phase column using a multiphasic elution gradient (5–40% acetonitrile from 0 to 100min, 80% from 100-110min, and 2% from 110-120min) over a total run duration of 2 hours. The remaining half of the sample was processed in the same manner. To prevent cross-contamination, each sample was analyzed on a freshly prepared reversed-phase column. Raw spectra files generated by the instrument software Xcalibur (Finnigan) were searched against a FASTA file containing the human NCBI sequences using a normalized implementation of SEQUEST. Search parameters were as followed: mass tolerance ± 2 Da; only full tryptic peptides were retained. Accuracy of the SEQUEST assignments of MS/MS spectra to peptide sequences was estimated by the PeptideProphet™ software based on a statistical model\(^{135}\). For each identified peptide, a probability score was computed on a scale of 0 (for "incorrect") to 1 (for "correct") based on match of the peptide sequence to the tandem mass spectra and the trypsin proteolytic pattern. These assigned peptides were then subjected to ProteinProphet™ analysis to assign a protein probability score for each identified protein or related protein group inferred
from the peptide data\textsuperscript{136}. The protein probabilities, again on a scale of 0 to 1, discriminate correct \((p = 1)\) from incorrect \((p = 0)\) protein identifications. Validation of initial database search results on the basis of statistical modeling allows the presentation of large-scale proteomics datasets with known sensitivity for positive identifications and error rates for false positive identifications. The SEQUEST searches and the analysis using peptide and proteinprophets were performed on a Sorcerer integrated data analysis system (SageNResearch). Only the proteins identified with a probability of 1.00 and with at least two independent peptides attributed were retained.

5.6: TOPFlash Luciferase Reporter Assays

Lentiviruses containing the superTOPFlash \(\beta\)-catenin dependent luciferase reporter (Firefly luciferase) and pRL-TK (Renilla luciferase) were produced and used to establish stable HEK293T, RKO, SW480 and HCT116 Wnt-reporter lines. Cells were seeded on 24-well plates, followed by cDNA transfection with Lipofectamine 2000 and/or reverse transfection with Lipofectamine RNAiMax for siRNA experiments. For experiments involving Wnt stimulation, media was replaced with a 1:1 mix of fresh DMEM:Wnt3A or DMEM:Control conditioned media. Cells were then assayed 24h after stimulation, performed in accordance with the Dual Luciferase assay specifications (Promega) using a Envision 2103 Multilabel Plate Reader (PerkinElmer).
5.7: Co-immunoprecipitation

For co-immunoprecipitation of endogenous proteins, HEK293T cells (5x10^6) were lysed in tandem affinity purification lysis buffer (TAP lysis: 10% glycerol, 50mM Hepes-KOH pH 8.0, 100mM KCl, 2mM EDTA, 0.1% NP40, 2mM DTT, 10mM NaF, 0.25mM Na_3VO_4, protease inhibitors (Sigma)). Lysates were cleared by centrifugation at 16,000g for 10min and immunoprecipitation was performed using polyclonal α-USP34 (Bethyl) or polyclonal α-Axin1 (#2074, Cell Signalling) antibodies and Protein A Sepharose (Sigma). Immunoprecipitated protein complexes were then analyzed by western blot, using antibodies noted in figure legends.

5.8: K48 Chain Cleavage

1μg of purified K48 chains from Boston Biochem (UC-230) were incubated in USP Assay Buffer (20mM Tris pH 8.0, 2mM CaCl_2, and 2mM β-mercaptoethanol) with 20nM USP2 core, 100nM USP34 core, 1μg of affinity-purified Axin Complex, or 1μg of Axin Complex (USP34 shRNA). The samples were incubated at 37°C for 30min and the reaction was stopped by addition of SDS sample buffer. The appearance of mono-ubiquitin was monitored by western blot using α-Ub antibody (Sigma U5379).

5.9: UBL-PLA2 Assay

Axin Activity Assay: 20nM USP2 core, 20nM USP34 core or 1μg of total protein from IPs was mixed with 30nM Ub-PLA2 and 20μM NBD C6HPC (PLA2 substrate, Invitrogen) in a total volume
of 100μL in a well in a black-walled 96-well-plate (Greiner Bio-One). Data were collected 45min after addition of Ub-PLA2 and NBD C6HPC on Perkin-Elmer Envision fluorescence plate reader with excitation and emission filters of 475nm and 555 nm respectively. Net RFU was then used to calculate signal (isopeptidase or IP + reporter) to background (reporter) ratio. **UBL-selectivity assays:** Relative isopeptidase activity against various UBLPLA2 fusions was determined by adding the USP34 core to a final concentration of 20nM in combination with 20μM NBD C6-HPC and 30nM of the individual UBL-PLA2 reporter constructs and expressed as a percentage of control isopeptidase: USP2 core (Ub-PLA2), Senp1core (SUMO3-PLA2), Den1 (NEDD8-PLA2), or PLpro (ISG15-PLA2). The UBL-PLA2 assay reagents are available from LifeSensors, Inc. (www.lifesensors.com) as CHOP reporter kits.

### 5.10: Immunofluorescence

Cells were seeded on poly-D-lysine treated coverslips and when indicated reverse transfected with siRNA. 48h after transfection cells were fixed with 4% paraformaldehyde/PBS for 20min, then permeabilized and blocked with 0.2% Triton X-100 and 10% normal donkey serum/PBS for 20 mins. Where indicated cells were treated with 5ng/ml Leptomycin B (LC Laboratories, MA) for 3 hours. Cells were then stained by indirect immunofluorescence using polyclonal α-Axin antibodies (provided by Dr. Woodget, Mount Sinai, Toronto) and Alexa488 conjugated anti-mouse antibodies. Cells were mounted with Vectorshield (Vector) and examined by laser scanning confocal microscope (Zeiss LSM 510).
5.11: β-catenin stabilization assay

RKO cells were reverse transfected with siRNA. 48 hours following transfection, cells were stimulated with control or Wnt3A CM treatment for different times (0.5h – 6h). Cells were washed and lysed with RIPA buffer for 15 mins then cleared by centrifugation at 16,000g for 10 mins before being resuspended in SDS sample buffer. β-catenin accumulation was monitored by western blot.

5.12: Axin Ubiquitination

HEK293T cells stably expressing human Axin1 (pGlue-Axin1) were transfected with Flag-Ubiquitin using calcium phosphate. Cells were lysed 48h after transfection using TAP lysis supplemented or not with 20mM N-Ethylmaleimide (Sigma), cleared by centrifugation at 16,000g for 10 mins. Axin was then purified by streptavidin affinity chromatography for 1h. Resin beads were then washed three times with lysis buffer (also supplemented with NEM when indicated) and the protein complexes eluted with 2x SDS sample buffer followed by SDS-PAGE electrophoresis and western blotting using FLAG antibodies to detect ubiquitin-conjugated Axin proteins.

5.13: Real-Time Quantitative-PCR (RT-QPCR)

Total RNA from SW480 cells treated with control or USP34 siRNAs were purified using Tri- Reagent (Biocompare). After DNasel (NEB) treatment, RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Primer sequences
used are: Cyclophilin 5'-GGAGATGGCACAGGAGAA-3', 5'-GCCCCGTAGTGCTTCAGTTT-3'; Nkd1 5'-TGAGAAGAAGATGGAGAGAGTGAGCGA-3', 5'-GCTGAACAATTTGCCTTCTG-3'; Tnfrsf19 5'-GGAGTTGTCTAAGGAATGTGG-3', 5'-GCTGAACAATTTGCCTTCTG-3'. Primer pair efficiencies were validated as previously described137. Quantitative RT-PCR analysis was carried out in triplicate using an Applied Biosystems Prism 7900HT instrument. Each reaction contained 12.5ng of cDNA, 150nM of each primer, and Power SYBR green PCR Master Mix (Applied Biosystems). Gene expression analysis was performed using the comparative CT method, normalized to Cyclophilin expression, and fold changes were calculated relative to control siRNA treated cells.

6.0: Results

6.1: Ubiquitin Specific Protease 34 (USP34) is a novel protein of the Axin protein complex, identified by mass spectrometry

Axin is a major scaffold protein for anchoring the Destruction Complex members together (APC, CK1α, GSK3β) and presenting β-catenin for phosphorylation. We set out to explore the Axin protein complex with a goal to identify and characterize novel proteins important for Axin function and Wnt signalling. We generated plasmids coding for Axin1 and Axin2 fusion proteins N-terminally tagged with streptavidin- and calmodulin-binding peptides along with an HA-epitope (SBP-HA-CBP-Axin1/2), stably expressed these constructs in human HEK293T cells, purified these baits and associated proteins by tandem affinity purification and analyzed the protein complexes by gel-free LC-MS/MS127,133.
Our approach was validated by successful detection of known Axin associated proteins such as β-catenin, APC, GSK3β, and PP2A (Fig. 4A: green; Table 2). Our method also detected new proteins associated with Axin (Fig. 4A: yellow), and most interestingly the uncharacterized protein USP34 (Fig. 4A: blue) associated with both Axin1 and Axin2. USP34 is a large protein (3546aa, ~400 kDa) with a centrally situated C19 Peptidase domain, characteristic of the USP family of DUBs\textsuperscript{138}.

The association of USP34 with Axin was verified by co-immunoprecipitation. Endogenous USP34 was co-purified with Axin from SBP-HA-CBP-Axin expressing HEK293T cell lysate using streptavidin affinity chromatography and subjected to western blot analysis using a polyclonal USP34 antibody (Fig. 4B: lanes 1, 3), while purification of an unrelated expressed protein (HA-Radil) did not show an interaction with USP34 (Fig. 4B: lanes 2, 4). This suggests that Axin and USP34 exist in the same protein complex.

6.2: USP34 confers ubiquitin specific protease activity to the Axin complex

The association of USP34 with Axin suggests it may confer de-ubiquitinase (DUB) activity to the Axin protein complex. To test this hypothesis we conducted ubiquitin protease assays using purified Axin protein complexes from SBP-HA-CBP-Axin HEK293T cells. Recombinant USP34 core (catalytic domain) and purified Axin complexes incubated with recombinant K48-linked ubiquitin chains produced a distinct mono-ubiquitin band detectable by western blot, indicative
of ε-amino deubiquitinase activity (Fig.5B: lanes 2, 3). Recombinant USP2 was included as positive control (Fig.5B: lane 1). Since depletion of USP34 by stable expression of USP34 shRNA in SBP-HA-CBP-Axin cells inhibited the DUB activity associated with purified Axin complexes, (Fig.5B: lane 4) we conclude that USP34 is largely responsible for this activity.

We observed similar results when employing the Ub-PLA2 assay as an alternative means to quantify DUB activity. The Ub-PLA2 consists of Ubiquitin N-terminally fused to Phospholipase A2 (PLA2)(α-amino linkage), which requires a free N-terminus to be active. Isopeptidase catalyzed cleavage of ubiquitin restores PLA2 activity which can then be measured by generation of a fluorophore. Recombinant USP34 core domain and purified Axin complex produced significantly more signal than Axin complex without USP34 (USP34 shRNA) and purified Radil complex (unrelated protein with no DUB activity)(Fig.5C: lane 2,3 vs. 4,5). shRNA depletion of endogenous USP34 was demonstrated by western blot (Fig.5A).

The PLA2 assay can also be fused with other ubiquitin-like proteins such as SUMO, NEDD8, and ISG15, and provides a means of testing the isopeptidase specificity of a particular enzyme. Using these variants of the Ub-PLA2 assay we determined that the isopeptidase activity of the recombinant USP34 core is ubiquitin specific (Fig.5D: lane 2 vs. 4, 6, 8).

The positive detection of DUB activity through the Ub-PLA2 assay (α-amino linked Ub) and through cleavage of poly-ubiquitin chains (ε-linked Ub) confirms USP34 (and purified Axin protein complex) has isopeptidase activity specific for ubiquitin and not other Ubls.
6.3: USP34 positively regulates β-catenin dependent transcription downstream of the Destruction Complex

Having established that USP34 is associated with Axin and conferred deubiquitinase activity to the Axin complex, we proceeded with assessing the functional role of USP34 in the context of canonical Wnt signalling. RNAi was employed to knockdown endogenous USP34 levels as the large size of USP34 was more amenable to siRNA-mediated knockdown than to overexpression since we have been unable to obtain a full length USP34 clone.

We first evaluated the knockdown efficiency of four independent siRNAs targeting USP34 mRNA by immunoblotting for endogenous USP34. RKO cells were transfected with individual siRNAs targeting USP34 (Fig.6A: lane 3-6), a non-targeting control (Fig.6A: lane 1-2), and a siRNA targeting β-catenin (Fig.6A: lane 7). Cells were lysed 48h after transfection and analyzed by western blot, probing with anti-USP34 antibody. All four siRNAs were able to knockdown endogenous USP34, and we selected siRNA “A” (Fig.6A: lane 3) for all subsequent experiments as it consistently yielded the best knockdown.

We then tested the effect of USP34 knockdown on the Wnt response in HEK293T and RKO cell lines stably expressing the TOPFlash β-catenin responsive luciferase reporter (Fig.6B). These cells were transfected with a non-targeting siRNA control, or siRNA targeting USP34 or β-catenin. 48 hours following transfection they were stimulated with Wnt3a conditioned media
for 18 hours and the state of pathway activation monitored using the TOPFlash reporter that is well characterized to report β-catenin mediated transcriptional activity. At resting state, in the absence of Wnt3a stimulation, siRNA treatments did not elicit any TOPFlash response. Upon Wnt3a stimulation, both cell lines treated with control siRNA generated a strong (~30-fold) response (Fig.6B: lane 1 vs. 4, 7 vs. 10), which could be strongly reduced by USP34 knockdown (Fig.6B: lane 6, 12). β-catenin knockdown was included as a positive control as the reporter activation is dependent upon β-catenin activity. Our TOPFlash luciferase reporter data thus suggests that USP34 functions as a positive regulator of canonical Wnt signalling.

The Wnt pathway can be artificially activated downstream of the ligand-receptor complex by ectopic introduction of positive regulators of signalling, such as overexpression of Dvl2, or a mutant pt.β-catenin resistant to degradation, and this can be detected by the TOPFlash assay (ex: pt.β-catenin, Fig.6C: lane 2-4). By asking whether USP34 depletion could still inhibit the activation of the pathway when activated at different levels along the signalling cascade we could test where in the pathway USP34 was acting. Interestingly, USP34 knockdown was able to antagonize the activation of the pathway by both Dvl2 (data not shown) and pt.β-catenin (Fig.6C: lane 4 vs. 2). These results suggest that USP34 functions downstream of the β-catenin stabilization step. SW480 and HCT116 cells are colon cancer cell lines with constitutive Wnt signalling due to inactivating mutation in APC (SW480), and an activating mutation in β-catenin (HCT116) respectively. We stably expressed the β-catenin responsive luciferase reporter TOPFlash along with a constitutively active Renilla luciferase under EF1α promoter control (for normalization) in these cells, and then tested the TOPFlash response upon USP34 knockdown.
by siRNA. We found that USP34 knockdown can inhibit the constitutively active Wnt pathway in these colon cancer cells (Fig.6E: lane 1 vs. 2, 4 vs. 5) again suggesting that USP34 functions downstream or at the level of the β-catenin stabilization step. We further confirmed these findings by showing that USP34 knockdown reduces expression of the endogenous Wnt target genes *Naked1* and *Tnfrsf19* (Fig.6F) that are both strongly upregulated by β-catenin in SW480 colon cancer cells. These results indicate that USP34 functions as a positive regulator of Wnt signalling, downstream or at the level of the β-catenin stabilization step.

To further confirm that USP34 functions downstream of the β-catenin stabilization step, we measured the rate of β-catenin stabilization following Wnt3a activation in RKO cells treated with control or USP34 siRNAs. RKO cells lack cadherin junctions and thus exhibit very little membrane bound β-catenin at resting state, simplifying the analysis of β-catenin stabilization induced by Wnt3a stimulation. Treatment with Wnt3a rapidly and robustly stabilizes β-catenin in the cytosol of these cells (Fig.6E: control siRNA) over a period of 6 hours and can be detected by western blot. Pre-treatment with USP34 siRNA does not alter the kinetics or the magnitude of β-catenin stabilization upon Wnt3a stimulation (Fig.6E: USP34 siRNA). Endogenous USP34 was probed to verify knockdown and Lamin-B levels were measured as loading control. Together, these data thus suggests a positive regulatory role for USP34 that operates downstream of β-catenin stabilization. Since USP34 is associated with the Axin complex our next objective was to explore the functional link between these two proteins.
6.4: A new positive regulatory role for Axin in the Wnt pathway, downstream of the Destruction Complex

A recent study employing a whole genome siRNA screen probing for new regulators of canonical Wnt signalling, found that depletion of Axin1/Axin2 reduced β-catenin mediated transcription. These results were not showcased in the original publication but suggest a positive regulatory role for Axin in the context of colon cancers, in contrast to its well known negative regulatory function in signalling. We replicated these results in our SW480 and HCT116 cells (stably expressing the β-catenin reporter) by testing TOPFLASH response upon Axin1/Axin2 knockdown by siRNA. SW480 and HCT116 colon cancer cell lines harbour mutations in APC and β-catenin respectively, and exhibit constitutively active Wnt signalling at resting state, which we have normalized to 100% (Fig.7A: lanes 1, 4). This aberrant pathway activity is β-catenin dependent as β-catenin knockdown abolishes the TOPFlash response (Fig.7A: lanes 3, 6). Axin1/Axin2 knockdown reduced TOPFlash to 64% and 30% of baseline (control siRNA) activity (Fig.7A: lanes 2, 5), confirming the siRNA screen results and suggesting that Axin1 and Axin2 are required for the constitutive β-catenin mediated Wnt signalling in colon cancer cells.

As stated previously, this positive role for Axin is completely opposite to its observed and well characterized negative regulatory role. For example, under resting condition HEK293T and RKO cells exhibit low basal TOPFlash activity (Fig.7B: lanes 1, 4) and can be stimulated by treatment with Wnt3A conditioned media (Fig.7B: lanes 2, 5). Pathway activation from Wnt3A treatment
can be potentiated by depleting Axin1/Axin2 (Fig.7B: lanes 3, 6), consistent with the established negative regulatory role that Axin serves within the Destruction Complex. However, when we introduced a degradation resistant form of β-catenin (S37A, loss of GSK3β phosphorylation site) to constitutively activate the normal pathway in HEK293T cells (mimicking the mutant β-catenin responsible for aberrant signalling in HCT116 colon cancer cells), Axin knockdown once again abrogated pathway activation (Fig.7C: lane 4 versus 2).

Since aberrant Wnt signalling in SW480 and HCT116 cells is due to loss of normal β-catenin regulation by the Destruction Complex, our results implied a new function for Axin impinging on β-catenin transcriptional activity downstream or independently of the Destruction Complex. This prompted us to examine Axin subcellular localization using a polyclonal Axin1 antibody using indirect Immunofluorescence in SW480 and HCT116 cells. We found strong nuclear staining for Axin in these cells, consistent with previous studies\textsuperscript{141,142}, while Axin depletion by siRNA eliminated this nuclear labelling with some residual diffuse immunostaining, verifying Axin antibody specificity (Fig.7D: left panels versus right panels).

In summary, in transformed colon cancer cells exhibiting aberrant Wnt signalling we found that Axin is required to maintain the constitutive pathway activation and that it was primarily localized in the nucleus. Given that USP34 functions downstream of the destruction complex and interacts with Axin, we hypothesized that USP34 could regulate the availability of nuclear Axin by controlling its stability.
6.5: USP34 controls the nuclear-accumulation of Axin

To assess whether USP34 controls the steady state levels of Axin in colon cancer cells, we treated SW480 cells that exhibit strong nuclear Axin labelling with control or USP34 siRNA. We found that USP34 knockdown almost completely abolished the immunodetection of nuclear Axin (Fig.8A). This suggests that USP34 is required for Axin nuclear accumulation in colon cancer cells.

In cells with normal Wnt pathway activation, Axin does not constitutively accumulate in the nucleus (Fig.8B: control siRNA without LMB). However, it has been previously shown that Axin undergoes nuclear-cytoplasmic shuttling as the nuclear export inhibitor Leptomycin B leads to its accumulation in the nucleus72. We thus hypothesized that during Wnt signalling USP34 is required to stabilize the pool of Axin entering/residing in the nucleus. To test this hypothesis, we examined the effect of USP34 knockdown on the LMB-induced nuclear Axin localization in HEK293T cells. Agreeing with previous reports, we show that LMB can induce the nuclear accumulation of endogenous Axin (Fig.8B: control siRNA; immunostaining verified by Axin1 siRNA knockdown). Depletion of USP34 abrogates the Axin nuclear localization. We have also found USP34 to be mainly cytoplasmic in nuclear/cytoplasmic fractions of different cell lines (data not shown). These findings strongly suggest that the deubiquitinase activity of USP34 is important for Axin nuclear localization not only in cancer cells but in the context of cells exhibiting normal Wnt signalling.
6.6: Axin ubiquitination is dynamic

Earlier studies have indicated that Axin is degraded upon initiation of Wnt signalling\textsuperscript{143} and this is dependent on LRP6 activation\textsuperscript{67,144,145}. Our detection of a DUB associated with Axin suggests that Axin degradation may be mediated by ubiquitination and that USP34 may act within this context. We introduced FLAG-tagged Ubiquitin (FLAG-Ub) by transfection into stable SBP-HA-CBP-Axin expressing HEK293T cells and 48 hours after transfection affinity purified Axin from cell lysates using streptavidin chromatography. We then assessed the presence of ubiquitinated-Axin conjugates by western blot using FLAG antibodies. Under these conditions minimal FLAG-Ub is present with purified Axin (Fig.9A: top panel lane 1).

However, since we detected significant Ubiquitin protease activity in Axin complexes (Fig.5C) we reasoned that this activity could actively deubiquitinate Axin and hinder our ability to detect ubiquitinated forms of Axin. We initially attempted to circumvent this issue by depletion of endogenous USP34 via siRNA knockdown, but this resulted in a significant reduction in Axin levels. While this complicated our analysis, destabilization of Axin upon USP34 knockdown indirectly implied that Axin is regulated by the ubiquitin proteasome system.

We next used a sulfhydryl alkylating agent, N-Ethylmaleimide (NEM, non-specific cysteine protease inhibitor), to inhibit all cysteine protease activity (including DUBs) at the time of cell lysis to prevent Ub-Axin from deubiquitination\textsuperscript{139}. In the presence of NEM we were able to detect robust poly-ubiquitination of Axin (Fig.9A: top panel lane 2). In addition to the high molecular weight ‘smear’ characteristic of poly-ubiquitin, distinct bands corresponding to Axin
conjugated with a one, two, or more ubiquitins were detected. Our data demonstrates that Axin is mono- and poly-ubiquitinated in HEK293T cells and that USP34 is involved in balancing this regulation by the UPS.

6.7: USP34 stabilizes Axin protein levels, likely via deubiquitination and inhibition of proteasomal mediated degradation.

The loss of nuclear Axin staining in colon cancer cells treated with USP34 siRNA (Fig.8A) could be explained by two separate ubiquitin-dependent functions. Ubiquitination of Axin (possibly monoubiquitination) could be important to control its subcellular localization in a proteasome independent way or alternatively ubiquitination of Axin could lead to its proteasome-dependent degradation. USP34 depletion would in these cases increase Axin ubiquitination and lead to decreased nuclear localization or increased proteolysis respectively. To distinguish between these two possibilities we took advantage of the well characterized proteasome inhibitor MG132 to test whether blocking proteasome functions could rescue the USP34 siRNA effect (loss of nuclear Axin staining). We pre-treated SW480 cells with USP34 siRNA, followed by an 18h treatment with the proteasome inhibitor MG132 to block general proteasome-mediated degradation. We demonstrate that MG132 can rescue the loss of nuclear Axin induced by USP34 as monitored by immunofluorescence (Fig.10A) and by western blot (Fig.10B). We conclude that the reduction of Axin staining in the nucleus upon USP34 knockdown is due to an enhanced degradation of Axin by the UPS system.
6.8: USP34 as a potential drug target in the Wnt pathway.

We demonstrated that knockdown of USP34 activity strongly antagonized the Wnt signalling pathway. Importantly, USP34 acts downstream of the destruction complex by controlling nuclear Axin levels. This makes USP34 an attractive drug target as most mutations leading to constitutive Wnt pathway activation occur at or downstream of the β-catenin stabilization step. We thus hypothesize that a small molecule inhibitor of USP34 would represent a specific Wnt pathway inhibitor. As a proof of concept for this hypothesis we treated SW480 colon cancer cells with the general cysteine protease inhibitor P5075 and asked whether Axin levels and state of constitutive Wnt pathway activation were affected. Incubation of SW480 cells with increasing doses of P5075 led to a dose dependent decrease in Axin protein levels (Fig.10C). When cells were treated with P5075 the TOPFlash reporter was also inhibited by 40%, similar to cells treated with USP34 siRNA (Fig.6E). These results suggest that inhibition of Ubiquitin protease activity leads to Axin degradation and inhibition of constitutive activity in SW480 cells.

7.0: Discussion

The requirement of the UPS system for the post-translational control of β-catenin stability has been well characterized. After identifying a DUB in complex with Axin, a protein critical for the orchestration of β-catenin phosphorylation and ensuing ubiquitination, our initial hypothesis was that USP34 counteracts β-catenin ubiquitination. In this context USP34 would de-ubiquitinate β-catenin, lead to its stabilization and promote signalling.
Consistent with this hypothesis, when using the β-catenin responsive luciferase reporter (TOPFLASH) we found that USP34 knockdown (by RNAi) significantly attenuated canonical pathway activation by Wnt3a in HEK293T and RKO cells that exhibit a normal canonical Wnt pathway. Moreover, USP34 knockdown also reduced the constitutively active signalling found in SW480 and HCT116 colon cancer cells that have mutations in regulatory components of the pathway. However, this reduction in pathway response was not due to a decrease in β-catenin protein levels, as would be predicted if USP34 controlled β-catenin stability. Moreover, USP34 knockdown could also inhibit ectopically induced signalling from expression of a constitutively active β-catenin that is resistant to ubiquitination. These findings led us to conclude that USP34 exerts a positive role in promoting canonical signalling, but this effect is not mediated through stabilization of β-catenin and likely operates downstream of the Destruction Complex.

Interestingly, using the TOPFLASH functional assays we observed that Axin knockdown paradoxically reduced the constitutive activity in SW480 and HCT116 colon cancer cells but, as expected, promoted the Wnt3a stimulation of the pathway in normal HEK293T and RKO cells. Recent work also indicates that Axin could be playing roles in addition to its well characterized function as a negative regulator of Wnt signalling. Indeed, a genome-wide integrative screen employing RNAi also revealed that Axin was a positive regulator (required for signalling) for β-catenin mediated Wnt signalling in colon cancer cells. Axin has also been repeatedly found in the nucleus of cancer cells but this has largely been ignored by the field. Furthermore, Axin accumulates in the nucleus of cells treated with the CRM1 nuclear export inhibitor LMB suggesting that it undergoes nuclear cytoplasmic shuttling. These results are in contrast to the
known negative regulation that Axin exerts on canonical Wnt signalling through its role as the cytoplasmic Destruction Complex scaffold.

One surprising finding of this study is that Axin fulfills a positive regulatory role for Wnt signalling in addition to its well characterized negative function in the Destruction Complex. We show that this positive role is masked under normal circumstances due to the dual role of Axin as a strong negative regulator but is uncovered in the context of colon cancer cells where the Destruction Complex is inactive and β-catenin signalling is hyperactivated. The finding that knocking down either Axin or USP34 leads to the inhibition of constitutive β-catenin activity in these colon cancer cells suggests that these proteins are needed downstream of the β-catenin Destruction Complex, possibly in the nucleus. Mounting evidence suggests multiple roles for other Wnt pathway components. For example, β-catenin has now been localized to at least three distinct subcellular pools to fulfill three disparate processes: adherens junction formation at the plasma membrane, canonical Wnt signalling in the cytoplasm and the nucleus, and the regulation of chromosomal integrity when localized to the centrosome\textsuperscript{149}. Dishevelled (Dvl) can form a nuclear complex with c-jun, β-catenin, and TCF4 and this interaction is important for canonical Wnt signalling\textsuperscript{82}. Dapper1, a Dvl interacting protein, promotes Dvl degradation in the cytoplasm, but can also repress TCF/LEF when in the nucleus\textsuperscript{150}. In \textit{D. melanogaster} APC has been recently found to have a positive regulatory role by promoting Axin downregulation\textsuperscript{80}, while nuclear APC has also been found to promote CtBP-mediated repression of canonical signalling as a form of negative feedback that is absent in colon cancer cells\textsuperscript{81}. In fact APC is a multifunctional protein serving other Wnt-independent functions such as in the regulation of
cell migration and cell cycle control through its ability to stabilize microtubule ends\textsuperscript{151,152}. Lastly, the kinase GSK3 has been found to serve opposing roles (in the context of Wnt signalling) depending on its localization at the plasma membrane, cytosol, or nucleus. At the plasma membrane, GSK3 has been shown to phosphorylate the Wnt co-receptor LRP6, a process that is important for signal amplification and transduction\textsuperscript{68}. Conversely, Axin-GSK3 within the cytoplasmic Destruction Complex negatively regulates β-catenin through phosphorylation\textsuperscript{54}. In the nucleus, GSK3 serves yet another negative regulatory role by modulating β-catenin transcription in a phosphorylation independent manner\textsuperscript{153}. The diverse and sometimes apparently contradictory functions of these major Wnt pathway components demonstrate the subtleties required for modulating pathway activity, and suggest that our observation of this novel positive regulatory role for Axin may in fact be an extension of a general trend for Wnt pathway components.

The dogma of canonical Wnt signalling has firmly entrenched Axin as a negative regulator of the pathway through its role as a cytoplasmic scaffold for the function of the β-catenin Destruction Complex. The initiation of intracellular signal transduction upon Wnt ligand activation of the Fz-LRP6 receptor complex leading to the dissolution of the Destruction Complex has been intensively studied in the work discussed in the introduction. However, Axin is capable of entering and leaving the nucleus (facilitated by CRM1 nuclear export\textsuperscript{72,148}), and has been found concentrated in the nucleus of colon cancer cells from tumour biopsies\textsuperscript{141,142}. The functional significance of nuclear Axin and the regulatory mechanisms controlling this localization have not been elucidated and these observations need to be reconciled with the established roles of
Axin within the Destruction Complex. Consistant with these observations, we and others\textsuperscript{156} found endogenous Axin to be strongly concentrated in the nucleus of SW480 or HCT116 cells, (Fig. 7D).

The question of how Axin exerts this positive role on signalling remains unresolved. Does it simply serve as an anchor to retain β-catenin in the nucleus or is it required for β-catenin transcription akin to the nuclear Dvl-c-Jun complex\textsuperscript{82}, perhaps by directly interacting with Wnt target gene promoters? One recent study points to Axin/APC influencing β-catenin localization based on retention rather than active transport\textsuperscript{74}. Surprisingly, USP34 knockdown abolished nuclear Axin staining. Together these results led us to test the hypothesis that USP34 may be functioning by regulating the accumulation of Axin in the nucleus where Axin may have a second role in canonical Wnt signalling impinging positively on β-catenin transcription.

Our working model thus advances the involvement of USP34 and the ubiquitin proteasome system in the control of Axin stability. We identified the ubiquitin protease involved in Axin de-ubiquitination but our work also implies that an as yet unidentified E3 ubiquitin ligase exists to control Axin ubiquitination. Furthermore, recent evidence suggests that Axin is sumoylated on its c-terminal tail and that this protects it from ubiquitination\textsuperscript{132}. This adds another degree of complexity to unravelling the mechanisms behind Axin regulation. New experiments are now needed to understand the interplay between USP34 and Axin sumoylation and ubiquitination.
Since nuclear localization of Axin and β-catenin are important for promoting canonical Wnt signalling, especially in maintaining constitutive signalling in colon cancer cells, controlling their nuclear residency by targeting USP34 may provide a new therapeutic approach towards diseases associated with aberrant Wnt signalling. Given that the majority of activating mutations in the pathway occur downstream of the Fz/LRP6 receptor complex (thus strategies involving receptor blockade would be ineffective) and the promiscuous nature of many core Wnt components being involved in cross-talk with other signalling pathways, the development of compounds specifically inhibiting the Wnt pathway has been largely unsuccessful. However, small molecules have been isolated and shown to inhibit protein-protein interactions between β-catenin and TCF but have met with challenges regarding the specificity and strength of such inhibition\textsuperscript{157}. As a result, currently there are no drugs targeting the Wnt pathway in clinical trials. Some progress has been made towards inhibiting Wnt secretion as well as prolonging Axin stability\textsuperscript{158}, but in light of our data suggesting a positive role for Axin, this strategy may not be as effective. The cysteine protease activity of USP enzymes are good targets for drug development, with over ten cysteine protease inhibitors currently at various stages of development and clinical trials\textsuperscript{159}. USP34 could therefore represent an alternative target for mitigating aberrant Wnt pathway activity through the use of cysteine protease inhibitors, provided such drugs are specific and exhibit high bioavailability (most available cysteine proteases are of peptide origin)\textsuperscript{160}. Our results with the general USP inhibitor are providing the proof of principle in that direction.
In summary, this thesis demonstrates nuclear Axin serving a positive regulatory role in propagating canonical Wnt signalling. Through tandem affinity purification and mass spectrometry analysis we found that the deubiquitinase enzyme Ubiquitin Specific Protease 34 (USP34) is associated with the Axin complex. We showed that USP34 is responsible for the ubiquitin protease activity present in the Axin complex and through loss of function studies we demonstrated that USP34 is a positive modulator of β-catenin transcription acting through the regulation of Axin stability. We predict that the identification of USP34 inhibitors could be used for the treatment of diseases associated with spurious Wnt pathway activation since the reduction of function for USP34 using siRNA or a non specific small molecule inhibitor led to inhibition of constitutive Wnt pathway activity found in colon cancer cells. Future in vivo studies are now needed to determine whether USP34 inhibition can inhibit or slow down the development or progression of diseases associated with pathological Wnt signalling.
References


134. Major, M.B., Roberts, B.S., Berndt, J.D., Marine, S., Anastas, J., Chung, N., Ferrer, M., Yi, X., Stoick-Cooper, C.L., von Haller, P.D., Kategaya, L., Chien, A., Angers, S., MacCoss,


Table 1: Wnt pathway major components

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<td>Adenomatous Poliposis Coli (APC)</td>
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Figure 1. Regulation of β-catenin by the Destruction Complex.
The Destruction Complex is a cytoplasmic protein complex responsible for the phosphorylation of free β-catenin, preparing it for ubiquitination by the SCFβ-TrCP complex and leading to its rapid degradation via the Proteasome. Axin acts as the main scaffold protein bringing CK1α and GSK3β into proximity to phosphorylate β-catenin. The presence of APC prevents PP2A from quickly dephosphorylating phospho-β-catenin, protecting it so it may reach the SCFβ-TrCP complex ubiquitin ligase complex. Within the SCF complex the Fbox protein β-TrCP acts as an E3 ligase specifically binding phospho-β-catenin. SKP1 is an adapter facilitating binding of β-TrCP to the Cul1 scaffold, which also carries the E2 conjugating enzyme RBX1. This assembly of proteins specifically binds and poly-ubiquitinates β-catenin, targeting it for destruction by the Proteasome.
Figure 2. Canonical Wnt Signalling.
The diagram depicts the pathway in both inactive (left side) and activated (right side) states and the steps involved in initiation of signalling upon Wnt3a ligand binding:

1. When the pathway is quiescent, β-catenin is actively degraded. Within the nucleus, transcriptional repressor Groucho is bound to TCF4 and inhibits transcription of Wnt target genes.

2. Wnt3a activation of Fz7 and LRP6 leads recruitment of Dvl to the plasma membrane along with Axin-GSK3β. This brings GSK3β into proximity of LRP6 and leads to its activation by phosphorylation. Activated LRP6 promotes further Axin-GSK3β docking and attracts more LRP6 units, amplifying the signal. Recruitment of Axin away from the cytoplasm inhibits formation of the Destruction Complex, allowing β-catenin levels to increase.

3. No longer constitutively degraded, β-catenin can freely enter the nucleus and displace the repressor Groucho from TCF4. Along with Dvl, β-catenin helps condense other transcriptional co-activators and chromatin modifiers to unwind DNA and initiate transcription of Wnt target genes.
**Figure 3.** The Ubiquitin Cycle.

The schematic describes the major aspects of Ubiquitin regulation within eukaryotes, including steps in which DUBs play a critical regulatory role:

1. Ubiquitin is always coded either as multimers or as fusion proteins, so one crucial role for DUBs is the cleave *de-novo* synthesized ubiquitins into monomers.
2. Free ubiquitin is unreactive and must be activated by E1 enzyme.
3. Activated ubiquitin is transferred from E1 to E2 conjugating enzymes, which can conjugate ubiquitin to proteins in close proximity when working in conjunction with E3 ligases. E3 ligases confer substrate specificity, presenting a high affinity substrate to the E2 for conjugation of ubiquitin. Typically E2 and E3 are found within the same protein complex (SCFβTrCP).
4. Following poly-ubiquitination, DUBs may operate in a chain editing capacity to ensure/modify a particular form of chain linkage (K48, K63, and perhaps other types).
5. DUBs may also rescue ubiquitinated proteins by completely removing ubiquitin. Following these modifications the proteins can proceed with their purpose, whether it is to signal or to localize in a particular subcellular compartment.
6. Substrates with four or more ubiquitin in K48 linked form are recognized by the proteasome and destroyed.
7. Ubiquitin conjugated to the substrate must be removed to facilitate substrate entry into the proteasome. The 19S regulatory particle (the proteasome “cap”) exhibits DUB activity for this process. Deubiquitinated proteins are then unfolded and enter the 20S catalytic particle of the proteasome for proteolysis into small peptides.
8. Free poly-ubiquitin chains (by-products of the proteasome) can be recycled by DUBs into monomers ready to begin the cycle anew.

**Degradation:**
K48 chains

**Signaling/Localization:**
Mono-ubiquitin
K63 chains
Figure 4. Identification of the Ubiquitin Specific Protease 34 (USP34) as an Axin interacting protein

Given the contradictory TOPFLASH results between colon cancer cells with defective Wnt signalling and cells with intact Wnt signalling, we employed a targeted proteomic approach to study Axin:

A. Mammalian Axin1 & Axin2 protein interaction network, arrows representing interactions found in Axin1 and Axin2 (red circles) pull-down experiments using LC-MS/MS. Yellow circles are previously described associated proteins.

B. Confirmation of the Axin-USP34 interaction using co-affinity purification: In HEK293T cells, endogenous USP34 associates with Axin1 (lane 3) but not with the unrelated protein Radil (lane 4).
Figure 5. USP34 confers ubiquitin protease activity to the Axin protein complex.

Characterization of USP34 enzymatic activity:

A. Western blot verification of endogenous USP34 knockdown in HEK293T strep-HA-Axin1 cells stably expressing USP34 shRNA. Lamin B was used as loading control.

B. Cleavage of K48-linked ubiquitin chains by recombinant USP2 and USP34 core domains and Axin1 protein complexes purified from HEK293T cells, but not from Axin1 protein complexes isolated from cells where USP34 expression was knockdown by shRNA. Cleavage is monitored with the appearance of mono-ubiquitin from the poly-ubiquitin chains.

C. Quantification of relative ubiquitin protease activity using the Ubiquitin-PLA2 assay: Purified Axin1 complexes from HEK293T cells but not from cells expressing USP34 shRNA exhibited protease activity. Similar amount of the unrelated Radil protein complex showed no activity. Recombinant USP2 and USP34 were used as positive controls in this assay.

D. Cleavage specificity of the USP34 core domains: Ub-, Sumo3-, ISG15- and Nedd8-PLA2 assays were used to demonstrate that the USP34 core domain preferentially cleaves ubiquitin.
Figure 6. USP34 has a positive regulatory function in Wnt signalling.
Since USP34 may be modulating Axin ubiquitination status, we explored its functional role in signalling using the TOPFLASH assay, and conducted a variety of experiments establishing where USP34 may function within the pathway activation sequence:

A. Validation of USP34 siRNAs: TOPFLASH assay in RKO cells treated with 4 independent siRNA targeting USP34. Lysates were then analyzed by western blot using anti-USP34 antibodies to monitor knockdown efficiency and anti-ERK antibodies as loading control. USP34 siRNA ‘A’ was used for subsequent experiments.

B. TOPFLASH assays in HEK293T and RKO cells treated with control siRNA (lanes 1,4,7,10) β-catenin siRNA (lanes 2,5,8,11) or USP34 siRNA (lanes 3,6,9,12), then stimulated with control conditioned media (lanes 1,2,3,7,8,9) or Wnt3A conditioned media (Lanes 4,5,6,10,11,12). USP34 knockdown inhibited Wnt3A mediated activation of the TOPFLASH reporter in both cell lines (lane 4 vs. 6, lane 10 vs. 12).

C. USP34 knockdown does not influence Wnt3A-induced stabilization of β-catenin. RKO cells treated with control or USP34 siRNA were stimulated with Wnt3A conditioned media for the indicated duration and lysates probed for β-catenin levels by western blot.
Figure 6. USP34 has a positive regulatory function in Wnt signalling.

Results from C,D, and E suggest USP34 is acting downstream of Destruction Complex, likely at the level of nuclear gene transcription:

D. Epistasis analysis of USP34 function: TOPFLASH assays in HEK293T cells showed that USP34 and β-catenin but not control siRNA antagonized the activation of the pathway by overexpression of the stabilized β-catenin mutant (lanes 2,3 and 4).

E. TOPFLASH assays in HCT116 and SW480 cells treated with USP34 siRNA showed that the aberrant activation of the β-catenin reporter in these cells requires USP34 function (lane 1 vs. 2, lane 4 vs. 5).

F. Quantitative RT-PCR analysis of SW480 cells treated with USP34 siRNA show reduced expression of the Wnt target genes Naked (NKD1) and Troy (Tnfsrf19) as a percentage of control siRNA, similar in effect to β-catenin siRNA knockdown. Gene expression normalized to cyclophilin.
**Figure 7.** Uncovering a positive regulatory role for Axin in pathological Wnt signalling.

We examined Wnt pathway activity using TOPFLASH assay in HCT116 and SW480 colon cancer cells that exhibit aberrant Wnt signalling, then in HEK293T and RKO, two cell lines with intact Wnt pathway:

A. Constitutively active Wnt signalling in HCT116 and SW480 colon cancer cells (lanes 1, 4) can be reduced by siRNA mediated knockdown of Axin1/2 (lanes 2, 5), β-catenin siRNA included as positive control (lanes 3, 6).

B. Axin1/2 knockdown potentiates Wnt response in HEK293T and RKO cells with intact Wnt pathway when stimulated by Wnt3A (lane 2 vs. 3, 5 vs. 6). Axin1/2 knockdown alone without Wnt3A stimulation does not change response vs. control (not shown).

TOPFLASH assay figures are representative of at least three independent experiments performed in duplicates where the error bars represent the standard errors.
Figure 7. Uncovering a positive regulatory role for Axin in pathological Wnt signalling.

We examined Wnt pathway activity using TOPFLASH assay in HCT116 and SW480 colon cancer cells that exhibit aberrant Wnt signalling, then in HEK293T and RKO, two cell lines with intact Wnt pathway:

C. Epistatic activation of the Wnt pathway in HEK293T cells expressing degradation resistant β-catenin mutant (pt.mutant.β-catenin; mimics HCT116) is also inhibited by Axin1+2 knockdown, suggesting that Axin1/2 is necessary for maximal pathway activation.

D. Immunofluorescence using polyclonal anti-Axin1 antibodies: Axin localizes to the nucleus of colon cancer cells (left panels). Specificity of the antibody was controlled using Axin1 siRNA (right panels).

TOPFLASH assay figures are representative of at least three independent experiments performed in duplicates where the error bars represent the standard errors.
Figure 8. USP34 controls the nuclear-cytoplasmic shuttling of Axin.
The strong nuclear localization of Axin in colon cancer cells (Fig. 7D), attenuation of aberrant signalling upon Axin/USP34 knockdown, and epistasis data showing USP34 function at the transcription level all suggested that USP34 may be maintaining Axin function which in turn modulated signalling. Using immunofluorescence microscopy we found USP34 knockdown altered Axin localization:

A. USP34 knockdown (middle panels) inhibits the strong nuclear localization of Axin seen in SW480 and HCT116 colon cancer cells transfected with control siRNA (left panels).

B. USP34 depletion (middle panel) also inhibits the nuclear accumulation of Axin in HEK293 cells observed when the CRM1 dependent nuclear export is blocked with Leptomycin B (left panel). In each experiment the specificity of the Axin antibody was controlled using Axin1 siRNA (right panels).
Figure 9. Ubiquitinated Axin is sensitive to ubiquitin specific protease activity (USP).
Because USP34 exhibited ubiquitin protease activity and was associated with Axin, we hypothesized and found that Axin is ubiquitinated (and hence a target for USP34):

A. Cells stably expressing Strep-HA-Axin1 were transfected with Flag-Ubiquitin. Input lysates prepared for streptavidin affinity purification were left untreated (lane 1) or treated with the non-specific USP inhibitor NEM (lane 2). Ubiquitin-linked Axin were resolved by SDS-PAGE and detected using anti-FLAG antibodies (top panel). The purification of Axin was controlled using anti-HA antibodies (bottom panel). The inhibition of USP activity robustly increase the amount of ubiquitinated Axin detected (compare lane 2 vs. 1).
Figure 10. USP34 knockdown destabilizes Axin.
Recently we acquired a very clean antibody specific for Axin1 which allowed us to measure endogenous Axin protein levels directly, providing a new tool to elucidate the effect of USP34 loss on Axin:
A. Immunofluorescence images of SW480 colon cancer cells: USP34 knockdown induced loss of nuclear Axin, which can be rescued by the proteasome inhibitor MG132.
B. Western blot on SW480 lysate demonstrating significant reduction of Axin upon USP34 knockdown, also rescued by MG132.
C. Dose-dependent Axin destabilization upon inhibition of DUBs by the general cysteine-protease inhibitor P5075 after 2h treatment in SW480 colon cancer cells.
### List of Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>APC</td>
<td>Adenomatous Polyposis Coli</td>
</tr>
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<td>BCL9</td>
<td>B-cell Lymphoma 9</td>
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<tr>
<td>β-TrCP</td>
<td>β-Transducin repeat-Containing Protein</td>
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<td>Cysteine Rich Domain</td>
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<td>Deubiquitinating Enzyme</td>
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<td>Extracellular Matrix</td>
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