Wnt receptor regulation by the tumor suppressor APC

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To my loving family.

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List of Abbreviations

AP2, Adaptor Protein 2

APC, Adenomatous Polyposis Coli

BafA, Bafilomycin A

BCL9, B-cell Lymphoma 9

CK1, Casein Kinase 1

CME, clathrin-mediated endocytosis

CRC, colorectal cancer

CRD, Cysteine Rich Domain

DKK, Dickkopf

DVL, Dishevelled

DUB, Deubiquitylase

E1, E1 activating enzyme

E2, E2 conjugating enzyme

E3, E3 ligase

FAP, Familial Adenomatous Polyposis

FZD, Frizzled

GSK3, Glycogen Synthase Kinase 3

Gro, Groucho

HDAC, Histone Deacetylase

HECT, Homologous to the E6-AP Carboxyl Terminus

HMG, High Mobility Group

LEF, Lymphoid Enhancer Factor

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

MBP, Maltose Binding Protein

MMTV, Mouse Mammary Tumor Virus

NDLB, Non-Denaturing Lysis Buffer

PIP2, Phosphatidylinositol (4,5)-biphosphate

PORCN, Porcupine

RING, Really Interesting New Gene

RNF43, Ring Finger 43

RSPO, R-spondin

SCF, Skp1-Cullin-F-box

sFRP, secreted Frizzled-Related Protein

TLE, Transducin-Like Enhancer of Split

UAF1, USP1-Associated Factor 1

UCH, Ubiquitin C-terminal Hydrolases

USP, Ubiquitin Specific Protease

WDR20, WD-repeat containing 20

Wg, Wingless

Wls, Wntless

WRE, Wnt Responsive Element

WT, Wild Type

XIAP, X-linked Inhibitor of Apoptosis Protein

ZNRF3, Zinc and Ring Finger 3

CHAPTER 1

The Canonical Wnt Signaling Pathway

Cell signal transduction

The capacity of cells to sense and respond to extracellular cues is critical to metazoan development and adult physiology (Basson, 2012). Chemical and physical cues arising from other cells, as well as the environment, direct cellular processes such as differentiation, growth, and proliferation (Nair et al., 2019). These cues are received and interpreted by various intracellular signaling pathways, each with varying levels of complexity. There are currently thought to be 17 signaling pathways active in metazoan physiology (Gerhart, 1999). Generally, signaling is initiated by a ligand (peptide, protein, small molecule, etc.), which is received on the extracellular surface by a receptor. Receptors then transduce the signal to the intracellular surface of the membrane, triggering molecular cascades, which propagate and amplify the signal and ultimately direct the final cellular process (Gerhart, 1999).

Developmental origins of Wnt signaling transduction

The Wnt signaling pathway is a highly conserved regulator of metazoan development, adult tissue homeostasis, and disease (Rim et al., 2022). Early observations in this signaling pathway were made using the model system *Drosophila* melanogaster. In 1973, R.P. Sharma performed mutagenesis screens and identified a gene in which mutations yielded wingless and halterless flies (Sharma & Chopra, 1976). These mutant flies also showed abnormalities that indicated this gene operated during early development. This was the initial discovery of the Wingless (Wg) gene (Sharma & Chopra, 1976).

In 1982, Roeland Nusse, while working in the Harold Varmus laboratory, was investigating the transformative properties of the mouse mammary tumor virus (MMTV), which induces carcinomas in mouse mammary tissues yet lacks an oncogene of its own (Nusse & Varmus, 1982). He identified and cloned the site of viral integration, terming it "Int-1" for "integration site 1" (Nusse et al., 1984; Nusse & Varmus, 1982). Harold Varmus was later awarded a Nobel Prize partially for this work, demonstrating normal cellular genes could be transformed into oncogenes by viral insertional mutagenesis (proto-oncogenes).

Sequencing and homology studies demonstrated that Int-1 is the vertebrate ortholog of the *Drosophila* gene, Wg (Fung et al., 1985; Rijsewijk, Wagenaar, et al., 1987). Both the vertebrate Int-1 gene and the *Drosophila* Wg gene code for 40 kDa, secreted, glycosylated proteins with multiple conserved cysteine residues (Baker, 1987; Fung et al., 1985). The Int-1 gene was also shown to have tumorigenic properties when transfected into cells (Rijsewijk, Deemter, et al., 1987). Furthermore, over time, the mouse int-1 gene was found to be homologous to at least ten additional genes in the mouse, identified by MMTV activation or sequence conservation, each encoding for secreted 40 kDa proteins (Nusse et al., 1991). With this explosion of newly identified homologous genes of the same function identified across model systems, a new nomenclature was adopted, combining Wg and Int-1, to form the portmanteau "Wnt" (Nusse et al., 1991). These events contributed significantly to the birth of the Wnt field.

Also in the early 1980s, around the same time as the initial discovery of Wg, Nusslein-Volhard and Wieschaus made the landmark discovery of segment polarity genes in *Drosophila*, for which they were later awarded a Nobel Prize (Nüsslein-Volhard & Wieschaus, 1980). When mutated, these genes show defects in the segmental pattern of *Drosophila* larva. One class of

these genes presented phenotypic similarities to the Wg gene, suggesting a shared biochemical pathway (Nüsslein-Volhard & Wieschaus, 1980; Peifer et al., 1991). Several canonical Wnt regulators were identified initially as segment polarity genes.

While multiple developmental model systems were critical in the early characterization of the Wnt signaling pathway, arguably, few were more impactful than *Xenopus laevis*, the African horned frog. *Xenopus* was an ideal system for investigating developmental signaling and embryogenesis, given their large egg size and number of embryos produced (Niehrs, 2022). Shortly after initial findings in *Drosophila* and mouse models, Wg/Int-1 signaling was shown to be involved in axis specification during early *Xenopus* development. *Xenopus* axes (dorsal-ventral, anterior-posterior axes) are established by a cluster of cells known as the Spemann organizer (Spemann & Mangold, 1924). The Spemann organizer secretes signaling molecules and acts as a dorsalizing factor during embryogenesis. At the time, it was known that a factor was required to induce the formation of the organizer, but the molecule itself was unknown. Andy McMahon and Randall Moon answered this outstanding question with microinjection of Int-1 mRNA into the early *Xenopus* embryo and the observation of the striking phenotype of axis duplication (the formation of two head structures) (De Robertis et al., 2000; McMahon & Moon, 1989). This implicated Wnt proteins in development and axis specification and also served as a powerful assay to study Wnt signaling molecules during early development. Numerous Wnt regulators were identified and characterized using this system.

Dysregulation of the Wnt pathway has been implicated in a wide variety of human diseases, the most notable of which is colorectal cancer (Klaus & Birchmeier, 2008). Familial adenomatous polyposis (FAP) is a hereditary disease in which an individual is predisposed to adenomas in the colonic epithelia (P. Lockhart-Mummery, 1925). The cloning of the FAP locus

led to the discovery of Adenomatous polyposis coli (APC), a negative regulator of the Wnt signaling pathway (Groden et al., 1991; Kinzler et al., 1991; Nishisho et al., 1991). Carriers of a mutant APC allele develop benign polyps that over time accumulate additional mutations and progress to malignancy. It was later identified that over 80% of sporadic colorectal cancers have mutations in APC (Kinzler & Vogelstein, 1996). Today, hyperactive Wnt has been implicated in the progression of multiple other cancers, including breast cancer, pancreatic cancer, and liver cancer (Björklund et al., 2009; de La Coste et al., 1998; Weivoda et al., 2016). Wnt pathway misregulation has also been implicated in a number of developmental defects, including those related to bone density and skeletal development, as well as neural and eye development (Gong et al., 2001; Jenkins et al., 2009; Kibar et al., 2007; Xu et al., 2004). This importance of Wnt pathway regulation in human development and disease underscores the need to better understand the molecular mechanisms regulating the Wnt signaling cascade.

Current model of Wnt signal transduction

Over the past 40 years, our knowledge and understanding of the Wnt signaling pathway has expanded significantly. The current model of canonical Wnt signaling is centered around controlling the cytosolic levels of the transcriptional regulator β-catenin. In the absence of Wnt pathway activation, β-catenin remains in a cycle of expression and degradation (Stamos & Weis, 2013). Wnt pathway activation acts to stabilize β-catenin, allowing it to translocate into the nucleus and activate downstream transcriptional programming (Stamos & Weis, 2013). Here, I will overview the current model of Wnt signal transduction. First, I will discuss β-catenin, the destruction complex, and the cycle of β-catenin degradation. I will then discuss the mechanisms of Wnt pathway activation.

Destruction Complex And Beta-Catenin Cycling

β-catenin

β-catenin is the central transcriptional regulator of the canonical Wnt signaling pathway (MacDonald et al., 2009; Saito-Diaz et al., 2013). β-catenin was also discovered independently for its role in cell-cell adhesion, where it associates with e-cadherin in gap junctions (Ozawa et al., 1989). In developmental signaling, β-catenin is the vertebrate homolog of the segment polarity gene, Armadillo (Nüsslein-Volhard & Wieschaus, 1980; Peifer et al., 1991). Armadillo is in the Wg family of segment polarity genes and thus has similar effects on pattern formation and embryonic development as Wg (Peifer et al., 1991). Studies in *Xenopus* embryos demonstrate a role for β-catenin as a signaling molecule in the Wnt pathway, as β-catenin antibodies and mRNA induce secondary axis formation (Funayama, 1995, 1995). The β-catenin protein is regulated by phosphorylation and ubiquitination, and N-terminal phosphorylation targets it for degradation by the ubiquitin proteasome system (Stamos & Weis, 2013).

Axin

Axin is a scaffold in the β-catenin destruction complex (Stamos & Weis, 2013). This protein was initially identified as the gene product of the fused locus in mice (Perry et al., 1995). Mutations at this locus cause multiple developmental effects including axis duplication (Perry et al., 1995; L. Zeng et al., 1997). Furthermore, injection of Axin mRNA into *Xenopus* embryos blocks dorsal axis formation, indicating it is an inhibitor of the Wnt pathway (L. Zeng et al., 1997). Axin is a large, intrinsically disordered protein (Noutsou et al., 2011; Spink et al., 2000), containing an α-helical binding site for β-catenin, and interacts with APC via its Regulator of G-protein Signaling (RGS) domain (Liu et al., 2002; Spink et al., 2000). Additionally, Axin has binding sites for the kinases GSK3 and CK1 (Ikeda et al., 1998; Rubinfeld et al., 2001; Stamos &

Weis, 2013). Axin also contains a DAX domain that is homologous to the DIX domain of Disheveled (discussed later) (Stamos & Weis, 2013). This scaffold is the rate limiting component of the destruction complex, and in the *Xenopus* system, its concentration is significantly lower than that of other signaling components (Lee et al., 2003). Axin is also notably regulated by the ubiquitin-proteasome system (Saito-Diaz et al., 2013).

APC

Adenomatous Polyposis Coli (APC) is a large (310kDa), intrinsically disordered scaffold in the β-catenin destruction complex (Minde et al., 2013). This protein was first identified after cloning of the familial adenomatous polyposis (FAP) locus in mice and was shown to bind and downregulate β-catenin in colorectal cancer cells (Kinzler et al., 1991; Nishisho et al., 1991). The central region of APC contains binding sites for β-catenin, a series of 15 or 20 amino acid repeats (Katharine Eklof Spink et al., 2001). β-catenin binding is also regulated by phosphorylation, as APC phosphorylation by CK1 and GSK3 increases its affinity for β-catenin (Ha et al., 2004; Xing et al., 2003). The central region of APC also contains multiple SAMP motifs, which are conserved binding sites for Axin (Behrens et al., 1998; Spink et al., 2000). Additionally, APC has an interaction surface for the E3-ligase β-TrCP, which ubiquitinates phosphorylated β-catenin (Su et al., 2008). While the exact molecular function of APC within the destruction complex has been elusive, recent findings using biochemical reconstitution of the destruction complex have indicated its role in coordinating β-catenin phosphorylation and ubiquitylation (Ranes et al., 2021).

GSK3

Glycogen synthase kinase (GSK)-3 is a serine-threonine kinase with multiple cellular functions. In mammals, there are two Gsk3 genes, GSK3-α and GSK3-β, and the GSK3-α

protein has an extended glycine-rich N-terminus (Woodgett, 1990). Within the destruction complex, however, these two kinases are functionally redundant (Doble et al., 2007). GSK3 was also found to be homologous to the segment polarity gene shaggy (a.k.a. zeste-white 3) (Siegfried et al., 1992). GSK3 has multiple cellular roles, including glycogen biosynthesis and cell cycle control (Wang et al., 2022). It has been demonstrated that GSK3 is differentially regulated by Wnt and other signaling pathways, as the inhibitory Akt phosphorylation of GSK3 on Serine 9 is not relevant to GSK3 function in the Wnt pathway (Ding et al., 2000; McManus et al., 2005).

CK1

Casien-kinase 1 is another serine-threonine kinase associated with the destruction complex. There are multiple isoforms of CK1, including α, δ, ε or γ (Amit et al., 2002). Studies have demonstrated that CK1α is most important for destruction complex regulation (Liu et al., 2002).

PP2A

Protein Phosphatase 2A (PP2A) is associated with the destruction complex, although its role is unclear. It has been demonstrated to bind and dephosphorylate APC and Axin in vitro (Hsu et al., 1999; Ratcliffe et al., 2000; Yamamoto et al., 2001).

β-TrCP

β-TrCP is the substrate recognition subunit of the Skp, Cullin, F-box containing (SCF) E3-ligase SCF-β-TrCP. (Henceforth, "β-TrCP"). This E3-ligase complex recognizes and ubiquitylates phosphorylated β-catenin in the destruction complex. β-TrCP is a WD-40 repeat-containing F-Box protein and recognizes β-catenin phosphorylated at Ser33 and Ser37 (Hart et al., 1999; Orford et al., 1997; Wu et al., 2003).

Cycle of β-catenin destruction

β-catenin and the destruction complex components are ubiquitously expressed (Saito-Diaz et al., 2013; L. Zeng et al., 1997). This complex functions to keep cytosolic β-catenin levels low when the pathway is inactive. β-catenin thus remains in a cycle wherein it is continuously synthesized and subsequently degraded by the destruction complex (Stamos & Weis, 2013). The destruction complex formation is initiated with Axin binding to GSK3 and CK1 (Dajani et al., 2003; Sobrado et al., 2005). Additionally, the scaffold APC binds the RGS domain of Axin (Spink et al., 2000). It is thought that β-catenin initially binds Axin via its N-terminal α helix and APC via its 15 amino acid repeats (Saito-Diaz et al., 2013). Axin binding brings β-catenin within close proximity of CK1 and GSK3, facilitating phosphorylation of the β-catenin N-terminus (Amit et al., 2002). CK1 phosphorylation of β-catenin on Ser45, then primes successive phosphorylation by GSK3 on β-catenin Thr31, Ser37, and Ser33 (Frame et al., 2001; Liu et al., 2002).

Both Axin and APC function in the destruction complex is regulated by phosphorylation. GSK3 phosphorylation of Axin increases its affinity for β-catenin, promoting the initial β-catenin recruitment to the complex (Ha et al., 2004). Phosphorylated 20 amino acid repeats of APC also have increased binding affinity for β-catenin and increased capacity to downregulate β-catenin (Ha et al., 2004; Rubinfeld et al., 1996). One model of APC phosphorylation in destruction complex function is that it functions to remove β-catenin from Axin and allows new molecules to bind (Kimelman & Xu, 2006; Rubinfeld et al., 1996). Following β-catenin phosphorylation by CK1 and GSK3 and its removal from Axin, the N-terminal phospho-sites (Ser33 and Ser37) of β-catenin are recognized by β-TrCP, which proceeds to ubiquitylate and target β-catenin for proteasomal degradation (Hart et al., 1999).

Wnt Pathway Activation

Wnt pathway activation stabilizes cytosolic β-catenin. I will discuss the pathway activation in three sections: 1) Wnt ligands and cell surface activation, 2) inhibition of the destruction complex, 3) activation of Wnt dependent transcription.

Cell surface activation: Ligands and secretion

Wnt ligands

There are 19 vertebrate Wnt ligands, and of those, Wnt3a, Wnt1 and Wnt8 generally stimulate the canonical β-catenin-dependent signaling pathway (Kikuchi et al., 2011; Kusserow et al., 2005). These ligands are secreted, cysteine rich, glycoproteins with a bilobular structure maintained by a network of disulfide bonds (Janda et al., 2012). Each Wnt ligand contains a signal sequence, which is required for secretion (Willert et al., 2003). Importantly, Wnt ligands are palmitoylated on a conserved serine residue, and this is required for both secretion and signaling at the cell surface (Bradley & Brown, 1990; Takada et al., 2006). This lipid modification also accounts for the hydrophobicity and insolubility of Wnt proteins, as they are difficult to purify without detergent or co-expression of a Wnt-binding molecule (Janda et al., 2012; Willert et al., 2003).

Porcupine

Porcupine (PORCN) is a membrane bound O-acyltransferase, located in the endoplasmic reticulum. PORCN adds a palmitoyl-group (16 carbon, monosaturated fatty acid) to the Ser209 of Wnt proteins (J. Li et al., 2015; Nusse & Clevers, 2017; Resh, 2017). This lipid modification is required for Wnt secretion and for the signaling capacity of Wnt molecules (Willert et al., 2003).

Wntless

Following palmitoylation, Wnt ligands are translocated from the ER to the Golgi apparatus (Buechling et al., 2011). Here, the lipid-modified Wnts are associated with the seven-pass-transmembrane receptor Wntless, which facilitates their plasma membrane transport and secretion (Port & Basler, 2010). Wntless receptors are then internalized and shuttled back to the Golgi apparatus (Coudreuse et al., 2006).

Cell surface activation: Receptors and signalosome components

Fzd

In 1996, Jeremy Nathans and Roeland Nusse identified the Frizzled family as general receptors for Wnt ligands and showed that Wg binding of Dfz2 stabilized Armadillo (Bhanot et al., 1996). Frizzled receptors are 7-transmembrane domain proteins, and thus have a topology similar to GPCRs (Saito-Diaz et al., 2013). While they share a similar structure, the role of the heterotrimeric G-protein complex in Wnt signaling is unclear. Frizzled receptors have an N-terminal cysteine-rich domain (CRD), and the production of soluble Wnts allowed for the biochemical characterization of the Wnt-Fzd interactions, demonstrating that Wnt ligands bind with high affinity to the CRD (Janda et al., 2012). Additionally, Frizzled receptors bind Disheveled via their C-terminal tail at the conserved KS/TxxxW motif (Wong et al., 2003).

LRP5/6

The product of the Arrow gene in *Drosophila* is a single pass-transmembrane protein required for Wg signaling and Armadillo stabilization (Wehrli et al., 2000). Arrow was shown to be homologous to the human LRP5/6 receptors, which act as co-receptors in the Wnt cascade (Tamai et al., 2000; Wehrli et al., 2000). LRP5/6 are 71% identical and are somewhat functionally redundant in Wnt pathway activation (Brown et al., 1998; Hey et al., 1998). LRP6 deletion exhibits more severe phenotypes during murine embryogenesis than LRP5 loss (O. G.

Kelly et al., 2004), and LRP6 overexpression in *Xenopus* and mammalian cells is a more potent Wnt activator than LRP5 overexpression (Mi & Johnson, 2005; Tamai et al., 2000). Because LRP6 appears to be the more efficacious of the two receptors, I will refer only to LRP6. The extracellular domain of LRP6 contains four β-propeller and epidermal growth factor (βP–E) domain repeats, along with three LDL-receptor type repeats (Nusse & Clevers, 2017; Saito-Diaz et al., 2013). The intracellular domain (ICD) of LRP6 is proline-rich and contains five PPPS/TP motifs, which are required for Wnt signaling and are phosphorylated upon activation (Tamai et al., 2004; X. Zeng et al., 2005).

Disheveled

Disheveled (DVL) is a signaling protein initially identified in *Drosophila*, where mutant flies displayed disoriented wing and body hairs (Fahmy & Fahmy, 1959). There are three Disheveled paralogs in humans (Dvl1, 2, and 3), and they are involved in both canonical and non-canonical Wnt signaling (Paclíková et al., 2021). DVL is a membrane regulator of Wnt signaling and, upon Wnt/Wg stimulation, is phosphorylated by CK1 and recruited to the cell membrane (Yanagawa et al., 1995). Three prominent domains make up the DVL protein: the DEP, DIX, and PDZ domains. The DEP is essential for Wnt membrane activation and interacts with the Fzd receptor, and dimerizes with itself via domain swapping (M. V. Gammons, Rutherford, et al., 2016a). The DIX domain is homologous to the DAX domain of Axin and binds Axin upon Wnt activation, as well as polymerizes with itself (M. V. Gammons, Rutherford, et al., 2016a). The PDZ domain recognizes the C-terminal tail of Fzd at the conserved KS/TxxxW motif (Wong et al., 2003).

Cell Surface Activation: Wnt Receptor Activation and Signalosome Formation

Wnt ligands trigger pathway activation by binding the Wnt co-receptors and forming a ternary complex. In 2012, Claudia Janda and K. Christopher Garcia provided the first structural model of Wnt ligand-receptor interactions, crystallizing *Xenopus* Wnt8 in complex with the mouse Frizzled-8 and revealing the mechanism of Wnt recognition by Frizzled receptors (Janda et al., 2012). The characteristic "hand" structure of the Wnt ligand contacts the CRD domain with the "index" and "thumb," which are projecting from a central "palm" domain. The N-terminal palmitoleic acid lipid group sits in a deep pocket of the CRD. The other point of contact is via hydrophobic amino acids (Janda et al., 2012). LRP6 recognizes ligands via its four βP–E (E1-E4) domains, with domains binding in pairs to a single ligand (Bourhis et al., 2010; S. Chen et al., 2011). Structural and biochemical studies suggest Wnt ligands have preferences for different binding sites, and ligands can be classified by their binding site (Cheng et al., 2011). CryoEM structures of the ternary complex revealed that canonical Wnts bind E1 and E2 within this complex (Tsutsumi et al., 2023).

The primary intracellular outcome of LRP6 receptor activation by Wnt ligands is the phosphorylation of the intracellular domain of LRP6 (LRP6-ICD) (Tamai et al., 2004; X. Zeng et al., 2005). Wnt induces LRP6 phosphorylation at multiple proline adjacent serine/threonine residues (X. Zeng et al., 2005). While multiple kinases can phosphorylate the LRP6 PPPSP motifs, GSK3 is the predominant proline-directed kinase to phosphorylate PPPSP motifs in response to Wnt (Tamai et al., 2004). GSK3 phosphorylation primes for CK1γ phosphorylation at the C-terminal serine in the PPPSPxS motif (X. Zeng et al., 2005). Phosphorylated LRP6 associates with Axin and the destruction complex, and receptor phosphorylation is critical for the stabilization of β-catenin (X. Zeng et al., 2008).

Wnt ligand stimulation of the co-receptors LRP6 and Fzd triggers the formation of "signalosomes," which are large receptor aggregates that potentiate the Wnt signal (Bilic et al., 2007; Colozza & Koo, 2021). Signalosome formation is mediated by the DVL adaptor protein, which is required for signalosome formation and LRP6 phosphorylation (X. Zeng et al., 2008). DVL affinity for the cytoplasmic tail of Fzd is increased upon receptor activation, and the protein then associates with Fzd via its PDZ domain (Wong et al., 2003). This increased concentration of DVL at the membrane promotes the oligomerization required for signalosome assembly (Ma et al., 2020). This oligomerization is via the DIX domain of DVL, which self-polymerizes through head-to-tail polymerization (Schwarz-Romond et al., 2007). The DEP domain of DVL also dimerizes with itself upon the protein binding to Fzd (M. V. Gammons, Renko, et al., 2016; M. V. Gammons, Rutherford, et al., 2016b).

The downstream effect of this assembly is the recruitment of Axin to the membrane, as DVL recruits and polymerizes with Axin via its homologous DIX domain (DAX) (Cliffe et al., 2003). Axin is recruited in complex with GSK3, and thus DVL recruitment of Axin promotes LRP6 phosphorylation and further Axin recruitment (Niehrs & Shen, 2010, 2010).

Membrane phospholipids also have a role in Wnt signalosome assembly. Wnt ligand stimulation triggers the formation of membrane phosphatidylinositol 4,5-bisphosphate (PIP2) by the kinases phosphatidylinositol 4-kinase type II (PI4KII) α and phosphatidylinositol-4-phosphate 5-kinase type I (PIP5KI) (Pan et al., 2008). The generation of membrane PIP2 by these kinases also regulates LRP6 phosphorylation (Pan et al., 2008). Membrane PIP2 recruits the protein APC membrane recruitment 1 or Wilms tumor gene on X (Amer1/WTX) (Major et al., 2007; Tanneberger et al., 2011). Amer1/WTX binds Axin, GSK3 and CK1, and thus contributes to their membrane recruitment (Tanneberger et al., 2011). Membrane PIP2 also

promotes signalosome assembly via the recruitment of the Clathrin endocytic machinery, as there is evidence Clathrin/AP2 are signalosome components and are required for LRP6 phosphorylation as well (I. Kim et al., 2013). Altogether, these large multiprotein signalosomes work to potentiate Wnt pathway activation by recruiting Axin and GSK3 to the membrane (Figure 1-2).

Wnt Antagonists

Multiple endogenous Wnt inhibitors have been identified. Secreted Frizzled Related Proteins (SFRPs) and Wnt Inhibitory Factors (WIFs) are classes of Wnt antagonists that bind and sequester Wnt ligands in solution. SFRPs are the largest family of secreted Wnt inhibitors and are homologous to the cysteine-rich Wnt binding domain of the Fzd receptor (Bovolenta et al., 2008).

Another secreted Wnt inhibitor is Dickkopf-1 (DKK1), which blocks signaling by directly binding and antagonizing the LRP6 co-receptor (Bovolenta et al., 2008). DKK1 was also instrumental in early structural studies examining LRP6-ligand interactions (Cheng et al., 2011). This inhibitor mediates LRP6 removal from the membrane by forming a ternary complex with the high-affinity DKK1 receptor, Kremen, and LRP6, inducing rapid endocytosis of LRP6 (Mao et al., 2002).

<u>Destruction complex inhibition</u>

The function of Wnt receptor activation is to ultimately stabilize cytosolic β-catenin by inhibiting the function of the β-catenin destruction complex. Multiple models have been proposed for this inhibition, and additional studies are required to resolve these competing models.

One set of competing models involves the dissociation of the destruction complex. Some studies have proposed that Wnt stimulation triggers the destabilization of the destruction complex, and this has been proposed to be mediated by CK1 phosphorylation of individual destruction complex components (Gao et al., 2002; Liu et al., 2002). Other studies, however, have demonstrated the opposite, that the destruction complex is recruited to the membrane as an intact complex (Bilic et al., 2007; X. Zeng et al., 2005).

The destruction complex is recruited to the membrane by phosphorylated LRP6 intracellular domain and by DVL. Multiple studies have shown that the phosphorylated PPPS/TP motifs of LRP6 can act as a competitive GSK3 inhibitor in vitro and in cells, and one model proposes GSK3 activity on β-catenin is inhibited by phosphorylated LRP6-ICD (Cselenyi et al., 2008; Hendriksen et al., 2008; Wu et al., 2009; Yamamoto et al., 2006). GSK3, however, is thought to be the predominant kinase in Wnt-dependent LRP6 phosphorylation (Tamai et al., 2004). It is unclear how membrane GSK3 retains its activity on PPPSP motifs, which also act as competitive inhibitors.

Axin is a rate-limiting component of the destruction complex function and is responsible for complex initiation (Lee et al., 2003). Axin is regulated by phosphorylation, poly-ADP-ribosylation, and ubiquitylation, which regulate its turnover (Kofron et al., 2007; Yamamoto et al., 1999). Some studies have shown that Axin levels are reduced following Wnt activation, and this has been proposed to be the model of destruction complex inhibition (Kofron et al., 2007). However, more recent findings have demonstrated that this Axin turnover occurs after destruction complex inhibition. Axin turnover is likely important for pathway activation, but is not the initial inhibition step for the destruction complex (Cselenyi et al., 2008; Saito-Diaz et al., 2013).

One study proposed that destruction complex inhibition is via GSK3 sequestration in multivesicular bodies, preventing its activity on β-catenin and other cytosolic substrates (Taelman et al., 2010). This study showed that Wnt stimulation leads to GSK3 localization in acidic vesicles with endosomal markers; however, this finding also occurs after β-catenin stabilization and pathway activation and is likely not responsible for the initial inhibition of the destruction complex (Saito-Diaz et al., 2013; Taelman et al., 2010).

In other signaling contexts, such as Akt signaling, phosphorylation of GSK3 on Ser9 negatively regulates the kinase's activity (Sutherland et al., 1993). Studies have shown that GSK3 phosphorylation at Ser9 is not stimulated by Wnt and is not the mechanism of GSK3 inhibition on LRP6 (McManus et al., 2005; Saito-Diaz et al., 2013).

β-catenin phosphorylation within the destruction complex targets it for ubiquitination by β-TrCP, and degradation by the proteasome system (Stamos & Weis, 2013). One study proposed a model in which the destruction complex containing β-TrCP, remains intact following Wnt stimulation and that Wnt activation blocks ubiquitination within the complex, rather than blocking GSK3 phosphorylation of β-catenin (V. S. W. Li et al., 2012). However, another study used quantitative measurements of β-catenin kinetics to demonstrate that β-catenin phosphorylation was the site of Wnt-dependent destruction complex inhibition (Hernández et al., 2012).

<u>β -catenin dependent transcriptional activation</u>

Following Wnt receptor activation and destruction complex inhibition, stabilized β-catenin is then translocated into the nucleus to control Wnt-dependent transcription (Anthony et al., 2020; Stamos & Weis, 2013).

The mechanisms regulating β-catenin nuclear localization are unclear. Nuclear import is generally mediated by nuclear localization signals (NLS), which is a basic peptide sequence on many proteins targeted to the nucleus (Fagotto et al., 1998). This NLS is recognized by the nuclear transport complex, a heterodimer consisting of the proteins Importin-α and Importin-β. Importin α recognizes the NLS, and Importin-β associates with α and the nuclear pore, translocating α and the cargo across the membrane (Anthony et al., 2020; Fagotto et al., 1998).

β-catenin does not contain an NLS sequence and is proposed to be transported by a different mechanism (Suh & Gumbiner, 2003). β-catenin armadillo domains are similar to the importin-β nucleoporin recognition domains, and thus it has been proposed that β-catenin can translocate by directly associating with nucleoporins. Studies have demonstrated, however, that β-catenin transport is independent of FG-nucleoporins (Suh & Gumbiner, 2003). Nucleoporin-independent models include "chaperone" models, in which a β-catenin binding protein regulates its transport across the nuclear membrane (Anthony et al., 2020). The proteins APC (localized to the cytosol and nucleus) and B-Cell CLL/lymphoma 9 (BCL9) (a nuclear β-catenin binding partner) have been proposed to control β-catenin nuclear translocation (Anthony et al., 2020; Henderson, 2000; Townsley et al., 2004).

Within the nucleus, β-catenin binding proteins mediate its transcriptional regulation. T-cell factor/lymphoid enhancer factors (TCF/LEF) are the most well-characterized transcription factors recruiting β-catenin to Wnt target genes (Cadigan & Waterman, 2012). Invertebrates have a single TCF gene, while four genes code for TCF gene products in vertebrates (Cadigan & Waterman, 2012). TCF1 (TCF7 gene) and LEF1 were the first of these genes to be identified, and were discovered in human T lymphocytes (Travis et al., 1991; van de Wetering et al., 1991). TCF 3 (TCF7L1 gene) and TCF4 (TF7L2 gene) were identified later via hybridization screens

(Cadigan & Waterman, 2012). These transcription factors bind β-catenin via their N-terminus and interact with the DNA via their high mobility group (HMG domain) (van de Wetering & Clevers, 1992).

In the pathway inactive state, TCF/LEFs associate with the transcriptional co-repressors Groucho/Transducing-like enhancer, which suppresses TCF/LEF dependent transcription (Cavallo et al., 1998; Roose et al., 1998). When associated TCF/LEF, Gro/TLE remodels and silences chromatin by the recruitment of histone deacetylases (HDACs) (Arce et al., 2009; G. Chen et al., 1999). To activate TCF/LEF dependent transcription, β-catenin must bind TCF/LEF and replace Gro/TLE (Anthony et al., 2020; Cadigan & Waterman, 2012). Multiple models have been proposed for this β-catenin-mediated switch from repression to activation. One model is one of simple competition, as β-catenin binding to TCF has been shown to directly compete with Gro/TLE for a shared binding site on TCF *in vitro* (Daniels & Weis, 2005). Recently, however, there has been evidence supporting a model in which ubiquitination regulates this transition. Both the E3-ligase XIAP and the deubiquitylase USP47 act on Gro/TLE to regulate its association with TCF/LEF and promote Wnt dependent transcription (Hanson et al., 2012; Kassel et al., 2023).

The TCF/LEF-associated β-catenin recruits the mediator complex that is required for Pol II-dependent transcription, and to recruit chromatin remodeling factors to Wnt response elements (Cadigan & Waterman, 2012; S. Kim et al., 2006). Additional non-TCF/LEF factors include the protein Pygopus, which is thought to participate in mediator complex recruitment, and Bcl9, which connects β-catenin to the WREs through an interaction with Pygo (Carrera et al., 2008; Städeli & Basler, 2005). Additionally, β-catenin interacts with various histone acetyltransferases and methyl transferases to remodel chromatin (Takemaru & Moon, 2000). Finally, a model has

been proposed in which β-catenin forms a large multiprotein complex with each of these factors, termed the "enhanceosome," which regulates Wnt-dependent transcription (Anthony et al., 2020; M. Gammons & Bienz, 2018).

Summary of Canonical Wnt Pathway

The Wnt signaling pathway is a master regulator of development and adult tissue homeostasis (Figure 1-1). Pathway misregulation contributes to multiple human disorders and diseases, most notably colorectal cancer. The transcriptional co-activator β-catenin regulates transcription of Wnt target genes, which are responsible for controlling cell proliferation and differentiation. In the pathway off state, β-catenin is associated with a cytosolic β-catenin destruction complex, which consists of the scaffolds Axin and APC, the kinases GSK3 and CK1. Axin initiates the destruction complex formation via its interactions with β-catenin, GSK3, and CK1, bringing them within close proximity. GSK3 and CK1 phosphorylate the N-terminus of β-catenin, generating a recognition surface for the SCF-βTrCP E3 ligase. APC coordinates the removal of phosphorylated β-catenin from Axin and delivers it to βTrCP for ubiquitylation and subsequent proteasomal degradation.

Canonical Wnt ligands, such as Wnt3a, are secreted, lipid-modified glycoproteins that signal in a paracrine manner. Wnt ligands are palmitoylated by the acyl-transferase Porcupine, and their secretion is mediated by the receptor Wntless. Ligands bind the co-receptors LRP6 and Fzd, forming a ternary complex. Wnt-Fzd interactions recruit the adaptor protein DVL to the membrane, and DVL polymerization mediates the formation of large receptor aggregates called signalosomes. Cytosolic Axin and GSK3 are then recruited to these signalosomes, wherein GSK3 phosphorylates the intracellular domain of the co-receptor LRP6. Phosphorylated LRP6

forms a docking site for further recruitment of Axin to the membrane and also inhibits GSK3 activity on β-catenin.

Cytosolic β-catenin levels rise, and the protein is translocated into the nucleus by an unknown mechanism. Nuclear β-catenin localizes Wnt response elements via its association with the transcription factor TCF/LEF. Absent β-catenin, TCF/LEF is associated with the transcriptional repressor Gro/TLE. β-catenin displaces Gro/TLE from TCF/LEF, recruits the mediator complex and chromatin remodeling proteins to activate Wnt dependent transcription.

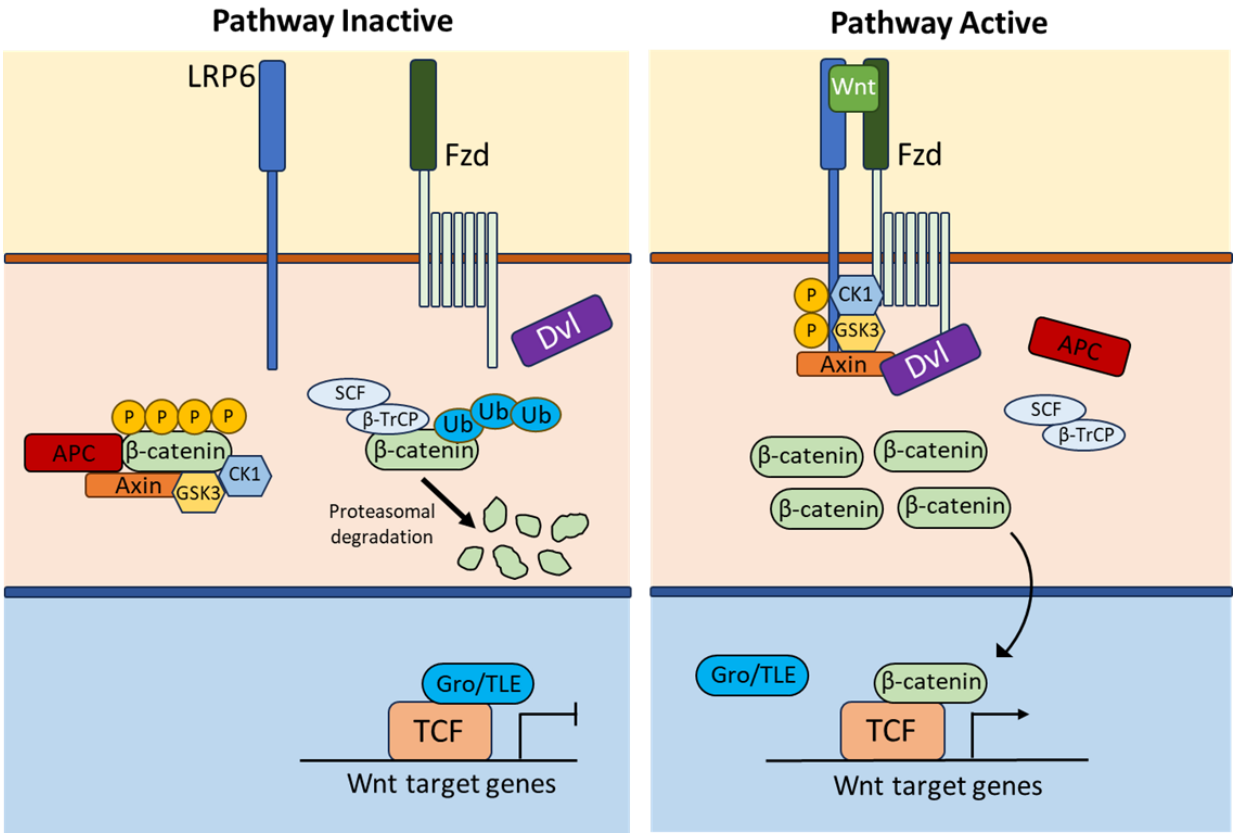


Figure 1-1: Schematic of canonical Wnt signaling pathway, as described in text. In the absence of Wnt ligand, β-catenin is associated with the cytosolic destruction complex, consisting of the scaffold proteins APC and Axin, and the kinases GSK3 and CK1. Destruction complex phosphorylation of β-catenin target it for ubiquitination by SCF-βTrCP, and subsequent proteasomal degradation. Wnt ligands bind the co-receptors LRP6 and FZD, leading to the recruitment of Axin and GSK3 to the membrane. GSK3 phosphorylation provides a docking site for Axin, which also polymerizes with the FZD-associated cytosolic adaptor protein, DVL. This inhibits destruction complex phosphorylation of β-catenin, stabilizing it, and allowing it to translocate into the nucleus. Nuclear β-catenin associates with TCF/LEF transcription factors, displacing the inhibitory Gro/TLE, and activating the Wnt transcriptional program.

Regulation of Wnt receptors: A novel role for the tumor suppressor APC

The most significant and well-characterized regulatory function of APC in the Wnt pathway is in the downregulation of β-catenin within the destruction complex. The central region of APC contains binding sites for β-catenin and Axin (20 a.a. repeats and SAMP motifs, respectively), and these are critical for APC destruction complex function (Behrens et al., 1998; Katharine Eklof Spink et al., 2001; Spink et al., 2000). Inactivating mutations in this region generate truncated proteins lacking these motifs, and these truncations have impaired destruction complex function and hyperactive Wnt (Fearnhead et al., 2001). This region is termed the mutation cluster region, as the majority of cancer-causing APC mutations cluster in this region of the protein (Fearnhead et al., 2001).

Saito-diaz et al. (2018) demonstrated an additional function for APC within the Wnt pathway: negative regulation of Wnt receptor activation (Figure 1-3). This study demonstrated that APC deletion in mammalian cells led to constitutive, ligand-independent receptor activation and signalosome formation and that this activation was dependent on clathrin-mediated endocytosis (Saito-Diaz et al., 2018). Findings in *Drosophila* and murine organoid models were consistent, indicating conservation. Based on these findings, a model was proposed in which membrane APC prevents constitutive, ligand-independent clathrin/AP2-mediated signalosome formation and receptor activation (Saito-Diaz et al., 2018).

In the subsequent chapters, I will discuss the two projects centered on APC regulation of Wnt receptor activation. One chapter will biochemically examine the mechanisms by which APC regulates Wnt receptors, and the other will examine the function of receptor homeostasis in APC-deficient cells.

Endocytosis and ubiquitylation are both critical to the regulation of Wnt receptor function and homeostasis, and an understanding of which is required for the ensuing chapters. Here, I discuss these two processes and their role in regulation of Wnt receptors.

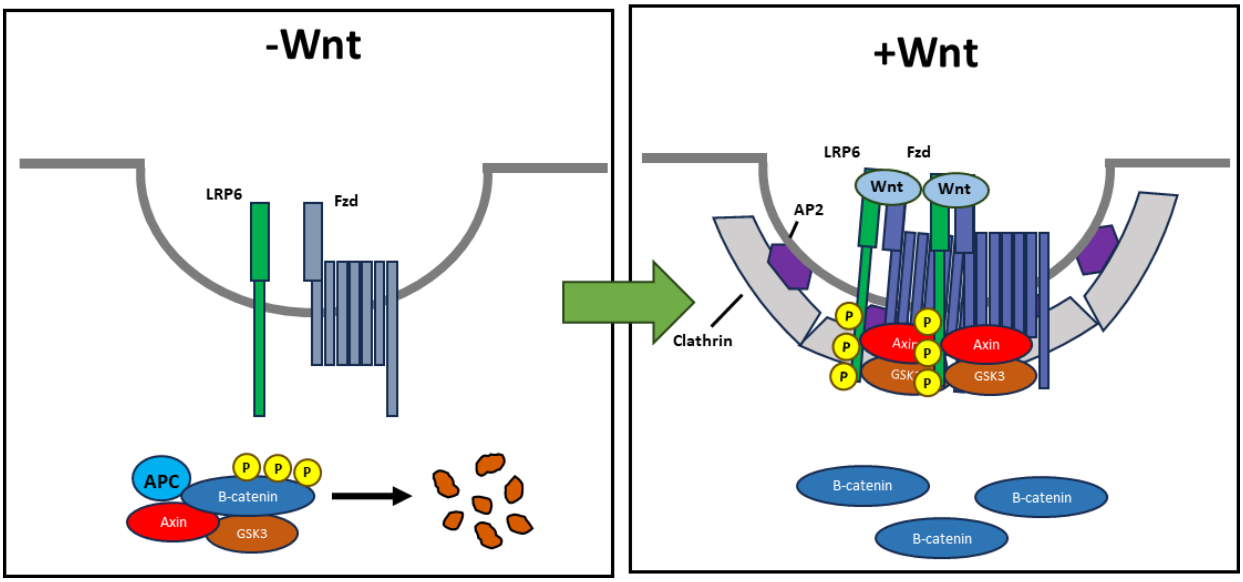


Figure 1-2: Schematic of Wnt ligand-dependent signalosome formation. Wnt ligands bind the co-receptors LRP6 and FZD, and this triggers receptor clustering. Receptor clustering and LRP6 phosphorylation are dependent on clathrin/AP2, and AP2 associates with LRP6 within the signalosome.

Regulation of Wnt receptors: Endocytosis

Clathrin-mediated endocytosis

Clathrin-mediated endocytosis (CME) is the primary mechanism of cell surface internalization in mammalian cells, controlling signaling via the uptake of cell surface receptors and transporters, as well as altering membrane composition in response to environmental cues (Mettlen et al., 2018). The main components are clathrin proteins and their adaptor protein complexes. Clathrin exists as a trimer with three heavy chains and three associated light chains and forms vesicles by first assembling as a "lattice-like" coat on the interior surface of membranes (Kirchhausen & Harrison, 1981; Ungewickell & Branton, 1981).

Clathrin is recruited to membranes via heterotetrameric adaptor proteins. Vertebrate genomes encode for five types of adaptors-proteins (AP1-AP5) (Mettlen et al., 2018). AP2 mediates endocytosis at the plasma membrane. AP1 and AP3 control intracellular trafficking between the golgi and endosomes, while AP4 and AP5 roles are unknown (Hirst et al., 2013; Kirchhausen et al., 2014).

AP2 adaptor complexes are comprised of four subunits. AP2α2 and AP2β2 are homologous, heavy subunits that interact with the membrane and clathrin (Kirchhausen et al., 2014). AP2μ2 and AP2sigma2, medium and small chains, respectively, interact with cargo. The adaptor protein undergoes conformation changes and has closed and open conformations (Jackson et al., 2010; Rapoport et al., 1997). In the close conformations, the main cargo recognition subunit, the C-terminal domain of AP2μ2, is hidden. In open conformations, the AP2μ2 C-term is exposed and in a position permissive to cargo recognition (Jackson et al., 2010).

Adaptor proteins are recruited to membranes by various phosphoinositide species, and AP2 is recruited to and oriented at the plasma membrane by PI(4,5)P2 (Kirchhausen et al., 2014). PIP2 also triggers a conformation change to the open conformation (Rapoport et al., 1997). AP2μ2 primarily recognizes cargo via the Y-x-x-φ tyrosine-based endocytic sorting motifs, where φ is a bulky hydrophobic residue (Owen & Evans, 1998). Additionally, AP2 recognizes cargo via acidic dileucine motifs ([DE]xxxL[LI]), which sit at the interface between AP2μ2 and AP2α2 subunits (B. T. Kelly et al., 2008).

Endocytic vesicles proceed through stages of initiation, maturation, and membrane fission. Various early endosomal adaptor proteins (EAPs) act as scaffolds and regulators of CME during the maturation process (Mettlen et al., 2018; Robinson, 2015; E. M. Schmid & McMahon, 2007). The large GTPase dynamin catalyzes membrane fission (S. L. Schmid & Frolov, 2011).

Caveolin dependent endocytosis

Caveola are flask-shaped invaginations in the plasma membrane that internalize membrane proteins and shuttle them to early and late endosomes (Hayer et al., 2010). These pits are characterized by their lack of a coat, as observed in clathrin-mediated endocytosis, and their enrichment in lipid species (Hayer et al., 2010). Caveola are formed through the assembly of caveolin and cavin proteins. Caveolins are integral membrane proteins that reach the membrane via the secretory pathway, and membrane caveolins form oligomeric structures enriched in cholesterol (Hayer et al., 2010; Khater et al., 2019). These structures recruit cavins, which are accessory proteins that are synthesized in the cytosol and are recruited to membrane phospholipids, such as PIP2 (Kovtun et al., 2014). The membrane clathrin and cavin-dependent membrane invaginations are excised from the membrane in a dynamin-dependent process, and sorted into early and late endosomes (Hayer et al., 2010).

Wnt regulation by endocytosis

Endocytosis has been implicated in the regulation of Wnt pathway activation, and various lines of evidence indicate Wnt signalosomes are endocytosed (Colozza & Koo, 2021). In the Drosophila embryo, electron microscopy detected Wg on the cell surface as well as in puncta within the cell, indicating uptake of the ligand (van den Heuvel et al., 1989). Additionally, Arrow and Fzd receptors are endocytosed in the wing disc, and Dynamin has been demonstrated to be required for Wg signaling in flies (Rives et al., 2006; Seto & Bellen, 2006).

The preferred endocytic route of the Wnt signalosome in mammalian cells is unclear. Some reports indicate caveolin is required for Wnt signaling and receptor activation, while others demonstrate this requirement is mediated by clathrin instead.

First, caveolin has been proposed to be required for Wnt signaling. Caveolin has been shown to co-localize with LRP6 and Wnt signalosomes, and siRNA silencing or small molecule inhibition of the caveolin endocytic pathway, but not clathrin, has been shown to block Wnt signaling transduction in some studies (Bilic et al., 2007). Furthermore, in HEK293 cells, some studies have found a requirement for caveolin in Wnt-dependent signaling, while clathrin was demonstrated to mediate inhibitory DKK1 signaling (Yamamoto et al., 2006, 2008).

Others reports have indicated a role for clathrin, but not the caveolin pathway. In murine L- cells, inhibition of CME was shown to block Wnt signaling (Blitzer & Nusse, 2006). Additionally, clathrin and AP2 have been demonstrated to be components of Wnt signalosomes and are required for signalosome formation and LRP6 phosphorylation in HEK293 cells (Bilic et al., 2007).

Various factors could account for these conflicting reports. One possibility is that the endocytic mechanism is cell type-specific, as Saito Diaz et al (2018) proposed epithelial cells

(eg. HEK293, RKO) endocytose via the caveolin pathway and fibroblast like cells (eg.. L cells, MEFs) endocytose via CME (Saito-Diaz et al., 2018). Additionally, as most studies utilize receptor overexpression, some have proposed that stoichiometry of the overexpressed protein is relevant, as Fzd overexpression alone endocytoses via clathrin, and Fzd-LRP6 co-expression is endocytosed via caveolin (Yamamoto et al., 2006).

Finally, recent reports suggest that neither endocytic pathway is required for Wnt ligand-dependent signaling. Using single-cell, nuclear imaging of β-catenin, the Roeland Nusse laboratory recently demonstrated that the knockdown of endocytic components in murine embryonic stem cells had no effect on β-catenin signaling (Rim et al., 2020). Given the conflicting reports, additional studies are required to elucidate the mechanisms by which endocytosis regulates Wnt pathway activation.

Wnt receptor regulation: Ubiquitylation

Ubiquitylation is a post-translation modification that regulates protein stability as well as various non-proteolytic functions, including membrane trafficking and DNA repair (Callis, 2014; Z. J. Chen & Sun, 2009). Ubiquitin is a small, 76 amino acid protein that is highly conserved across species (Callis et al., 1989). Three enzymes catalyze the covalent addition of ubiquitin to the substrate protein. The activating enzyme, E1, first forms a thioester bond linking the C-terminus of ubiquitin to a cysteine within itself, in an ATP-dependent process (Haas & Rose, 1982). Next, the E2 conjugating enzyme associates with the E1 enzyme and catalyzes a transesterification reaction linking the ubiquitin to a cysteine in the E2 active site (Stewart et al., 2016). Finally, the E3 ligase binds E2 and transfers the ubiquitin molecule to the substrate protein, forming an isopeptide bond between the C-terminal glycine of ubiquitin and a substrate lysine (Cruz Walma et al., 2022).

Ubiquitylated substrates are differentially regulated, depending on the type of ubiquitylation. Monoubiquitylation regulates various cellular processes, including receptor transport and cellular repair (Di Fiore et al., 2003; Hicke, 2001). Polyubiquitylaion, the formation of chains of different shapes and lengths, provides further diversification (Di Fiore et al., 2003; Hicke, 2001). Linkages between ubiquitin chains occur on conserved lysine residues, and Lys48 and Lys63 are the most well-characterized. Lys48 regulates protein stability via the 26S proteasome, and Lys63 has various signaling roles (Z. J. Chen & Sun, 2009; Jacobson et al., 2009; H. T. Kim et al., 2007; Komander et al., 2009).

The two most common types of E3 ligases are HECT domain and RING-finger E3 ligases (Huibregtse et al., 1995). HECT (homologous to the E6AP carboxyl terminus) E3 ligases contain a C-terminal HECT domain, and ubiquitin is transferred to the active site of this HECT domain before transfer to the substrate (Sluimer & Distel, 2018). Substrates are recognized by their protein N-terminus (Sluimer & Distel, 2018).

RING-finger E3 ligases contain a RING (Really Interesting New Gene) domain (Deshaies & Joazeiro, 2009; Zheng & Shabek, 2017). The RING domain binds the E2 enzyme and, unlike the HECT domain, transfers the ubiquitin directly to the substrate (Bulatov & Ciulli, 2015). There are monomeric and multimeric RING E3 ligases. Multimeric RING E3 ligases contain a catalytic RING box protein, a substrate recognition subunit, and an adaptor. SCF-βTrCP is an example of a multimeric RING (Cruz Walma et al., 2022; Stamos & Weis, 2013).

Ubiquitylation is a reversible post-translational modification. Deubiquitylases (DUBs) mediate the removal of ubiquitin molecules from substrates, and counteract the action of E3 ligases (Komander et al., 2009). There are 79 DUBs in the human genome. These DUBs have been shown to have three main functions: the generation of free ubiquitin from ubiquitin

transcribed and translated as linear fusions, the removal of ubiquitin from modified proteins, and the editing of ubiquitin chains by pruning (Komander et al., 2009).

The two largest classes of DUBs are the 20-30 kDa ubiquitin C terminal hydrolases (UCH) and the 100 kDa ubiquitin-specific proteases (USPs). Both of these classes catalyze the removal of ubiquitin via a catalytic triad containing a cysteine residue (D'Andrea & Pellman, 1998, 1998; Komander et al., 2009).

Ubiquitylation plays a critical role in the regulation of Wnt receptor homeostasis. The primary E3 ligases mediating the ubiquitylation and turnover of the co-receptors LRP6 and Fzd, are the paralog RING-type E3 ligases RNF43 and ZNFR3 (Hao et al., 2012; Koo et al., 2012). These are single-pass transmembrane E3 ligases with a C-terminal, intracellular RING domain that catalyzes the ubiquitylation of LRP6 and Fzd, targeting them for lysosomal degradation (Hao et al., 2012; Koo et al., 2012). The membrane levels of these E3 ligases are regulated by R-spondins and the LGR5 receptor. R-spondin ligands form a ternary complex with ZNFR3/RNF43 and LGR5, triggering auto-ubiquitylation and turnover of the E3 ligases and stabilizing the receptors (Koo et al., 2012). ZNFR3 and RNF43 loss-of-function mutations, or R-spondin activating mutations, lead to elevated Wnt signaling and are implicated in various cancer types (Koo et al., 2015). Recently, the DUB counteracting these E3 ligases was identified as USP46 (Ng et al., 2023; Spencer et al., 2023). In both human cells and Drosophila, USP46 was shown to deubiquitylate and stabilize the LRP6 and Arrow, promoting Wnt/Wg activation. Furthermore, it is proposed that USP46 regulates Wnt signalosome formation via the pruning of ubiquitin molecules from LRP6.

Two projects examining novel aspects of Wnt receptor regulation

Wnt receptor homeostasis and function are carefully regulated by both ubiquitination and endocytosis. Here, I explore two projects that examine novel modes and contexts in which Wnt receptors are regulated by these processes. Previously, we have demonstrated that APC can regulate Wnt receptor activation and proposed to be via APC association with the clathrin/endocytic machinery (Saito-Diaz et al., 2018). Chapter Three biochemically examines this model of Wnt receptor regulation. Additionally, we have previously shown that LRP6 is required for APC-deficient pathway activation (Saito-Diaz et al., 2018), and separately we have demonstrated that Wnt receptor stability is controlled by the deubiquitiylase USP46 (Ng et al., 2023). In Chapter Four, I test a model in which this DUB regulates APC-deficient pathway activation via its control over LRP6 homeostasis.

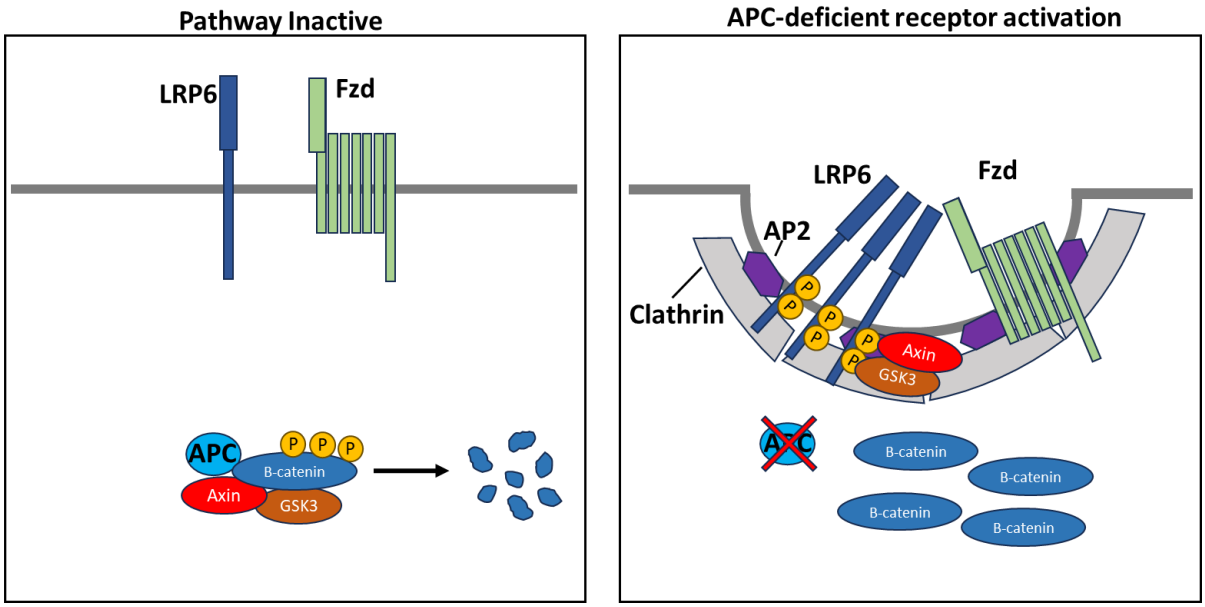


Figure 1-3: Hypothetical model of APC-deficient Wnt receptor activation. APC deletion triggers ligand-independent receptor activation and signalosome formation. Clathrin/AP2 are required for this activation. Our laboratory proposes a model in which APC association with AP2 blocks constitutive receptor clustering in the absence of Wnt ligands.

CHAPTER 2

Materials and Methods

Cell lines

The cell lines HEK293 (human embryonic kidney) and HEK 293STF (stably expressing the TOPFLASH luciferase reporter) were purchased from American Type Culture Collection (ATCC). The colorectal cancer cell line SW480 was also purchased from ATCC. The colorectal cancer cell line, DLD1, was a generous gift from the Anna Means laboratory (Vanderbilt University Medical Center). SW480 and DLD1 cells stably expressing the 7TFP luciferase reporter were generated using lentiviral transduction of the 7TFP plasmid acquired from the Roeland Nusse laboratory (Addgene). HEK293 and HEK293STF cells were maintained in DMEM supplemented with 8% FBS, and SW480 and DLD1 cells were maintained in RPMI supplemented with 10% FBS.

DNA plasmid transfections and RNAi knockdown

Plasmid transfections in HEK293 cells were performed using Calcium Phosphate transfection, whereas Lipofectamine 3000 (Invitrogen) was used for SW480 and DLD1 cancer cell transfections. For calcium phosphate transfections, cells were seeded at 60% confluency and incubated overnight. For transfection of a single well, 1-2.5 micrograms of plasmid DNA was mixed with 6.1 microliters of CaCl2 per microgram, along with sterile, DNAse-free water. This mix was added to a tube of 2X HBS (280mM NaCl, 10mM KCl, 1.5mM Na2HPO4-2H2O, 12mM Dextrose (D-glucose) and 50mM HEPES). Following mixing via bubbling air and vortexing, the transfection mix was added to cells. Lipofectamine transfections were performed

according to the manufacturer's protocol. SiRNA transfections were performed using Dharmafect 1 (HEK293) and Dharmafect 2 (SW480 and DLD1), according to manufacturer's protocol.

Table 2-1: siRNA sequences

siRNA	**Sense Sequence (5'-> 3')**	Source
NT	UAAGGCUAUGAAGAGAUACUU	Saito-Diaz et al., 2018
Axin #1	GCGUGGAGCCUCAGAAGUUUU	Saito-Diaz et al., 2018
Axin #2	CCGAGGAGAAGCUGGAGGAUU	Saito-Diaz et al., 2018
APC #1	CUGAAGAUGAUGAAAGUAAUU	Saito-Diaz et al., 2018
APC #2	UGAUAAUGAUGGAGAACUAUU	Saito-Diaz et al., 2018
USP46 #1	GCAGGAUGCUCAUGAAUUUUU	Ng et al., 2023
USP46 #2	UCCGAAACAUCGCCUCCAUUU	Ng et al., 2023
UAF1 #1	CAGCAGAGAUGUAUAGCAAUU	Ng et al., 2023
UAF1 #2	CCUAAGAAACCCUGACAUUUU	Ng et al., 2023

Cell Lysis and immunoblotting

Following respective treatments, cells were lysed in non-denaturing lysis buffer (NDLB) (50mM Tris-HCL (pH 7.4), 300mM NaCl, 5mM EDTA, 1% (w/v) Triton X-100, 1mM PMSF, and phosphatase inhibitor cocktail (Roche)). Following the pelleting of cell debris, supernatants were analyzed by SDS-PAGE and immunoblotting. Chemiluminescent signal was detected using C-DiGit blot scanner (LI-COR), and fluorescent signal was detected using an Odyssey M Imager (LI-COR).

Co-immunoprecipitation studies

Cell lysates harvested in NDLB were diluted to 1mg/mL and incubated with antibodies overnight at 4°C. Samples were incubated with Protein A/G magnetic beads (Pierce) or anti-

FLAG agarose beads (Sigma) for two hours. Following 5x washes, bound proteins were eluted from the beads with sample buffer (90°C for 10 minutes).

Lentiviral production and transduction

Stable 7TFP reporter cell lines were generated via lentiviral transduction. Lentiviral media was collected from HEK293FT cells transfected with the 7TFP lentiviral plasmid (Addgene), along with the PAX2 (packaging) and pMD2G (envelope) plasmids. SW480, and HCT116 cells were cultured in viral media with 8 mg/mL Polybrene for 24 hours. Antibiotic selection was performed with puromycin.

Detection of *in vivo* ubiquitination

HEK293T cells were co-transfected with HA-ubiquitin and FLAG-Arrow plasmids, along with either empty vector or V5-tagged Drosophila USP46, UAF1, and WDR20 plasmids (2 ug or each plasmid, or the equivalent amount of empty vector was used for each transfection). Transfected cells were treated with either 100 nM Bafilomycin A (Pierce) for 16 hours or 10 uM MG132 (Sigma) for 6 hours prior to lysis. Cell lysates were immunoprecipitated with HA antibody (12CA5) conjugated to Protein A/G (Pierce) agarose beads, and elutions were probed with anti-FLAG antibody (Proteintech) and anti-V5 antibodies (CST).

TOPFLASH Reporter Assay

TCF/LEF transcriptional activity was measured using TOPFLASH/7TFP reporter systems. Following experimental treatment, reporter cells were lysed in 1x Passive Lysis Buffer (Biotium) and incubated on a shaker for 15 minutes. Each lysate was assayed for luciferase

reporter activity using Stready-Glo Luciferase Assay (Promega) and for cell viability using Cell Titer Glo (Promega). Luciferase activity was normalized to cell viability. FLUOstar Luminometer was used to measure luminescence. Assays were performed in triplicate and repeated three times.

RNA isolation and qRT-PCR

SW480, DLD1, and HCT116 cells were harvested in Trizol reagent (Thermo) on ice and homogenized. A chloroform extraction was then performed, the aqueous phase precipitated with 70% ethanol, and total RNA was purified using RNeasy kits (Qiagen). Complementary DNAs were prepared using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCR was performed using TaqMan Fast Advanced Master Mix (Applied Biosystems) and analyzed using CFX96 Real-time PCR system (BioRad). The following validated TaqMan probes (Thermo) were used: GusB (Hs00939627_m1), Axin2 (Hs00610344_m1), DKK1 (Hs00183740_m1), and Myc (Hs01031255_m1).

Expression and Purification of recombinant protein

BL-21 DE3 competent cells were transformed with plasmids for his-LRP6-ICD or MBP-AP2µ2. Bacterial cultures were inoculated and incubated at 37°until the optical density was between OD600=0.6 and 0.8. Expression was induced with 1 mM IPTG overnight at 18°C. Cells were lysed in a buffer containing 50mM Tris-HCl, 150mM NaCl, and 2mM EDTA, supplemented with 1mM PMSF and Roche Protease inhibitor cocktail tablets. For the purification of his-LRP6-ICD, cells were lysed in the previous buffer sans EDTA. Cell lysates were incubated with either Nickel IMAC resin (Pierce; for his-tagged proteins) or Amylose resin (Pierce; for MBP-tagged proteins). Following washes with the lysis buffer, proteins were eluted

with either lysis buffer plus 200mM imidazole or lysis buffer plus 50mM maltose to purify his- and MBP-tagged proteins, respectively.

Affinity pulldown assays

Recombinant MBP-AP2 was immobilized on Amylose beads and incubated with lysates expressing various APCT constructs at 4°C for one hour on a TOMY shaker. MBP-AP2μ2 beads were pelleted and washed with buffer. Bound protein was eluted in the aforementioned lysis buffer with 50mM maltose, and the eluates precipitated with methanol. Precipitates were pelleted at 12,000 rpm for 15 minutes at room temperature and resuspended in sample buffer. For competition binding assays, affinity pulldowns were performed in the presence of increasing amounts of recombinant his-LRP6-ICD. For pulldown assays using immobilized APCT, V5-APCT was immunoprecipitated from HEK293 lysates by incubation with anti-V5 antibody and Protein A/G agarose beads. Beads were pelleted with centrifugation, washed with NDLB, and incubated with recombinant MBP-AP2. Following binding, beads were pelleted, washed, and eluted with sample buffer

Table 2-2: Antibodies

Antibodies	**Concentration**	**Source**
Rabbit V5	1:1000	CST
Mouse V5	1:1000	Genscript
Mouse MBP	1:1000	VAPR
Rabbit USP46	1:1000	Proteintech
Rabbit UAF1	1:1000	Proteintech
Mouse Myc	1:1000	VAPR
Mouse Tubulin	1:2500	Proteintech
Mouse β-catenin	1:1000	VAPR
Mouse HA (12CA5)	1:1000	VAPR
Rabbit DYKDDDDK(FLAG)	1:1000	Proteintech
Mouse DYKDDDDK(FLAG)	1:1000	VAPR

CHAPTER 3

Wnt receptor regulation by the tumor suppressor APC

Introduction

The canonical Wnt pathway is regulated by the cytoplasmic levels of the transcriptional co-activator β-catenin. In the pathway inactive state, β-catenin levels are kept low via the cytosolic "destruction complex." The destruction complex consists of the scaffold proteins APC and Axin and the kinases GSK3 and CK1. Destruction complex-mediated phosphorylation of β-catenin (the rate-limiting step in β-catenin turnover) targets it for degradation by the ubiquitin-proteosome system. Wnt ligands bind the co-receptors LRP6 and Fzd, forming an active ternary complex, which forms large multiprotein aggregates known as "signalosomes." Signalosome formation is mediated by the cytoplasmic protein Dishevelled and has been proposed to occur via clathrin-mediated endocytosis(Colozza & Koo, 2021). In a process that is not well characterized, components of the destruction complex are recruited to the Wnt signalosomes, thereby inhibiting destruction complex activity (via blocking its capacity to phosphorylate β-catenin). Consequently, cytoplasmic β-catenin levels rise and enter the nucleus to bind TCF/Lef transcriptional factors, thereby initiating a Wnt-specific transcriptional program.

The prevailing model for APC's predominant function in the Wnt pathway is in coordinating the downregulation of cytoplasmic levels of β-catenin through its role in the destruction complex. Our laboratory has recently identified a role for APC in the regulation of receptor function, as work by Saito-Diaz et al. (2018) demonstrated that APC functions to inhibit constitutive LRP6 signalosome formation and that APC loss leads to ligand-independent LRP6

activation, clustering, and endocytosis. This APC-deficient membrane signaling requires clathrin-mediated endocytosis. Furthermore, APC co-localizes and co-immunoprecipitates with Clathrin and AP2, the cargo component of the Clathrin complex.

Herein, I test a model in which APC regulation of Wnt receptors is mediated by its direct association with AP2. I demonstrate a direct and specific interaction between APC and AP2 and identify the regions of APC required for this interaction. Furthermore, I demonstrate that the cytoplasmic tail of the LRP6 receptor (LRP6-ICD) displaces APC from AP2 *in vitro*, suggesting a mutually exclusive interaction. Finally, I demonstrate that overexpression of APC suppresses Wnt ligand-dependent receptor activation and that this is dependent on the interaction between APC and AP2. These findings provide strong evidence to support a model in which APC regulates physiological Wnt receptor activation via inhibition of signalosome formation through a mechanism involving displacement of LRP6 within the Clathrin endocytic complex.

Results

The central fragment of APC interacts with the cargo recognition subunit of AP2, AP2μ2

Previously, we demonstrated that endogenous APC co-immunoprecipitated with both Clathrin and the cargo recognition subunit of the AP2 adaptor complex (AP2μ2) (Saito-Diaz et al., 2018). Furthermore, we showed that the central fragment of APC ($APC^{1265\text{-}2060}$), which is responsible for downregulating β-catenin in the destruction complex and for suppressing APC-deficient LRP6 activation, also co-immunoprecipitated with Clathrin/AP2 (Saito-Diaz et al., 2018).

To confirm that $APC^{1265\text{-}2060}$ associates with the cargo recognition subunit of AP2, I performed affinity pulldown assays of $APC^{1265\text{-}2060}$ with recombinant N-terminal MBP-tagged

AP2-μ2 expressed and purified from bacteria. I demonstrated that immobilized recombinant MBP-AP2-μ2 pulled down *in vitro* transcribed and translated $APC^{1265-2060}$ in a dose-dependent manner (Figure 3-1A). Similarly, immobilized MBP-AP2-μ2 pulled down from whole cell lysate $APC^{1265-2060}$ overexpressed in HEK-293, which also displayed dose-dependent binding (Figure 3-1B)**.** Finally, we performed an orthogonal pulldown assay in which we immobilized V5-epitope tagged $APC^{1265-2060}$ (from lysates of V5-$APC^{1265-2060}$ overexpressing HEK-293 cells) and demonstrated pulldown of recombinant MBP-AP2-μ2 with V5-$APC^{1265-2060}$ beads (Figure 3-1C). Together, these results provide evidence supporting an interaction between the central fragment of APC and the cargo recognition subunit of AP2.

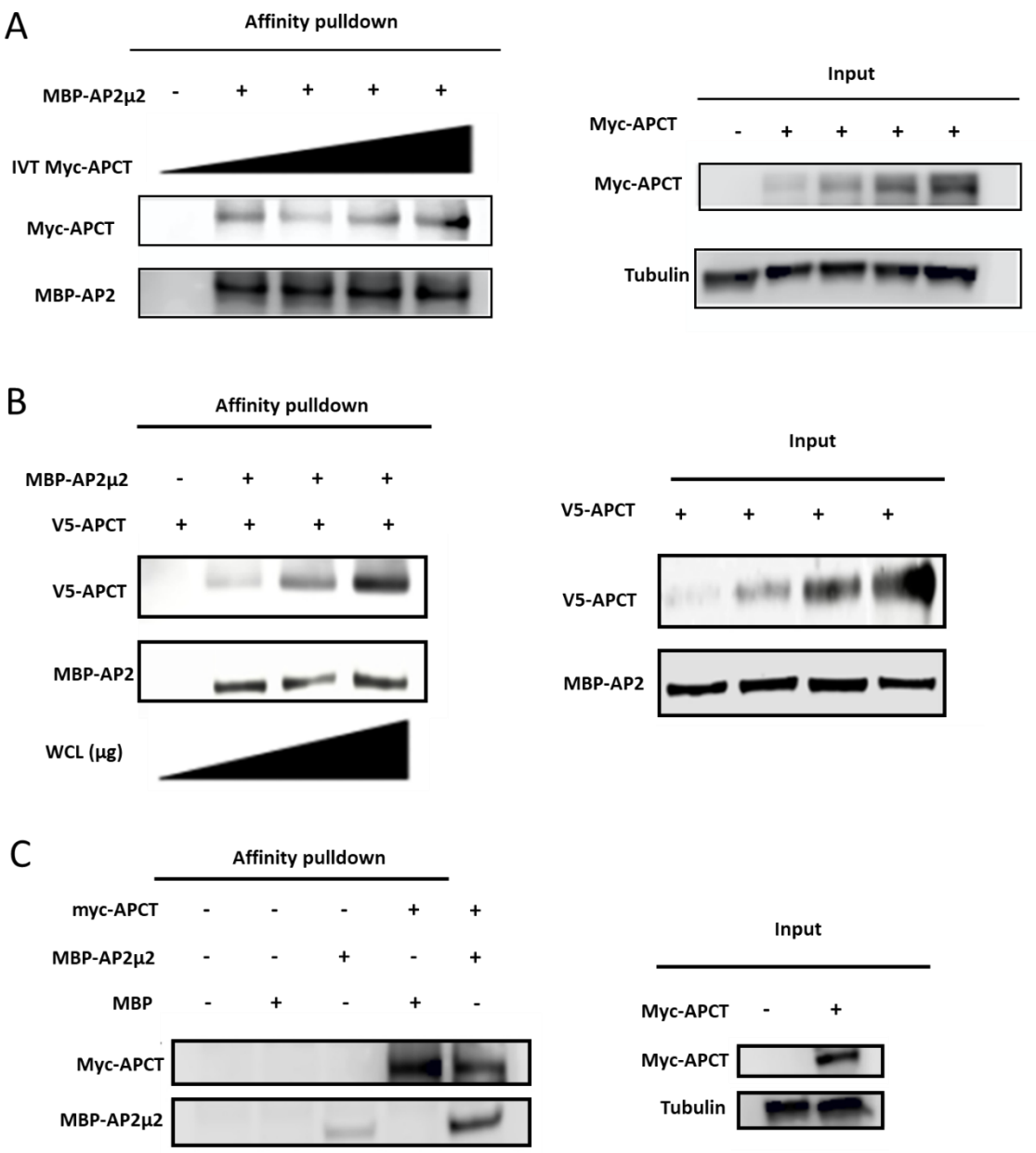
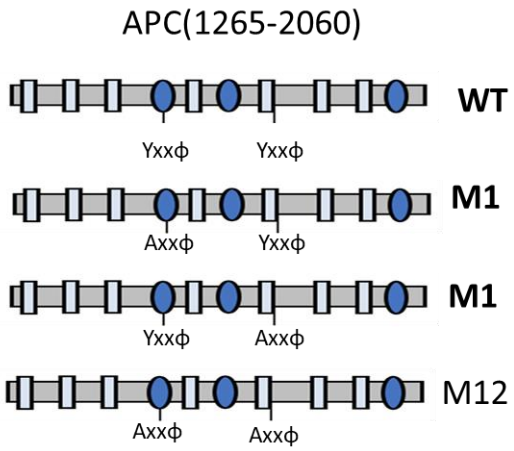


Figure 3-1: The central fragment of APC interacts with the cargo recognition subunit of AP2, AP2µ2. A-B) Affinity pulldown assays, wherein recombinant MBP-tagged AP2µ2 is incubated with crude lysates expressing epitope tagged APCT(APC1265-2060), and bound APCT is detected via immunoblotting. Pulldowns were performed from A) *in vitro* translation reactions expressing myc-tagged APCT, and B) HEK293-whole cell lysates expressing V5-tagged APCT. C) V5-APCT was immunoprecipitated from HEK293 WCL with V5-epitope conjugated beads. These V5-APCT bound beads were then incubated with recombinant MBP-AP2µ2, and the amount of AP2µ2 that pulled down detected via immunoblotting. Blots are representative of at least 3 independent experiments.

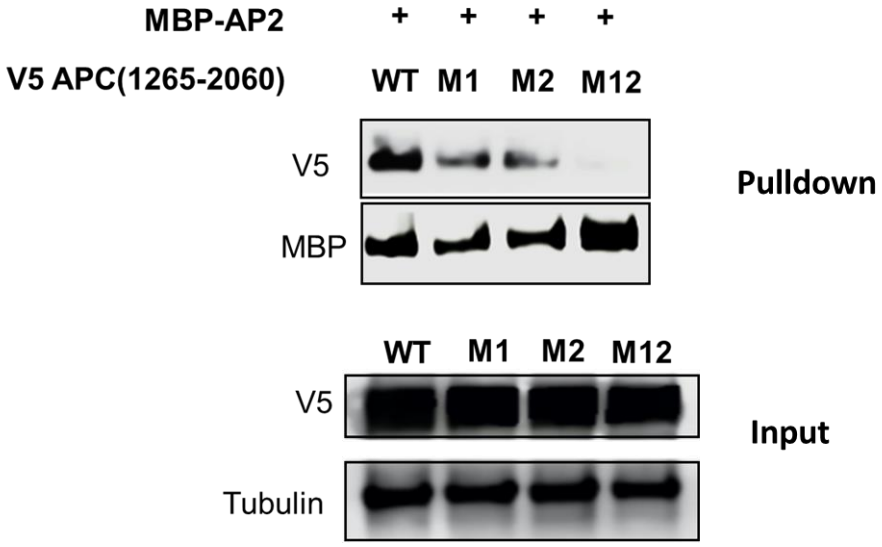
APC contains the Y-x-x-φ endocytic sorting motifs, which are required for its interaction with AP2

To determine the specificity of the APC- AP2-μ2 interaction, we performed mutagenesis of $APC^{1265\text{-}2060}$. The C-terminus of AP2-μ2 has been shown to recognize membrane cargo via its four amino acid tyrosine-based endocytic motifs (Bonifacino & Dell'Angelica, 1999; Rapoport et al., 1997). The canonical motif is Y-x-x-φ, where x is any amino acid, and φ is a bulky, hydrophobic residue (F, I, L, M, and W). AP2-μ2 can also recognize non-canonical motifs of the sequence Y-x-x-G-φ. Mutating the tyrosine residue in the sorting motif to alanine has been previously shown to abrogate the binding of cargo to the AP2-μ2 subunit (Bonifacino & Dell'Angelica, 1999). The $APC^{1265\text{-}2060}$ fragment encodes two canonical endocytic motifs. We thus sought to determine if the AP2-μ2 and $APC^{1265\text{-}2060}$ interaction is dependent on these endocytic motifs on $APC^{1265\text{-}2060}$ by introducing tyrosine to alanine mutations and performing AP2-μ2 affinity pulldown assays with wildtype, single mutant, and double mutant $APC^{1265\text{-}2060}$. We found that mutating individual Y-x-x-φ motifs in $APC^{1265\text{-}2060}$ attenuated binding with AP2-μ2, and mutations of both motifs almost completely abrogated binding (Figure 3-2A). The requirement for the canonical sorting motifs for this interaction provides further evidence to support a direct and specific interaction between these two proteins. We also found that the interaction between AP2-μ2 and the central fragment of APC is conserved. The corresponding central portion of Drosophila APC ($dAPC^{830\text{-}1504}$) pulls down with AP2-μ2 in affinity pulldown assays (Figure 3-2B). $DAPC^{830\text{-}1504}$ has a single endocytic sorting motif, and mutating this motif attenuates pulldown. This finding demonstrates that interaction is conserved and is dependent on these endocytic motifs.

A


APC(1265-2060)
WT
Yxxϕ
Yxxϕ
M1
Axxϕ
Yxxϕ
M1
Yxxϕ
Axxϕ
M12
Axxϕ
Axxϕ

B


MBP-AP2 + + + +
V5 APC(1265-2060) WT M1 M2 M12
V5
MBP
Pulldown
WT M1 M2 M12
V5
Tubulin
Input

C

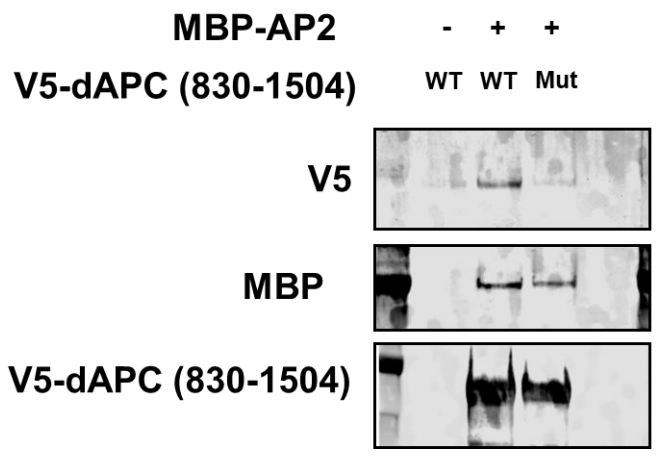

MBP-AP2 - + +
V5-dAPC (830-1504) WT WT Mut
V5
MBP
V5-dAPC (830-1504)

Figure 3-2: APC contains the Y-x-x-φ endocytic sorting motifs, which are required for its interaction with AP2. A) Schematic of APC1265-2060 WT, and sorting motif mutants. M1 and M2 are single mutants, and M12 is a double mutant. B) Affinity pulldown assays, wherein recombinant MBP-tagged AP2-μ2 is incubated with crude lysates expressing V5-APC1265-2060 WT and sorting motif mutants, and bound APCT is detected via immunoblotting.

Truncation mutants reveal regions of APC$^{1265\text{-}2060}$ required for AP2 interaction

To determine the minimal fragments of APC$^{1265\text{-}2060}$ required for mediating the interaction with AP2-μ2, I generated multiple APC$^{1265\text{-}2060}$ truncation mutants, overexpressed them in HEK293 cells, and performed affinity pulldown assays from their lysates. Affinity pulldown assays with these APC$^{1265\text{-}2060}$ truncation mutants indicate that different fragments of APC$^{1265\text{-}2060}$ possess different capacities to bind AP2-μ2 (Figure 3-3). Figure 3-3A shows the schematic of the truncations. Truncations A and B represent the N-terminal third of the APC$^{1265\text{-}2060}$, with and without the endocytic sorting motif, respectively. Truncation A retains the capacity to pulldown with AP2-μ2 at a level comparable to the WT APC$^{1265\text{-}2060}$, and the deletion of the sorting motif attenuates (but not completely blocks) that binding (Figure 3-3B). Truncation C represents the central third of the APC$^{1265\text{-}2060}$, and Truncations D and E represent the central third of APC$^{1265\text{-}2060}$, but the endocytic sorting motifs have either been mutated (D) or deleted (E). The central region of APC$^{1265\text{-}2060}$ retains some capacity to pulldown with AP2-μ2, and as expected, deletion or mutation of the endocytic sorting motifs abrogate this interaction. Truncations F and G represent the C-terminal third of APC$^{1265\text{-}2060}$, and F contains a sorting motif, while this motif has been deleted in G. Interestingly, no binding with MBP-AP2 was detected with the C-terminal fragments of APC$^{1265\text{-}2060}$ (F and G), even though they were expressed at comparable levels as the N-terminal fragments (Truncations A and B). This would suggest the N-terminus of APC$^{1265\text{-}2060}$ mediates the interaction with AP2-μ2, and the C-terminus is dispensable for this interaction. Furthermore, the N-terminal region of APC$^{1265\text{-}2060}$ retains some capacity to associate with AP2-μ2 after deletion of the endocytic sorting motif. This is unexpected and could suggest that the region of APC$^{1265\text{-}2060}$ has an additional interaction surface, and that surface could potentially have been uncovered upon deletion of the central and C-terminal domains of APC$^{1265\text{-}2060}$.

Additional studies are required to further examine the binding regions required for this interaction, as well as determine the quantitative difference between $APC^{1265\text{-}2060}$ fragment and domain binding capacity.

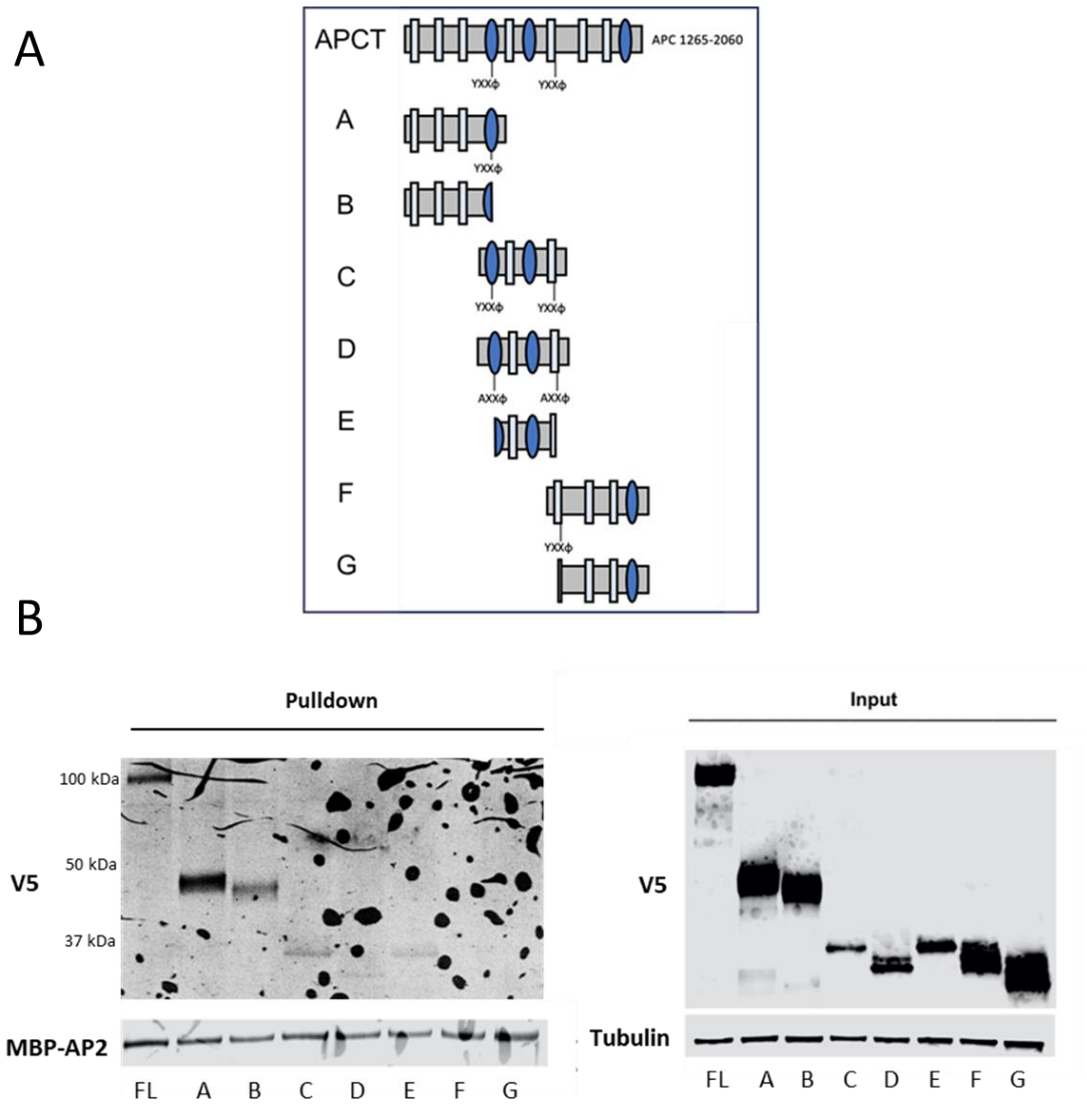


Figure 3-3: Truncation mutants reveal regions of APC1265-2060 required for AP2 interaction. A) Series of APC1265-2060 truncation mutants generated for this study. Truncations A, C and F retain their respective sorting motifs. Truncations B, E, and G have their motifs deleted, and Truncation E contains tyrosine to alanine mutations within the sorting motifs. B) Affinity pulldown assays with MBP-AP2 were performed as previously described, with the indicated APC1265-2060 truncations. Binding was assessed via immunoblotting. Representative of n=3 independent experiments.

Competitive binding between the intracellular domain of LRP6 and APC for AP2-μ2

Clathrin-mediated endocytosis (CME) has been implicated in the regulation of Wnt receptor activation (Colozza & Koo, 2021; I. Kim et al., 2013; Saito-Diaz et al., 2018). These reports suggest a model in which, upon ligand binding, LRP6 association with clathrin-AP2 is required for receptor activation and signalosome formation. We previously proposed a model for Wnt activation in APC mutant cells, in which membrane APC association with AP2 prevents constitutive receptor activation in the absence of Wnt ligand (Saito-Diaz et al., 2018). How, then, do we reconcile these models? I hypothesize a simple competition mechanism in which binding between LRP6 and APC to AP2-μ2 are mutually exclusive that incorporates both of these two models: 1) In the absence of Wnt ligand activation, APC physically block clathrin-AP2 interactions with LRP6, thereby prevent Wnt receptor activation. 2) Wnt ligands trigger a switch in AP2 association from APC to LRP6, allowing for the formation of Wnt signalosomes and subsequent pathway activation. To test for mutually exclusive binding between $APC^{1265\text{-}2060}$ and LRP6 for AP2-μ2 binding, we pulled down $APC^{1265\text{-}2060}$ from crude lysate with AP2-μ2 in the presence of increasing amounts of recombinant intracellular domain of LRP6 (LRP6-ICD). In a dose-dependent manner, LRP6-ICD reduced binding between AP2-μ2 and $APC^{1265\text{-}2060}$ (Figure 3-4). This is the first evidence supporting competitive binding between LRP6 and APC for AP2-μ2. Additional studies will be required to test the relevance of this competitive model in cells.

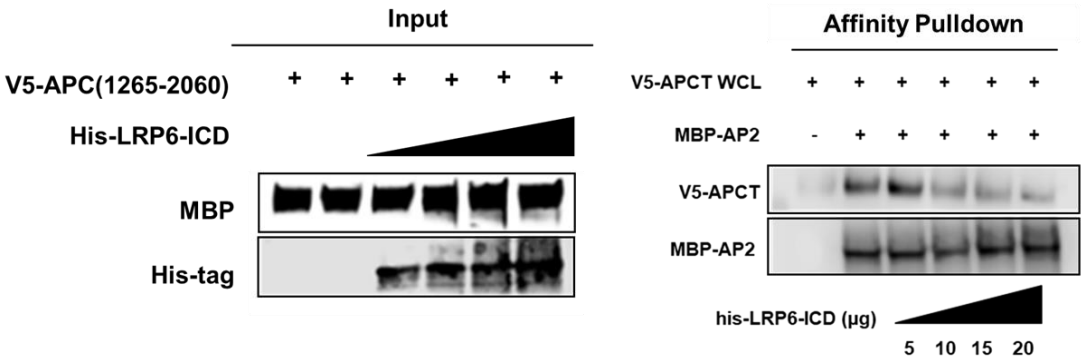


Figure 3-4: Competitive binding between the intracellular domain of LRP6 and APC for AP2-µ2. Affinity pulldown assays between V5-APCT and MBP-AP2-µ2 performed as previously described, but here with increasing amounts of recombinant histidine-tagged LRP6-intracellular domain (his-LRP6-ICD). V5-APCT(APC1265-2060) pulled down by MBP-AP2 was detected by immunoblot. Representative immunoblots of n=3 independent experiments.

APC interaction with AP2 is required for the suppression of Wnt ligand-dependent receptor activation

LRP6-AP2 interaction has been proposed to be required for Wnt ligand-dependent receptor activation and signalosome formation. My competitive binding experiments between LRP6 and APC for AP2-μ2 binding raise the possibility that sufficiently elevated levels of APC could block physiological Wnt ligand-dependent activation in cells. To test this possibility, I overexpressed $APC^{1265\text{-}2060}$ in HEK293 cells, stimulated with Wnt3a, and monitored phosphorylation of LRP6 at Serine 1490 (an indicator of Wnt receptor activation) (Bilic et al., 2007; Niehrs & Shen, 2010; X. Zeng et al., 2005). Control cells treated with Wnt3a showed an increase in LRP6 phosphorylation at S1490, and overexpression of $APC^{1265\text{-}2060}$ suppressed LRP6 phosphorylation (Figure 3-5). This result shows that $APC^{1265\text{-}2060}$ overexpression can suppress Wnt ligand-dependent receptor activation.

I found that the putative canonical cargo recognition motifs in $APC^{1265\text{-}2060}$ are required for its interaction with AP2-μ2. To test if this interaction is required for $APC^{1265\text{-}2060}$ suppression of Wnt3a ligand-dependent LRP6 phosphorylation, I overexpressed the $APC^{1265\text{-}2060}$ sorting motif double-mutant and found that LRP6 phosphorylation was similar to the level of controls. To my knowledge, this is the first evidence indicating that the central region of APC can regulate ligand-dependent Wnt receptor phosphorylation, and this regulation is dependent on its interaction with AP2-μ2**.** Additional studies are required to validate this finding and to examine additional indicators of receptor activation.

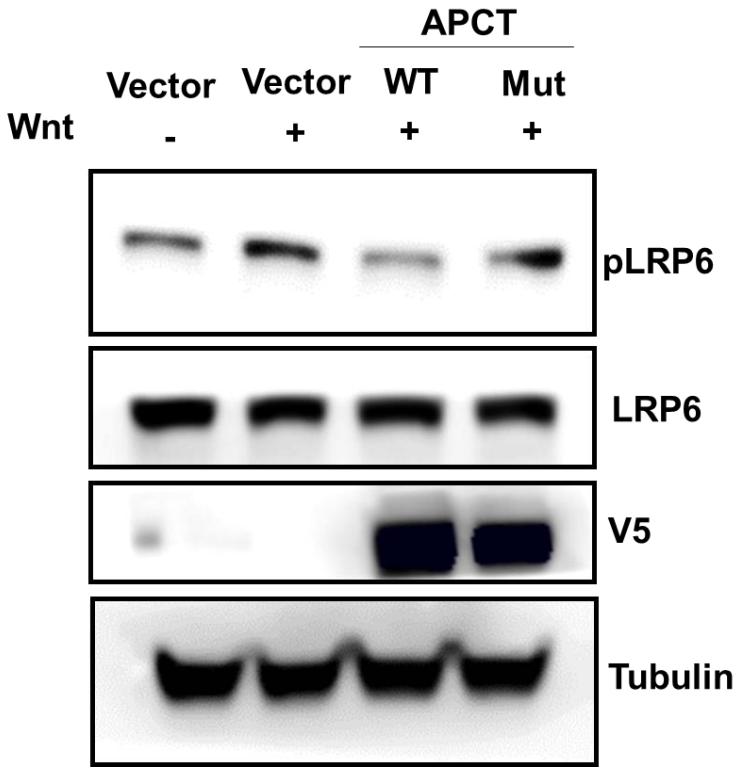


Figure 3-5: APC interaction with AP2 is required for the suppression of Wnt ligand-dependent receptor activation. HEK293 cells were transfected with either vector or V5-APC1265-2060. Forty-eight hours after transfection, cells were treated with either control or 50ng/mL of Wnt3a for 2 hours. Phosphorylated LRP6 was detected using an antibody for 52hosphor-Ser1490, an indicator of Wnt receptor activation. Representative of at least n=3 independent experiments.

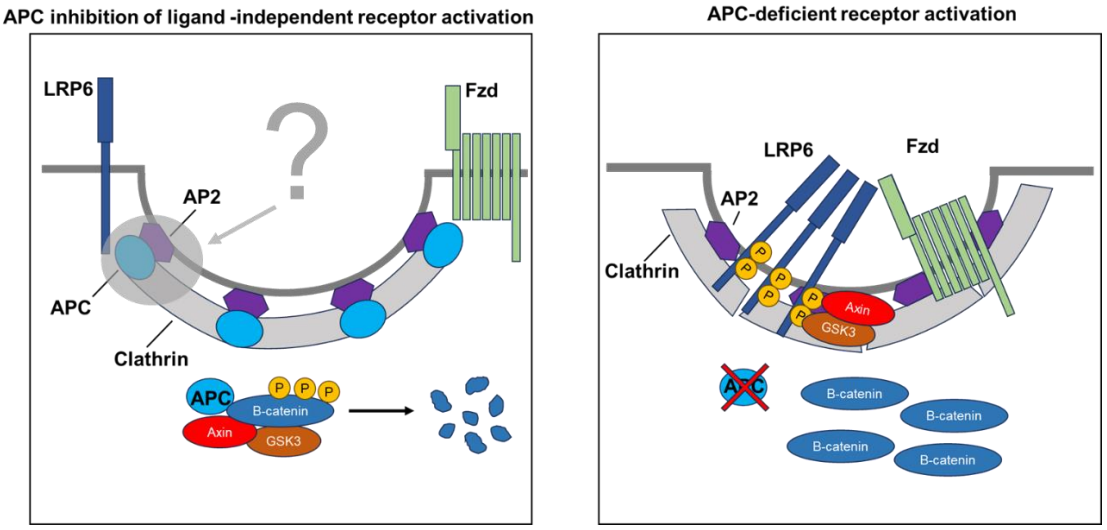


Figure 3-6: Proposed model of APC regulation of Wnt receptor activation. In the pathway inactive state, membrane AP2 blocks constitutive signalosome formation via association with its Clathrin/AP2. APC-depletion leads to APC-deficient receptor clustering and activation. Here, we are biochemically examining the APC interaction with AP2.

Discussion

The most well-characterized role for APC in regulating the Wnt pathway is as a component of the destruction complex, where APC coordinates the degradation of β-catenin. Our laboratory has recently identified a role for APC in the regulation of receptor activation (Saito-Diaz et al., 2018). We proposed a model in which membrane APC suppresses constitutive receptor activation and signalosome formation in the absence of Wnt ligands. Herein, I propose that this regulation is via APC's association with the Clathrin endocytic machinery, which is required for signalosome formation, involving a competitive mechanism with LRP6.

I have demonstrated an interaction between the cargo recognition subunit of the Clathrin/AP2 adaptor protein (AP2-μ2) and the central region of APC ($APC^{1265\text{-}2060}$) using affinity pulldown assays. These assays were performed using recombinant protein and cellular lysates and *in vitro* translated proteins generated from wheat germ extracts. Thus, there is a possibility that the observed APC-AP2-μ2 is indirect. AP2-μ2 recognizes specific peptide motifs on potential cargo. The central fragment of APC contains two of these motifs, and mutations of these residues attenuate pulldown. The observation of a dose-dependent pulldown from lysates, coupled with the finding that individual point mutations attenuate and abrogate this pulldown, is strong evidence supporting a direct interaction. Competition with the known AP2-μ2 interactor, LRP6-ICD, also supports this model. Given these results, however, a conclusive demonstration showing the direct interaction between APC and AP2-μ2 will require binding studies using purified recombinant APC and AP2-μ2 in future studies.

I have initiated studies to identify the structural elements of APC required for its interaction with AP2-μ2 outside its canonical binding motif. Initial findings suggest the N-terminal fragment of APCT is also required for this interaction. Furthermore, this fragment

exhibits a higher affinity for AP2-μ2 than its central or C-terminal regions. This raises the possibility of auto-inhibition of N-terminal binding by the C-terminus, and that the deletion of the C terminus exposes an additional interaction surface (or conformation). Additional truncation studies are required to test this possibility. Additionally, it is unclear why mutation of the C-terminal sorting motif exhibits reduced binding, yet deletion of the C-terminus does not abrogate binding. Perhaps the putative N-terminal interaction surface can compensate for the loss of the C-terminal Y-x-x-φ. Additional studies are required to illuminate the potential involvement of an as-yet-unidentified N-terminal element in regulating APC-AP2-μ2 interaction.

In my studies, I sought to reconcile the prevailing models of Wnt receptor activation with our proposed model of the APC-mediated mechanism of Wnt receptor inhibition. I demonstrated that LRP6-ICD displaces APC from AP2 *in vitro*, suggesting a mutually exclusive interaction. This result supports a model of simple competition upon ligand stimulation, in which Wnt3a-activated LRP6 displaces APC from the AP2 adaptor complex within the Clathrin endocytic complex, allowing for Wnt signalosome formation. I also demonstrate that $APC^{1265\text{-}2060}$ overexpression suppresses Wnt ligand-dependent receptor phosphorylation at S1490, and this is dependent on the capacity of APC to interact with AP2-μ2. My studies demonstrating APC suppression of Wnt3a ligand-stimulated receptor activation in an AP2-dependent manner is novel and would add to our current model of Wnt pathway activation. Additional studies are required to test and validate this finding.

One limitation of my study is that it does not address the function of the full-length APC protein. Several questions remain: 1) How does the large, intrinsically disordered APC protein interact with the cargo recognition subunit of AP2? 2) Is there autoinhibition between domains in AP2 binding, and how is this regulated? 3) We showed that APC overexpression in cells

moderately suppresses the phosphorylation of LRP6. How do the endogenous proteins respond to Wnt stimulation in cells? 4) Can we observe Wnt3a dependent competition between APC and LRP6 for AP2 binding in cells?

Altogether, the findings here further clarify and characterize a novel mechanism of Wnt receptor regulation by the tumor suppressor APC and define a path for future studies. APC is mutated in 80% of non-hereditary colorectal cancer and has been called the "gatekeeper" of colon cancer (Fearnhead et al., 2001; Kwong & Dove, 2009). Elucidating its mechanism of action will thus have important implications for our understanding of its role in human disease.

CHAPTER 4

The deubiquitylase USP46 is required for APC-deficient Wnt signaling

Introduction

The canonical Wnt pathway is regulated by the cytoplasmic levels of the transcriptional co-activator β-catenin. In the pathway inactive state, β-catenin levels are kept low via the cytosolic "destruction complex". The destruction complex consists of the scaffold proteins APC and Axin, and the kinases GSK3 and CK1. The destruction complex mediates the phosphorylation of β-catenin, targeting it for degradation by the ubiquitin-proteasome system. Wnt ligands bind the co-receptors LRP6 and Fzd, forming an active ternary complex. These activated Wnt receptors, in turn, form large multiprotein aggregates known as "signalosomes." Signalosome formation is mediated by the polymerization of the cytoplasmic proteins Dishevelled and Axin. The prevailing model proposes that the recruitment of Axin and associated GSK3 and CK1 to the intracellular domains of activated Wnt receptors ultimately inhibit the phosphorylation of β-catenin by the destruction complex, resulting in stabilized β-catenin.

Wnt pathway activity is regulated by ubiquitylation at multiple levels, including Wnt receptor homeostasis. The transmembrane E3 ligases RNF43 and ZNFR3 promote receptor turnover, and their loss leads to elevated Wnt receptor levels and hyperactive Wnt signaling. Our laboratory has recently identified USP46 as the de-ubiquitinase (DUB) that counteracts RNF43 and ZNFR3 in the Wnt signaling pathway (Ng et al., 2023; Spencer et al., 2023).

The USP46 complex is composed of the catalytic USP46, as well as the WD40 repeat-containing proteins, WDR20 and UAF1. These accessory proteins regulate USP46 localization and are required for USP46 catalytic activity (Cohn et al., 2009; Olazabal-Herrero et al., 2019). Ng et al. (2023), and Spencer et al. (2023) demonstrated that USP46 de-ubiquitylates LRP6, increasing its surface levels to promote Wnt pathway activation. In addition, Ng et al., (2023) propose a model in which USP46 de-ubiquitination of LRP6 is required for signalosome formation via a "pruning" step to prevent steric hindrance by the polyubiquitin chains of LRP6 in receptor signalosome formation.

Previously, our laboratory reported the unexpected finding that LRP6 receptors are required for Wnt pathway activation in APC-deficient cells. Saito-Diaz et al., (2018) showed that LRP6 knockdown attenuates Wnt pathway activation and β-catenin stabilization in APC knockdown and mutant cells. Because LRP6 is required to activate Wnt signaling in APC-deficient cells, and USP46 regulates LRP6 levels, I tested whether USP46 regulates pathway activation in APC-depleted cells. I demonstrate that USP46 is required for Wnt activation in APC mutant and knockout cells. Shen Chen (Georgetown University) and Hassina Benchabane (Dartmouth University) demonstrated that USP46 is required for signaling in APC^{min} organoids and the APC mutant *Drosophila* midgut, respectively (personal communications). These findings are consistent with my hypothesis that USP46 regulates Wnt signaling in APC mutant cells by altering LRP6 levels. However, I also found that USP46 is required for Wnt pathway activation upon Axin silencing, which suggests that USP46 acts downstream of the destruction complex and downstream of LRP6. Furthermore, additional epistasis experiments would be required to test a possible role for USP46 in the nuclear regulation of Wnt activation. In summary, my

findings suggest an additional role for USP46 in Wnt activation independent of receptor regulation.

Results

Drosophila USP46 complex co-immunoprecipitates with and deubiquitylates Arrow in cells

In a pair of studies with the Yashi Ahmed laboratory at Dartmouth University, our group demonstrated that the deubiquitylase USP46 stabilizes the LRP6 receptor and is required for Wnt activation (Ng et al., 2023; Spencer et al., 2023). Victoria Ng demonstrated that the mammalian USP46 complex can co-immunoprecipitate with LRP6 in HEK293 cells. Furthermore, it was shown that overexpressed USP46 deubiquitylated LRP6 in cells and *in vitro*. To test if the association between USP46 and LRP6 is evolutionarily conserved, I first co-expressed the *Drosophila* homolog of LRP6 (Arrow) and the *Drosophila* USP46 complex (dTri46) in HEK293 cells and demonstrated that the dTri46 complex co-immunoprecipitates with Arrow (Figure 4-1A). I next tested the capacity dTri46 to deubiquitylate Arrow in cells. Thus, I co-expressed Arrow, dTri46, and HA-ubiquitin in HEK293 cells and performed in vivo ubiquitylation assays. I found that dTri46 overexpression stabilizes Arrow and reduces the ubiquitylation of Arrow in HEK293 cells (Figure 4-1B). Ubiquitylated surface LRP6 is degraded by the lysosome, and this degradation is thus sensitive to lysosomal inhibition (Ng et al., 2023). Intracellular, misfolded LRP6 is degraded by the proteasome via the endoplasmic reticulum-assisted pathway (ERAD) and is thus sensitive to proteasomal inhibition (Perrody et al., 2016). Ubiquitylated Arrow is more enriched in cells incubated with either Bafilomycin A (BafA), a lysosome inhibitor, or MG132, a proteasome inhibitor. However, the reduction in Arrow ubiquitylation upon dTri46

overexpression is greater in BafA-treated cells, indicating drosophila USP46 deubiquitylation stabilizes surface LRP6, which would otherwise be degraded by the lysosome. Together, these findings indicate that the USP46 association and deubiquitylation of Arrow/USP46 is conserved across phyla. This data was included in the Spencer et al., (2023) *Nature Communications* publication, which demonstrated the role of the *Drosophila* USP46 complex in regulating Wg signaling by stabilizing Arrow during development.

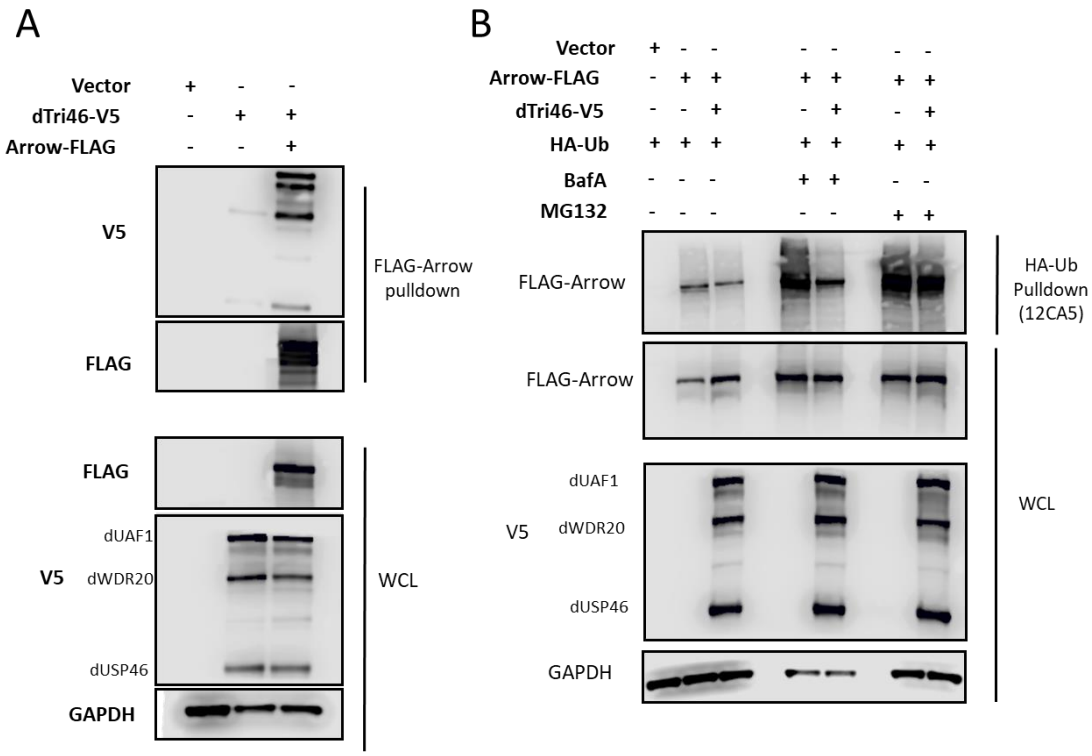


Figure 4-1: Drosophila USP46 (dTri46) complex co-immunoprecipitates and de-ubiquitylates Arrow in HEK293 cells. A) Co-immunoprecipitation of V5-dUSP46 and FLAG-Arrow. HEK293 cells were co-transfected with V5-tagged *Drosophila* USP46 complex and FLAG-Arrow, and FLAG-Arrow was immunoprecipitated (IP) with anti-FLAG conjugated beads, and V5-tagged dUSP46 complex were detected via immunoblot. B) *In vivo* detection of Arrow deubiquitylation by dTri46. HEK293 cells were transfected with V5-dUSP46, FLAG-Arrow, and HA-ubiquitin. HA-Ubiquitin was pulled down and FLAG-Arrow was detected by immunoblot. Bafilomycin (BafA) is a lysosome inhibitor, and MG132 is a proteosome inhibitor. WCL = Whole cell lysates. Blots are representative of at least 3 independent experiments.

USP46 complex is required for reporter activation upon both Wnt3a stimulation and APC silencing

Our group has previously shown that the Wnt co-receptor LRP6 is required for signaling in APC mutant and knockdown cells and that Wnt receptors are activated upon APC loss (Saito-Diaz et al., 2018). Additionally, we have shown that USP46 stabilizes surface LRP6 (Ng et al., 2023). Here, I aim to test if the USP46 deubiquitylase is required for signaling upon APC loss. First, I confirmed that USP46 knockdown blocks Wnt ligand-dependent activation. Using HEK293 cells stably expressing the TOPFLASH luciferase reporter system, I found that siRNAs against USP46 attenuated reporter activation (Figure 4-2A). This result is consistent with our previously reported findings (Ng et al., 2023).

Significantly, I found that USP46 was also required for Wnt activation upon APC loss, as USP46 knockdown blocked APC siRNA-induced Wnt reporter activation (Figure 4-2B). This result raises the possibility that USP46 stabilization of LRP6 is required for APC-deficient Wnt activation. Alternatively, USP46 may have a role in the Wnt pathway that is independent of receptor activation. Immunoblots for LRP6 and β-catenin levels will be helpful in deciphering the role of USP46 in Wnt signaling in APC-deficient cells.

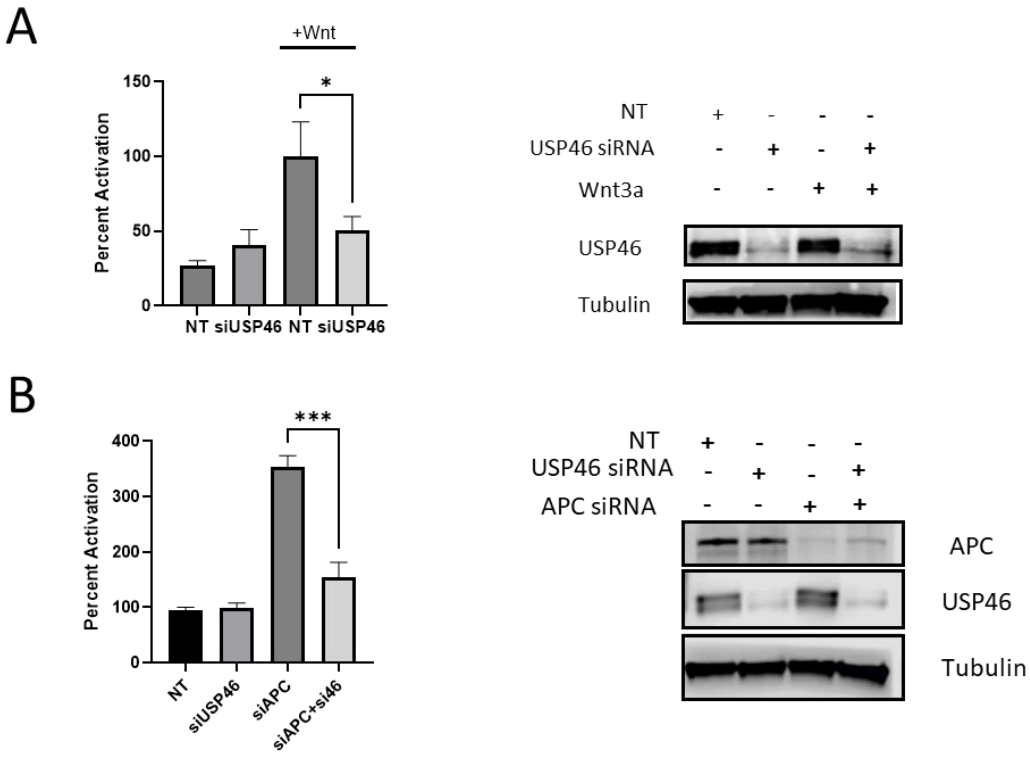


Figure 4-2: USP46 complex is required for reporter activation upon both Wnt3a activation and APC silencing. A) HEK293-STF Wnt reporter cells were transfected with non-targeting (NT) or USP46 siRNA (72hours), and treated with 25ng/mL of Wnt3a for 16 hours. TOPFLASH luciferase assays monitored Wnt reporter activation. B) HEK293-STF Wnt reporter cells were transfected with nontargeting (NT) or USP46 siRNA, either alone or in combination with APC siRNA for 72 hours. TOPFLASH assays monitored Wnt reporter activation. Representative data from n=3 independent biological experiments performed in triplicate. Mean +/- SD of percent activation, normalized to NT control + treatment. Significance analyzed by two tailed student's t-test. P-values compare NT or siUSP46 incubated in the presence of experimental treatment.

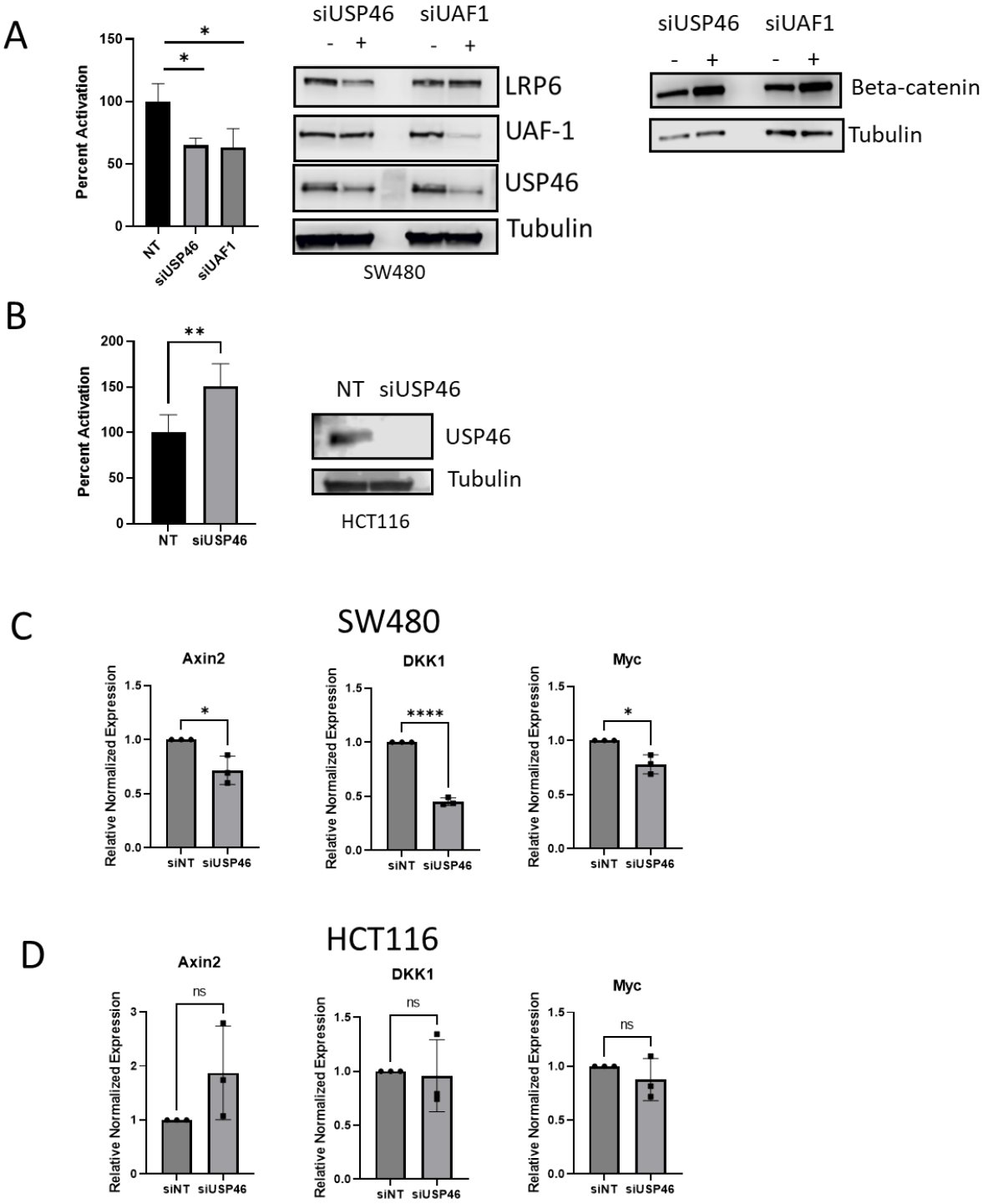
USP46 is required for Wnt signaling in APC but not β-catenin mutant colorectal cancer cells

To test the USP46 requirement for Wnt pathway activation in colorectal cancer cells, we knocked down USP46 and UAF1 in SW480 cells, stably expressing the 7TFP luciferase reporter. SW480 is a colorectal cancer cell line expressing a truncated APC, and β-catenin levels and TCF-Lef reporter activity are thus elevated in these cells. Not surprisingly, given my results with HEK293 cells in which APC is knocked down, I found that USP46 and UAF1 knockdown in these cells attenuated Wnt reporter activity, indicating a requirement for the USP46 complex for Wnt activation in this APC-truncated cell line (Figure 4-3A). Surprisingly, while USP46 knockdown attenuated Wnt reporter activity, β-catenin levels were not significantly reduced, however, in these cells. In fact, I found that they were elevated upon USP46 knockdown (Figure 4-3A). The observed increase in β-catenin levels and the reduction in TOPFLASH activity upon USP46 knockdown in the SW480 cell line suggest possible impairment of β-catenin nuclear localization.

To test whether this finding is specific to APC mutant cells, I next knocked down USP46 in HCT-116 WTKO cells and monitored Wnt reporter activation. HCT116 cells express a non-degradable mutant copy of β-catenin, as well as a wild-type copy. In the HCT-116 WTKO cell line, the wild-type β-catenin copy is knocked out, and only the mutant is expressed (Saito-Diaz et al., 2018). USP46 knockdown in these cell cells did not significantly reduce Wnt reporter activation (Figure 4-3B). Consistent with my findings using the firefly luciferase-based Wnt reporter assay, USP46 knockdown attenuated Wnt target gene expression in APC mutant SW480 cells but not HCT116 WTKO cells (Figure 4-3C and D). Wnt target gene expression was

assessed by qRT-PCR of Axin2, DKK1, and Myc, all of which have been previously well-characterized.

These findings provide evidence consistent with USP46 having a role in Wnt regulation that is specific to APC loss-of-function. β-catenin localization studies, as well as immunoblotting for LRP6 and β-catenin upon USP46 knockdown, will be useful in further interrogating USP46's role in Wnt signaling in APC mutant colon cancer cells.


A
Percent Activation
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NT
siUSP46
siUAF1
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siUSP46 siUAF1
- + - +
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USP46
Tubulin
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Beta-catenin
Tubulin
B
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200
150
100
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USP46
Tubulin
HCT116
C
SW480
Axin2
DKK1
Myc
Relative Normalized Expression
siNT siUSP46
*
****
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D
HCT116
Axin2
DKK1
Myc
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ns
Relative Normalized Expression
siNT siUSP46

Figure 4-3: USP46 required for Wnt target gene expression, and Wnt reporter activity in APC mutant colorectal cancer cells, but not β-catenin mutant cells. A) 7TFP reporter activation, LRP6 and β-catenin levels in SW480-7TFP reporter cells. Cells were treated with siRNAs targeting UAF-1 and USP46, and reporter activity was measured by TOPFLASH. B) HCT116-WTKO cells were transfected with siRNAs targeting USP46, and reporter activity measured by TOPFLASH. Knockdown for A and B were assessed by immunoblotting. C) RT-qPCR in SW480 and HCT116-WTKO cells, monitoring target gene expression of Axin2, DKK1 and Myc upon USP46 siRNA knockdown. TOPFLASH, representative date from n=3 independent experiments performed in triplicate. RT-qPCR, mean and standard error of the mean from n=3 independent experiments performed in triplicate. Mean +/- SD of percent activation for TOPFLASH, normalized to NT control + treatment. Significance analyzed by two tailed student's t-test. P-values compare NT or siUSP46 incubated in the presence of experimental treatment.

Epistasis experiments indicate a potential role for USP46 downstream the destruction complex

To further test the specificity of my findings, I asked if USP46 is also required for Wnt activation upon silencing of Axin in HEK293-STF cells. Axin knockdown significantly activates the Wnt reporter, and surprisingly, USP46 knockdown attenuated this activation (Figure 4-4A). This suggests that USP46 is required for signaling downstream of Axin and the destruction complex.

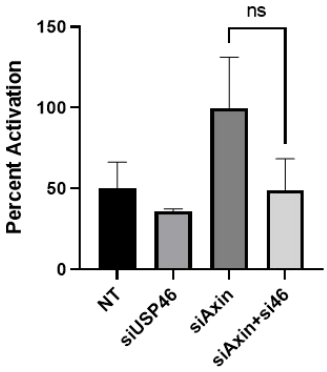
In the Wnt pathway inactive state, GSK3 (a component of the destruction complex) negatively regulates the pathway by phosphorylating β-catenin and targeting it for proteasomal degradation. Thus, GSK3 inhibitors, such as CHIR99021 acts as Wnt pathway activators by inhibiting the destruction complex. To test whether USP46 is required for Wnt pathway activity upon destruction complex inhibition, I knocked down USP46 in the presence of CHIR99021 or vehicle control (Figure 4-4B). I found that USP46 knockdown did not significantly reduce Wnt reporter activation upon CHIR99021-mediated GSK3 inhibition. Similarly, USP46 was not required for GSK3 inhibition-induced stabilization of β-catenin.

Conversely, Wnt pathway activation upon overexpression of non-degradable β-catenin did show a requirement for USP46 activation. GSK3 phosphorylation of the N-terminus of β-catenin targets it for ubiquitination and proteasomal degradation. Serine-to-alanine mutations of GSK3 phospho-sites stabilizes β-catenin. Overexpression of non-degradable β-catenin is sensitive to USP46 expression, as USP46 knockdown attenuates its Wnt reporter activity (Figure 4-4C). This result would indicate a role for USP46 downstream β-catenin stabilization, contrary

to the findings from my GSK3 inhibition studies. This difference could likely be due to the multiple cellular roles of GSK3, independent of Wnt pathway activation.

USP46 is localized to both the cytosol and the nucleus. Previous studies have demonstrated a role for nuclear USP46 in chromatin remodeling (Joo et al., 2011). Thus, I test whether USP46 has a role in regulating β-catenin-independent transcription. The transcription factor LEF1 interacts with β-catenin via its N-terminus, and β-catenin binding promotes Wnt target gene transcription. Thus, overexpression of ΔN-LEF1 with a fused VP16 transactivation domain activates Wnt reporter independent of β-catenin. To test whether USP46 is required for Wnt activation downstream LEF1-dependent transcription, I knocked down USP46 upon overexpression of ΔN-LEF1-VP16 (Figure 4-4D). USP46 knockdown did not significantly reduce LEF1-dependent Wnt reporter activation. This would indicate that any nuclear role of USP46 is likely upstream of LEF1 transcriptional regulation.

A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Percent Activation
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100
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0
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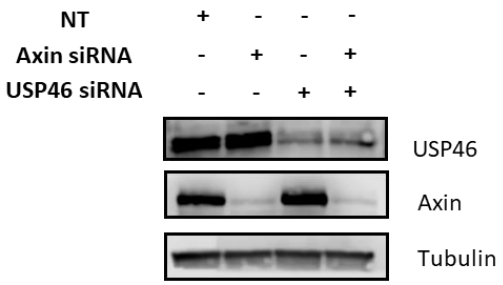
siUSP46
siAxin
siAxin+si46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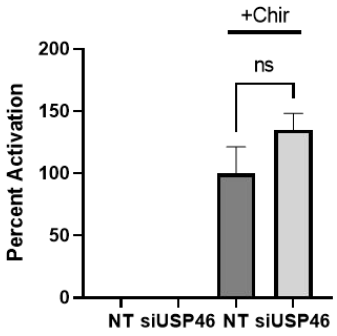
Axin siRNA - + - +
USP46 siRNA - - + +
USP46
Axin
Tubulin

B


+Chir
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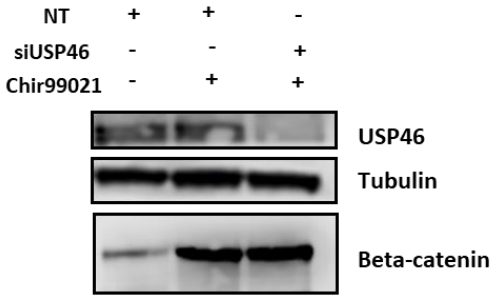
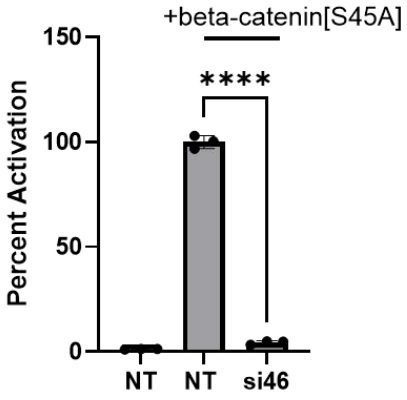
NT siUSP46 NT siUSP46


NT + + -
siUSP46 - - +
Chir99021 - + +
USP46
Tubulin
Beta-catenin

C


+beta-catenin[S45A]
****
Percent Activation
150
100
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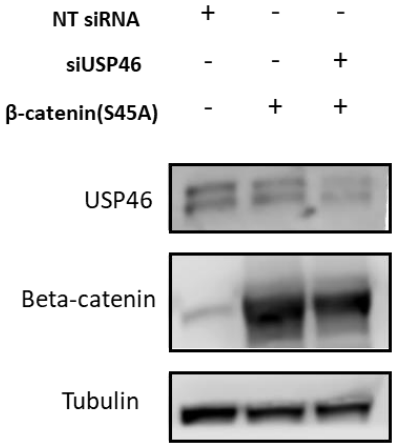
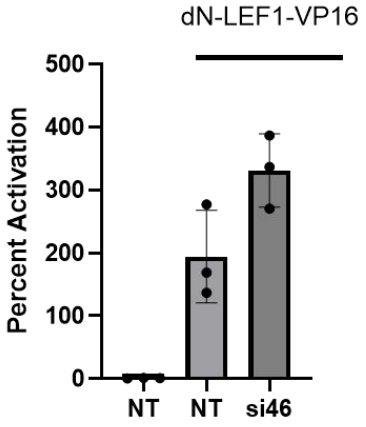
0
NT
NT
si46


NT siRNA + - -
siUSP46 - - +
β-catenin(S45A) - + +
USP46
Beta-catenin
Tubulin

D


dN-LEF1-VP16
Percent Activation
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400
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200
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0
NT
NT
si46

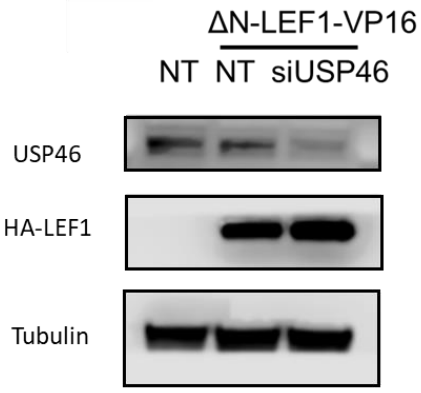
ΔN-LEF1-VP16
NT NT siUSP46
USP46
HA-LEF1
Tubulin

Figure 4-4: Epistasis experiments indicate a potential role for USP46 downstream the destruction complex. A) USP46 siRNA knockdown in HEK293-STF with and without siRNAs targeting Axin, for 72 hours. B) HEK293-STF transfected with either NT or USP46 siRNA and treated with Chir99021 for 16 hours. C) HEK293-STF cells were transfected with NT or USP46 siRNAs, followed by overexpression of C) β-catenin S45A, a non-degradable mutant form of β-catenin, or D) dN-LEF1-VP16, a constitutively active LEF-1 mutant. TOPFLASH was performed to measure reporter activity. Representative data from n=3 independent experiments, assayed in triplicate. Mean +/- SD of percent activation, normalized to NT control + treatment. Significance analyzed by two tailed student's t-test. P-values compare NT or siUSP46 incubated in the presence of experimental treatment.

Drosophila USP46 is required for Wnt target expression in the fly midgut

Wnt/Wg signaling controls development and tissue homeostasis in the *Drosophila* midgut, maintaining intestinal stem cell populations. Escargot (esg) is an intestinal stem cell marker and a proxy for active Wg signaling in *Drosophila* (Korzelius et al., 2014). To test whether USP46 is required for Wg signaling in the APC-deficient *Drosophila* midgut, in collaboration with Hassina Benchabane (Yashi Ahmed laboratory, Dartmouth University), RNA interference was performed to silence USP46 and WDR20 in an APC1 mutant background. In APC1 mutant midguts, increased esg positive cells indicate elevated Wg signaling (Figure 4-5). USP46 and WDR20 RNA interference attenuated the number of esg-positive cells in the midgut, suggesting USP46 is required for pathway activation upon APC1 loss in this system. These findings are consistent with results observed upon silencing of other membrane signaling factors, such as Dsh, in APC1 mutant cells and indicate that USP46 has a conserved role in signaling downstream APC function.

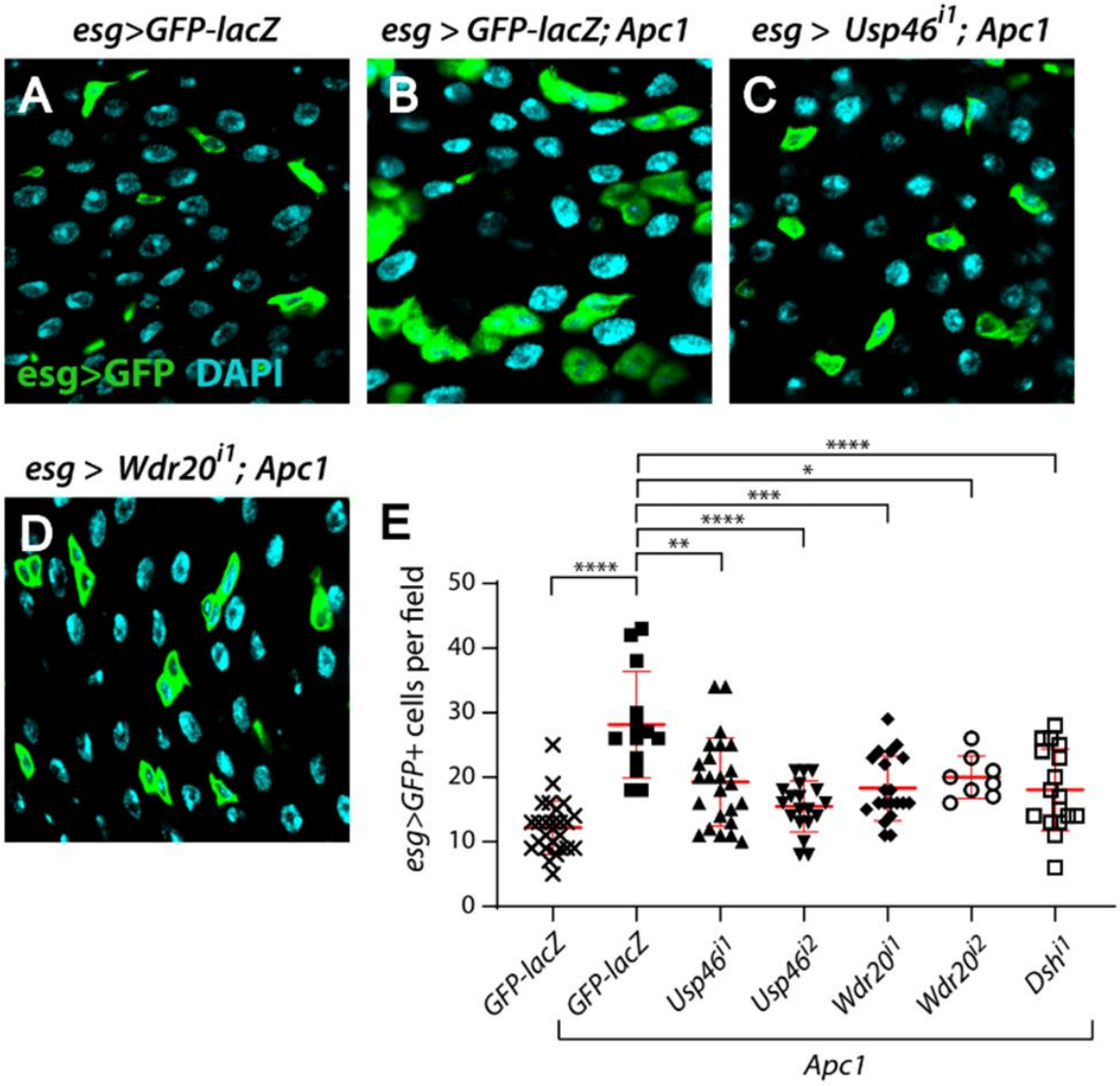

Figure 4-5: Drosophila USP46 is required for Wnt target expression in the fly midgut. A) Few escargot (esg)-GFP positive cells in the normal *Drosophila* midgut. B-D) Esg-positive cells in APC1 mutant *Drosophila* midgut, upon RNA interference of B) control, C) USP46, and D) WDR20. E) Quantification of esg-GFP cells per field. Dsh (Disheveled) is a positive control for inactivation of Wnt membrane signaling. These experiments were performed by Hassina Benchabane.

USP46 is required for organoid viability, Wnt target gene expression

The mouse model APC^{min} harbors a mutant allele of APC and displays multiple intestinal neoplasia (min). Intestinal organoids derived from these mice have elevated β-catenin-dependent signaling and represent a powerful model for examining APC-deficient Wnt pathway activation. To test if USP46 is required for the signaling and viability in these APC mutant organoids, we collaborated with Chen Shen (David Robbins laboratory, Georgetown University) to knock down USP46 in this system. She found that APC^{min} organoids exhibit elevated basal β-catenin levels and that USP46 shRNA treatment reduced β-catenin levels in the APC^{min} organoids (Figure 4-6A and B). Her data also demonstrates an accompanying reduction in LRP6 protein levels. Similarly, she found that USP46 shRNA treatment reduced Wnt target gene expression in the APC^{min} organoids and that organoid viability was also significantly decreased (Figure 4-6C). These findings are consistent with USP46 having a role downstream of APC.

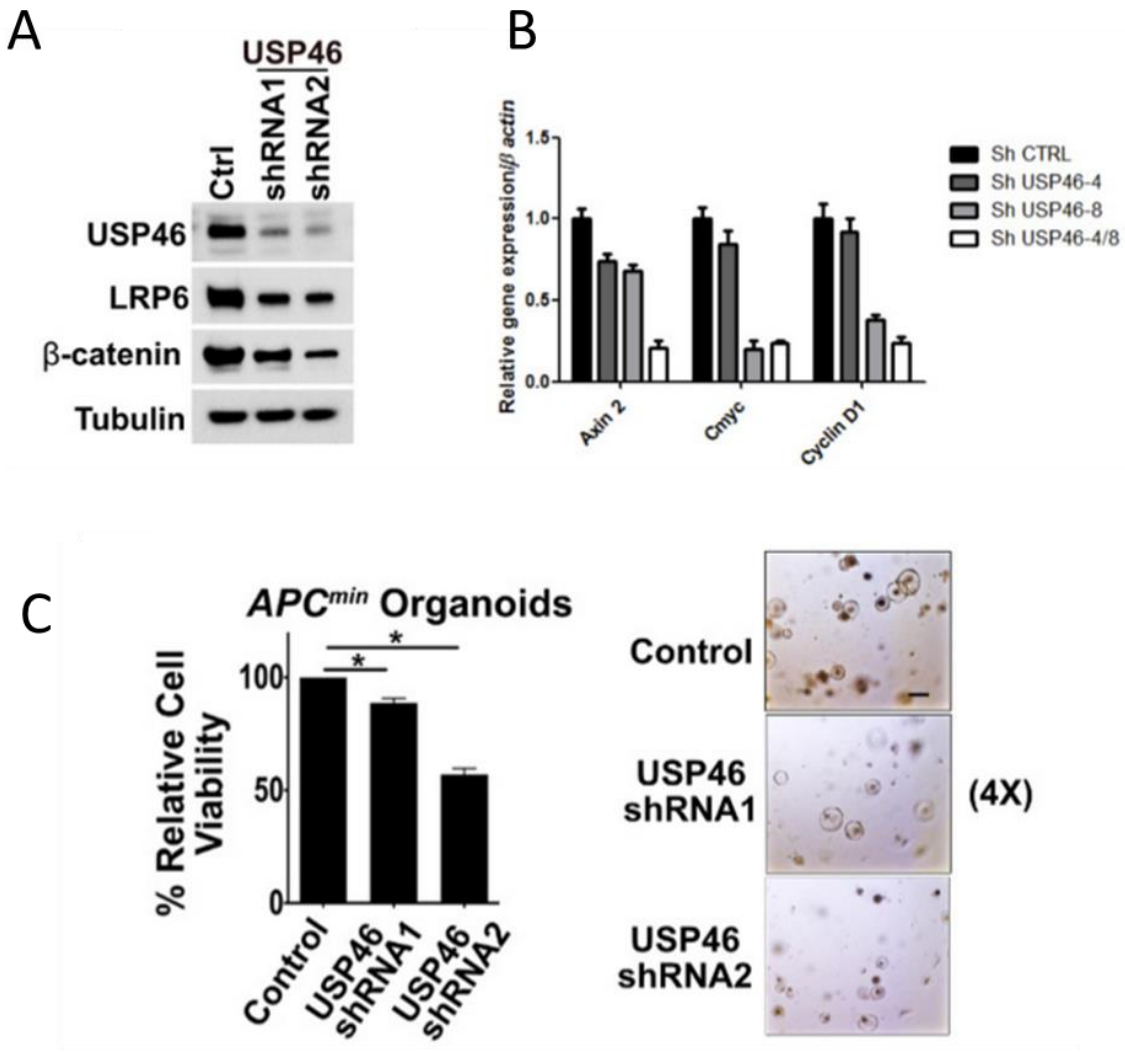

Figure 4-6: USP46 is required for organoid viability, Wnt target gene expression. A) β-catenin and LRP6 levels in APCmin organoids upon USP46 shRNA silencing. B) Axin2, C-myc, and CyclinD1 relative target gene expression as measured by RT-qPCR. C) APCmin organoid viability quantification and light microscopy images. These experiments were performed by Chen Shen.

Discussion

We have previously demonstrated the Wnt co-receptor LRP6 is required for signaling in APC mutant and knockdown cells (Saito-Diaz et al., 2018). Additionally, we showed that USP46 is required for the stabilization of surface LRP6 (Ng et al., 2023; Spencer et al., 2023). Based on these studies, I test the potential role for USP46 in regulating Wnt signaling in APC deficient cells (Figure 4-7).

I demonstrated that USP46 silencing blocks Wnt reporter activation and target gene expression in HEK293 cells depleted of APC and the previously well-characterized APC mutant colorectal cancer cell line, SW480. Studies in two other APC mutant model systems, APC1 mutant *Drosophila* midgut and organoids derived from APCmin mice, also indicate that the USP46 requirement for Wnt activation upon APC loss is evolutionarily conserved.

Surprisingly, the findings from my epistasis experiments suggest the requirement for USP46 in Wnt signaling is not specific to the Wnt ligand-mediated or APC-depleted state; USP46 knockdown blocked activation due to Axin silencing and to overexpression of a non-degradable β-catenin mutant. However, my findings indicate that USP46 is not required for LEF1-dependent transcription.

It is possible that USP46 has a previously undescribed role in Wnt signaling downstream of the destruction complex and upstream of TCF/Lef-mediated transcriptional activation. One possibility is that USP46 regulates β-catenin localization. This could potentially explain why USP46 knockdown reduced Wnt reporter activity in SW480 cells while β-catenin levels increased. This possibility, however, does not account for the inability of USP46 silencing to block Wnt reporter activity and target gene expression in HCT116 cells. Further studies involving β-catenin localization may help resolve these seemingly contradictory findings.

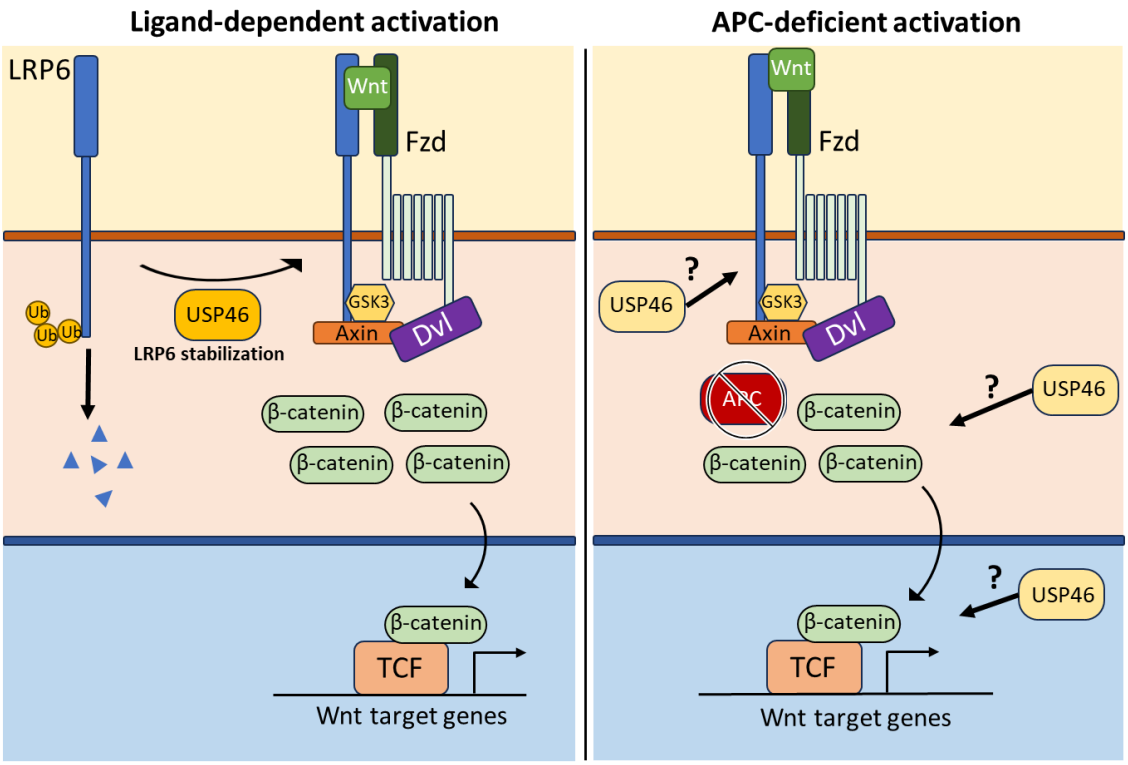

Figure 4-7: Model of USP46 potential regulation of Wnt signal transduction. USP46 regulates Wnt stabilization. Indicated here are the known role for USP46 in Wnt ligand stimulated signal transduction, as well as potential LRP6-dependent and -independent functions identified in APC-deficient Wnt activation.

Additionally, mass spectrometry could be useful in the identification of a target interaction partner.

Finally, my epistasis findings do not preclude a potential role for USP46 in regulating β-catenin levels via LRP6 stabilization in APC-deficient cells. I was unable to determine if β-catenin levels were regulated upon APC and Axin knockdown in HEK293 cells. Previously, Ng et al. (2023) showed that USP46 knockdown did not reduce β-catenin accumulation due to Axin silencing. It would be interesting to determine if β-catenin levels are regulated by USP46 in APC knockdown cells. Furthermore, I only examined one APC mutant colorectal cell line. Future studies are needed to test the requirement of USP46 in Wnt signaling using additional APC mutant cell lines, such as DLD1 and DiFi cells. These additional experiments will be useful in further dissecting the function(s) of USP46 in regulating the Wnt signaling pathway.

CHAPTER 5

Future Directions and Concluding Remarks

Wnt receptor regulation by the tumor suppressor APC

Previously, we demonstrated that APC negatively regulates Wnt receptor activation and signalosome formation and proposed this is via an interaction with Clathrin/AP2 endocytic machinery (Saito-Diaz et al., 2018). Here, we characterized the interaction between AP2-μ2 and the central region of APC and began to explore the functional significance of this interaction in regulating receptor activation. Future studies examining Wnt receptor regulation by APC will aim to further examine the interaction between $APC^{1265\text{-}2060}$ and the μ subunit of AP2 biochemically and to test the functional significance of this interaction in cells and model systems. Here, we presented evidence supporting $APC^{1265\text{-}2060}$ interaction with AP2μ2 using affinity pulldown assays from crude lysates. To confirm these findings, future studies will require *in vitro* binding assays with full-length recombinant protein.

Additionally, our studies presented here demonstrated the tyrosine based endocytic sorting motifs present on $APC^{1265\text{-}2060}$, and recognized by AP2μ2, are required for their interaction. The heterotrimeric AP2 complex *in vitro* also recognizes acid dileucine motifs that are not recognized by the μ2 subunit alone (B. T. Kelly et al., 2008). $APC^{1265\text{-}2060}$ contains three of these motifs. GST-pulldown assays of endogenous clathrin/AP2 machinery with recombinant $APC^{1265\text{-}2060}$ (WT, tyrosine sorting motif mutant, and dileucine motif mutants) will be beneficial in determining which motifs are most important for mediating $APC^{1265\text{-}2060}$ interaction with endogenous adaptor proteins.

We performed initial characterization of the regions of $APC^{1265\text{-}2060}$ required to mediate AP2μ2 binding. Additional truncation studies will be required to determine the minimal binding

motif. Competitive binding assays between distinct $APC^{1265-2060}$ fragments could also be useful in confirming specificity of the interactions and in demonstrating varied capacity in competitive capacity.

While we showed here that $APC^{1265-2060}$ overexpression can suppress LRP6 phosphorylation at Ser1490 in cells, additional studies examining the functional relevance of the $APC^{1265-2060}$ interaction with AP2μ2 in cells are required. It would be interesting to test if $APC^{1265-2060}$ can regulate other Clathrin/AP2-dependent facets of Wnt receptor function such as clustering/signalosome formation and internalization (Colozza & Koo, 2021; I. Kim et al., 2013).

We have demonstrated that the AP2μ2 interaction with the central region of APC is conserved with *Drosophila* APC1, and the tyrosine-based sorting motifs on APC are required for this interaction. It would be interesting to determine if the central fragment of *Drosophila* APC1 can rescue APC-deficient signaling in the fly and to examine the functional relevance of blocking the APC1 interaction with AP2 with sorting motif mutants. Similar rescue studies can be performed in APCmin organoids to determine the functional significance of the AP2μ2 interaction with $APC^{1265-2060}$ *in vivo.*

The studies discussed here provide further cellular and biochemical insight into a novel role for the tumor suppressor APC in Wnt pathway regulation. The most well-characterized function of APC is in the downregulation of β-catenin as a scaffold in the destruction complex (Fearnhead et al., 2001; Stamos & Weis, 2013). As such, the region of APC most critical to Wnt regulation has been demonstrated to be the central region of the protein consisting of the β-catenin and Axin binding sites (Katharine Eklof Spink et al., 2001). We have demonstrated biochemical evidence indicating this region of the protein has an additional function in regulating Wnt receptor function.

Inactivating mutations of APC are the initiating mutations of 80% of colorectal cancers (Kinzler & Vogelstein, 1996). Over 60% of somatic mutations in APC occur between codons 1286 and 1513 (Miyoshi et al., 1992), and thus this region is termed the "mutation cluster region" (Fearnhead et al., 2001). These mutations generate APC truncations lacking most β-catenin binding sites and all Axin binding sites (Fearnhead et al., 2001), and thus, the conventional thinking is that the primary result of truncating mutations is a loss in destruction complex function.

Our findings present another potential function lost by APC truncation: the capacity for membrane APC to associate with the clathrin/AP2 endocytic machinery. The full-length APC contains eight tyrosine-based cargo sorting motifs recognized by AP2μ2, and of those, three are within or to the C-terminus of the mutation cluster region. Our findings raise the possibility that APC mutations within the mutation cluster region partially drive Wnt activation by impairing APC's capacity to associate with AP2, thus reducing the protein's capacity to block constitutive receptor activation.

Previous findings from our laboratory, combined with my findings here, have uncovered a conserved function for APC in regulating Wnt receptor activation. These studies could potentially open the door to new therapeutic approaches in which receptors are targeted in APC mutant colorectal cancers.

The deubiquitylase USP46 is required for APC-deficient Wnt signaling

Previously, we have demonstrated that the Wnt receptor LRP6 is required for Wnt pathway activation in APC-depleted cells (Saito-Diaz et al., 2018). We also have demonstrated that the deubiquitylase USP46 stabilizes LRP6 (Ng et al., 2023). Here, we have demonstrated USP46 is required for APC-deficient pathway activation. We have also found evidence that

USP46 has an additional regulatory role in the Wnt pathway independent of Wnt receptor regulation, yet the target of this regulation is unknown. Future studies will aim to confirm the requirement for USP46 in APC-depleted cells via Wnt receptor regulation, in addition to identifying the points at which USP46 regulates the Wnt pathway downstream of the destruction complex.

Immunoblotting for β-catenin in APC- or Axin-depleted cells upon USP46 knockdown will be beneficial in determining if there is differential regulation of β-catenin levels by USP46 in these two contexts. Previously, we demonstrated that USP46 knockdown does not block the accumulation of β-catenin in cells in which Axin has been knocked down (Ng et al., 2023). If USP46 blocks APC-deficient β-catenin accumulation, then epistasis and rescue experiments can be performed upstream β-catenin stabilization to determine if this phenotype is via USP46-mediated Wnt receptor regulation. If there isn't differential regulation of β-catenin levels by USP46 in APC- and Axin-depleted cells, then this further supports a model in which USP46 regulates APC-deficient activation via a receptor-independent downstream mechanism.

To further examine the potential roles of USP46 in the Wnt pathway downstream receptor function requires the identification of an additional USP46 target. We have previously performed candidate co-immunoprecipitation (co-IP) experiments, as well as IP-mass spec experiments (data not shown), and these have failed to yield a putative receptor-independent target for USP46 in the Wnt pathway. Additional studies will be required to determine Wnt-relevant USP46 interactors in cells.

The studies discussed here provide evidence of a second receptor-independent function for USP46 in the regulation of the Wnt pathway. Here, I have demonstrated a conserved requirement for USP46 in APC-deficient Wnt pathway regulation. While the findings presented

here do not support the initial hypothesis of this regulation being via USP46 control of Wnt receptor levels, they do raise the possibility of an appealing alternative: USP46 having multiple conserved functional roles within the Wnt pathway. Various Wnt regulators have multiple Wnt-related functions, including APC, GSK3, and CK1 (MacDonald et al., 2009). The identification of an additional point of regulation for the deubiquitylase USP46 in Wnt regulation would be a significant advance in our understanding of Wnt pathway regulation by ubiquitin post-translational modifications.

Together, these two projects centered on APC regulation of Wnt receptor activation have begun to uncover novel cellular and molecular insights into the normal, conserved function of the Wnt signaling pathway.

CHAPTER 6

References

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