REGULATION OF WNT RECEPTOR ACTIVATION BY THE TUMOR SUPPRESSOR APC

By

Vicente Kenyi Saito-Diaz

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Approved:

William Tansey, Ph.D.

Andrea Page-McCaw, Ph.D.

Jin Chen, M.D., Ph.D.

Scott Hiebert, Ph.D.

Ethan Lee, M.D., Ph.D.

For my mother.

Y para Mollie, por su apoyo incondicional.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

AID, Auxin-inducible degron

AP-1, Adaptor protein 1

APC, Adenomatous Polyposis Coli

Arr, arrow

βP-E, β-propellers and Epidermal Growth Factor-like domain

Cav1, Caveolin-1

Cav2, Caveolin-2

Cav3, Caveolin-3

CCP, clathrin-coated pits

CCV, clathrin-coated vesicles

CK1, Casein Kinase 1

CME, clathrin-mediated endocytosis

CMV, cytomegalovirus

CRC, colorectal cancer

CRD, Cystein-rich domain

C-terminal, Carboxy terminal

D, aspartic acid

DKK, Dickopff

dn, dominant-negative

Dvl, dsh, Dishevelled

ECD, extracellular domain

EGFR, Epidermal Growth Factor Receptor

EM, electron microscopy

ER, Endoplasmic reticulum

esg, escargot

Fab, fragment antigen-binding

FAP, familial adenomatous polyposis

Fc, fragment crystallizable

FL, Full-length

Fz, Frizzled

GFP, green fluorescent protein

GPCR, G-protein coupled receptor

gRNA, guide RNA

Gro, Groucho

GSK3, Glycogen Synthase Kinase 3

HA, hemagglutinin

HEK, Human Embryonic kidney

ICD, Intracellular domain

IgG, Immunoglobulin G

IgM, Immunoglobulin M

int-1, integration-1

ISC, Intestinal stem cell

KO, knock out

LDL, low-density lipoprotein

LRP5/6, low-density lipoprotein receptor related 5 or 6

mAb, monoclonal antibody

MAP, mitogen-activated protein

MBP, Maltose Binding Protein

MEF, Mouse embryonic fibroblast

MVB, multivesicular bodies

MVE, multivesicular endosomes

NDLB, non-denaturing lysis buffer

NES, nuclear export signal

NLS, nuclear localization signal

N-terminal, Amino terminal

P, Proline

PIP2, phosphatidyl inositol-4,5-bisphosphate

PIP3, phosphatidyl inositol-3,4,5-trisphosphate

PORCN, Porcupine

Pygo, Pygopus

qRT-PCR, Quantitative Reverse trancriptase polymerase chain reaction

RNAi, RNA interference

RPE, Retinal Pigment Epitheilium

SCF, Skp1-Cullin-F-box

SDS-PAGE, Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis

Ser/S, Serine

SHC, SH2 domain-containing transforming protein

shRNA, short-hairpin RNA

siRNA, short interference RNA

STF, Super TOPflash

TCF, T-cell factor

TGFβ, Transforming Growth Factor β

Thr/T, Threonine

TKO, triple knock out

TLE, Transducing-like enhancer

TLR4, Toll-like receptor 4

TNBC, Triple negative breast cancer

ts, temperature sensitive

W, tryptophan

Wg, wingless

WT, wild-type

Y, tyrosine

YFP, yellow fluorescent protein

CHAPTER I

INTRODUCTION TO THE WNT SIGNALING PATHWAY

Introduction

The canonical Wnt signaling pathway is an evolutionary conserved pathway required for proper development of all metazoans. Wnt signaling plays a fundamental role in the determination of cell fate, proliferation, polarity, and cell death during embryonic development, as well as in tissue homeostasis in adults. Misregulation of the Wnt pathway leads to disease including osteoporosis and multiple types of cancer (Saito-Diaz et al. 2012). Understanding the mechanism of Wnt signal transduction is critical for developing new therapeutic approaches. In this chapter, I establish the background to understand my cell biology and biochemical studies of the Wnt pathway as outlined in Chapters II, III and IV. I conclude my work with future directions (Chapter V) based on the results presented in Chapters III and IV.

The cell: the basic unit of life

Signaling transduction is the process in which cells detect changes in their environment and orchestrate their intracellular response (Gerhart 1999; Alberts et al. 2002; Gomperts et al. 2009). Signaling transduction pathways can be characterized as a cascade of events triggered by binding of a ligand to a “receptor” (often embedded in the plasma membrane). When the appropriate stimulus (e.g. chemically encoded information) is detected and binds the receptor, a series of biochemical reactions occur that, ultimately, result in a wide range of events that include changes in cell metabolism,

cell motility, regulation of gene transcription, or even initiation of cell death (Gomperts et al. 2009). Signals sent to and from other cells activate signaling transduction pathways which promote cell-cell communication and allow cells to become organized in a more complex fashion, giving rise to tissues, organs and, ultimately multicellular organisms (Alberts et al. 2002).

Despite the legion of metazoans and their significant diversity in morphology, reproductive strategy, nutrient requirement, and the ecological niche they occupy, there are only 18 known signaling pathways in metazoans as distinguished by their transduction intermediates. These signaling pathways are required during early development, late development, and in differentiated cells in adults (Gerhart 1999; Meng et al. 2016). Nearly all of these pathways contribute to human disease when their normal functions are disrupted. Because of this, it is necessary to understand how signaling pathways work and how they interact with each other (cross-talk) during animal developmental in order to develop better treatment in cases where they are misregulated in human disease.

History of the Wnt pathway

The Wnt pathway, named for its ligands, the Wnt family of secreted glycoproteins, was discovered more than 30 years ago, and the historical events that led to the discovery and naming of Wnt ligands highlight its importance in development and in human disease. In 1976, Sharma and Chopra described a *Drosophila melanogaster* mutant that exhibited reduced or absent wings and halteres (Sharma and

Chopra 1976). Based on the mutant phenotype, they named this locus *wingless* (*wg*) and suggested that it played an important role in development. A few years later, Nusse and Varmus conducted a forward genetic screen to identify genes in mice that could lead to tumorigenesis (Nusse et al. 1984). Using mouse mammary tumor virus (MMTV) insertion sites, they identified a locus termed int-1, short for integration 1, which induced mouse mammary tumors. Comparative genomic studies revealed that wg and int-1 were homologs, and the names were merged into the mnemonic Wnt (Nusse et al. 1991). Overexpression of int-1 in *Xenopus* embryos induced the formation of an ectopic axis, demonstrating that it not only acts as an oncogene but also plays a critical role in early axis specification (McMahon and Moon 1989). These studies collectively drew an implicit connection between the physiological role for Wnts in development and a potential pathophysiological role in carcinogenesis.

Forward genetic studies in *Drosophila* have been crucial in identifying Wnt pathway components. In 1980, Eric Wieschaus and Christiane Nusslein-Volhard identified a series of *Drosophila* mutants that controlled patterning of the early embryo (Nüsslein-Volhard and Wieschaus 1980). This work was a watershed moment in developmental biology, for which they were awarded a Nobel Prize in 1995. The 15-year period after their initial publication produced a number of genetic and molecular studies that elucidated the role of these mutants within various signaling pathways and resulted in the discovery of key members of the Wnt pathway, including *armadillo* (the vertebrate homolog of β-catenin), *disheveled* (Dvl/Dsh), *shaggy* (the vertebrate version of glycogen synthase kinase 3 or GSK3), *frizzled*, and *arrow* (Riggleman et al. 1989;

Riggleman et al. 1990; Siegfried et al. 1992; Klingensmith et al. 1994; Bhanot et al. 1996; Wehrli et al. 2000).

The activation of the Wnt signaling pathway on the future dorsal side of the early *Xenopus* embryo is a critical event in the formation of the Spemann organizer, a tissue-organizing center found in vertebrates (Spemann and Mangold 1938). The role of Wnt in organizer formation was uncovered when mRNA of Wnt-1 and Xwnt8 was injected into *Xenopus* blastomeres. Ectopic activation of Wnt signaling on the future ventral side of the embryo was shown to induce a second organizer that coordinates the formation of a complete secondary body axis (Smith and Harland 1991; Sokol et al. 1991). Embryonic axis duplication was also found to be induced by overexpression of positive downstream components of the pathway (i.e. Dvl and β-catenin) or by inhibiting negative components of the pathway (i.e. inhibiting GSK3 activity or overexpressing dominant-negative Axin) (Dominguez et al. 1995; Guger and Gumbiner 1995; Fagotto 1999).

Numerous genetic and environmental perturbations of the Wnt pathway can lead to a variety of human diseases, ranging from birth defects to cancers (MacDonald et al. 2009). One well-established connection between the Wnt pathway and human disease is a genetic lesion that occurs early in the onset of colon cancer. In 1991, a germline mutation in the Wnt pathway component adenomatous polyposis coli (APC) was identified in patients with familial adenomatous polyposis (FAP), a form of hereditary cancer (Kinzler et al. 1991; Nishisho et al. 1991). FAP patients inherit one defective allele of APC, and upon stochastic loss of the second allele develop colon adenomas (polyps) at an early age. These benign polyps frequently acquire other mutations and

develop into invasive colon carcinomas. Later studies showed that loss of both APC alleles occurs in the large majority (>80%) of nonhereditary, sporadic colorectal cancers as well (Kinzler and Vogelstein 1996). Following this work, inappropriate activation of Wnt signaling was subsequently found in other cancers, including liver cancer, skin cancer, lung cancer, Wilms' tumor, prostate cancer, and breast cancer. A variety of developmental genetic defects were also shown to occur as a result of Wnt pathway misregulation, including defects in limb formation (tetra-amelia), bone ossification, eye vascularization, and tooth development (Gong et al. 2001; Boyden et al. 2002; Niemann et al. 2004; Xu et al. 2004). Understanding the basis of the numerous human diseases resulting from misregulation of Wnt signaling and designing therapies for their treatment obviously require a detailed understanding of the molecular mechanism of the Wnt pathway.

The current model of the Wnt pathway

Wnt signals can direct a wide variety of cellular responses in development, physiology, and disease. Originally, it was thought that a variety of cellular responses to Wnt signaling were mediated by the different transcriptional targets modulated in different cellular contexts. This original model, in which Wnt signaling alters transcription, is referred to as "canonical" Wnt signaling. It is now widely accepted that Wnt signaling can also activate distinct pathways that do not involve the nucleus or transcription, but rather signals cytoplasmic changes involving the actin cytoskeleton and intracellular calcium stores. These non-transcriptional Wnt pathways are loosely known as "noncanonical" Wnt signaling. My work focuses exclusively on the "canonical"

Wnt signaling, so I will only discuss this pathway. For clarity, I will use the term Wnt/β-catenin signaling to identify what has been commonly referred to as “canonical” signaling. This nomenclature specifies the ligand (Wnt) and the essential downstream transcriptional effector (β-catenin).

Wnt/β-catenin signal transduction, at its simplest, is a pathway that results in the cytoplasmic protein β-catenin entering the nucleus to modulate transcription. When the pathway is not activated, β-catenin is subject to a “futile cycle” of continual synthesis and destruction by the β-catenin destruction complex, comprised of the scaffold proteins Axin and APC and the kinases GSK3 and casein kinase 1 (CK1) (Figure 1.1). Wnt signaling removes APC from the complex and relocalizes the other components to the plasma membrane via the adaptor Dvl, thus stabilizing β-catenin which enters the nucleus to mediate transcription (Figure 1.1). Thus, Wnt/β-catenin signaling can be divided into three general molecular events: (1) surface receptor activation, (2) inhibition of the β-catenin destruction complex, and (3) activation of a Wnt-specific nuclear transcriptional complex. The next sections of this chapter consider each of these steps more closely.

Surface receptor activation

Wnt ligands

Wnt proteins are cysteine-rich morphogens of ~350–400 amino acids that can act in short- and long-range signaling. There are 19 vertebrate Wnts, and all appear to be able to activate the pathway. The crystal structure of Xenopus Wnt8 (XWnt8) bound to

the cysteine-rich domain (CRD) of the mouse Frizzled (Fz) 8 receptor has been solved to 3.25 Å. The Wnt8 structure is bilobular with an N-terminal helical domain and a C-terminal extended β-hairpin stabilized by extensive disulfide bonds. Interactions between XWnt8 and Fz8 occur via extensions from each lobe (“thumb” and “finger”) to grasp the CRD of Fz8 on two distinct sites (Janda et al. 2012). All Wnts contain an N-terminal signal peptide for secretion and are N-linked glycosylated (Smolich et al. 1993; Willert et al. 2003; Takada et al. 2006). N-glycosylation of *Wg* (*Drosophila* Wnt homolog) has been shown to be stimulated by lipid modifications (Tanaka et al. 2002). Although an early study suggests that glycosylation is dispensable for Wnt secretion and activity (Mason et al. 1992), more recent studies demonstrate that mutating the glycosylation sites on Wnts blocks their secretion (Komekado et al. 2007; Kurayoshi et al. 2007).

Wnts contain several polar amino acids and undergo a series of lipid modifications that affect their activity and secretion (Bradley and Brown 1990). Wnts have been shown to undergo acylation at Cys77 and Ser209 with palmitate and palmitoleate, respectively (Willert et al. 2003; Takada et al. 2006). Interestingly, the co-crystal structure of XWnt8–Fz8 CRD indicates that Cys77 is engaged in disulfide bonding, whereas Ser209 is acylated (likely palmitoleic acid). The palmitoleic acid lipid group was shown to dock within a hydrophobic groove on the CRD of Fz8 and, thus, plays a direct role in Wnt–Fz interaction (Janda et al. 2012).

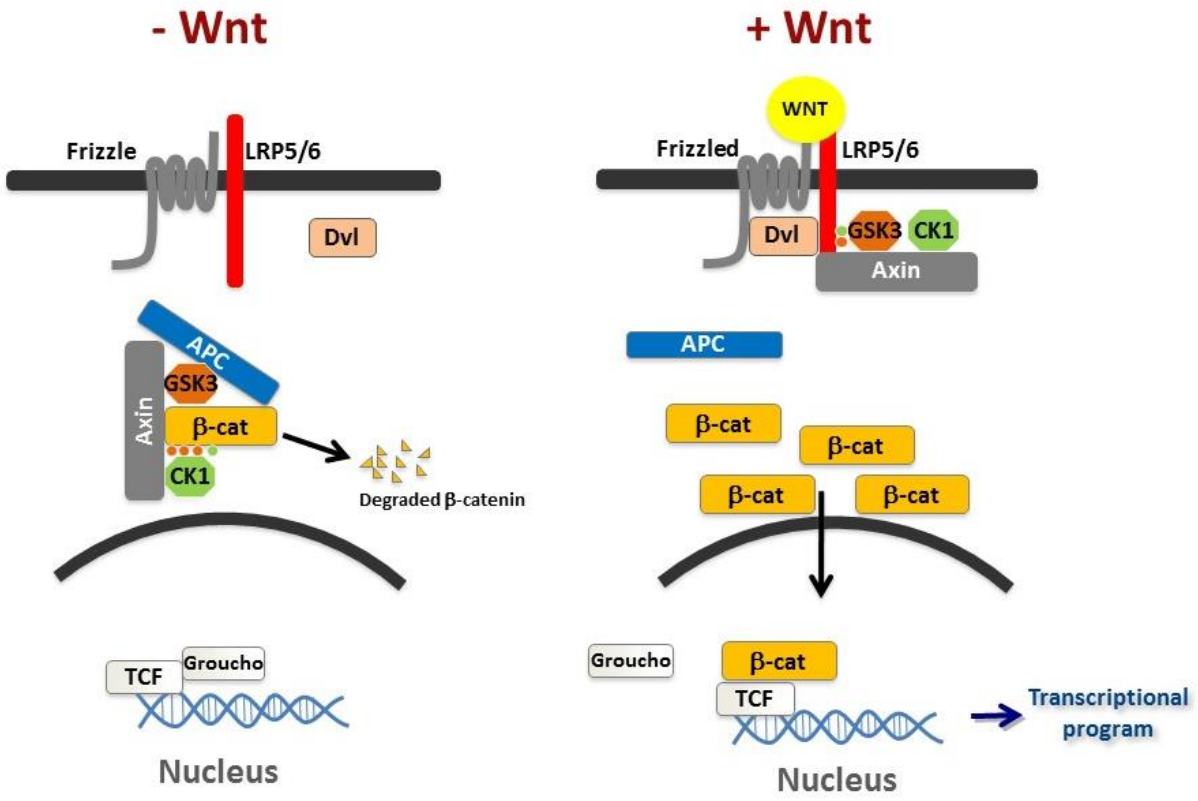


Figure 1.1. Schematic of the canonical Wnt signaling pathway

Left panel: In the absence of a Wnt ligand, β-catenin associates with the β-catenin destruction complex composed by APC, Axin, GSK3, and CK1. Within this complex, β-catenin is sequentially phosphorylated by CK1 and GSK3. Phosphorylated β-catenin is recognized by the E3 ligase β-TRCP and targeted for proteasome-mediated degradation.

Right panel: Binding of a Wnt ligand to the co-receptors Fz and LRP6, promotes LRP6 phosphorylation by GSK3. Axin is recruited to the plasma membrane by Dvl and binds phospho-LRP6 which results in the disassembly of the β-catenin destruction complex. In consequence, β-catenin accumulates in the cytoplasm and translocates to the nucleus where it binds to TCF to activate the transcription of Wnt target genes.

The endoplasmic reticulum (ER)-embedded, multi-pass transmembrane O-acetyltransferase protein Porcupine (PORCN) is the enzyme that mediates lipid modifications of Wnt (Port and Basler 2010). PORCN was initially identified in *Drosophila* as a segment polarity gene and was the first gene shown to be required in Wnt-secreting cells (van den Heuvel et al. 1993). Loss of PORCN function causes Wnts to accumulate in the ER (van den Heuvel et al. 1993; Kadowaki et al. 1996), whereas PORCN over-expression results in a larger fraction of Wnts that are modified by lipids (Galli et al. 2007). PORCN is directly responsible for Ser209 palmitoylation of Wnts, however, it is not known whether other acetyltransferases are involved (Gao and Hannoush 2014). Modified Wnts are translocated from the ER to the Golgi apparatus in a process mediated by the p24 protein family (Buechling et al. 2011; Port et al. 2011). Once in the trans-Golgi network, the seven-pass transmembrane protein Wntless (Wls) is thought to provide further transport of Wnts to the plasma membrane for release outside the cell (Port and Basler 2010) . Consistent with the necessary role of Wls in Wnt signaling, loss of Wls resembles a Wnt loss-of-function phenotype (Bänziger et al. 2006). Wls has been shown to bind Wnts at the conserved palmitoylated Ser209, explaining the accumulation of Wnts in the ER of PORCN mutants (Herr and Basler 2012). Wls is recycled from the plasma membrane via a multiprotein complex called the retromer. The retromer is responsible for routing Wls into a retrograde pathway that transports transmembrane proteins from endosomes back to the trans-Golgi network (Coudreuse et al. 2006; Port and Basler 2010). In the absence of the retromer complex, Wls is trapped in endosomes and subsequently degraded (Yang et al. 2008). This requirement of the retromer for Wnt signaling can be bypassed by providing additional

Wls (Franch-Marro et al. 2008; Port et al. 2008), further confirming the role of the retromer in Wnt secretion via its regulation of Wls (Figure 1.2).

Wnt extracellular transport

Several mechanisms have been proposed for how Wnt ligands traverse the extracellular space to bind their target cells. It is possible that these mechanisms are tissue specific and influenced by the extracellular environment in which the cell resides (Port and Basler 2010). The mechanisms underlying the graded distribution of extracellular Wnt/Wg ligands have been best delineated in *Drosophila* wing discs, where a gradient of Wg protein patterns the boundary between the developing dorsal and ventral wing surfaces. These studies suggest a restricted diffusion model. In this model, Wg diffuses across cells extracellularly while interacting with receptors and cell-surface heparan sulfate proteoglycans (HSPGs), which act generally as positive regulators of Wg signaling (Strigini and Cohen 2000; Baeg et al. 2004; Han et al. 2005; Yan and Lin 2009). HSPGs are comprised of a protein core decorated with long glycosaminoglycan (GAG) chains (Häcker et al. 2005). The importance of GAG chains of HSPGs has been demonstrated by studies showing that when enzymes involved in heparan sulfate synthesis are mutated, extracellular Wg does not accumulate and Wg signaling is reduced (Binari et al. 1997; Häcker et al. 1997; Haerry et al. 1997; Lin and Perrimon 1999; Lin 2004). The HSPGs most studied as modulators of morphogen activity are glypicans, which are anchored to the cell surface by a glycosylphosphatidylinositol (GPI)

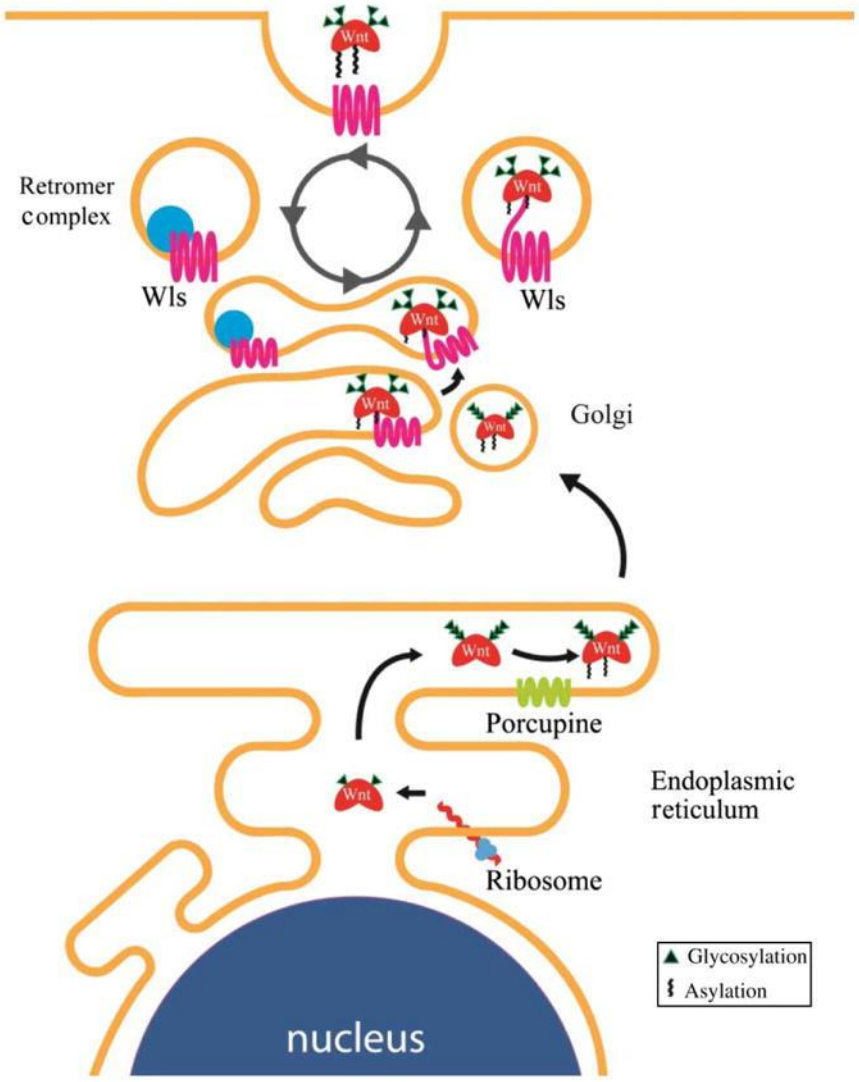


Figure 1.2. Synthesis and export of Wnt ligand.

Wnt ligand undergoes multiple posttranslational modifications in the ER. Glycosylation and palmitoylation of Wnt ligand (the latter mediated by the transmembrane protein Porcupine) are required for its translocation to the Golgi apparatus. Palmitoylation of Wnt allows it to bind Wls, which provides a mechanism for transportation to the plasma membrane. The retromer complex recycles Wls from the plasma membrane back to the Golgi.

anchor. There are two glypicans in Drosophila, known as division abnormally delayed (Dally) and Dally-like protein (Dlp), and both bind Wg in cell culture (Franch-Marro et al. 2005). Dlp overexpression in wing discs leads to extracellular Wg accumulation, whereas Dally has little effect (Franch-Marro et al. 2005; Han et al. 2005), suggesting that Dlp binds Wg with higher affinity than Dally. Genetic studies suggest that Dally mainly functions as a co-receptor to present Wg to the Fz2 receptor (Lin and Perrimon 1999).

In contrast to Dally, Dlp has a more complex influence on Wg signaling. Dlp inhibits Wg signaling close to the Wg source (short-range Wg signaling) but promotes the range of Wg signaling distant from the source (long-range Wg signaling) (Kirkpatrick et al. 2004; Kreuger et al. 2004; Franch-Marro et al. 2005); this biphasic activity of Dlp serves to reduce the morphogen gradient. Various models have been proposed to explain Dlp's biphasic activity. An early model focused on proteolytic cleavage of Dlp by Notum, an α/β-hydrolase expressed at the Wg source, capable of shedding Dlp at its GPI anchor and releasing it from the cell surface. One possibility to account for Dlp biphasic activity is that Notum cleavage converts Dlp into a short-range Wg antagonist (Kreuger et al. 2004). However, in the wing, neither the ectopic expression nor the loss of Notum alters Dlp levels, suggesting that the biphasic activity of Dlp is independent of Notum (Han et al. 2005; Gallet et al. 2008). A more recent model proposes that Dlp mediates transcytosis of Wg from the apical cell surface to the basolateral surface, where it is spread to the next distal cell, so that Dlp effectively siphons Wg away from regions of high Wg expression toward distal regions (Gallet et al. 2008). Another model, based on wing-disc and cell culture studies, suggests that competition between Dlp and

Fz2 for binding Wg at the cell surface is responsible for the biphasic activity of Dlp (Yan et al. 2009), such that when Wg concentration is high (in short-range signaling), Dlp sequesters Wg from Fz2, but when Wg concentration is lower (in long-range signaling), Dlp concentrates Wg in the vicinity of Fz2 to promote signaling.

The Fz receptor family

The soluble Wnt ligands bind to members of the Fz (Fz) family of seven transmembrane domain receptors, which have structural similarities to G-protein-coupled receptors (GPCRs). Biochemical evidence indicates that Wnts bind to the CRD of the Fz receptor, and that the affinity of Wnt for Fz is in the low nanomolar range (Bhanot et al. 1996; Hsieh et al. 1999).

The topological similarities of Fz to GPCRs have led to the suggestion that heterotrimeric G proteins may be required for Wnt signal transduction, and several studies propose a link between G proteins and Wnt pathway activation. Genetic studies in Drosophila suggest that Gαo transduces signaling from Fz, and that Gαo interacts with the scaffold protein Axin to promote its localization to the plasma membrane (Katanaev et al. 2005; Egger-Adam and Katanaev 2009). In cultured mammalian cells, depletion of Gαo or Gαq has been shown to inhibit Wnt signaling, possibly via disruption of GSK3β–Axin complexes (Liu et al. 2005). More direct evidence for a role of G proteins in Wnt pathway activation comes from reconstitution studies indicating that Gαo, Gαi2, Gαq, and Gβγ have the capacity to inhibit both β-catenin phosphorylation by GSK3 and β-catenin turnover in Xenopus egg extract. In the case of the latter, it was

proposed that Gβγ promotes the recruitment of GSK3 to the plasma membrane to enhance low-density lipoprotein receptor-related protein 6 (LRP6) phosphorylation and activation (Jernigan et al. 2010). Whether heterotrimeric G proteins are bona fide mediators of Wnt ligand-mediated signaling, or whether other pathways act through them to modulate Wnt signaling, remains unclear.

The co-receptor LRP5/6

LRP5 and LRP6 are functionally redundant single-pass transmembrane receptors that act as co-receptors for Wnt ligands (Pinson et al. 2000; Tamai et al. 2000; Wehrli et al. 2000). In *Drosophila*, there is only one family member, *Arrow*. In some assays, LRP6 is more potent than LRP5. There are thought to be no qualitative differences in their mechanism of action in mediating Wnt pathway activation, although they likely play different roles during development (He et al. 2004; Mi and Johnson 2005). Biochemical studies of LRP6 indicate that different Wnts may bind to different extracellular domains of the LRP5/6 protein (Bourhis et al. 2010). Specifically, the LRP6 extracellular domain contains four repeating sequences of β-propeller and epidermal growth factor-like (βP–E) domains. The crystal structures of the extracellular LRP6 regions indicate that the βP–E repeats represent two discrete, compact, rigid structures, each containing two βP–E pairs. Wnt9b binds the first two βP–E repeats on the extracellular domain of LRP6, whereas Wnt3a binds the last two βP–E domains (Ahn et al. 2011; Chen et al. 2011; Cheng et al. 2011).

Binding of Wnt ligands to Fz and LRP5/6 results in the production of phosphatidylinositol (4,5)-bisphosphate (PIP2) (Pan et al. 2008). Increased PIP2 induces oligomerization and clustering of LRP5/6. Although hydrodynamic studies suggest that Fz and LRP6 oligomerize and form clusters of “signalosomes” upon Wnt signaling, the *in vivo*, physiological significance of such events in Wnt pathway activation remains to be determined (Cong et al. 2004; Bilić et al. 2007).

Increased PIP2 also induces recruitment of Axin to LRP5/6. This recruitment may be due, in part, to the action of Amer1/WTX (APC membrane recruitment 1 or Wilms tumor gene on the X chromosome), a tumor suppressor mutated in Wilms’ tumor that binds to Axin, CK1γ, and GSK3. Amer1/WTX is recruited to the plasma membrane in a PIP2-dependent manner (Major et al. 2007; Tanneberger et al. 2011).

The interaction between LRP6 and Axin is critical for activation of the Wnt pathway, and the recruitment of Axin and the associated destruction complex to the plasma membrane upon Wnt ligand binding initiates a chain of events that leads to the phosphorylation of the intracellular domain of LRP5/6. This initial recruitment of Axin to LRP6 in a Wnt–Fz-dependent manner is referred to as the “initiation step” of Wnt pathway activation (Baig-Lewis et al. 2007).

The LRP5/6 receptor contains five PPPSPxS motifs on its intracellular domain that are required for signal transmission. Each of these five motifs alone can activate the Wnt/β-catenin pathway: when transferred to heterologous receptors, the PPPSPxS motif is sufficient for pathway activation (Tamai et al. 2004; Zeng et al. 2005). Mutational analyses of these motifs indicate that they act in a cooperative manner to mediate downstream signaling (MacDonald et al. 2008; Wolf et al. 2008).

The predominant kinases involved in PPPSPxS phosphorylation have been identified as GSK3 and CK1 (Davidson et al. 2005; Zeng et al. 2005). Wnt binding to LRP5/6 has been shown to induce PPPSP phosphorylation by GSK3, and this event primes LRP6 for subsequent xS phosphorylation by CK1 (Zeng et al. 2005; Pan et al. 2008). Another study, however, suggests that CK1 phosphorylates conserved S/T clusters outside the PPPSPxS motif, and this phosphorylation primes phosphorylation of LRP6 by GSK3 (Davidson et al. 2005). Phosphorylated LRP6 has a high affinity for Axin and promotes further recruitment of cytoplasmic Axin-bound GSK3 complexes to the cell surface (Mao et al. 2001; Zeng et al. 2008). The recruitment of additional Axin-bound GSK3 complexes further promotes the phosphorylation of additional LRP5/6 PPPSP motifs in a positive feedback mechanism and has been referred to as the "amplification step" in Wnt pathway activation (Baig-Lewis et al. 2007). Once the Axin-bound β-catenin destruction complex is recruited by LRP6, the phosphorylated cytoplasmic domain of LRP6 is capable of directly inhibiting GSK3 activity, blocking β-catenin phosphorylation and subsequent ubiquitin-mediated proteasomal degradation (Cselenyi et al. 2008; Piao et al. 2008). The capacity of phosphorylated LRP6 to limit GSK3 activity by direct inhibition appears to limit the capacity of Axin-bound GSK3 to promote further LRP6 phosphorylation (amplification step). It is possible that these two events are temporally regulated such that direct inhibition of GSK3 may be blocked during the amplification step.

The cytoplasmic adaptor Dvl

Dishevelled (Dvl/Dsh) has long been known to be required genetically in the Wnt/β-catenin pathway (Klingensmith et al. 1996). In vertebrates, there are three Dsh isoforms encoded by distinct genes (Dvl1–3) (Sussman et al. 1994; Semënov and Snyder 1997). Upon Wnt–receptor interaction, Dsh is phosphorylated and recruited to the cytoplasmic side of the receptor complex (Yanagawa et al. 1995; Semënov and Snyder 1997; Rothbächer et al. 2000). Several studies suggest that physical interaction between the Fz receptor and Dvl is important for transduction of the Wnt signal. Dvl contains three major domains: the DEP, the PDZ, and the DIX domains. Biochemical and structural studies have implicated both the PDZ and the DIX domains of Dsh in binding to the Fz receptor (Wong et al. 2000; Wong et al. 2003; Tauriello et al. 2012). Dvl phosphorylation upon Wnt signaling appears to be independent of LRP6 activation (González-Sancho et al. 2004). Dvl and Axin share DIX domains that can polymerize and are required for receptor clustering (Schwarz-Romond et al. 2007). Loss-of-function studies show that Dvl acts upstream of LRP6 (Tolwinski et al. 2003). Consistent with this observation, Dsh has been shown to bind and activate PI4KIIa and PIP5KI to promote the synthesis of PIP2, which is required to promote oligomerization and clustering of LRP5/6 (Pan et al. 2008). In overexpression studies in Drosophila and Xenopus egg extracts, however, Dsh has been shown to activate β-catenin signaling independently of Arrow/LRP6 (Salic et al. 2000). The precise involvement of Dsh in receptor activation and Axin recruitment remains to be determined.

Both the stability and the activity of Dsh appear to be regulated by ubiquitylation. Three ubiquitin ligases, NEDL1 and ITCH of the HECT-type ligase and KLHL12 of the

Cullin3-type ligase, have been implicated in ubiquitylating Dvl to promote its degradation (Miyazaki et al. 2004; Angers et al. 2006; Wei et al. 2012). One study implicates the Naked2 protein as a necessary co-factor for Dvl ubiquitylation (Hu et al. 2010). Finally, the deubiquitinating enzyme, CYLD (encoded by the familial cylindromatosis tumor suppressor gene), has been shown to be a negative regulator of Wnt signaling (Tauriello et al. 2010). CYLD was shown to remove a regulatory Lys63-linked ubiquitin from Dvl. Thus, ubiquitylation of Dvl via Lys63 linkages appears to be necessary for efficient activation of signaling by Dvl. The ubiquitin ligase that mediates Lys63-linked ubiquitylation of Dvl, however, is still unknown.

Inhibition of the β-catenin destruction complex

The β-catenin destruction complex is a macromolecular machine that efficiently acts to phosphorylate β-catenin, targeting it for degradation. We will first describe the players involved in the formation of the β-catenin destruction complex (Figure 1.1) and follow with our current understanding of the behavior of the pathway upon receptor activation.

The transcriptional regulator β-catenin

β-Catenin is the primary effector of Wnt signaling. In the absence of signaling, the destruction complex targets β-catenin for ubiquitin-mediated proteasome degradation by $SCF^{\beta\text{-}TRCP}$, a member of the Skp1-Cullin-F-box (SCF) E3 ubiquitin ligase complex. In the presence of signaling, β-catenin is spared destruction and translocates

from the cytoplasm to the nucleus to activate signaling. β-Catenin was first identified in *Drosophila* as the segment polarity gene armadillo and also as a component of the adherens junction in *Xenopus* (Nüsslein-Volhard and Wieschaus 1980; McCrea et al. 1991). The structure of β-catenin consists of a central core of 12 helical 42 amino acid armadillo repeats that form a superhelical structure (Huber et al. 1997). Analysis of full-length β-catenin protein indicates that the N- and C-terminal domains are unstructured and form dynamic interactions with the armadillo repeats of the protein (Xing et al. 2008). Notably, the armadillo repeats form a positively charged groove that mediates the interaction of β-catenin with other components of the Wnt pathway [e.g. APC, Axin, and T-cell factor (TCF)/LEF] as well as with E-cadherin (Huber et al. 1997; Graham et al. 2000; Xing et al. 2003; Xing et al. 2004).

The cellular factors that coordinate whether nascent β-catenin mediates Wnt target gene transcription or plays a structural role in maintaining integrity of the adherens junction are not completely understood. A large number of studies using a variety of model systems have shown that overexpression of cadherins is sufficient to inhibit Wnt target gene transcription and to promote relocalization of β-catenin to the membrane (Heasman et al. 1994; Sadot et al. 1998; Gottardi et al. 2001; Stockinger et al. 2001). Furthermore, evidence to support potential influence of cadherins on Wnt signaling comes from studies demonstrating that proteolytic cleavage of cadherins by proteases such as ADAM10 and presenilin-1/γ-secretase is sufficient to release bound β-catenin, to increase soluble cytoplasmic β-catenin, and to activate Wnt target gene transcription (Marambaud et al. 2002; Maretzky et al. 2005; Reiss et al. 2005). Multiple studies in which E-cadherin is knocked down in cells with wild-type Wnt pathway

components, however, fail to demonstrate activation of Wnt signaling, suggesting a lack of a significant interaction between cadherin-mediated cell adhesion and Wnt signaling, compensatory regulation of β-catenin levels with E-cadherin, or that turnover of β-catenin by the degradation complex in the wild-type situation is capable of compensating for the increased flux of β-catenin (Kuphal and Behrens 2006; Herzig et al. 2007). Support for the existence of distinct pools of β-catenin comes from a study demonstrating that β-catenin can exist as a monomeric or dimeric form bound to α-catenin (Gottardi and Gumbiner 2004). Biochemical studies indicate that the monomeric form preferentially participates in Wnt signaling, whereas the dimeric form preferentially binds cadherins. Surprisingly, little is known about the mechanism of β-catenin nuclear translocation.

The scaffold protein Axin, the limiting component of the destruction complex

The scaffold protein Axin is a critical component of the β-catenin destruction complex, acting as a limiting negative regulator of Wnt/β-catenin signaling. It was first identified as the gene product of the locus *fused* in mice (Zeng et al. 1997). Axin plays a role as a scaffold protein that directly binds to many of the other components of the destruction complex and brings them within close proximity to each other (Figure 1.1). The sites of interactions have been visualized in co-crystal structures of Axin and APC proteins (Spink et al. 2000), Axin and β-catenin (Xing et al. 2003), and Axin and GSK3β (Dajani et al. 2003). Studies of Axin in fly embryos suggest that Axin complexes may form oligomers in vivo, and that Axin may also act as a cytoplasmic anchor to restrict armadillo/β-catenin import into the nucleus (Tolwinski and Wieschaus 2001; Peterson-

Nedry et al. 2008). Axin was initially found to be present at low concentrations and is the limiting component of the β-catenin degradation complex in *Xenopus* (Lee et al. 2003). A long-standing puzzle about Wnt signaling is how it maintains specificity because many Wnt components also play biological roles in other cellular processes; for example, GSK3 is a node for many types of cell signaling (Forde and Dale 2007). The low concentration of Axin has been proposed to isolate the Wnt pathway from affecting other intracellular pathways (Lee et al. 2003). The low level of intracellular Axin is due, in part, to its ubiquitin-mediated turnover that is promoted by LRP5/6 (Yamamoto et al. 1999; Cselenyi et al. 2008). Degradation of Axin has been shown to be regulated by GSK3 phosphorylation, which inhibits its rate of degradation (Yamamoto et al. 1999). In addition, the turnover of Axin requires the tumor suppressor APC, and studies in Xenopus egg extract, as well as in flies, suggest that this may represent a mechanism to compensate for fluctuations in levels of APC in order to maintain low levels of β-catenin in cells (Lee et al. 2003).

Smad ubiquitin regulatory factor 2 (Smurf2) has been shown to be an E3 ubiquitin ligase that targets Axin for degradation (Kim and Jho 2010). Due to its key role in Wnt signaling, it is likely that Axin is tightly regulated. Tankyrase has been shown to promote poly ADP-ribosylation (PARsylation) and ubiquitylation and further degradation of Axin through the addition of polyADP-ribose moieties onto proteins through PARsylation (Huang et al. 2009a). The importance of Axin turnover is demonstrated by the identification of tankyrase inhibitors IWR-1 and XAV939 that have been shown to potently inhibit Wnt signaling by increasing the steady-state level of Axin (Chen et al. 2009; Huang et al. 2009). These tankyrase inhibitors prevent PARsylation of Axin and

thus reduce Axin turnover. RNF146 has been identified as the polyADP-ribose-directed E3 ubiquitin ligase that ubiquitylates Axin (Callow et al. 2011; Zhang et al. 2011). RNF146 binds directly to the covalently linked poly(ADP-ribose), targeting Axin for degradation and maintaining low steady-state levels of Axin. A deubiquitinating enzyme, ubiquitin-specific protease (USP) 34, has been identified to catalyze the deubiquitylation of Axin and increase steady-state levels of Axin in cells (Lui et al. 2011). In contrast to PARsylation, SUMOylation of Axin at its C-terminus has been shown to confer stability by inhibiting Axin ubiquitylation (Kim et al. 2008). Recently, quantitative measurements of Axin concentration in a variety of mammalian cells suggest that its levels vary significantly to alter the dynamics of Wnt signaling (Tan et al. 2012). Thus, the regulation of Axin levels and stability may be a major mechanism by which cells control the response to Wnt signals.

The kinase GSK3

GSK3 is a ubiquitous serine/threonine protein kinase involved in numerous cellular processes (Forde and Dale 2007). Antagonizing GSK3 activity is central to all models of Wnt signaling mechanisms. The homolog of GSK3 in Drosophila is *shaggy*, aka *zeste white 3* (Siegfried et al. 1992). In mammals, there are two distinct genes, α and β, that are likely to have redundant functions in the Wnt pathway (Doble et al. 2007). GSK3 was first identified for its role in the regulation of glucose metabolism, targeting muscle glycogen synthase (Embi et al. 1980). GSK3 has both positive and negative roles in Wnt signal transduction, which will be described in further detail later in this review. GSK3 often recognizes substrates that have been previously

phosphorylated (primed), and thus GSK3 is often found to act in concert with other kinases. β-Catenin phosphorylation by GSK3 (at Ser33, Ser37, and Thr41) leads to its ubiquitin-mediated degradation (Peifer et al. 1994; Yost et al. 1996). The crystal structure of GSKβ has been solved and conforms to a typical protein kinase bilobed structure topology consisting of an amino-terminal β-sheet domain linked to a carboxy-terminal α-helical domain. Similar to other activated kinases, the structure of unphosphorylated GSK3β shows an activation loop that is responsible for its unique priming mechanism (Haar et al. 2001). Furthermore, phosphorylation of Ser9, which has been shown to inhibit GSK3 activity, is predicted to act in an auto-inhibitory fashion by blocking access to the catalytic site (Dajani et al. 2001). In addition to β-catenin, other major Wnt pathway substrates of GSK3 include APC, Axin, and LRP6 (Rubinfeld et al. 1996; Willert et al. 1999; Zeng et al. 2005).

The kinase CK1α

The CK1 family of kinases is comprised of a group of serine/threonine kinases encoded by seven distinct genes in mammals (α, β, γ1, γ2, γ3, δ, and ε; although β was identified in bovine and has not been found in humans) (Knippschild et al. 2005). As with GSK3, CK1 is a widely-expressed family of kinases with a large number of substrates. All CK1 members have highly similar catalytic domains, but differ significantly in both the length and the sequence of their C-terminal non-catalytic domains. CK1α, with its short (~24 amino acid) C-terminal domain, appears to be an outlier compared with the other family members, which have much longer C-terminal tails (~200 amino acids). CK1α, γ, δ, and ε have been implicated in positively regulating

the Wnt pathway by phosphorylating Dsh, LRP5, TCF/LEF, and Axin (Peters et al. 1999; Kishida et al. 2001; Cong et al. 2004; Zeng et al. 2005). In contrast, CK1 family members have also been implicated as negative regulators of the Wnt pathway by phosphorylating β-catenin, APC, Axin, and TCF/LEF (Kishida et al. 2001; Gao et al. 2002; Hämmerlein et al. 2005). CK1α has been proposed to be the *in vivo* priming kinase for GSK3 and phosphorylates β-catenin at Ser45 (Liu et al. 2002). The activation of CK1α has emerged as a potential therapeutic drug target, and a recent study reported that the antihelminthic drug, pyrvinium, inhibits Wnt signaling by activating CK1α to enhance β-catenin phosphorylation and degradation (Thorne et al. 2010).

β-catenin degradation cycle

The ubiquitous expression of β-catenin and other pathway members suggests that all metazoan cells express the β-catenin destruction complex. Although commonly described as having cytoplasmic localization, the core components can also be found in the nucleus where the β-catenin destruction complex is likely to reside and function (Cong and Varmus 2004; Wiechens et al. 2004; Sierra et al. 2006). This machine is in a constitutively active state and contains a number of enzymes that target β-catenin for degradation. Thus, in the absence of a Wnt signal, β-catenin is caught in a cycle of synthesis followed by rapid destruction. Axin is the scaffold protein that nucleates the formation of the β-catenin destruction complex. It binds with high affinity to the two kinases GSK3 and CK1α. A well-defined α-helix in the central portion of Axin anchors GSK3, whereas the site of CK1 binding has been mapped to a more C-terminal region of Axin (Dajani et al. 2003; Sobrado et al. 2005). APC binds the RGS domain of Axin,

an N-terminal region that has structural homology to domains found in regulators of G-protein signaling (Spink et al. 2000). β-Catenin enters the complex by binding both 15 amino acid repeats of APC and a single α-helix on Axin on the C-terminal side of the GSK3 binding site. The exact order and kinetics of binding are unknown, and it is unclear whether this binding is ordered or stochastic (Lee et al. 2003).

Evidence suggests that phosphorylation of Axin, likely by GSK3, increases its affinity for β-catenin (Willert et al. 1999). Upon binding to Axin, the N-terminal region of β-catenin becomes positioned for rapid phosphorylation by CK1 at serine 45. This creates a priming site for subsequent and successive phosphorylation of β-catenin by GSK3 at Thre41, Ser37, and Ser33 (Amit et al. 2002; Liu et al. 2002). Twenty amino acid repeats on APC are also phosphorylated by CK1 and GSK3 (Ha et al. 2004). This phosphorylation increases the affinity of the 20 amino acid repeats for β-catenin by 140-fold and competes β-catenin off of the α-helix binding site on Axin. Based on these observations, it has been proposed that APC phosphorylation triggers β-catenin removal from Axin, allowing a new β-catenin species to enter the destruction complex (Kimelman and Xu 2006). The action of APC is also thought to prevent the action of the phosphatase, PP2A, from acting on the phosphorylated β-catenin (Su et al. 2008). Once β-catenin is phosphorylated at Ser33 and Ser37, a destruction consensus sequence is recognized by β-TRCP, a specificity subunit of the SCF ubiquitin ligase (Jiang and Struhl 1998; Kitagawa et al. 1999; Liu et al. 1999). Binding of SCFβ-TRCP to β-catenin catalyzes its polyubiquitylation (via K48 linkages) and subsequent degradation through the proteasome. These events ensure that newly synthesized, free cytosolic levels of β-catenin are kept below the threshold necessary for gene regulation.

Pathway behavior on activation

Elevation of β-catenin levels in response to the presence of Wnt is a hallmark of the Wnt/β-catenin pathway. The precise mechanism of destruction complex inhibition is under intense investigation and a number of proposed mechanisms exist. At the core of all the current models is the inhibition of GSK3 anti-catenin activity. This has been proposed to arise through several distinct mechanisms: (1) dissociation of components of the β-catenin destruction complex, (2) phosphorylation of GSK3 at Ser9, which inhibits its activity, (3) LRP6 binding and direct inhibition of GSK3 activity against β-catenin, and (4) Axin degradation upon activation of signaling, which prevents formation of the destruction complex necessary for GSK3 to phosphorylate β-catenin. We discuss these models individually below.

Several studies have described Axin–GSK3 dissociation upon signaling (Liu et al. 2002; Luo et al. 2007). Early studies seem to suggest that Dvl recruits GSK3 binding protein (GBP) to Axin where GSK3 is directly inhibited and dissociates from Axin (Yost et al. 1998; Farr et al. 2000). One caveat to these studies is the apparent lack of requirement for GBP in Wnt signaling in *Drosophila* and mice. Drosophila does not have a GBP ortholog, and genetic knockout studies in mice show no requirement for GBP in development or Wnt signaling (van Amerongen et al. 2005). In contrast to these studies, other studies have shown that the complex remains intact and localizes to the Fz/LRP6 co-receptors rapidly after Wnt stimulation (Mao et al. 2001; Bilić et al. 2007).

GSK3α/β is phosphorylated at Ser9 and Ser21, and when phosphorylated at these sites, kinase activity is greatly reduced (Sutherland et al. 1993; Cross et al. 1995). Wnt-induced stimulation of Ser9/21 phosphorylation has been speculated about by

several groups (Ding et al. 2000; Yokoyama and Malbon 2007). However, studies in mice in which non-phosphorylatable forms of GSK3α/β were knocked-in produced animals with no developmental defects and no observable perturbation in Wnt pathway activation (McManus et al. 2005).

A number of mammalian cell culture and biochemical studies suggest that the β-catenin destruction complex moves to the cell surface upon Wnt stimulation, and this migration is followed by the direct inhibition of GSK3 by LRP6 (Yamamoto et al. 2006; Bilić et al. 2007; Cselenyi et al. 2008). The collective evidence from these studies suggests a mechanism in which ligand-stimulated LRP6 phosphorylation by GSK3 and CK1 creates a docking site for Axin. This phosphorylation event in turn recruits the Axin–GSK3 complex to the receptor. Recruitment of Axin–GSK3 to phosphorylated LRP6 has been proposed to be facilitated in part by Dsh, which binds directly to the Fz receptor and Axin (Schwarz-Romond et al. 2007). A problem for this model, however, is the requirement for GSK3 to phosphorylate the PPPSPxS motifs on LRP5/6 to create docking sites for Axin–GSK3 (amplification step) and the fact that phosphorylated PPPSPxS motifs can directly inhibit GSK3 activity, further limiting LRP6 phosphorylation. High-resolution structural data are essential to clarify this model and to explain how PPPSPxS motifs are sufficient for both Axin docking and GSK3 inhibition.

A compendium of studies from cell culture, *Xenopus* embryos and egg extracts, and *Drosophila* suggest that a conserved and critical event is the degradation of Axin upon Wnt signaling (Yamamoto et al. 1999; Tolwinski et al. 2003; Cselenyi et al. 2008). Because Axin is a concentration-limiting factor, regulation of Axin stability is likely to have a dramatic effect on signaling (Lee et al. 2003). Genetic studies in *Drosophila*

designed to elucidate the requirement of Axin regulation show that flies genetically null for GSK3 have elevated levels of β-catenin as expected, yet unexpectedly they still have near wild-type Wg-based embryonic patterning that is dependent on dynamic levels of Axin. These results indicate that regulation of Axin stability is sufficient to pattern the embryo, as β-catenin nuclear translocation only occurs when Axin levels are low, and Axin may act as a cytoplasmic anchor to regulate β-catenin-mediated transcription (Tolwinski et al. 2003). Other work has shown that Axin degradation appears to lag behind β-catenin stabilization and is not necessary for β-catenin stabilization (Willert et al. 1999; Hino et al. 2005; Cselenyi et al. 2008). These studies promote the model that the block in β-catenin destruction that occurs upon Wnt signaling does not require Axin turnover, and that degradation of Axin is a distinct event that may modulate the character of the response.

Activation of a Wnt-specific nuclear transcriptional complex

Nuclear translocation of β-catenin

β-catenin is normally constitutively transcribed and translated. Thus, a signal-induced block in proteolysis leads to rapid rise in cytosolic β-catenin protein levels upon receptor activation. In addition to increased cytosolic accumulation, β-catenin also accumulates in the nucleus. Remarkably, studies indicate that it is not the absolute increase in concentration of β-catenin that results in activation of a Wnt transcriptional program (Goentoro and Kirschner 2009). Rather, it is the relative fold change (~2 ×) in the concentration of β-catenin that is recognized by the Wnt transcriptional machinery.

Such a mechanism is expected to minimize cell–cell variation caused by fluctuations in the basal level of β-catenin as well as allowing for activation of the transcriptional program when the signal is sufficiently elevated in proportion to the background noise (Goentoro et al. 2009).

β-catenin does not contain any recognizable nuclear localization signal (NLS) or nuclear export signal (NES). Nuclear accumulation of β-catenin has been attributed to its cytoplasmic retention, nuclear retention, and nuclear export. Nuclear entry of β-catenin is thought to be independent of classic import factors (e.g. RanGTPase and importins) (Fagotto et al. 1998; Yokoya et al. 1999). The armadillo repeats of β-catenin, however, are structurally related to importin-β HEAT repeats and may interact directly with the nuclear pore complex during nuclear entry (Kutay et al. 1997; Malik et al. 1997). Surprisingly, deletion of β-catenin armadillo repeats 3–6 in Drosophila, which presumably disrupts binding to major cytoplasmic Wnt components, results in constitutive nuclear localization of the armadillo/β-catenin mutant (Orsulic and Peifer 1996). Based on this result, it has been suggested that β-catenin is normally regulated by its cytoplasmic retention. Consistent with this data, both cadherins and Axin have been shown to sequester β-catenin in the plasma membrane and cytoplasm, respectively (Heasman et al. 1994; Sadot et al. 1998; Gottardi et al. 2001; Tolwinski and Wieschaus 2001). The nuclear proteins TCF, Pygopus, and BCL9 (described below) have been proposed to similarly act as anchors for β-catenin in the nucleus (Townsley et al. 2004; Krieghoff et al. 2006). It has been proposed that nuclear export of β-catenin may play a critical role in Wnt signaling, and Axin, APC, and RanBP3 have been implicated in regulating the export of β-catenin from the nucleus (Henderson and

Fagotto 2002; Cong and Varmus 2004). Finally, Rac1 GTPase and Jun N-terminal kinase 2 (JNK2) have also been shown to promote β-catenin nuclear localization upon Wnt signaling, although the mechanism by which this is accomplished is unclear (Wu et al. 2008).

β-catenin-mediated gene transcription

Wnt-induced nuclear β-catenin accumulation leads to an interaction with the TCF/LEF family of DNA-bound transcription factors that are critical for Wnt-mediated gene regulation (Behrens et al. 1996; Molenaar et al. 1996). Invertebrates appear to have only one TCF gene, whereas mammals have four: TCF1, LEF1, TCF3, and TCF4. In the absence of β-catenin, TCF interacts with the co-repressor Groucho/transducin-like enhancer (Gro/TLE1–3 in vertebrates) to repress gene transcription. TCF binds at the DNA consensus sequence CCTTTGWW (W can be either T or A), termed the Wnt-responsive element (WRE). It has been predicted that there are greater than 6000 high-confidence WREs in at least one studied colorectal cancer cell line, and these WREs collectively regulate 300–400 genes (Hatzis et al. 2008).

Several studies suggest that TCF proteins are phosphorylated to regulate Wnt signaling. CK1 has been shown to phosphorylate TCF to positively and negatively regulate its interaction with β-catenin (Lee et al. 2001; Hämmerlein et al. 2005). The Nemo-like kinase (NLK) has been shown to phosphorylate TCF and inhibit Wnt signaling by reducing the affinity of β-catenin–TCF for DNA (Ishitani et al. 2003; Smit et al. 2004). Finally, phosphorylation of LEF-1, TCF4, and TCF3 by the homeodomain-

interacting protein kinase 2 has been shown to promote their dissociation from Wnt target gene promoter (Hikasa et al. 2010; Hikasa and Sokol 2011). TCF proteins are also modified by ubiquitylation. The NLK-associated RING finger protein promotes the ubiquitylation of TCF/LEF, targeting it for degradation in a manner dependent on NLK activity (Yamada et al. 2006). Similarly, the deubiquitinase USP4, which deubiquitylates K48 and K63 ubiquitin linkages, has been shown to act on TCF4 to inhibit Wnt signaling (Zhao et al. 2009). In the canonical model of Wnt target gene activation, displacement of Gro/TLE by β-catenin converts TCF/LEF into a transcriptional activator. The X-linked inhibitor of apoptosis has been shown to monoubiquitinate Gro/TLE, decreasing its affinity for TCF/LEF and allowing for unrestricted binding of β-catenin to TCF/LEF (Hanson et al. 2012).

Two notable nuclear co-factors found in Drosophila and vertebrates, Pygopus (Pygo) and BCL9, have been shown to facilitate Wnt pathway-mediated transcription (Belenkaya et al. 2002; Thompson et al. 2002). BCL9 binds and bridges β-catenin with the Pygo protein. Pygo has been shown to bind the multiprotein transcriptional co-activator mediator complex and contains a plant homology domain that interacts with dimethylated histone 3 lysine 4, an indicator of transcriptional activation (Fiedler et al. 2008). Studies in Drosophila indicate that TCF, β-catenin, BCL9, and Pygo represent a core transcriptional complex that is necessary for Wnt-mediated gene transcription. In mammals, the situation is more complex, and there appears to be functional redundancy and cell type-specific roles for BCL9 and Pygo (Schwab et al. 2007; Sustmann et al. 2008).

It is important to note an often-underappreciated role for TCF/β-catenin signaling in transcriptional repression. Studies have shown a number of potential mechanisms of repression, including competition of TCF/β-catenin with transcriptional activators, recruitment of co-repressors to WREs, or TCF binding to a novel consensus sequence that specifically mediates repression (Jamora et al. 2003; Theisen et al. 2007; Blauwkamp et al. 2008). In addition, there are multiple studies showing that many DNA-binding transcription factors (e.g. Smad3, AP-1, RXR, and Kaiso) bind to β-catenin to activate or repress Wnt/β-catenin target genes. In light of the large number of TCF binding sites and numerous transcriptional co-regulators, it is clear that the Wnt/β-catenin gene expression program is vast and induces dramatic changes in the physiological state of the cell, many of which are still not well understood (Cadigan 2012). Thus, Wnt signaling leads to a transcriptional program that includes downregulation and upregulation, rather than to simply transcriptional activation.

The scaffold protein APC

APC is a scaffold protein consisting of 2843 amino acids with a mass of approximately 310 kDa, and it acts as a negative regulator of Wnt/β-catenin signaling. Vogelstein and colleagues first identified the gene in 1991 as the site of a mutation found in FAP, a familial form of colon cancer (Kinzler et al. 1991; Nishisho et al. 1991). APC plays diverse roles in cellular functions, including Wnt signaling, migration, mitotic spindle alignment, and apoptosis, which are likely carried out through different APC subpopulations (Faux et al. 2008). The C-terminal third of APC contains a region involved in microtubule binding that interacts with the proteins EB1 and Discs large (Su

et al. 1995; Matsumine et al. 1996). These regions have been demonstrated to be involved in microtubule dynamics in mitosis and cell migration and are thought to be independent of the role of APC in Wnt signaling (Näthke et al. 1996). The connection between APC and Wnt signaling was identified in a series of studies that showed that APC binds to β-catenin, and that mutations in APC caused elevated levels of β-catenin in cancer cells (Rubinfeld et al. 1993; Su et al. 1993). APC binds β-catenin, GSK3, and Axin in several regions within the central portion of the protein (Rubinfeld et al. 1996; Ikeda et al. 1998; Fagotto 1999). Despite numerous studies published on the function of APC, a clear mechanistic picture of the role of APC in regulating the Wnt pathway remains elusive. Indeed, loss of APC leading to elevated β-catenin levels and activation of the Wnt pathway can be overcome by overexpression of Axin, which is the limiting component of the β-catenin destruction complex (Lee et al. 2003). Furthermore, overexpression of an Axin mutant lacking its APC binding domain (RGS) is capable of promoting β-catenin degradation and inhibiting Wnt signaling to a similar extent as overexpression of wild-type Axin (Hart et al. 1998).

How APC acts as a negative regulator of Wnt/β-catenin signaling is something of a mystery. Several models have been proposed, including (1) exporting β-catenin from the nucleus (Hamada and Bienz 2004), (2) repressing Wnt target genes (Sierra et al. 2006), (3) retaining β-catenin in the cytoplasm (Tolwinski and Wieschaus 2001), (4) targeting the β-catenin destruction complex to the cell cortex, where its E3 ubiquitin ligase ($SCF^{\beta\text{-}TRCP}$) resides (Näthke et al. 1996; McCartney et al. 1999), (5) coordinating the phosphorylation and release of β-catenin from the destruction complex to allow its ubiquitylation (Kimelman and Xu 2006), (6) blocking dephosphorylation of β-catenin by

protein phosphatase 2A (PP2A) (Su et al. 2008), and (7) shielding the β-catenin degradation complex from the inhibitory action of Dvl (Mendoza-Topaz et al. 2011).

None of the proposed models for the role of APC in the Wnt pathway are mutually exclusive, although the most compelling experimental evidence (4–6) strongly supports APC's role in negatively regulating steady-state levels of cytoplasmic β-catenin. It is likely that APC, similar to other components of the Wnt pathway (e.g. GSK3 and CK1), may participate in multiple events in the Wnt signaling pathway.

Several kinases, including CK1, protein kinase A (PKA), and GSK3, have been shown to phosphorylate APC (Rubinfeld et al. 1996; Morin et al. 1997). Phosphorylation of APC by GSK3 was shown to enhance binding of β-catenin by APC (Salic et al. 2000). In addition to phosphorylation, the APC protein is ubiquitinated. In cells, APC is stabilized by the COP9 signalosome-associated deubiquitinase, USP15, which binds to the β-catenin degradation complex (Huang et al. 2009). The deubiquitinase Trabid, which removes K63-linked regulatory ubiquitylation chains from APC, has been identified as a positive regulator of Wnt signaling (Tran et al. 2008). Although it is not clear if APC is the sole target of Trabid, this finding is consistent with the suggestion that K63-linked ubiquitylation of APC antagonizes Wnt signaling, presumably by potentiating APC activity. The E3 ligase that mediates ubiquitylation of APC and the exact mechanism by which these K63-linked ubiquitin chains regulate APC activity are unknown.

The interaction between cells and their extracellular environment

The outer layer of the plasma membrane is the surface from which cells communicate with their environment. Among the many membrane components, there are membrane receptors which are required to activate signaling pathways in response to environmental perturbations. In order to maintain homeostasis, cells often control the abundance of receptors at the surface of the plasma membrane via their internalization (thereby removing them from participation in signaling) through a process called “endocytosis” (Doherty and McMahon 2009; Sorkin and von Zastrow 2009).

Over 100 years ago, Ilya Metchnikoff made the first observation of cell internalization. In 1882, he reported that specific cells used vesicles to engulf and degrade pathogens. He called this process “phagocytosis” (Tauber 2003). In 1931, Warren Lewis observed that cells used similar vesicles to uptake fluids in a process he described as “pinocytosis”. In the following years, Christian de Duve discovered lysosomes and while studying their function coined the term “endocytosis” (Sabatini and Adesnik 2013). The development of electron microscopy (EM) allowed for further discoveries in the process of endocytosis and greatly advanced the field. In 1953, George Palade identified “cave-like” invaginations of the cell membrane, and named them “caveolae” (Latin for little caves). For their work, both de Duve and Palade received the Nobel Prize in 1974 (Nassoy and Lamaze 2012). Finally, in 1975 Joseph Goldstein, Michael Brown, and Richard Anderson showed that the low-density lipoprotein (LDL), binds to a cell surface receptor leading to formation of “coated pits” (Goldstein and Brown 2009). These pits are then internalized and the LDL receptor is endocytosed. Goldstein and Brown coined this process “receptor-mediated endocytosis”

and were awarded the Nobel Prize in 1985. During the same year, Barbara Pearse isolated and identified the major component of those pits as clathrin, giving rise to the term “clathrin-mediated endocytosis” (CME) (Robinson 2015).

Clathrin mediated endocytosis

Most of the early work performed to understand the process of endocytosis focused on CME. The main player involved in CME is the clathrin protein. Clathrin is a trimer composed of three 190kDa heavy chains bound to a 25kDa light chain which, together assemble a cage-like structure and form invaginations on the cytoplasmic side of the plasma membrane called clathrin-coated pits (CCP) (Figure 1.3) (Kirchhausen 2000; Takei and Haucke 2001; Schütze et al. 2008). Several adaptor proteins such as AP-1 through AP-5, ARH, Dab2, Fe65, AP180, Epsin, and Snx17 interact with clathrin and their specific cargo proteins (Traub and Bonifacino 2013). Of these clathrin-associated proteins, the best studied are the AP family of adaptors. AP-2 is the major AP family member required for trafficking at the plasma membrane and plays a pivotal role in clathrin-mediated endocytosis. AP-2 links clathrin to the membrane and coordinates the assembly of the clathrin coat. The AP-2 complex comprises two large subunits, α and β2, and two smaller subunits, μ2 and σ2 (Takei and Haucke 2001). The α subunit targets the AP-2 complex to the membrane by binding to the phospholipids, phosphatidyl inositol-4,5-bisphosphate (PIP2) and phosphatidyl inositol-3,4,5-trisphosphate (PIP3) (Chang et al. 1993; Gaidarov et al. 1996; Gaidarov and Keen 1999). The β2 and μ2 subunits recognize specific motifs on the cytoplasmic domain of

transmembrane receptors and provide cargo specificity to the CCPs (Ohno et al. 1995; Nesterov et al. 1999).

In CME, AP-2 associates with PIP2 at the plasma membrane and recruits clathrin molecules resulting in the formation of clathrin-coated buds. These clathrin-coated buds eventually form clathrin-coated vesicles (CCV), which are irreversibly released into the cytoplasm by a large GTPase called Dynamin. Inhibition of dynamin function by small molecules or a kinase-dead form of Dynamin prevents release of CCV and blocks endocytosis (Damke et al. 1994; Doherty and McMahon 2009; Kirchhausen et al. 2014). Clathrin is released from vesicles by Auxilin and Hsc70, and naked vesicles are transported (along with their cargo) to their designated cellular compartment (Doherty and McMahon 2009). Internalization of a variety of receptors are regulated by CME and include G-protein coupled receptors (GPCRs), the transferrin receptor, and the LDL receptor. Thus, CME plays a role in regulating homeostasis of a diverse number of receptors.

Caveolin-mediated endocytosis

Among the many clathrin-independent endocytic pathways, endocytosis by caveolae is the best characterized. Caveolae are pits 60-80 nm in diameter in the plasma membrane that are enriched for caveolins, sphingolipids, and cholesterol (Figure 1.3). Three caveolin isoforms are expressed in mammals, and expression of each one is cell type dependent. Caveolin-1 (Cav1, the most common isoform) and caveolin-2 (Cav2) are expressed in non-muscle cells, whereas caveolin-3 (Cav3) is present only in muscle

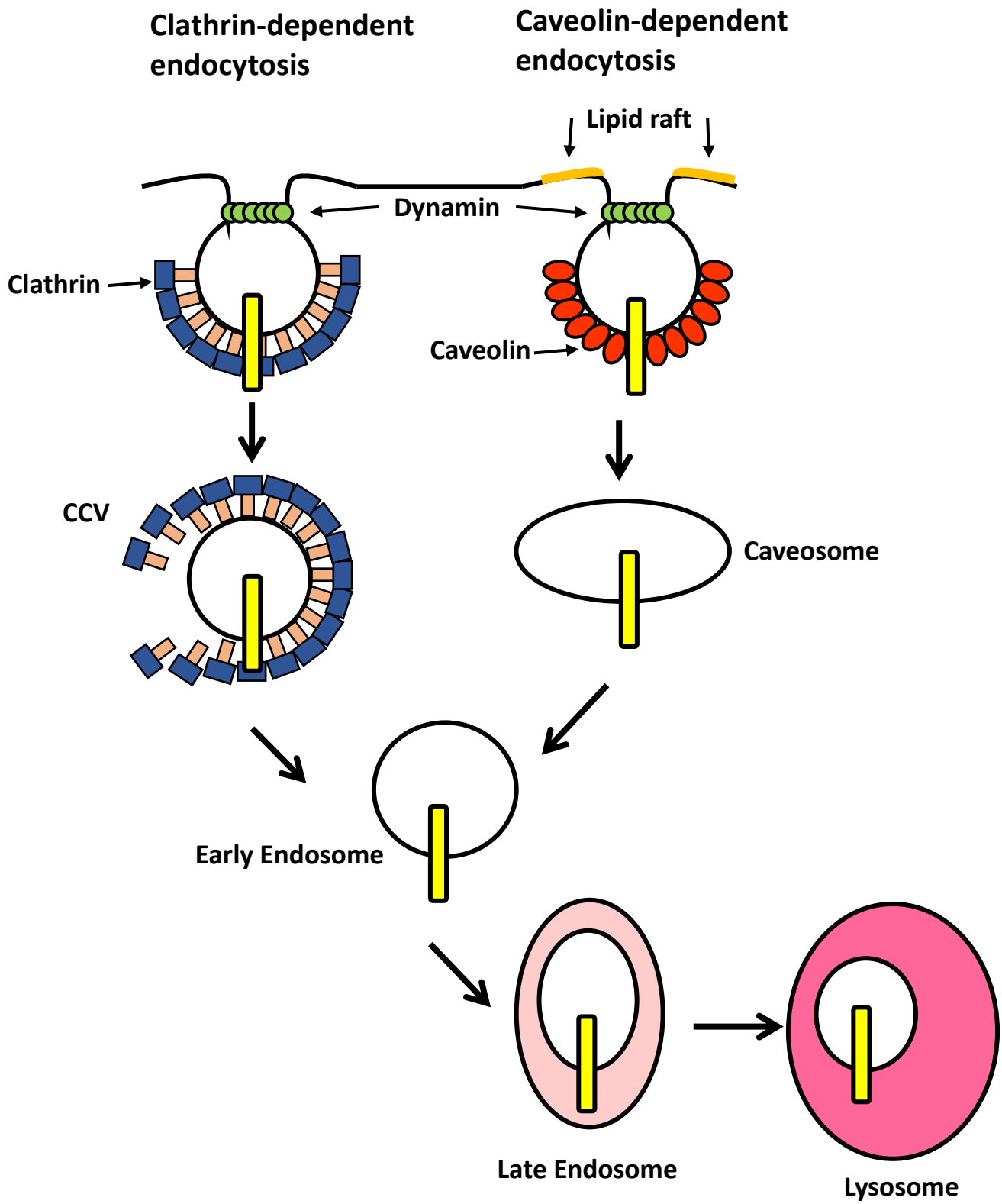


Figure 1.3. Schematics of major endocytic pathways in the cell.

cells (Nassoy and Lamaze 2012). Cav1 and Cav3 are required for caveolae formation (Drab et al. 2001; Galbiati et al. 2001). The requirement of Cav2 in caveolae formation is not clear because loss of Cav2 function has no observable effect *in vivo* (Razani et al. 2002; Sowa et al. 2003). All caveolin proteins encode cytoplasmic N- and C-terminal domains connected by an intramembrane hairpin. Caveolin palmitoylation at Cys133, Cys145, and Cys156 facilitate their binding to the plasma membrane (Dietzen et al. 1995; Parton and Simons 2007). Caveolins oligomerize and interact with cholesterol-rich domains in the plasma membrane known as lipid rafts (Murata et al. 1995). Interaction with lipid rafts is critical for caveolae formation, and it has been shown that cholesterol depletion increases Cav1 motility and disrupts the function of caveolae (Rothberg et al. 1992).

Once caveolae are released from the plasma membrane by the GTPase, Dynamin (Henley et al. 1998), they can either fuse with the early endosome in a process mediated by Rab5, or fuse with a large non-endosomal organelle (caveosome) in a Rab5-independent manner. The caveolar vesicles are then recycled back to the membrane for reuse (Razani et al. 2002; Pelkmans et al. 2004). Two early studies identified a large number of proteins involved in signal transduction within caveolae (Sargiacomo et al. 1993; Lisanti et al. 1994). This observation, led to formation of the "caveolae/raft hypothesis", that proposed that localization of signaling molecules in a compartment could facilitate cross-talk between signaling pathways. Although the molecules found in caveolae do not exhibit a common feature that specifically targets them to caveolae, they all undergo lipid modification. Indeed, many signal transduction proteins found in caveolae (e.g., the Epidermal Growth Factor Receptor (EGFR), H-

Ras, Src family of tyrosine kinases, and α subunit of heterotrimeric G-proteins) have one or more myristoyl, palmitoyl, or prenyl groups (Platta and Stenmark 2011).

Endocytosis and signaling

Although receptor internalization is often thought of being associated with a decrease in sensitivity to stimulus by controlling the number of receptors available at the plasma membrane, it also promotes signal propagation within the cell. Upon their activation, receptors typically undergo post-translational modifications, which promote recruitment of proteins required for signaling and/or endocytosis (Sorkin and von Zastrow 2009). Internalized receptors are subsequently sorted to specific compartments depending on the requirement of the cell. For example, if decreased signaling is needed, receptors are sorted to late endosomes and lysosomes for degradation. If further signaling is required, receptors are shuttled back to the plasma membrane (Platta and Stenmark 2011).

The prevailing model for receptor endocytosis proposes that receptors of essentially all major signaling pathways undergo CME. On other hand, certain receptors (e.g., some GPCRs, Transforming Growth Factor β (TGFβ) receptors, Wnt receptors, and Notch) undergo clathrin-independent endocytosis such as caveolin-mediated endocytosis (Sorkin and von Zastrow 2009). The concentration of ligands (e.g., EGF) at the plasma membrane modulate whether the signaling is regulated by clathrin-or caveolin-mediated endocytosis (Platta and Stenmark 2011). Because both clathrin- and

caveolin-mediated endocytosis have a direct impact on the qualitative behavior of the output, preference for one or the other may depend on the type of response required.

Upon CME, receptors are sorted to early endosomes, where they continue to interact with downstream molecules to propagate the response in order to maintain proper signal duration and intensity (Sigismund et al. 2008). EGFR has been the most extensively studied and exemplifies this mode of action. Stimulation of cells with EGF, result in rapid clustering of EGFR in CCPs and CCVs. Recruitment of downstream effectors such as GRB2, SH2 domain-containing transforming protein (SHC), Ras, and MAP kinase, occur at the plasma membrane and maintain their interactions in early endosomes (Vieira et al. 1996; Sorkin and von Zastrow 2009).

Some signaling complexes are exclusively sorted into early endosomes where the signaling output is modulated. The lipid composition of the early endosomal membrane, which is rich in phosphatidylinositol-3-phosphate (PIP3), define their specificity (Funderburk et al. 2010). This phenomenon occurs in TGFβ signaling where the TGFβ receptor associates with the PIP3-binding proteins, SARA and endofin. SARA binds to internalized TGFβ-receptor and SMAD2 and facilitates the phosphorylation of the latter. Phosphorylated SMAD2 interacts with SMAD4, and the resultant complex translocates to the nucleus to promote gene transcription. Similarly, endofin interacts with TGFβ-receptor to promote assembly of the SMAD2-SMAD4 complex (Chen et al. 2007).

Change in phospholipid composition in early endosomes can also result in the disassembly of protein complexes. In the case of the Toll-like receptor 4 (TLR4),

activated TLR4 interacts with the PIP2-associated protein TIRAP and MyD88. After translocation to the endosome, the low concentration of PIP2 results in complex dissociation. TLR4 is subsequently free to associate with TRAM and TRIF, thereby activating endosome-specific TLR4 signaling to promote the secretion of type I interferons (Platta and Stenmark 2011).

Recycling of receptors from the early endosome back to the plasma membrane can modulate the response of signaling pathways. For example, the β2-adrenergic receptor is sorted into early endosomes that are shuttled back to the plasma membrane following a fast recycling route mediated by Rab4-positive endosomes. Alternatively, receptors are transported to recycling endosomes in a Rab11-dependent manner to follow a slow recycling pathway (Puthenveedu et al. 2010). In the case of the transferrin receptor, export to the plasma membrane can occur via bulk membrane recycling or sequence-dependent recycling, which relies on local actin polymerization. Studies indicate that the β2-adrenergic receptor is also recycled by this mechanism (Stenmark 2009; Puthenveedu et al. 2010).

Finally, receptors transported to late endosomes or multivesicular endosomes (MVE, also known as multivesicular bodies (MVB)) are usually targeted to lysosomes for degradation. One well-studied example is EGFR, which is ubiquitylated and targeted for lysosomal degradation, thereby, downregulating the EGFR signaling cascade (Wiley et al. 1985; Renfrew and Hubbard 1991). MVEs can also promote specific signaling events. For instance, the adaptors p14 and MP1 recruit MEK1 to MVEs in order to propagate ERK signaling (Teis et al. 2006). It is clear that signal transduction relies on multiple cellular mechanisms to effectively activate and regulate downstream effectors.

For the Wnt pathway, there is a growing body of evidence supporting the necessary role of endocytosis in Wnt signal transduction although our knowledge of the role of endocytosis in Wnt signaling is currently limited.

Regulation of the Wnt pathway by endocytosis

The first evidence of the role of endocytosis in Wnt signaling was the observation that Wg (Drosophila Wnt) localizes by electron microscopy in receiving cells in small vesicles as well as multivesicular bodies (MVBs) (van den Heuvel et al. 1989). MVBs are the last compartment receptors are sorted into prior to lysosomal degradation. Furthermore, *shibire* mutants, the *Drosophila* homolog of dynamin, resulted in accumulation of Wg at the extracellular surface of the plasma membrane (Bejsovec and Wieschaus 1995; Strigini and Cohen 2000). Consistent with these findings, the co-receptors Arrow and Fz mediate Wg internalization in *Drosophila* (Rives et al. 2006).

Initial studies suggested that Wg internalization resulted in downregulation of the Wnt pathway in *Drosophila* embryos (Dubois et al. 2001). Thus, blocking internalization should enhance Wnt signaling. However, further evidence showed that inhibition of endocytosis by loss of dynamin or Rab5 function not only affected Wg endocytosis, but also inhibited Wg signaling (Seto and Bellen 2006). These results were confirmed in mammalian cells where loss of dynamin function and inhibition of clathrin- or caveolin-mediated endocytosis blocked Wnt activation in the presence of Wnt ligands (Blitzer and Nusse 2006; Yamamoto et al. 2006). The contradicting evidence highlights the complexity behind the regulation of membrane components of the Wnt pathway.

Both clathrin and caveolin have been proposed to mediate LRP6 internalization. LRP6 interacts with clathrin as well as the adaptor protein AP-2 (as assessed by sucrose density gradient experiments). The interaction between LRP6 and AP-2 appear to be strengthened by Wnt ligands. Reports show that the number of clathrin clusters around LRP6 at the cell membrane significantly increases upon Wnt3a treatment (Kim et al. 2013). Also, Wnt3a promotes PIP2 formation leading to LRP6 phosphorylation and AP-2 recruitment (Pan et al. 2008). Furthermore, the μ subunit of AP-2 has been shown to interact with Dvl (Yu et al. 2007). These studies agree with a recently proposed model in which LRP6 resides in nascent clathrin-coated pits, and, in the presence of Wnt3a, the Wnt signalosome is assembled in CCV in a process mediated by AP-2 (Gammons et al. 2016).

In contrast to the clathrin-based model, another model proposes that LRP6 is internalized by caveolae upon Wnt activation. In this model, LRP6 resides in lipid rafts that interact with caveolin in the presence of Wnt3a, allowing for Axin recruitment and β-catenin accumulation. LRP6 subsequently recycles back to the plasma membrane via Rab5- and Rab11-positive vesicles (Yamamoto et al. 2006). Interestingly, LRP6 phosphorylation still occurs upon dynamin inhibition (blocks LRP6 internalization) in the presence of Wnt, and LRP6 is internalized in the absence of Wnt. In the case of the latter, LRP6 phosphorylation required the presence of Wnt ligand. β-catenin accumulation, however, required both LRP6 phosphorylation and internalization, suggesting that caveolin-mediated endocytosis prevent spontaneous activation of the Wnt pathway. The same investigators showed that the Wnt antagonist, DKK1, promotes CME of LRP6, suggesting that different endocytic pathways control Wnt signaling

(Yamamoto et al. 2008). Fz has also been shown to internalize upon Wnt activation. Wnt3a induces rapid Fz5 internalization into Rab5-dependent vesicles, and it is shuttled back to the membrane by Rab11-positive endosomes (Yamamoto et al. 2006). Similar to LRP6, the interaction between AP2μ and Dvl is essential for clathrin-mediated Fz endocytosis and Wnt activation (Yu et al. 2007). In summary, there is compelling evidence to support a role for both clathrin and caveolin-mediated receptor endocytosis in Wnt signaling. It remains unclear, however, how a cell chooses to undergo Wnt signaling by utilizing either mechanisms, how Wnt signaling may lead to distinct outputs depending on whether it is via the clathrin or caveolin pathways, and how signaling via the clathrin or caveolin pathways are distinctly controlled.

In the current body of work, I address two major questions in the Wnt field and demonstrate that they are linked: the role of APC in Wnt signaling and the role of the endocytic pathway in Wnt receptor activation. In the latter case, because endocytosis has been shown to be absolutely required for Wnt signaling and because its requirement is evolutionarily conserved, understanding its role in receptor activation will be extremely important for our understanding of the basic mechanism of Wnt pathway activation. Because mutations in the tumor suppressor, APC, occur in 80% of all cases of sporadic CRC (accounting for 600,000 deaths annually worldwide) our understanding of the former case has obvious important clinical implications.

In Chapter III, I describe the initial characterization of an inhibitory monoclonal antibody (mAb7E5) developed against the extracellular domain of LRP6, and demonstrate that mAb7E5 potently inhibited Wnt signaling. In Chapter IV, using mAb7E5, I show that mutations in APC lead to clathrin-dependent, ligand-independent

activation of the Wnt pathway. Furthermore, I demonstrate that whether Wnt signaling occurs via the clathrin or via caveolin-mediated endocytosis is dictate by the cell type of the responding cell. Finally, in Chapter V, I discuss the biological significance of my findings and how it has revised our current model of the Wnt pathway.

CHAPTER II

MATERIALS AND METHODS

Cell lines

The following cells were generous gifts: HEK293 STF (J. Nathans, Johns Hopkins University), RPE (I. Kaverina, Vanderbilt University), MEF and MEF PORCN^{KO} (C. Murtaugh, University of Utah), 293FT (D. Carbone, Ohio State University), HEK293T Dvl TKO (S. Angers, University of Toronto), and HCT116 WTKO (B. Vogelstein, Johns Hopkins University). HEK293, RKO, SW480, DLD1, L-Wnt3a, L-cells, and MEF Caveolin-1^{KO} were purchased from the American Type Culture Collection. HEK293 CMV-luc was reported previously (Thorne et al. 2010). Cell lines were maintained in DMEM except for SW480 and DLD1 cells (RPMI) and HCT116 cells (McCoy's) with 10% FBS and 1% penicillin/streptomycin. See also table 2.1.

Drosophila RNAi lines, Gal4 driver, and progenitor cell reporter

The following Drosophila strains were used: *Apc1*^{Q8} (Ahmed et al. 1998), *Apc1* RNAi line (Vienna Drosophila Resource Center [VDRC] #51469), four *Arr* RNAi lines (*Arr*^{i1}, VDRC#4819; *Arr*^{i2}, VDRC #6707; *Arr*^{i4}, VDRC #6708; and *Arr*^{i3}, Bloomington Drosophila Stock Center (BDSC) #53342), two *dsh* RNAi lines (*dsh*^{i1}, BDSC #31306; *dsh*^{i2}, VDRC #101525), *UAS-dicer2,* VDRC #60010, and *esg*^{ts} (*esg-gal4 tubgal80*^{ts} *UAS-GFP/CyO*) (Micchelli and Perrimon 2006; Guo et al. 2013).

Transfections

Plasmid transfections were performed using $CaCl_2$, Lipofectamine 3000 (Invitrogen), or FuGene HD (PROMEGA). siRNA transfections were performed using Dharmafect1 (HEK293 and RKO cells), Dharmafect2 (SW480, DLD1, and HCT116 cells) (both from Dharmacon), or HiPerfect (RPE cells) (Qiagen) according to manufacturer's protocol. siRNAs were transfected in a pool consisting of two distinct siRNAs in all experiments except where noted.

Immunoblots and immunoprecipitation

Whole cell lysates were obtained using non-denaturing lysis buffer (NDLB) (50 mM Tris-Cl (pH 7.4), 300 mM NaCl, 5 mM EDTA, 1% (w/v) Triton X-100, 1mM PMSF, 1 mM sodium orthovandate, 10 mM NaF, and phosphatase inhibitor cocktail (Roche). Soluble fractions were prepared for immunoblotting. Cytoplasmic fractionation was performed as previously describe (Thorne et al. 2010). Cells were incubated on ice in lysis buffer (10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM MgCl2, 0.1 mM EDTA, 1mM PMSF, 1 mM sodium orthovandate, 10 mM NaF, and phosphatase inhibitor cocktail (Roche)), scraped, transferred to microfuge tubes, and NP-40 was added to 0.5%. Lysates were vortexed and sheared 8 times using a 23-gauge needle. Lysates were centrifuged at 16,000Xg for 2 minutes. Supernatants (cytoplasmic fractions) were recovered. For co-immunoprecipitations, cell lysates were prepared using NDLB. Lysates were diluted to 1 mg/ml and incubated with antibody for 1 hour in a TOMY shaker at 4°C followed by addition of Protein G magnetic beads (New England Biolabs) for 1 hour. Beads were

then washed 3 times with 10X volume of NDLB. Proteins were eluted from beads with protein sample buffer and analyzed by SDS-PAGE and immunoblotting. Chemiluminescence signal was detected using a C-DiGit blot scanner (LI-COR). Obtained images and band intensity were analyzed using Image Studio (LI-COR).

Generation of the mAb7E5 antibody

A protein fragment corresponding to the fragments LRP6-1, LRP6-2, and LRP6-3 of the extracellular domain of human LRP6 was used to inoculate mice for monoclonal antibody production. Positive clones were initially identified based on their capacity to inhibit Wnt3a-mediated signaling in HEK293 STF cells. Clones were then screened for their capacity to inhibit Wnt signaling in the SW480 CRC cell line.

Generation of RKO APC^{KO}

Two APC gRNA sequences (5'-CCCCCTATGTACGCCTCCC-3' and 5'-CTTTGACAAACTTGACTTT-3') were cloned into pCAS-Guide-eGFP (Origene) using the restriction enzymes BamHI and BsmBI. APC gRNA oligos were designed with a 5'-GATCG overhang and an extra guanidine nucleotide on the 3'-end of the sequence (Sigma). Oligonucleotides were annealed as per manufacturer's protocol using a 4-step PCR procedure. Digested pCAS-Guide-eGFP vector was ligated to the annealed oligos using T4 ligase. Successful clones were sequence-verified. Plasmids encoding the two APC gRNA sequences were transfected into RKO cells at 80% confluence using Fugene HD. Transfected cells were sorted by flow cytometry (Vanderbilt Flow

Cytometry Core) as single cells into a 96-well plate. Single colonies were grown to confluence and split into 24-well plates. Clones were propagated and screened by TOPflash. Clones that showed activation as compared to a vector control were further screened by immunofluorescence and stained for β-catenin. Clones with high levels of β-catenin were further screened by immunoblotting.

Generation of stable cell lines

RKO, DLD1, HCT116 WTKO, and RPE cells were incubated with 1.5 mL of TOPflash or APC shRNA lentivirus, and RKO APC^{KO} cells were incubated with 1.5 mL TIR1-9myc retrovirus with 12 μg of polybrene (hexadimethrine bromide) for 4 hours. Media was removed and cells were incubated for 48 hours prior to selection with puromycin or G418.

Sucrose density gradients

Lysates from RKO, RKO incubated for 2 hours with 20ng recombinant Wnt3a (Times Bioscience), and RKO APC^{KO} were prepared using NDLB as previously described. Lysates were added on top of sucrose gradients containing equal volumes of 5%, 10%, 20%, and 30% sucrose in PBS (137 mM NaCl, 7 mM Na_2HPO_4, and 3 mM NaH_2PO_4, pH 7.2). Gradients were centrifuged for 4 hours at 46,000 rpm in a SW55Ti swinging-bucket rotor (Beckman Coulter) along with molecular weight standards (Bio-Rad). After centrifugation, fractions were collected and precipitated using a protein precipitation kit

(National Diagnostics) according to manufacturer's protocol. Precipitated proteins were resuspended in sample buffer and analyzed by immunoblotting.

Endocytosis assays

Cells were plated in four 35 mm dish and incubated at 37°C with L-cells conditioned media (L CM), Wnt3a Conditioned Media (Wnt3a CM) or 20 ng/ml recombinant Wnt3a (Time Bioscience) for 24 hours. Cells were then incubated at 4°C for 3 hours. Cells were lysed in NDLB at 0, 1, 2, and 3 hours post temperature shift. For recovery assays, dishes were then shifted back to 37°C, and lysates were taken 0, 1, 2, and 3 hours later. For small molecule experiments, cells were pre-incubated for 30 minutes with specified concentrations of chloroquine, pitstop-2, and nystatin. Cells were then incubated with 20 ng/ml recombinant Wnt3a (Time Bioscience) for 2 hours. Cells were then washed with ice cold PBS and lysed with NDLB or analyzed by confocal microscopy.

Microscopy

For β-catenin staining, cells were fixed in methanol at -20°C followed by washing with TBS plus 0.1% Triton X-100. Cells were then blocked in Abdil buffer (TBS, 0.1% Triton X-100, 2% BSA, and 0.1% sodium azide) overnight at 4°C. Cells were washed with TBS plus 0.1% Triton X-100 and incubated with anti-β-catenin antibody (1:250, Vanderbilt Protein and Antibody Resource). Cells were incubated with anti-mouse secondary antibody conjugated to Alexa Fluor 488 in a 1:1000 dilution. Cells were mounted in ProLong Gold Antifade Reagent with DAPI (Invitrogen). Images were acquired using a

CoolSNAP ES camera mounted on a Nikon Eclipse 80i fluorescence microscope using 40X and 60X objectives. For immunostaining with APC and clathrin antibodies, RPE cells were seeded on 12 mm glass coverslips and cultured for 24 hours. Cells were then either untreated or treated with Wnt Ligand (20 ng/ml) and incubated in tissue culture for additional 2 hours. Cells were fixed at 37°C for 20 minutes in 4% paraformaldehyde in PBS. The reaction was quenched by rinsing cells 3X with 100 mM glycine in PBS followed by blocking and permeabilization for 1 hour with 0.1% TX-100 in PBS containing 5% glycine and 5% goat serum for 1 hour at room temperature. Cells were then incubated with mouse anti-APC antibody (Santa Cruz) and rabbit anti-clathrin heavy chain monoclonal antibody (1:200, Cell Signaling) or rabbit anti-caveolin-1 polyclonal antibody (1:200, BD Transduction Laboratories) in blocking buffer with 0.05% Tx-100 for 2 hours at room temperature. Cells were rinsed with PBS and incubated for 1 hour in a 1:300 dilution of Alexa Fluor 488- and Alexa Fluor 546-conjugated secondary antibodies. After washing in PBS, the coverslips were mounted on glass slides using Prolong Gold Antifade Reagent. Fluorescent images were acquired using a Zeiss LSM 510 confocal microscope (Carl Zeiss Microscopy, Inc.; Thornwood, NY) using a Plan-Apochromat 100X/1.4 oil Neofluar oil immersion objective with Argon/2 30mW laser (458, 488, 514 nm) or HeNe lasers (543 or 633 nm).

Live cell imaging

Cells were plated in 35 mm glass bottom dishes (MatTek Corporation) at ~50% confluency and transfected with LRP6-eYFP for 6h. Media was then removed and replaced with red phenol-free DMEM + 10% FBS. Cells were pre-incubated for 30

minutes with small molecules or mAb7E5 and/or incubated with 20ng recombinant Wnt3a for 2 hours. Images were obtained using a Leica TCS SP5 confocal microscope with 63X objective. For time-lapse live cell imaging one control stack was taken of 7-10 cells using “mark and find” (Leica Application Suite). Wnt3a was added to the MatTek dish, refocusing was done, and subsequent z-stacks of the same cells were taken every 9 minutes for 3 hours. Imaging, was done on a Leica SP5 confocal microscope using the Live Cell Controller by Live Cell at 37°C and 85-90% relative humidity. The 561nm laser was used for excitation, and the laser power remained the same during acquisition for each experiment. This eliminated cells not visible under a certain threshold of brightness to account for heterogeneity in plasmid expression of transiently transfected plasmids.

Line scans analysis

Images or maximum-intensity projections of z-stacks were analyzed using ImageJ (National Institutes of Health, Bethesda, MD). Line scans analyses to quantify nuclear β-catenin or LRP6-eYFP signal intensity were performed using ImageJ. At least five representative cells were measured per condition.

Drosophila RNAi experiments

To induce temporal knockdown in progenitor cells, RNAi-mediated knockdown was performed using the temperature-sensitive progenitor cell driver *esg*^{ts}. For knockdown, crosses with *Arr* RNAi lines were maintained at 18°C until late third instar, when larvae

were shifted to the restrictive temperature of 29°C to allow RNAi-mediated knockdown during pupation. Crosses with the *dsh* RNAi lines, were maintained at 18°C and shifted to 29°C during the second instar larval stage.

Genotype for Arrow knockdown in *Apc1* null mutant: *esg-Gal4 tubGal80^{ts} UAS-GFP/ UAS-Arrow RNAi; Apc1^{Q8}*

Genotype for simultaneous knockdown of Arrow and Apc1: *UAS-dicer2/+; esg-Gal4 tubGal80^{ts} UAS-GFP/+* or *UAS-Arrow RNAi; UAS-Apc1 RNAi/UAS-Arrow RNAi* or +.

Genotype for simultaneous knockdown of Dsh and Apc1: *UAS-dicer2/+; esg-Gal4 tubGal80^{ts} UAS-GFP/+* or *UAS-dsh1 RNAi; UAS-Apc1 RNAi/UAS-dsh1 RNAi* or *+.*

Drosophila Immunohistochemistry

Two-day-old adult intestines were dissected in PBS and fixed in 4% paraformaldehyde in PBS for 45 minutes. Tissues were washed with PBS, 0.1% Triton X-100, followed by incubation in PBS, 0.1% Tween-20 and 10% BSA for 1 hour at room temperature. The samples were incubated with chicken anti-GFP antibody (1:10000, Abcam) at 4°C overnight in PBS, 0.5% Triton X-100. Samples were stained with secondary antibody goat anti-chicken Alexa Fluor 488 in a 1:400 dilution for 2 hours at room temperature, stained with DAPI (2 µg/ml), and mounted in Prolong Gold (Invitrogen). Confocal images were obtained from the R4 region of the posterior midgut with a Nikon A1RSi confocal microscope with 60x lens, and the total number of *esg*-positive cells in a field of 0.051 mm^2 was counted. For all quantification, more than 15 posterior midguts of

each genotype were analyzed. Images were processed using Adobe Photoshop software.

Auxin-dependent degradation assay

RKO APC^{KO} TIR1 cells were plated in 35 mm dishes, and transfected with pCS2 Myc-APC^{T} or pCS2 AID-Myc-mCherry-APC^{T}. At 24 hours post-transfection, cells were incubated with 500□M auxin analog 1-Naphthaleneacetic acid (Sigma). Timepoints were taken every 30 minutes for 2 hours, and cells were lysed in NDLB and immunoblotted.

Reporter assay

For cell-based luciferase assays, HEK293 STF, RKO STF, SW480 STF, DLD1 STF, HCT116 STF, HCT116 WTKO STF, and HEK293 CMV-Luc cells were seeded into 24-well plates at ~50% confluency. Cells were incubated with L CM, Wnt3a CM, 20 ng/ml purified Wnt3a (Time Bioscience), or 30 mM LiCl prior to lysis with 1X Passive Lysis buffer. Luciferase expression was measured using Steady-Glo Luciferase Assay (Promega). For inhibitor studies, cells were incubated with mAb7E5, Wnt-C59 (Cellagen Technology), IWP-2 (StemRD) or Fz8-Fc (R&D Systems) for 24 hours (with the exception of 48 hours for SW480, DLD1, and HCT116 cells) prior to lysis. Luciferase signal was normalized to viable cell number using CellTiter-Glo Assay (Promega). Alternatively, TOPflash experiments in HEK293, HEK293 Dvl TKO, MEF, MEF PORCN^{KO}, MEF Caveolin-1^{KO}, RKO, and RKO APC^{KO} cells were performed as follows.

Cells were plated and co-transfected with TOPflash and Renilla expression plasmids 24 hours later. Cells were lysed using Dual-Glo Assay (Promega), and luciferase signal was normalized to co-transfected Renilla expression. For each experiment, data was normalized to the signal obtained from positive controls (plotted as 100% activation). Assays were performed in quadruplicate and repeated at least 3X.

RNA isolation and qRT-PCR

HEK293 cells were treated with LRP6 siRNA and APC siRNA for 72 hours. Cells were then harvested using STAT60 reagent (Amsbio) according to manufacturer's protocol and further purified using RNeasy purification kit (Qiagen). RNA (2 μg) was reverse transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kit (Thermo Scientific). qRT-PCR was performed on a Bio-Rad C1000 thermocycler using predesigned and revalidated TaqMan probes that span exons (Thermo Scientific). GAPDH was used as reference control. Technical duplicates were performed for each gene.

Statistical analysis

All graphs were made using Prism (GraphPad Software, Inc.). Statistical analyses were performed using a two-tailed, unpaired Student's t-test, except for colocalization analyses. A value of $p<0.05$ is considered statistically significant.

Colocalization Analysis

For colocalization analysis, Intensity Correlation Analysis plugin in Fiji was used. Pearson's Correlation coefficients were calculated using 4X zoom images. 10-19 images were analyzed for each condition. Box and Whisker plot was plotted for reporting Pearson's Correlation Coefficients. A two-tailed, unpaired, non-parametric Mann-Whitney U-test was used for calculating p-values.

CHAPTER III

INHIBITION OF THE WNT PATHWAY BY A MONOCLONAL ANTIBODY AGAINST THE CO-RECEPTOR LRP6

Introduction

Given the fact that there are nineteen Wnt ligands, ten Frizzled receptors, and only two LRPs, it would be logical to target LRP5 or 6 as the most efficient way to globally inhibit Wnt signaling. LRP5 and LRP6 are 70% identical, however LRP6 has been proposed to play a more significant role in Wnt signaling transduction (He et al. 2004). Indeed, LRP6 overexpression but not LRP5 results in Wnt pathway activation *in vivo* and in mammalian cells (Mi and Johnson 2005; MacDonald et al. 2011). The extracellular domain (ECD) of LRP6 contains four YWTD β-propellers followed by four Epidermal Growth Factor-like domains (E1 through E4). These domains bind different Wnt ligands. For instance, Wnt1 binds E1 and E2, whereas Wnt3a bind E3 and E4. Wnt antagonists such as DKK1 compete for the same domains that Wnt ligands bind to, thereby supporting a model for Wnt inhibition by DKK1 through ligand competition (Chen et al. 2011; Cheng et al. 2011). Wnt-binding to LRP6 induces a conformational change in the ECD due to a hinge-like structure found between E2 and E3 (Cheng et al. 2011). This conformational change is thought to act as a regulatory step that prevents constitutive Wnt activation. Agreeing with this model, expression of a truncated LRP6 missing the extracellular domain leads to higher levels of Wnt activation compared to

that of full-length LRP6, suggesting that the extracellular domain of LRP6 auto-inhibits its activation (Liu et al. 2003).

Multiple antibodies targeting the LRP6 ECD that can inhibit or potentiate ligand-dependent Wnt activation have been reported (Ettenberg et al. 2010; Gong et al. 2010). Because Wnt ligands bind to the two distinct domains of LRP6 ECD (E1E2 and E3E4), treatment with at least two distinct antibodies may be necessary to prevent all Wnt activation. Using a bioinformatics approach, we identified three regions from the LRP6 ECD that are highly conserved across phyla. We developed an antibody (mAb7E5) against those three regions, and we show that mAb7E5 inhibits ligand-dependent Wnt activation of the Wnt1 and Wnt3a class. Furthermore, we found that mAb7E5 inhibits Wnt activation in the triple-negative breast cancer cell line MDA-MB-231, which expresses high levels of LRP6, suggesting that mAb7E5 can potentially be developed as a therapeutic agent for the treatment of Wnt-driven cancers.

Results

Development of an antibody that inhibits LRP6

To identify LRP6 antibodies that regulate Wnt signaling we performed alignments of DNA sequences of LRP6 across multiple species to find highly similar regions. We identified three highly conserved regions (LRP6-1, LRP6-2, and LRP6-3) located between E2 and E3 (Figure 3.1A and B). These regions were of particular interest because the LRP6 hinge, which facilitates LRP6 activation, connects E2 and E3 (Chen et al. 2011). Monoclonal antibodies against a fusion of LRP6-1, LRP6-2, and LRP6-3

(LRP6-1,2,3) fragments were generated and tested for their capacity to suppress Wnt signaling in HEK293 cells stably expressing the Wnt reporter TOPflash (HEK293 STF) (Thorne et al. 2010) (Thorne et al. 2010). We identified the clone 7E5 (herein referred as mAb7E5) as the most potent Wnt inhibitor as measured by TOPflash (Figure 3.2A). Interestingly, mAb7E5 was expressed as an IgM. To confirm that the observed inhibition was not due to the multivalent nature of IgMs, we cloned the variable region of mAb7E5 into an IgG backbone. We found that both mAb7E5 IgM and IgG inhibited Wnt activation in HEK293 cells (Figure 3.2B). Furthermore, we tested whether the Fab fragment of mAb7E5 (mAb7E5^{Fab}) could also inhibit Wnt signaling. Similar to mAb7E5 IgM, and mAb7E5 IgG, mAb7E5^{Fab} inhibited Wnt3a-mediated TOPflash activity and β-catenin accumulation (Figure 3.2C). These results confirm that mAb7E5 effectively inhibits Wnt signaling.

mAb7E5 inhibits ligand-mediated Wnt activation

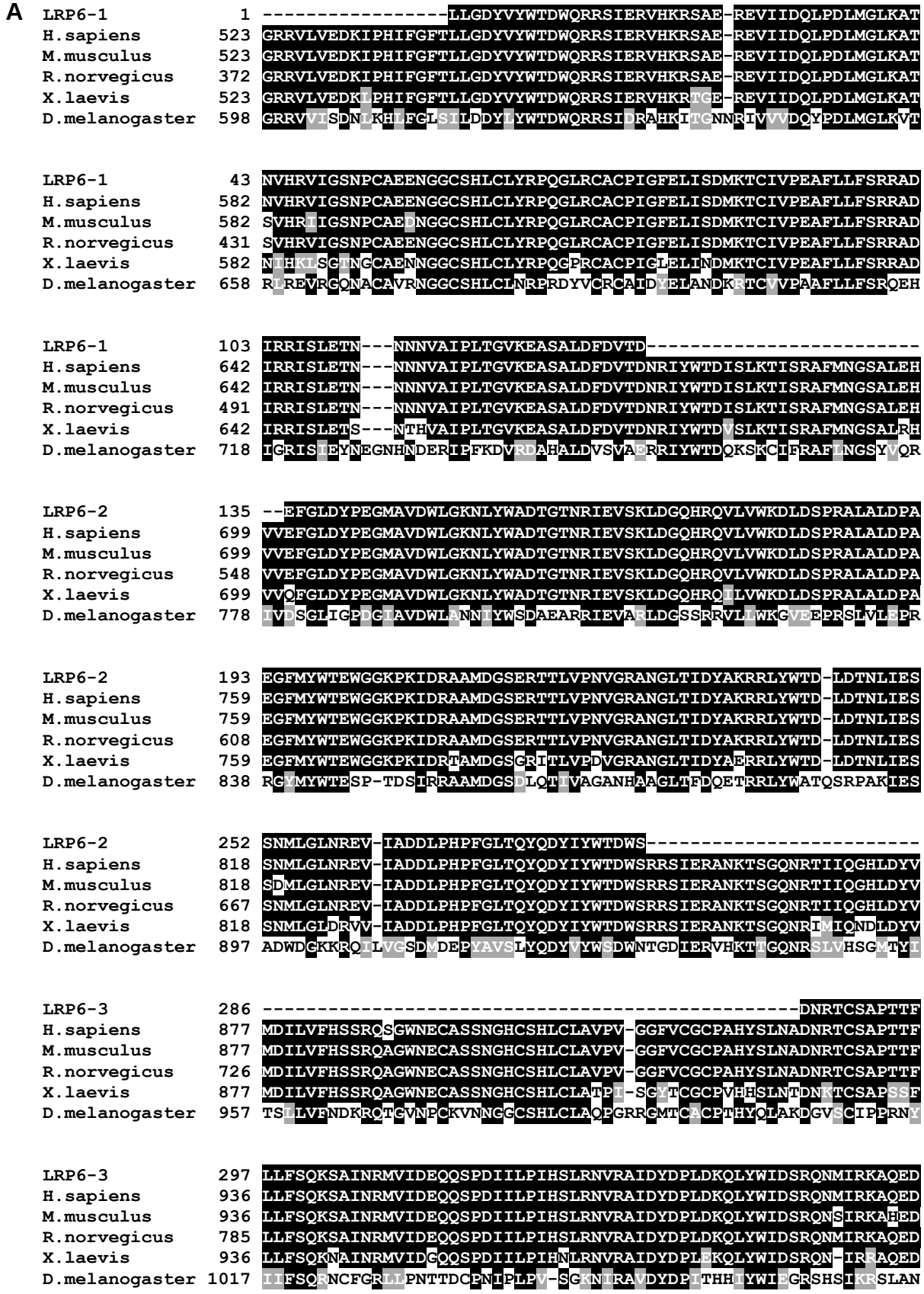
Activation of the Wnt pathway involves multiple coordinated events that occur downstream of the Wnt receptors. Key steps are activation of LRP6 (measured by an increase in LRP6 phosphorylation at Thr1572) and accumulation of cytoplasmic β-catenin (Saito-Diaz et al. 2012). Because mAb7E5 is a potent inhibitor of the Wnt pathway, we further characterized its effects on the various steps required for Wnt activation. Agreeing with our initial screening results, mAb7E5 blocked Wnt3a-dependent TOPflash activity in the human cell lines HEK293 STF and RKO STF, which correlated with decreased cytoplasmic β-catenin levels and decreased Wnt-mediated

LRP6 phosphorylation (Figure 3.3A and B). These results were confirmed by immunofluorescence in the Retinal Pigment Epithelium (RPE) cell line. mAb7E5 decreased the number of RPE cells with elevated levels of nuclear β-catenin staining, compared to RPE cells treated with Wnt3a alone (Figure 3.3C). Furthermore, mAb7E5 inhibited Wnt activation by Wnt3a and Wnt1, which bind different domains of LRP6 (Figure 3.1B and Figure 3.3D). Taken together, these results confirm that mAb7E5 inhibits Wnt signaling at the level of the co-receptor, LRP6.

mAb7E5 binds specifically to LRP6

To confirm that mAb7E5 interacts with the LRP6 ECD and to identify its binding region on the LRP6 ECD, we added a Histidine (His) tag to the C-terminal domain of the IgG heavy chain. We performed pull-down studies with the His-tagged mAb7E5 and purified maltose binding protein (MBP)-tagged LRP6-1, LRP6-2, and LRP6-3 fragments. We found that mAb7E5 binds to LRP6-2 and LRP6-3, as well as the fusion LRP6-1,2,3 (Figure 3.4A and B).

To confirm that mAb7E5 inhibited Wnt signaling via direct interaction with LRP6, cells were transfected with full-length LRP6 (LRP6 FL) or a constitutively active truncated-LRP6 missing the extracellular domain (LRP6ΔN) (Liu et al. 2003). As predicted, mAb7E5 inhibited Wnt activation by LRP6 FL overexpression but not by LRP6ΔN overexpression (Figure 3.5A). To rule out effects by mAb7E5 downstream of LRP6, we inhibited GSK3 using lithium chloride in HEK293 STF. As predicted, mAb7E5


A
LRP6-1 1 ----------LLGDYVYWTDWQRRSIERVHKRSAE-REVIIDQLPDLMGLKAT
H.sapiens 523 GRRVLVEDKIPHIFGFTLLGDYVYWTDWQRRSIERVHKRSAE-REVIIDQLPDLMGLKAT
M.musculus 523 GRRVLVEDKIPHIFGFTLLGDYVYWTDWQRRSIERVHKRSAE-REVIIDQLPDLMGLKAT
R.norvegicus 372 GRRVLVEDKIPHIFGFTLLGDYVYWTDWQRRSIERVHKRSAE-REVIIDQLPDLMGLKAT
X.laevis 523 GRRVLVEDKLPHIFGFTLLGDYVYWTDWQRRSIERVHKRTGE-REVIIDQLPDLMGLKAT
D.melanogaster 598 GRRVVISDNLKHLFGLSIILDDYLYWTDWQRRSIDRAHKITGNNRIVVVDQYPDLMGLKVT
LRP6-1 43 NVHRVIGSNPCAEENGGCSHLCLYRPQGLRCACPIGFELISDMKTCIVPEAFLLFSRRAD
H.sapiens 582 NVHRVIGSNPCAEENGGCSHLCLYRPQGLRCACPIGFELISDMKTCIVPEAFLLFSRRAD
M.musculus 582 SVHRIIGSNPCAEDNGGCSHLCLYRPQGLRCACPIGFELISDMKTCIVPEAFLLFSRRAD
R.norvegicus 431 SVHRVIGSNPCAEENGGCSHLCLYRPQGLRCACPIGFELISDMKTCIVPEAFLLFSRRAD
X.laevis 582 NTHKLSGTNGCAENNGGCSHLCLYRPQGPRCACPIGLELINDMKTCIVPEAFLLFSRRAD
D.melanogaster 658 RLREVRGQNACAVRNGGCSHLCLNRPRDYVCRCAIDYELANDKRTCVVPAAFLLFSRQEH
LRP6-1 103 IRRISLETN---NNNVAIPLTGVKEASALDFDVTD-------------------------
H.sapiens 642 IRRISLETN---NNNVAIPLTGVKEASALDFDVTDNRIYWTDISLKTISRAFMNGSALEH
M.musculus 642 IRRISLETN---NNNVAIPLTGVKEASALDFDVTDNRIYWTDISLKTISRAFMNGSALEH
R.norvegicus 491 IRRISLETN---NNNVAIPLTGVKEASALDFDVTDNRIYWTDISLKTISRAFMNGSALEH
X.laevis 642 IRRISLETS---NTHVAIPLTGVKEASALDFDVTDNRIYWTDVSLKTISRAFMNGSAIRH
D.melanogaster 718 IGRISIEYNEGNHNDERIIFKDVRDAHALDVSVAERRIYWTDQKSKCIERAFLNGSYVQR
LRP6-2 135 --EFGLDYPEGMAVDWLGKNLYWADTGTNRIEVSKLDGQHRQVLVWKDLDSPRALALDPA
H.sapiens 699 VVEFGLDYPEGMAVDWLGKNLYWADTGTNRIEVSKLDGQHRQVLVWKDLDSPRALALDPA
M.musculus 699 VVEFGLDYPEGMAVDWLGKNLYWADTGTNRIEVSKLDGQHRQVLVWKDLDSPRALALDPA
R.norvegicus 548 VVEFGLDYPEGMAVDWLGKNLYWADTGTNRIEVSKLDGQHRQVLVWKDLDSPRALALDPA
X.laevis 699 VVQFGLDYPEGMAVDWLGKNLYWADTGTNRIEVSKLDGQHRQILVWKDLDSPRALALDPA
D.melanogaster 778 IVDSGLIGPDGIAVDWLANNTYWSDAEARRIEVARLDGSSRRVLLWKGVEEPRSLVLEPR
LRP6-2 193 EGFMYWTEWGGKPKIDRAAMDGSERTTLVPNVGRANGLTIDYAKRRLYWTD-LDTNLIES
H.sapiens 759 EGFMYWTEWGGKPKIDRAAMDGSERTTLVPNVGRANGLTIDYAKRRLYWTD-LDTNLIES
M.musculus 759 EGFMYWTEWGGKPKIDRAAMDGSERTTLVPNVGRANGLTIDYAKRRLYWTD-LDTNLIES
R.norvegicus 608 EGFMYWTEWGGKPKIDRAAMDGSERTTLVPNVGRANGLTIDYAKRRLYWTD-LDTNLIES
X.laevis 759 EGFMYWTEWGGKPKIDRTAMDGSGRITLVPDVGRANGLTIDYAERRLYWTD-LDTNLIES
D.melanogaster 838 RGYMYWTESP-TDSIRRAAMDGSDLQTTVAGANHAAGLTFDQETRRLYWATQSRPAKIES
LRP6-2 252 SNMLGLNREV-IADDLPHPFGLTQYQDYIYWTDWS-------------------------
H.sapiens 818 SNMLGLNREV-IADDLPHPFGLTQYQDYIYWTDWSRRSIERANKTSGQNRTIIQGHLDYV
M.musculus 818 SDMLGLNREV-IADDLPHPFGLTQYQDYIYWTDWSRRSIERANKTSGQNRTIIQGHLDYV
R.norvegicus 667 SNMLGLNREV-IADDLPHPFGLTQYQDYIYWTDWSRRSIERANKTSGQNRTIIQGHLDYV
X.laevis 818 SNMLGLDRVV-IADDLPHPFGLTQYQDYIYWTDWSRRSIERANKTSGQNRIMIQNDLDYV
D.melanogaster 897 ADWDGKKRQILVGSDMDEPYAVSLYQDYVYWSDWNTGDIERVHKTTGQNRSLVHSGMTYI
LRP6-3 286 --DNRTCSAPTTF
H.sapiens 877 MDILVFHSSRQSGWNECASSNGHCSHLCLAVPV-GGFVCGCPAHYSLNADNRTCSAPTTF
M.musculus 877 MDILVFHSSRQAGWNECASSNGHCSHLCLAVPV-GGFVCGCPAHYSLNADNRTCSAPTTF
R.norvegicus 726 MDILVFHSSRQAGWNECASSNGHCSHLCLAVPV-GGFVCGCPAHYSLNADNRTCSAPTTF
X.laevis 877 MDILVFHSSRQAGWNECASSNGHCSHLCLATPI-SGYTCGCPVHHSLNTDNKTCSAPSSF
D.melanogaster 957 TSLLVFNDKRQTGVNPCKVNNGGCSHLCLAQPGRRGMTCACPTHYQLAKDGVSCIPERNY
LRP6-3 297 LLFSQKSAINRMVIDEQQSPDIILPIHSLRNVRAIDYDPLDKQLYWIDSRQNMIRKAQED
H.sapiens 936 LLFSQKSAINRMVIDEQQSPDIILPIHSLRNVRAIDYDPLDKQLYWIDSRQNMIRKAQED
M.musculus 936 LLFSQKSAINRMVIDEQQSPDIILPIHSLRNVRAIDYDPLDKQLYWIDSRQNSIRKAHED
R.norvegicus 785 LLFSQKSAINRMVIDEQQSPDIILPIHSLRNVRAIDYDPLDKQLYWIDSRQNMIRKAQED
X.laevis 936 LLFSQKNAINRMVIDGQQSPDIILPIHNLRNVRAIDYDPLEKQLYWIDSRQN-IRRAQED
D.melanogaster 1017 IIFSQRNCFGRLLPNTTDCENIPLPV-SGKNIRAVDYDPITHHIYWIEGRSHSIKRSLAN

```
LRP6-3           357 GSQGFTVVVSSVPSQNLEIQPYDLSIDIYSRYIYWTCEATNVINVTRLDGRSVGVVLKGE
H.sapiens        996 GSQGFTVVVSSVPSQNLEIQPYDLSIDIYSRYIYWTCEATNVINVTRLDGRSVGVVLKGE
M.musculus       996 GGQGFNVVANSVANQNLEIQPYDLSIDIYSRYIYWTCEATNVIDVTRLDGRSVGVVLKGE
R.norvegicus     845 GGQGFTVVVSSVPNQNLEIQPYDLSIDIYSRYIYWTCEATNVIDVTRLDGRSVGVVLKGE
X.laevis         995 GSQSMTIVASTIPNQNMDMQPYDLSIDIYSRLIYWTCEATNIINVTRLDGRAIGVVLKGE
D.melanogaster  1076 GTKVS-LLAN-------SGQPFDIAIDIIGRLLFWTCSQSNSINVTSFLGESVGVIDTGD

LRP6-3           417 QDRPRAIVVNPE--------------------------------------------------
H.sapiens       1056 QDRPRAIVVNPEKGYMYFTNLQERSPKIERAALDGTEREVLFFSGLSKPIALALDSRLGK
M.musculus      1056 QDRPRAIVVNPEKGYMYFTNLQERSPKIERAALDGTEREVLFFSGLSKPIALALDSKLGK
R.norvegicus     905 QDRPRAIVVNPEKGYMYFTNLQERSPKIERAALDGTEREVLFFSGLSKPVALALDSKISR
X.laevis        1055 QERPRAILVNPERGYMYFTNLQERSPKIERAALDGSEREVLFFTGLSKPVALALDNKMGK
D.melanogaster  1128 SEKPRNIAVHAMKRLLFWTDVGSHQ-AIIRARVDGNERVELAY-KLEGVTALALDQQSDM
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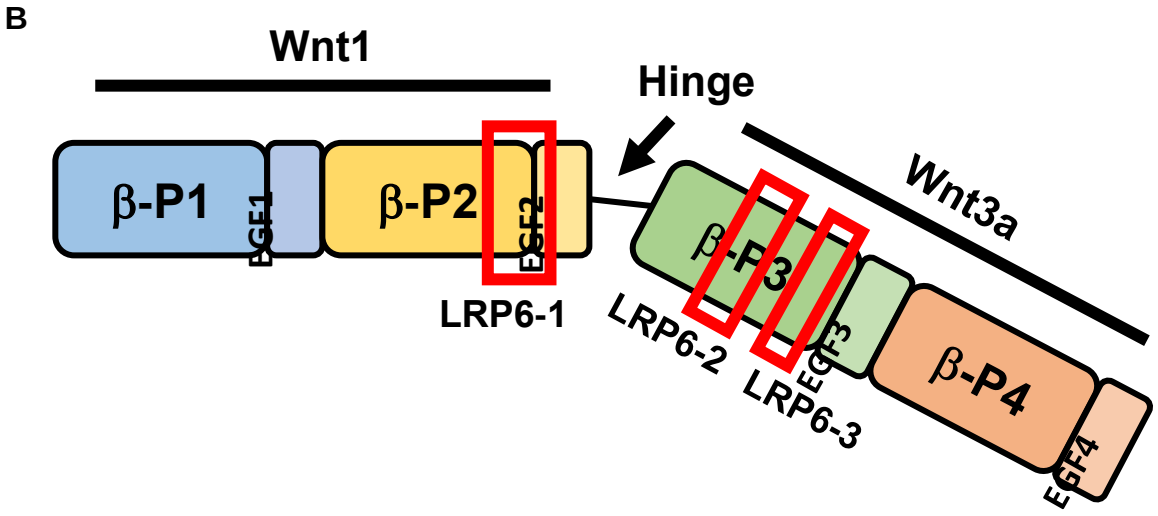

Figure 3.1. **LRP6 domains selected as antigen for antibody development.**

(A) DNA sequences of LRP6-1, LRP6-2, and LRP6-3 aligned with LRP6 sequence of multiple species.

(B) Approximate location of LRP6-1, LRP6-2, and LRP6-3 within full-length LRP6. β-P, β-propeller; EGF, epidermal growth factor-like domain. Wnt1 and Wnt3a binding sites are shown.

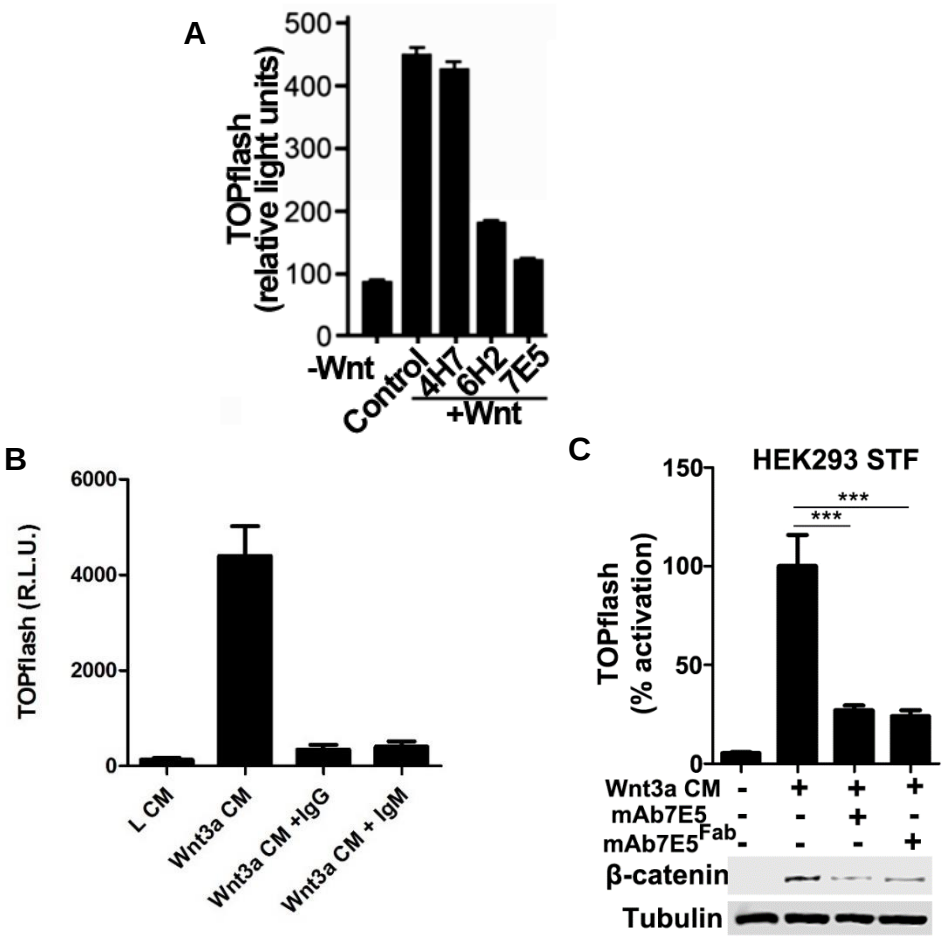

Figure 3.2. The monoclonal antibody mAb7E5 is an inhibitor of the Wnt pathway.

(A) Screening of multiple clones of monoclonal antibodies. HEK293 STF cells were incubated with Wnt3a and monoclonal antibodies.

(B) The monoclonal antibody mAb7E5 inhibits Wnt activation regardless of its isotype. HEK293 STF cells were incubated with L (L CM) or Wnt3a (Wnt3a CM) conditioned media and mAb7E5 IgG or mAb7E5 IgM.

(C) mAb7E5Fab can inhibit ligand-dependent Wnt signaling. HEK293 STF cells were incubated with full-length or Fab mAb7E5 and Wnt3a CM.

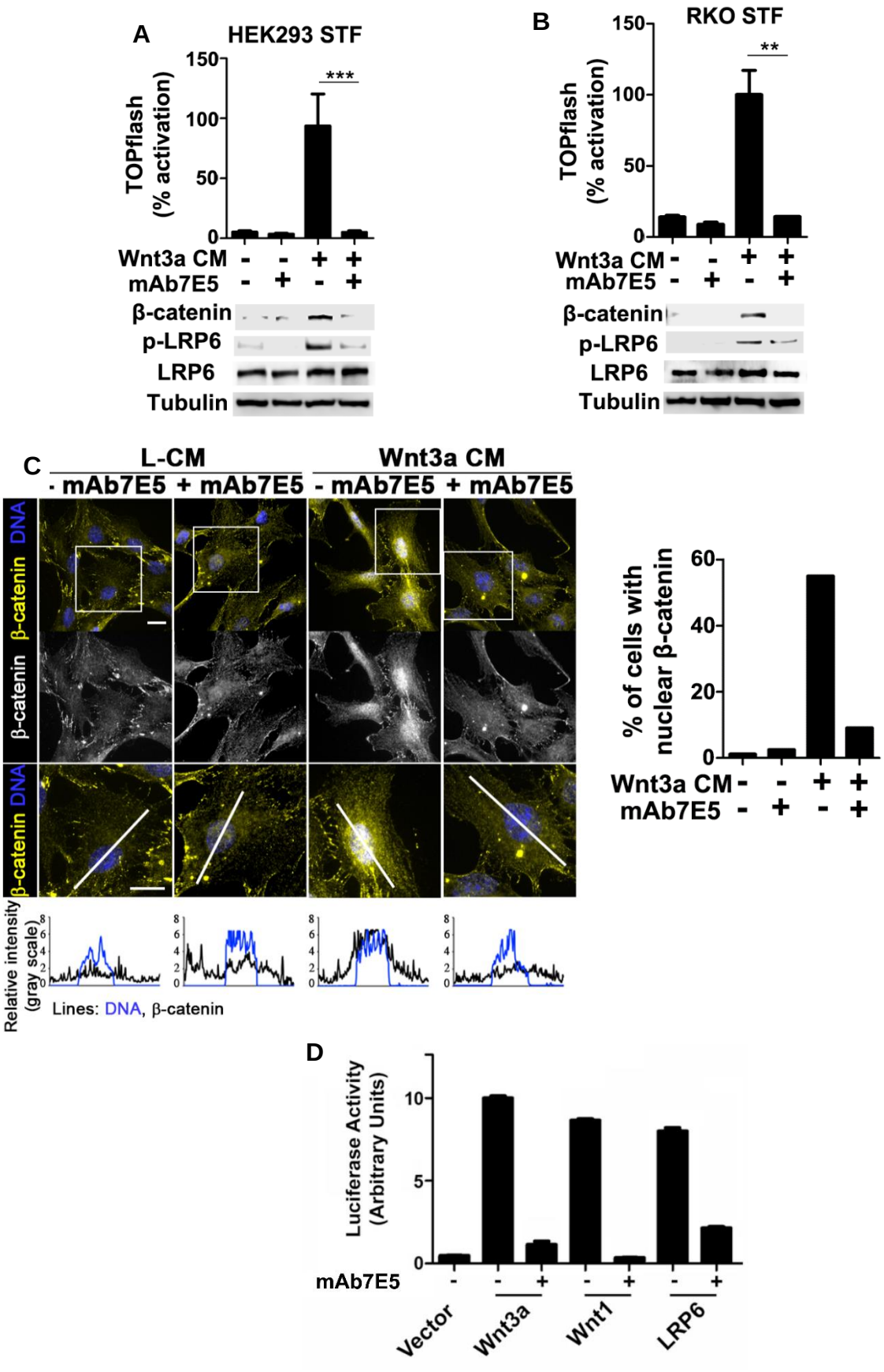
A
HEK293 STF
TOPflash (% activation)
150
100
50
0
***
Wnt3a CM - - + +
mAb7E5 - + - +
β-catenin
p-LRP6
LRP6
Tubulin
B
RKO STF
TOPflash (% activation)
150
100
50
0
**
Wnt3a CM - - + +
mAb7E5 - + - +
β-catenin
p-LRP6
LRP6
Tubulin
C
L-CM
- mAb7E5 + mAb7E5
Wnt3a CM
- mAb7E5 + mAb7E5
DNA
β-catenin
β-catenin DNA
Relative intensity (gray scale)
Lines: DNA, β-catenin
% of cells with nuclear β-catenin
60
40
20
0
Wnt3a CM - - + +
mAb7E5 - + - +
D
Luciferase Activity (Arbitrary Units)
10
5
0
mAb7E5 - - + - + - +
Vector
Wnt3a
Wnt1
LRP6

Figure 3.3. mAb7E5 inhibits ligand mediated Wnt activation.

(A and B) mAb7E5 blocks Wnt ligand-mediated signaling (A) HEK293 STF and (B) RKO STF cells were incubated with Wnt3a and mAb7E5.

(C) mAb7E5 prevents nuclear accumulation of β-catenin. RPE cells were incubated with Wnt3a and mAb7E5 followed by fixation and immunostaining. Scale bar: 5 μm. Inset: Line scans of selected cells. Scale bar: 10 μm. Cells (>100/condition) showing nuclear β-catenin staining were quantified and plotted.

(D) mAb7E5, inhibits Wnt signaling mediated by the Wnt3a and Wnt1 classes of ligands and by elevated LRP6. HEK293 STF were transfected with plasmids encoding Wnt3a, Wnt1, LRP6 or vector control in the presence or absence of mAb7E5.

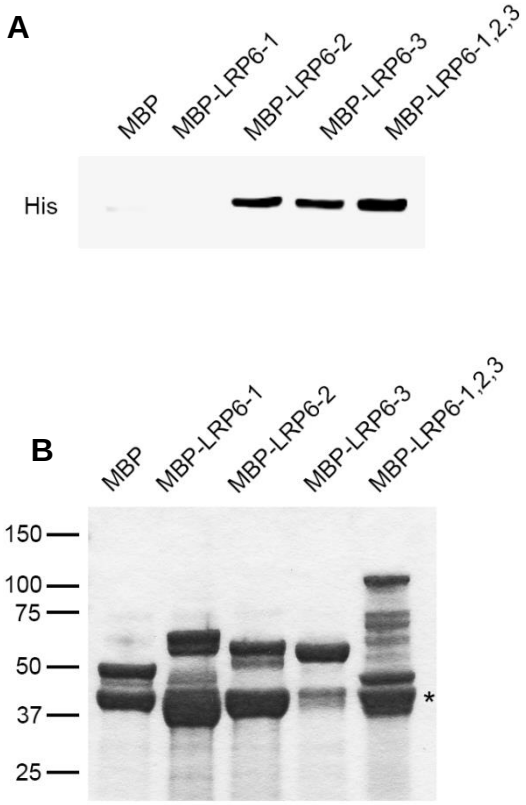

Figure 3.4. mAb7E5 binds to LRP6-2 and LRP6-3 domains *in vitro*.

(A) MBP-tagged LRP6-1, LRP6-2, and LRP6-3 fragments were purified and incubated with His-mAb7E5. Samples were analyzed by SDS-PAGE electrophoresis and interaction between mAb7E5 and each LRP6 fragment was assessed by immunoblotting.

(B) Coomassie staining of (A) to confirm equivalent amounts of protein were used. Asterisk shows non-specific band.

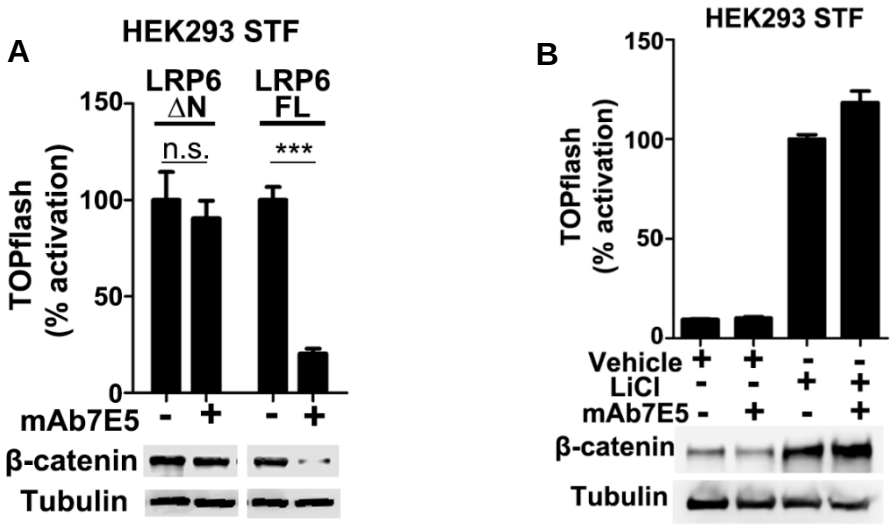

Figure 3.5. mAb7E5 inhibits Wnt activation at the level of the co-receptor LRP6

(A) The extracellular domain of LRP6 is required for mAb7E5 to inhibit LRP6-mediated Wnt signaling. HEK293 STF cells were transfected with LRP6ΔN or LRP6 FL and incubated with mAb7E5.

(B) mAb7E5 blocks Wnt signaling upstream of the degradation complex. HEK293 STF cells were incubated with mAb7E5 and 30 mM LiCl.

did not block Wnt reporter activity or accumulation of cytoplasmic β-catenin upon lithium treatment (Figure 3.5B). These results provide strong evidence that mAb7E5 inhibits Wnt signaling at the level of LRP6.

mAb7E5 inhibits Wnt signaling in breast cancer cells

Three major markers are currently used for guiding clinical diagnosis and treatment of breast cancer: estrogen receptor expression, epidermal growth factor receptor expression, and aberrant HER2 expression. Triple-negative breast cancer (TNBC) is defined as the subtype of breast cancer lacking expression of all three markers, and is considered highly aggressive and patients exhibit poor prognosis (Banerjee et al. 2006; Bianchini et al. 2016).

MDA-MB-231 is a TNBC cell line, which exhibits constitutive Wnt signaling due to an autocrine signaling loop. Incubation with the Wnt inhibitors DKK1 and SFRP1 prevent β-catenin accumulation and Wnt activation in this cell line (Bafico et al. 2004). MDA-MB-231 cells exhibit high levels of LRP6, and depletion of LRP6 by siRNA decreases Wnt signaling, viability, and proliferation of MDA-MB-231 *in vitro* and in mouse xenograft models (Bafico et al. 2004; Liu et al. 2010). Loss of Fz7 in MDA-MB-231 cells also inhibits tumor growth *in vivo* (Yang et al. 2011). Because mAb7E5 targets LRP6, we decided to test whether mAb7E5 inhibits Wnt signaling in MDA-MB-231 cells. We found that both mAb7E5 and mAb7E5^{Fab} decrease nuclear β-catenin accumulation in MDA-MB-231 cells (Figure 3.6). In titration studies, we found that mAb7E5 is effective

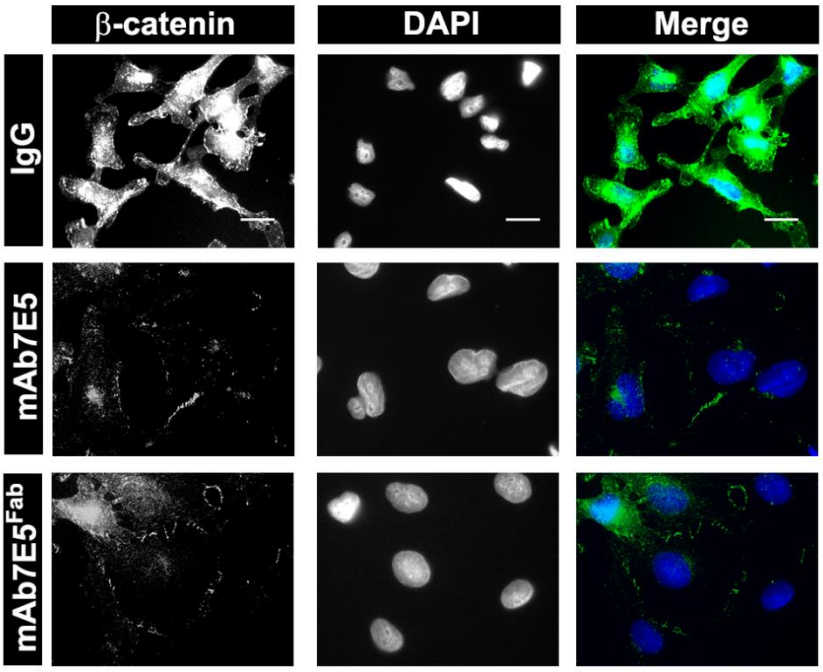

Figure 3.6. mAb7E5 downregulates β-catenin levels in MDA-MB-231 cells.

MDA-MB-231 cells were incubated with full length mAb7E5 or mAb7E5^{Fab}, followed by immunostaing.

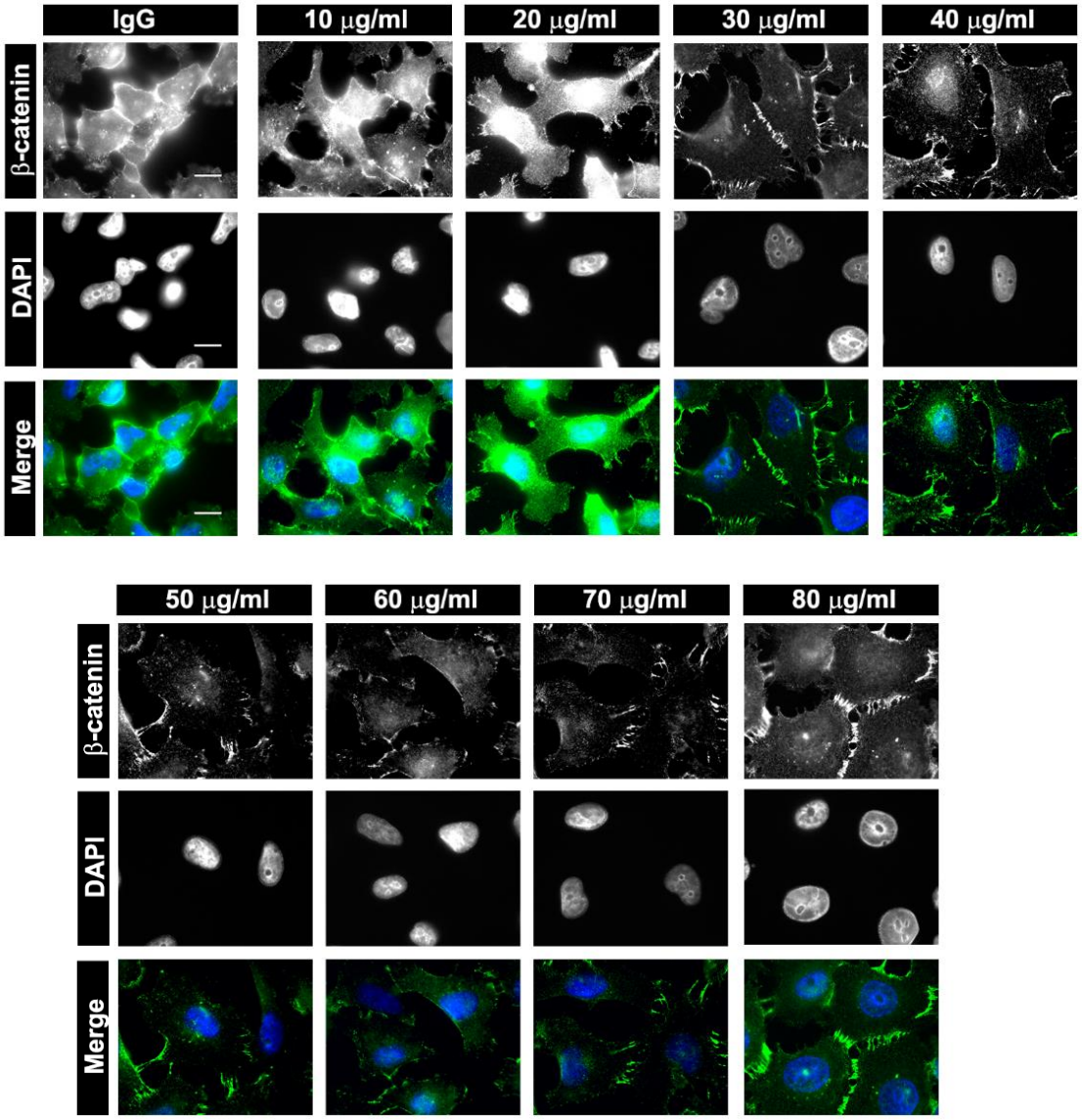

Figure 3.7. Minimum mAb7E5 concentration required to inhibit β-catenin accumulation in MDA-MB-231 cells.

MDA-MB-231 cells were incubated with indicated concentrations of mAb7E5 followed by immunostaining.

at a concentration of 30μg/ml (Figure 3.7). These results suggest that mAb7E5 can potentially be used as a therapeutic agent in treating TNBC.

Discussion

LRP6 is a very attractive target for developing inhibitors of the Wnt pathway for several reasons: (1) LRP6 is located at the plasma membrane and binds to Wnt ligands, (2) there are only two members of the LRP receptor family (LRP5 and LRP6), and (3) LRP6 is only involved in the canonical Wnt pathway. Thus, any perturbation in LRP6 function would not have significant effects in other non-canonical functions of the Wnt pathway, thereby limiting toxicity (Saito-Diaz et al. 2012).

Previously reported antibodies have been generated against the extracellular domain of LRP6 using antibody libraries (Ettenberg et al. 2010) or by producing them against purified E1-E2 and E3-E4 fragments of the LRP6 ECD (Gong et al. 2010). As a result, these studies identified multiple antibodies that uncovered new properties of LRP6. In contrast, our approach focused on bioinformatically identifying highly conserved regions of LRP6 ECD important for LRP6 function and Wnt signal transduction. Furthermore, in contrast to the reported anti-LRP6 antibodies, we found that mAb7E5 inhibits both Wnt1 and Wnt3a class of ligand.

Interestingly, we found that mAb7E5 also inhibits ligand-independent Wnt activation. Our data shows that mAb7E5 blocks Wnt activation upon LRP6 overexpression in human cells. This suggests that mAb7E5 may target a region of LRP6 that is undergoes conformational change in response to Wnt ligand binding. It has

been reported that ECD of LRP6 prevents constitutive LRP6 activation and, upon Wnt ligand binding, LRP6 undergoes a conformational change that relays the signal to downstream effectors. It is possible that binding of mAb7E5 blocks this conformational change to block LRP6 activation. Thus, the regions of LRP6-2 and LRP6-3 (targeted by mAb7E5) may mediate structural changes in the coreceptor that are induced by Wnt ligand binding.

The Wnt pathway is constitutively active in many diseases, including colorectal and breast cancer. Thus, Wnt inhibitors would be potentially beneficial for the treatment of these diseases. Multiple studies have reported several small molecules as candidate therapeutics (Blagodatski et al. 2014). These small molecules, however, suffer from low efficacy and selectivity, thereby limiting their potential use as therapeutics. In contrast, monoclonal antibodies have multiple advantages as therapeutics as they are highly selective and have longer half-lives than small molecules.

Antibodies against Fz have been developed as potent Wnt inhibitors. Two antibodies, vantictumab and OTSA101, which targets Fz7 and Fz10, respectively, are currently undergoing Phase II clinical trials (Blagodatski et al. 2014). Fz7 is up regulated in a wide range of cancers, including CRC, TNBC, and hepatocellular carcinomas, whereas Fz10 is highly expressed in synovial sarcomas (King et al. 2012). Although vantictumab was initially identified as in inhibitor of Fz7, it has been shown to interact with five other members of the Fz family (Gurney et al. 2012). As a result, patients taking vantictumab demonstrate moderate-severe side effects, including osteoporosis (a side of effect of significant Wnt inhibition) (Blagodatski et al. 2014).

Our results suggest that antibodies against LRP6 also have the potential for being developed further for the treatment of Wnt-driven diseases. Just as importantly, these antibodies can also be used as a tool to probe how LRP6 controls Wnt pathway activation and possibly give us insight of unappreciated regulatory mechanisms. In the case of the latter, the role of mAb7E5 in deciphering the mechanism of action of the tumor suppressor, APC, in the Wnt pathway will be described in Chapter IV

CHAPTER IV

APC INHIBITS LIGAND-INDEPENDENT WNT SIGNALING BY THE CLATHRIN ENDOCYTIC PATHWAY

Introduction

In the absence of a Wnt ligand, levels of cytoplasmic β-catenin are maintained at low levels by its association with a destruction complex consisting of the scaffold protein Axin, the tumor suppressor adenomatous polyposis coli (APC), and the kinases casein kinase 1α (CK1α) and glycogen synthase kinase (GSK3). Within this complex, β-catenin is phosphorylated and targeted for degradation by the ubiquitin-proteasome system (Saito-Diaz et al. 2012). Wnt ligand binding promotes the formation of a trimeric complex of Wnt, the co-receptors Frizzled (Fz), and the low-density lipoprotein receptor 5/6 (LRP5/6) (Tamai et al. 2000). The formation of aggregated receptors (the signalosome) promotes the recruitment of Axin in a process mediated by Dishevelled (Dvl) (Tamai et al. 2004; Bilić et al. 2007; Zeng et al. 2008). Consequently, degradation of β-catenin is inhibited, its cytoplasmic levels rise, and it is translocated to the nucleus. Within the nucleus, β-catenin associates with the TCF family of transcription factors to initiate a Wnt transcriptional program (Saito-Diaz et al., 2012).

In the classical model, the role of APC is limited to β-catenin proteolysis. Herein, we report that APC is also essential to inhibit Wnt receptor activation in the unstimulated state. We have identified a constitutive, clathrin-mediated pathway that activates Wnt signaling independent of Wnt ligands upon APC inactivation. These studies provide a

new model for the mechanism by which the Wnt pathway is aberrantly activated upon APC loss and offer critical insight for the development of novel therapeutics targeting Wnt-driven cancers.

Results

LRP6 is required for Wnt pathway activation in APC mutant cells but not in cells with mutant β-catenin

Loss of APC has been proposed to induce the formation of a Wnt autocrine loop in colorectal cancer (CRC) (Bafico et al. 2004; Voloshanenko et al. 2013). We tested the effects of mAb7E5 on the CRC lines SW480 and DLD1, which contain mutations in APC, and HCT116, which expresses stabilized β-catenin. mAb7E5 inhibited Wnt signaling only in SW480 and DLD1 cells (Figure 4.1A and B). We found that all the CRC cells tested exhibit elevated levels of phospho-LRP6 that were decreased by treatment with mAb7E5 (Figure 4.1A). To confirm these results, we knocked down APC using siRNA in HEK293 and RKO cells, which increased Wnt reporter activity, β-catenin accumulation, and phospho-LRP6 levels (Figure 4.2A-C). mAb7E5 treatment blocked all of the effects of APC depletion (Figure 4.2B and C). As control, mAb7E5 had no effect on a reporter line (HEK293-CMV) under the control of a non-Wnt regulated promoter (Thorne et al., 2010) (Figure 4.2D). To rule out effects of residual APC activity, we used CRISPR-Cas9 to generate a cell line (RKO APC^{KO}) null for APC protein (Figure 4.3E). RKO APC^{KO} cells exhibited elevated levels of phospho-LRP6 and cytoplasmic β-catenin

(Figure 4.2E), that was downregulated with mAb7E5 treatment (Figure 4.2F). These findings indicate that the effects of APC loss on Wnt pathway activation are mediated partly through LRP6.

To confirm that the constitutive activation of Wnt signaling resulting from APC loss requires LRP6, we performed siRNA knockdown of LRP6 in HEK293 cells (Figure 4.3A). The activation of both the Wnt reporter and endogenous Wnt target genes resulting from APC knockdown (on our Wnt reporter line and endogenous Wnt target genes) was reversed by concomitant knockdown of LRP6 (Figures 4.3B-E). A similar result was observed with either combined siRNA knockdown of APC in RKO cells or siRNA knockdown of LRP6 in RKO APC^{KO} cells (Figure 4.3F and G). Furthermore, in SW480 and DLD1 cells, but not in HCT116 cells, knocking down LRP6 also inhibited Wnt signaling (Figure 4.3H). Together, these findings provide further evidence that the activation of Wnt signaling resulting from APC depletion is dependent on LRP6.

Requirement for surface receptor activation but not Wnt ligands for pathway activation in APC mutant cells

We hypothesized that like LRP6, other components of the signalosome are required for the constitutive activation of Wnt signaling upon APC loss. Thus, inhibiting other membrane components (e.g., Fz and Dvl) should inhibit Wnt signaling in APC mutant cells. To block multiple Fz and Dvl isoforms, we expressed their dominant-negative forms, Fz1-ER and Xdd1, respectively (Wallingford et al. 2000; Kaykas et al.

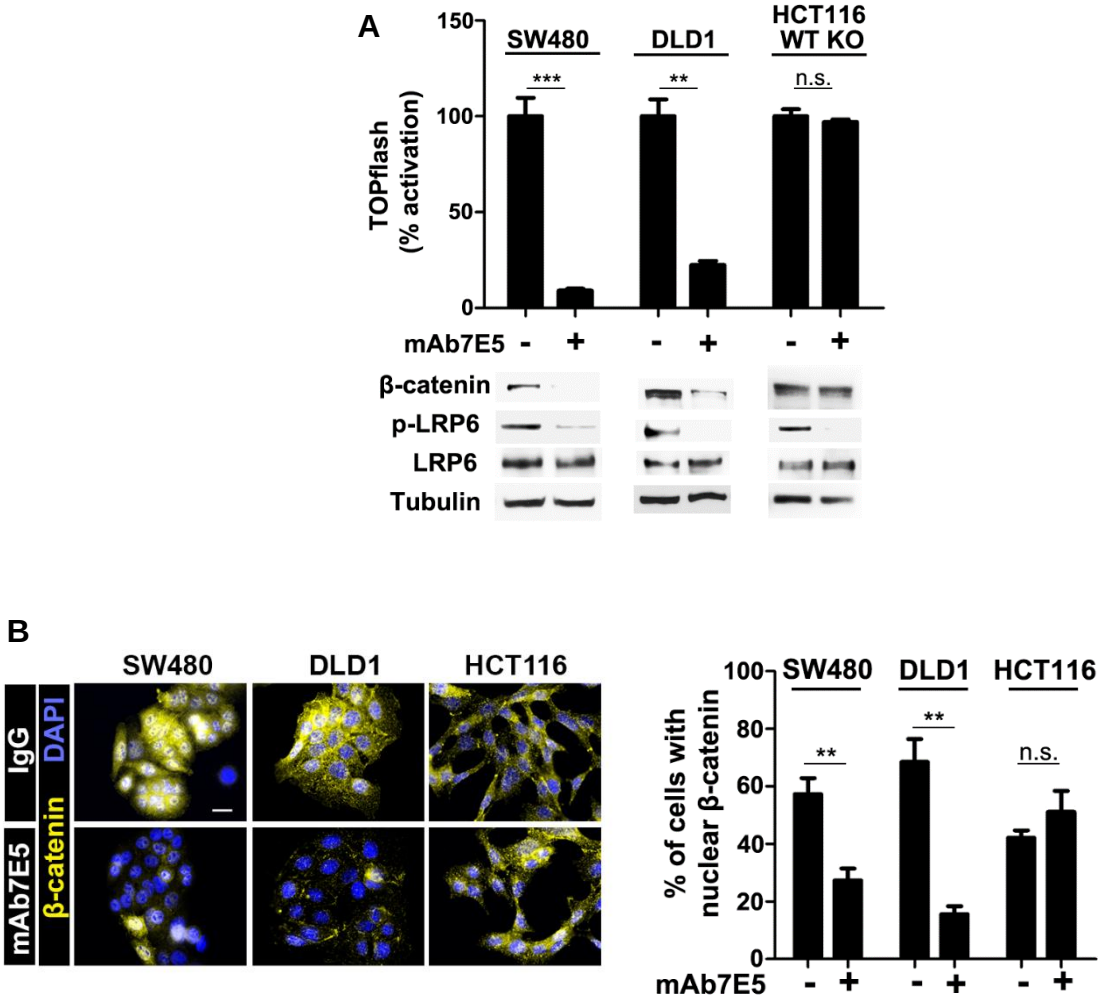

Figure 4.1 mAb7E5 inhibits Wnt signaling in APC-mutant CRC cells.

(A) mAb7E5 inhibits Wnt signaling in APC mutant CRC cells. CRC cells were incubated for 48h with mAb7E5.

(B) mAb7E5 prevents accumulation of nuclear β-catenin in APC mutant CRC cells. Scale bar: 40 μm.

Graph shows mean ± s.e.m. n.s., non-significant, **p<0.01, ***p<0.001.

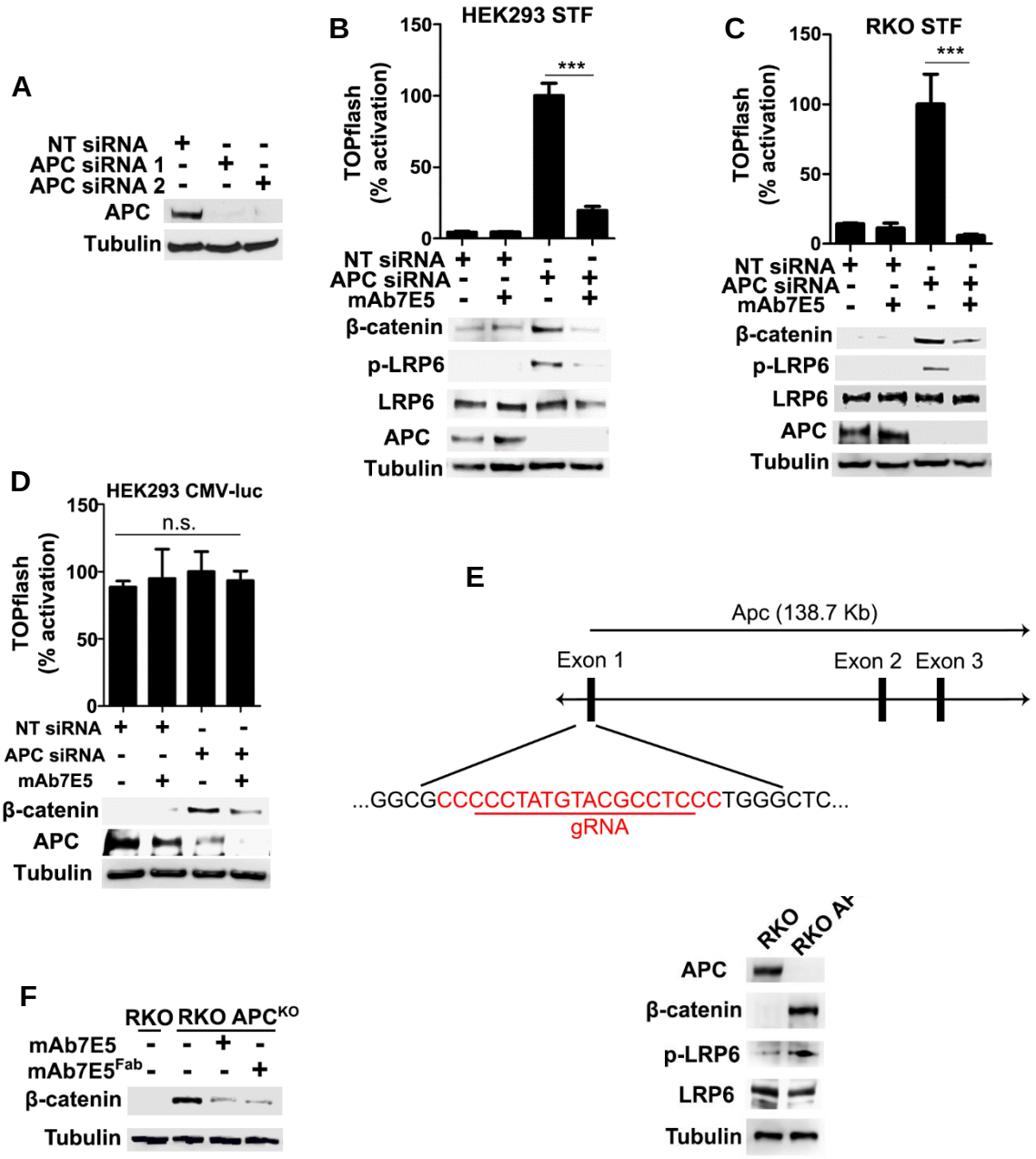

Figure 4.2. mAb7E5 blocks Wnt activation in APC-deficient cells

(A) Downregulation of APC by two distinct siRNAs.

(B and C) mAb7E5 inhibits Wnt signaling upon APC loss. (B) HEK293 STF and (C) RKO STF cells were incubated with APC siRNA and mAb7E5.

(D) Specificity of Wnt signaling inhibition by mAb7E5 in APC-deficient cells. A non-Wnt-regulated reporter cell line, HEK293 CMV-luc, was incubated with APC siRNA and mAb7E5.

(E) Generation of RKO APC^{KO}. Schematics of selected gRNA and characterization of RKO APC^{KO} cells.

(F) RKO APC^{KO} cells were incubated with mAb7E5 or mAb7E5Fab followed by immunoblotting.

Graph shows mean ± s.e.m. n.s., non-significant, ***p<0.001.

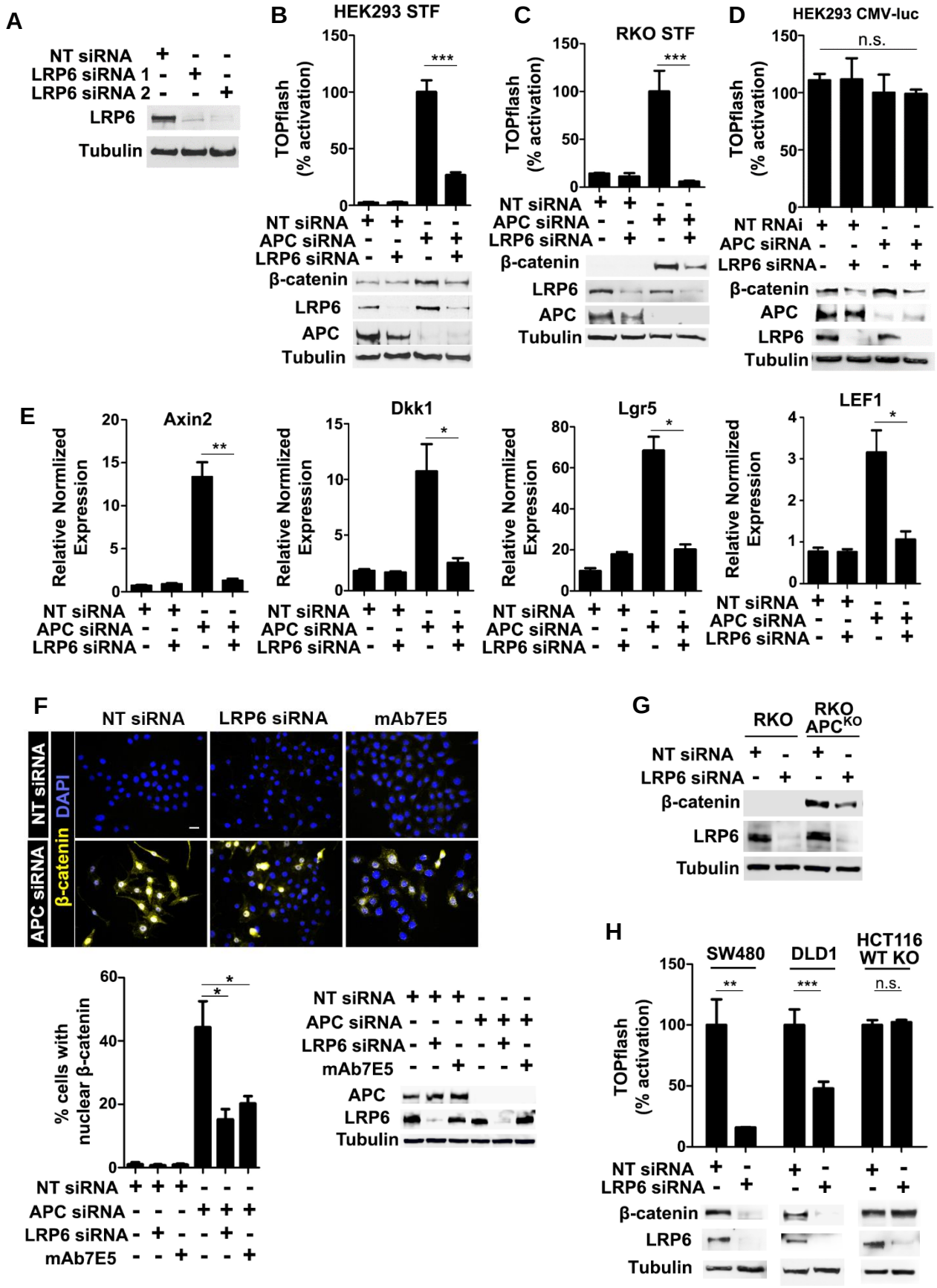
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Figure 4.3. LRP6 is required for Wnt signaling in APC-deficient cells.

(A) Downregulation of LRP6 by two distinct siRNAs.

(B and C) LRP6 is required for Wnt pathway activation upon loss of APC. (B) HEK293 STF and (C) RKO STF cells transfected with cells were transfected with APC and LRP6 siRNA.

(D) Specificity of Wnt signaling inhibition by downregulating LRP6 in APC-deficient cells. The non-Wnt-regulated reporter cell line, HEK293 CMV-luc, was incubated with APC and LRP6 siRNAs.

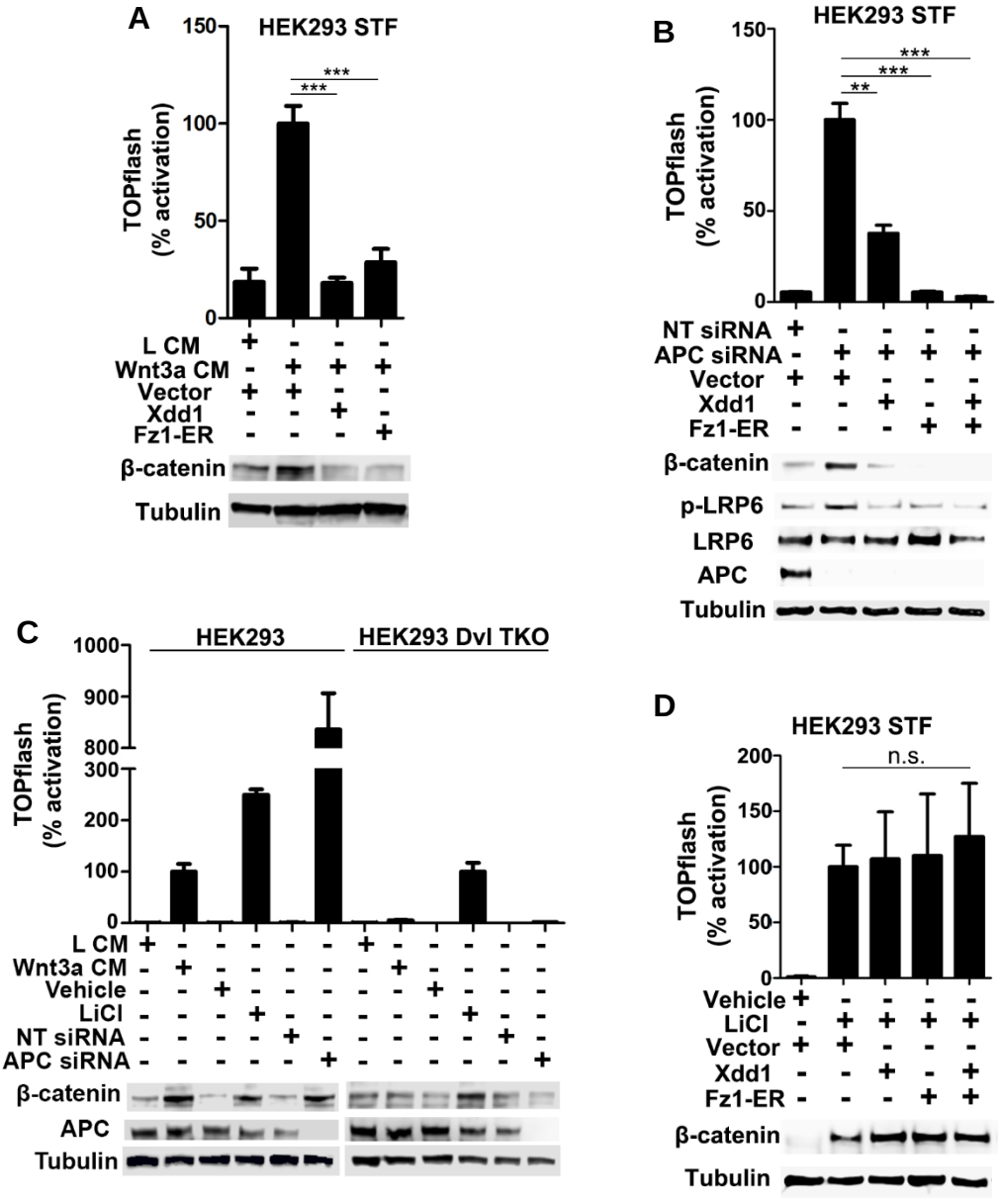
(E) Stimulation of endogenous Wnt target gene expression upon APC loss is inhibited by downregulating LRP6. HEK293 cells were transfected with APC and LRP6 siRNA, mRNAs isolated, and qRT-PCR performed.

(F) LRP6 is required nuclear β-catenin accumulation upon loss of APC. RKO cells were treated with APC siRNA and either LRP6 siRNA or mAb7E5 followed by immunostaining. Scale bar: 40 μm.

(G) RKO APC^{KO} cells were incubated with LRP6 siRNA. Lysates were collected and analyzed by immunoblotting.

(H) CRC cells were transfected with LRP6 siRNA followed by immunoblotting and TOPflash.

Graph shows mean ± s.e.m. n.s., non-significant, *p<0.05, **p<0.01, ***p<0.001.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β-catenin
Tubulin
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17kDa
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LRP6
Control
Wnt3a
APC^KO

Figure 4.4 Loss of APC promotes ligand-independent signalosome formation.

(A) Fz and Dvl are required for ligand-mediated Wnt activation. HEK293 STF cells were transfected with Xdd1 and Fz1-ER for 24 hours and then incubated with either L-cell (L CM) or Wnt3a (Wnt3a CM) conditioned media.

(B) Dvl and Fz are required for Wnt activation in APC-deficient cells. HEK293 STF cells were transfected with APC siRNA plus Xdd1 or Fz1-ER.

(C) APC depletion fails to activate the Wnt pathway in Dvl null cells. HEK293 and HEK293 Dvl TKO were transfected with APC siRNA or treated with Wnt3a CM or 30 mM LiCl.

(D) Wnt signaling due to β-catenin stabilization is not dependent on Fz or Dvl. HEK293 STF cells were transfected with Xdd1 or Fz1-ER and incubated with 30 mM LiCl.

(E) Similar to Wnt ligand-activated cells, LRP6 forms high molecular complexes in the absence of APC on sucrose density gradient.

Graphs show mean ± s.e.m., n.s. non-significant, **p<0.01, ***p<0.001.

2004) (Figure 4.4A). Fz1-ER and Xdd1 expression inhibited Wnt reporter activity and reduced phospho-LRP6 levels in APC-depleted cells (Figure 4.4B). Furthermore, knocking down APC in a cell line null for all three Dvl genes (Dvl TKO) (Jiang et al. 2015) failed to activate Wnt signaling (Figure 4.4C). As control, inhibiting Fz and Dvl function did not inhibit Wnt signaling induced by lithium treatment (Figure 4.4D).

These findings suggested that multiple signalosome components mediated the aberrant signaling resulting from APC loss. We therefore tested whether loss of APC induced signalosome formation. The receptor aggregates or signalosomes that form upon Wnt ligand activation are detectable as multi-protein complexes in sucrose density gradients (Bilić et al., 2007). As expected, we observed a shift in the migration of LRP6 into the denser fractions of lysates from RKO cells treated with Wnt3a compared to untreated cells (Figure 4.4E). However, LRP6 was also detected in the denser fractions of lysates from RKO APC^{KO} cells even in the absence of Wnt3a, suggesting aggregate formation (Figure 4.4E). Based on these findings, we conclude that APC loss induces signalosome assembly.

We next tested if Wnt ligands were necessary for the aberrant activation of signaling in APC mutant cells. A chimeric protein encoding the immunoglobulin Fc domain fused to the cysteine-rich region of Fz8 (Fz8-Fc) sequesters Wnt ligands and thereby blocks Wnt pathway activation (DeAlmeida et al. 2007). Fz8-Fc inhibited Wnt reporter activity and β-catenin accumulation in Wnt3a-treated cells (Figure 4.5A). Fz8-Fc, however, had no effect on Wnt reporter activity in APC-deficient cells (Figures 4.5B-D). Interestingly, APC-deficient cells were still responsive to exogenous Wnt3a activation, as indicated by the increase of phospho-LRP6, which was inhibited by Fz8-

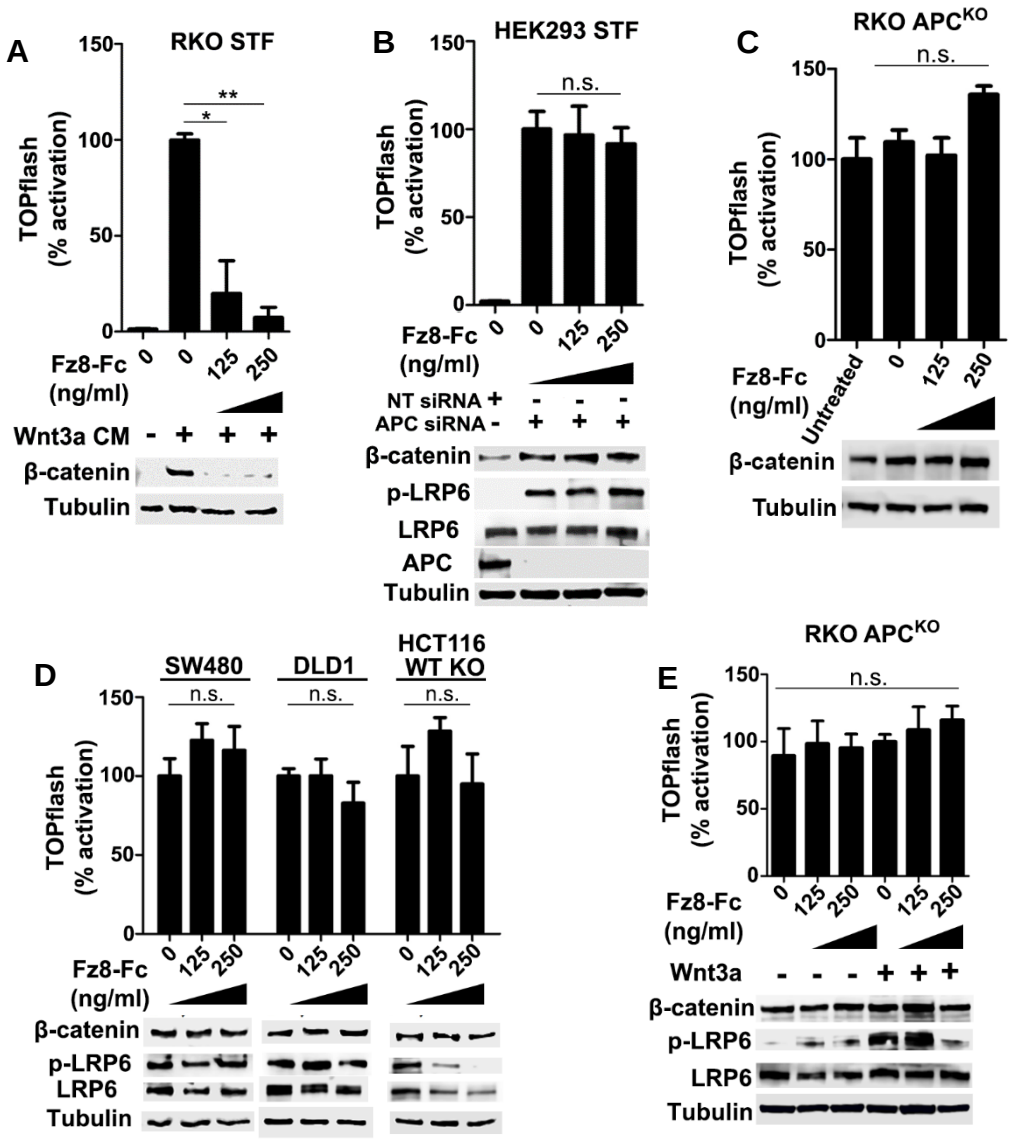


Figure 4.5. Wnt receptor activation in APC-deficient cells is Wnt ligand independent.

(A-D) Fz8-Fc blocks Wnt ligand-mediated signaling but not signaling in APC-deficient cells. (A) RKO STF cells were incubated with Wnt3a and Fz8-Fc at the indicated concentrations. (B) HEK293 STF cells transfected with APC siRNA and (C) RKO APC^{KO} cells were incubated with Fz8-Fc. (D) Wnt activation in CRC cells is not dependent on Wnt ligand. CRC cells were incubated with Fz8-Fc at the indicated concentrations for 48h.

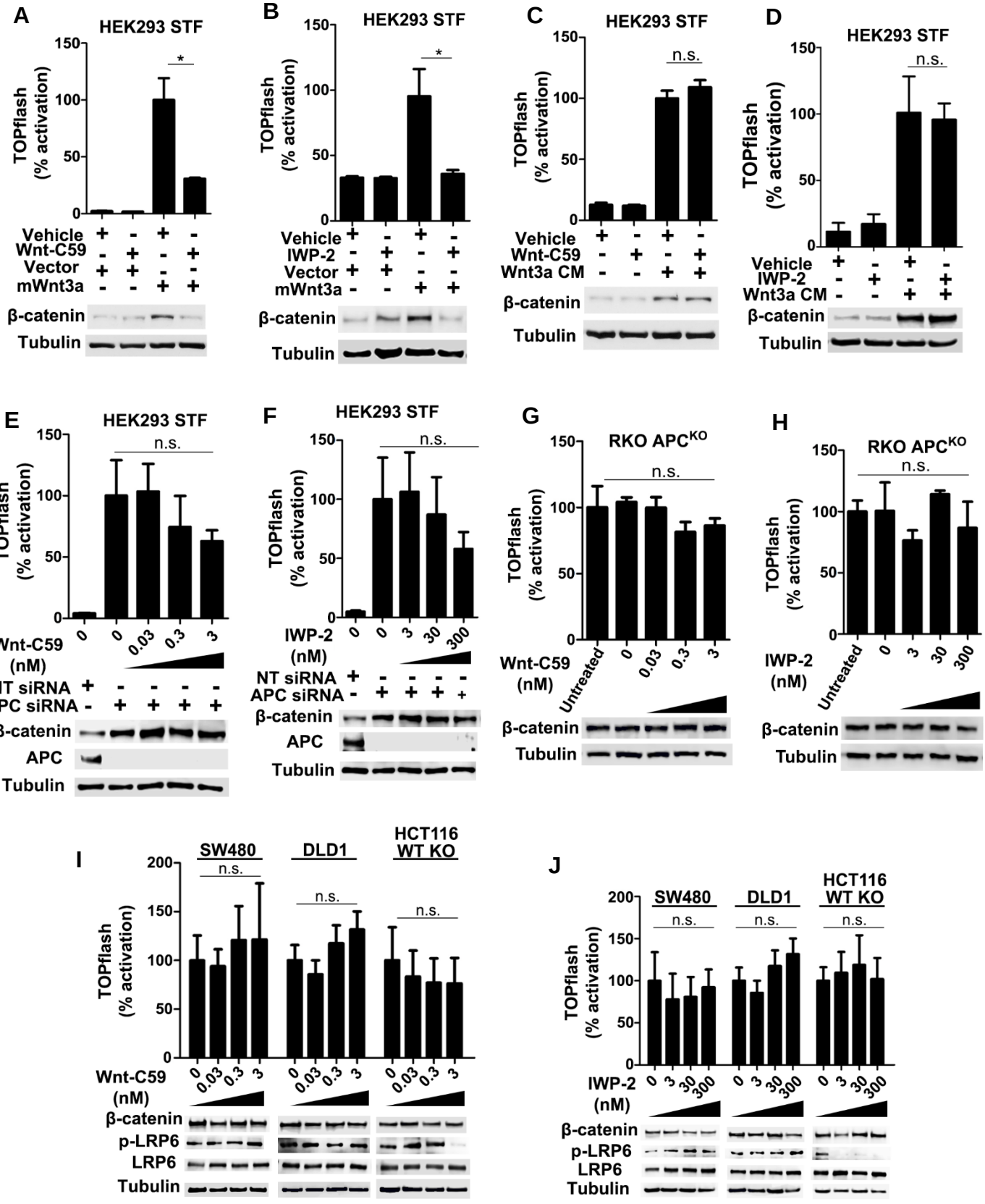
(E) APC-depleted cells are still responsive to Wnt ligand. RKO APC^{KO} cells were pre-incubated for 30 min with Fz8-Fc followed by incubation with Wnt3a.

Graphs show mean ± s.e.m., n.s. non-significant, *p<0.05, **p<0.01.

Fc (Figure 4.5E). We conclude that Wnt ligands are dispensable for pathway activation resulting from APC loss.

To test the possible requirement for Wnt ligands using an independent approach, we inhibited their secretion. Palmitoylation of Wnt ligands by the O-acyltransferase Porcupine (PORCN), is required for their secretion and receptor binding (Gao and Hannoush, 2014; Janda et al., 2012). As expected, two PORCN inhibitors, Wnt-C59 (IC50=0.1nM) and IWP-2 (IC50=27nM) (Chen et al. 2009; Proffitt et al. 2013), inhibited Wnt signaling in cells transfected with Wnt3a (Figure 4.6A and B) but not when Wnt3a was added exogenously (Figure 4.6C and D). Wnt-C59 and IWP-2 also had no observable effect on Wnt signaling in APC-deficient cells (Figure 4.6E-H). In contrast to APC mutant CRC cells, we observed a reduction in phospho-LRP6 in HCT116 cells upon Fz8-Fc, Wnt-C59, and IWP-2 treatment, suggesting that an autocrine feedback circuit occurs in HCT116 cells; reporter activity, however, was not noticeably impaired (Figure 4.6I and J).

To rule out any residual PORCN activity, we knocked down APC in PORCN null cells, which are non-responsive to transfected Wnt3a (Barrott et al. 2011) (Figure 4.7A). In contrast to Dvl TKO cells, knocking down APC in PORCN null cells activated Wnt signaling, which was inhibited by LRP6 knockdown (Figure 4.7B). These results are consistent with a model in which loss of APC leads to ligand-independent activation of the Wnt receptors and downstream signaling.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p-LRP6
LRP6
Tubulin
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DLD1
HCT116 WT KO
TOPflash (% activation)
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0
n.s.
IWP-2 (nM) 0 3 30 300
β-catenin
p-LRP6
LRP6
Tubulin

Figure 4.6. PORCN is not required for Wnt activation in the absence of APC.

(A and B) Inhibition of secreted Wnt3a by the PORCN inhibitors Wnt-C59 and IWP-2. HEK293 STF were transfected with Wnt3a and treated with (A) Wnt-C59 (0.3 nM) or (B) IWP-2 (30 nM).

(C and D) Wnt pathway activation by exogenously added Wnt3a is not blocked by PORCN inhibitors. HEK293 STF cells were pre-incubated with (C) Wnt-C59 or (D) IWP-2 for 30 minutes followed by incubation with Wnt3a.

(E-H) PORCN inhibition does not block Wnt activation upon loss of APC. (E and F) HEK293 STF cells transfected with APC siRNA and (G and H) RKO APC^{KO} cells were incubated with (E and G) Wnt-C59 or (F and H) IWP-2.

(H and I) PORCN inhibition does not block Wnt activation in CRC cells. CRC cells were incubated with (H) Wnt-C59 or (I) IWP-2 for 48h.

Graphs show mean ± s.e.m., n.s. non-significant, *$p<0.05$.

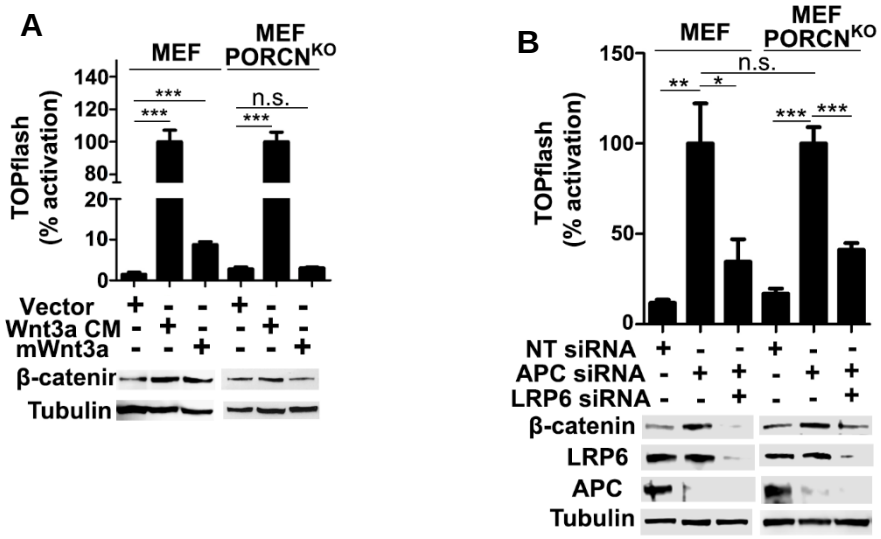


Figure 4.7. LRP6 is required for Wnt activation upon loss of APC in PORCN null cells

(A) PORCNKO cells are responsive to exogenous Wnt3a but not transfected Wnt3a. PORCNKO MEF and wild-type MEF were transfected with Wnt3a or incubated with exogenous Wnt3a.

(B) LRP6 is required for Wnt activation upon loss of APC in the absence of PORCN. PORCNKO MEF and wild-type MEF were transfected with APC and LRP6 siRNA.

Graphs show mean ± s.e.m., n.s. non-significant, *p<0.05, **p<0.01, ***p<0.001.

LRP6 and Dishevelled are required for the aberrantly increased proliferation of Apc1 mutant adult intestinal stem cells in Drosophila

Similar to the consequences of APC loss in the mammalian intestine (Kinzler and Vogelstein 1996; Korinek et al. 1997; Sansom et al. 2004), inactivation of *Drosophila* Apc1 in adult intestinal stem cells (ISCs) results in their aberrantly increased proliferation (Cordero et al. 2009; Lee et al. 2009). We tested whether the ISC overproliferation and epithelial cell polarity defects resulting from *Apc1* inactivation is dependent on the Drosophila homolog of LRP6 (Arrow; *Arr*) (Wehrli et al., 2000). By comparison with controls (Figure 4.8A, B, and M), a significantly increased number of progenitor cells (ISCs and enteroblasts), marked by their expression of *esg*>GFP (esg-gal4,UAS-GFP) (Micchelli and Perrimon 2006), was observed in the posterior midgut of 1- to 2-day-old *Apc1* null mutant adults (Figure 4.8E, F, and M). In contrast, RNAi-mediated *Arrow* knockdown in *Apc1* null mutant intestinal progenitor cells during development of the adult midgut resulted in a significant reduction in progenitor cell number (Figure 4.8I, J, and M). Furthermore, the disrupted epithelial cell polarity in *Apc1* null mutants, as revealed by the aberrant subcellular localization of adherens junction marker *Armadillo* (*Arm*) (compare Figures 4.8C and 4.8D with Figures 4.8G and 4.8H) was also rescued by *Arrow* depletion (Figures 4.8K and 4.8L). To rule out off-target effects resulting from expression of the RNAi constructs, we tested two independently-derived Arrow knockdown lines and obtained similar results (Figure 4.8M).

To further test these findings, we examined the effects RNAi-mediated knockdown of Apc1 either singly or with concomitant knockdown of Arrow or Dvl

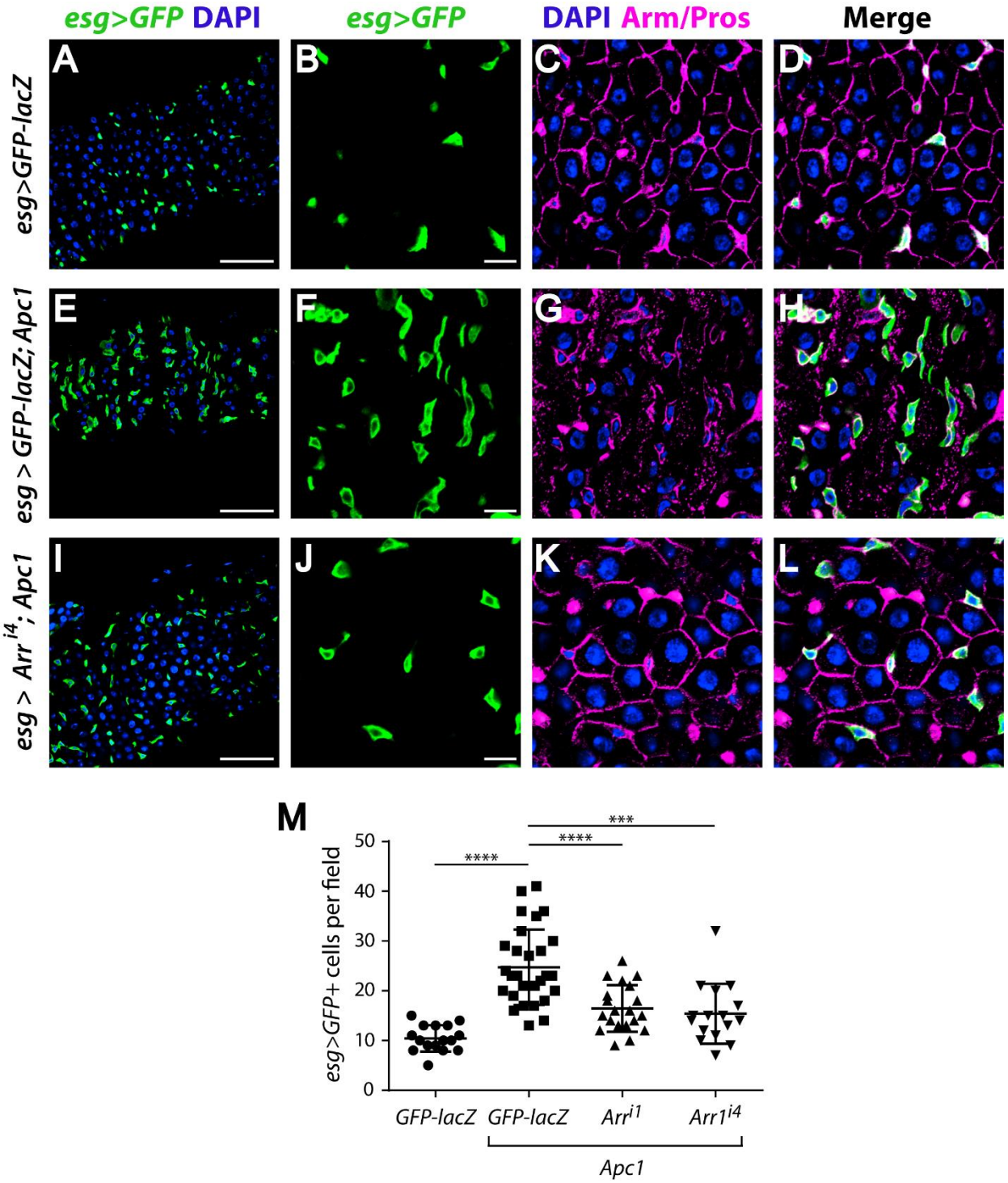


Figure 4.8. Both ISC overproliferation and epithelial cell polarity defects in *Drosophila Apc1* null mutant midguts are rescued by *Arrow* depletion.

(A-H, and M) By comparison to controls (A-D, and M), loss of Apc1 leads to increased progenitor cell number (E, F, H, and M) and deregulated epithelial cell polarity in the

adult posterior midgut (G, H). Intestinal progenitor cells are marked with esg>GFP (green). Adherens junctions are marked with Arm (magenta) and nuclei with DAPI (blue). Enteroendocrine cells are marked with Prospero (Pros, magenta).

(I-L, and M) Expression of Arrow RNAi constructs in Apc1 mutants leads to a significant reduction in progenitor cell numbers (I, J, L, and M) and rescues defects in cell polarity (K and L).

(A, E, and I) Lower magnification view (scale bars: 50 μm) (B-D, F-H, and J-L) higher magnification view (scale bar: 10 μm).

Graphs show mean ± s.e.m., ****p<0.0001, ***p<0.001.

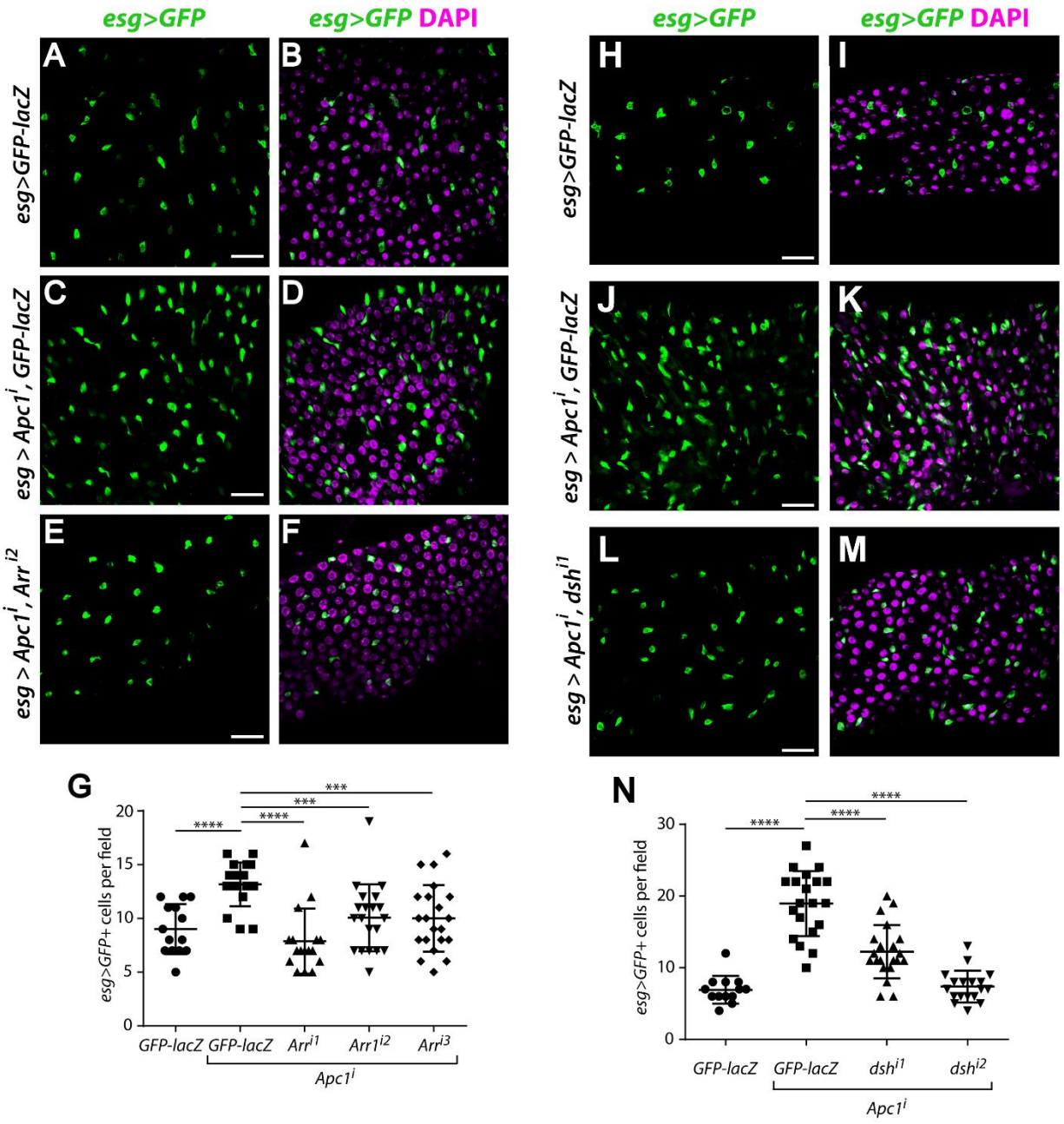


Figure 4.9. Arrow and Dishevelled are required for overproliferation of midgut stem cells resulting from depletion of Apc1 in *Drosophila*.

(A-D, and G) By comparison to controls (A, B and G), RNAi-mediated knockdown of Apc1 during pupation in ISCs using esgts (temperature sensitive driver) leads to an increase in adult midgut progenitor cell number (C, D, and G). Progenitor cells are marked with esg>GFP (green), and nuclei are marked with DAPI (magenta). (E-G) Concomitant expression of the Apc1 RNAi construct with any one of three different arr RNAi constructs leads to a significant reduction in progenitor cell numbers (E-G). Scale bar: 30 μm

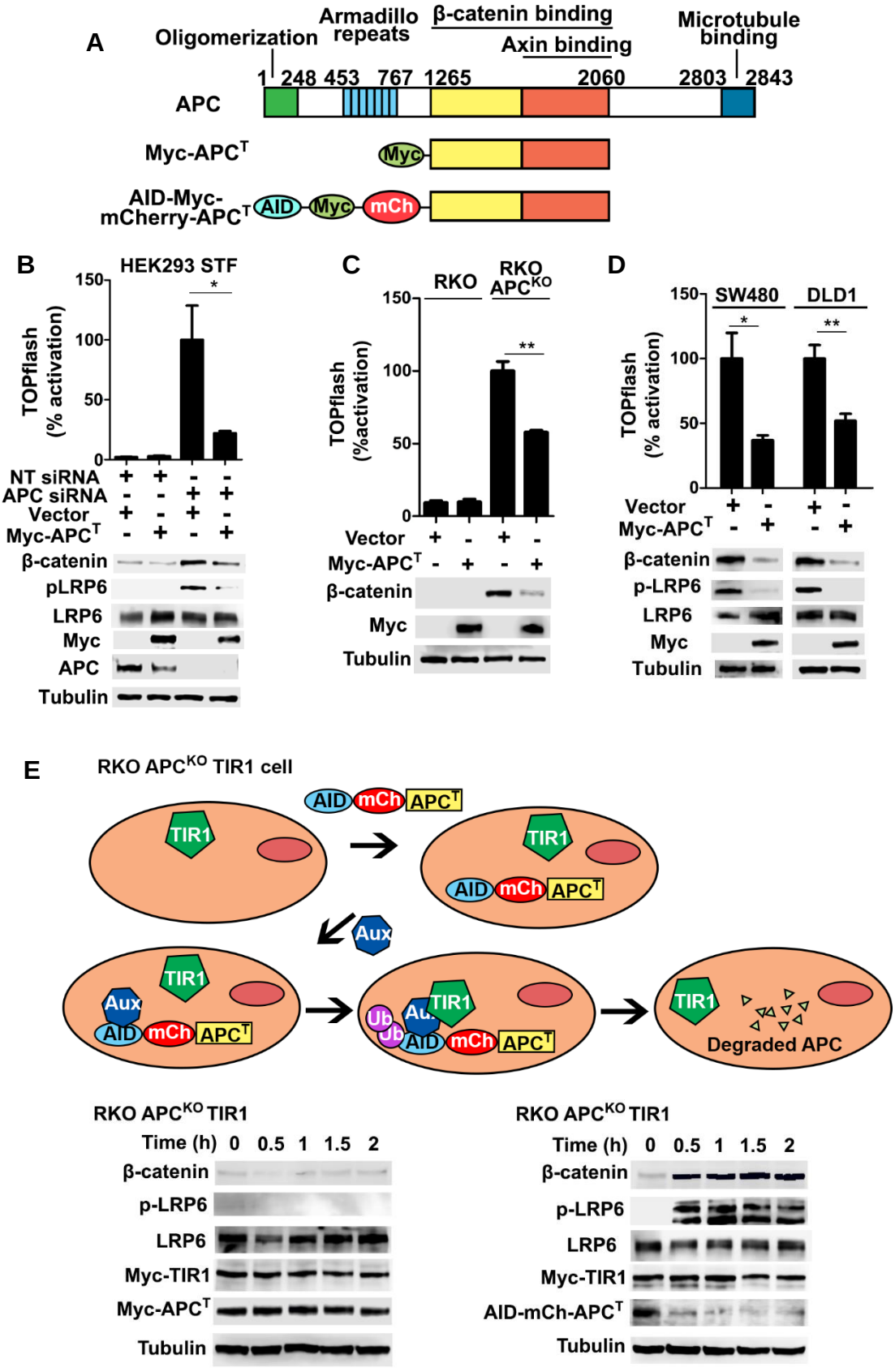
(H-K, and N) By comparison to controls (H, I, and N), RNAi-mediated knockdown of Apc1 in ISCs results in an increase in progenitor cell number (J, K, and N). Apc1 knockdown in progenitor cells during pupation was achieved by the temperature-sensitive ISC driver, esgts. Intestinal progenitor cells are marked with esg>GFP (green). Nuclei are marked with DAPI (magenta). (L-N) Concomitant expression of the Apc1 RNAi construct with either of two different dsh RNAi constructs results in a significant reduction in progenitor cell numbers. Scale bar: 30 μm.,

Graphs show mean ± s.e.m., *** $p<0.001$, **** $p<0.0001$.

(Dishevelled; *dsh*) (Klingensmith et al. 1994; Noordermeer et al. 1994; Siegfried et al.1994). *Apc1*, *Arrow*, or *dsh* were knocked down in ISCs during formation of the adult midgut using the temperature-sensitive progenitor cell driver esg^{ts}. Consistent with the *Apc1* null mutant phenotype, knockdown of *Apc1* resulted in an increase in progenitor cell number in the posterior midgut of 1- to 2-day-old adults (compare Figure 4.9A, B, and G with Figure 4.9C, D, and G). In contrast, concomitant RNAi-mediated knockdown of Apc1 and Arrow resulted in a significant reduction in this number (Figure 4.9E, F, and G). Similarly, concomitant knockdown of *dsh* and *Apc1* also resulted in a significant reduction of ISC number as compared to knockdown of *Apc1* alone (Figure 4.9H-N). To rule out RNAi off-target effects, we tested multiple independently-derived *arrow* and *dsh* knockdown lines and obtained similar results (Figure 4.9G and Figure 4.9N). These findings provide in vivo evidence that the requirement for plasma membrane components of the Wnt pathway for the aberrantly increased signaling in APC-deficient cells is evolutionarily conserved.

Rapid accumulation of phospho-LRP6 and β-catenin stabilization occur upon immediate loss of APC

We asked if receptor activation following APC loss was a secondary consequence of a simple positive feedback mechanism resulting from the regulation of Wnt target genes. We found that lithium treatment stabilized β-catenin and promoted Wnt reporter activity, but, in contrast to loss of APC, did not promote phospho-LRP6 accumulation (Figure 4.10A). We also tested the effects of a dominant-negative form of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Armadillo repeats
β-catenin binding
Axin binding
Microtubule binding
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Myc-APC^T - + - +
β-catenin
p-LRP6
LRP6
Myc
Tubulin
E
RKO APC^KO TIR1 cell
AID
mCh
APC^T
TIR1
Aux
Ub
Degraded APC
RKO APC^KO TIR1
Time (h) 0 0.5 1 1.5 2
β-catenin
p-LRP6
LRP6
Myc-TIR1
Myc-APC^T
Tubulin
RKO APC^KO TIR1
Time (h) 0 0.5 1 1.5 2
β-catenin
p-LRP6
LRP6
Myc-TIR1
AID-mCh-APC^T
Tubulin

Figure 4.10. LRP6 activation and β-catenin accumulation occur rapidly upon loss of APC function.

(A) Schematics of APC structure and truncations tested.

(B-D) β-catenin and Axin binding domains of APC are sufficient to rescue loss-of-APC phenotype. (E) HEK293 STF treated with APC siRNA, (F) RKO APCKO, and (G) CRC cells were transfected with Myc-APCT followed by TOPflash analysis and immunoblotting.

(E) Loss of APC correlates with β-catenin and phospho-LRP6 accumulation. RKO APCKO TIR1 cells were transfected with Myc-APCT or AID-Myc-mCherry-APCT followed by incubation with auxin. AID, auxin-inducible degron; mCh, mCherry; Aux, auxin.

Graphs show mean ± s.e.m., n.s. non-significant, *p<0.05, **p<0.01, ***p<0.001.

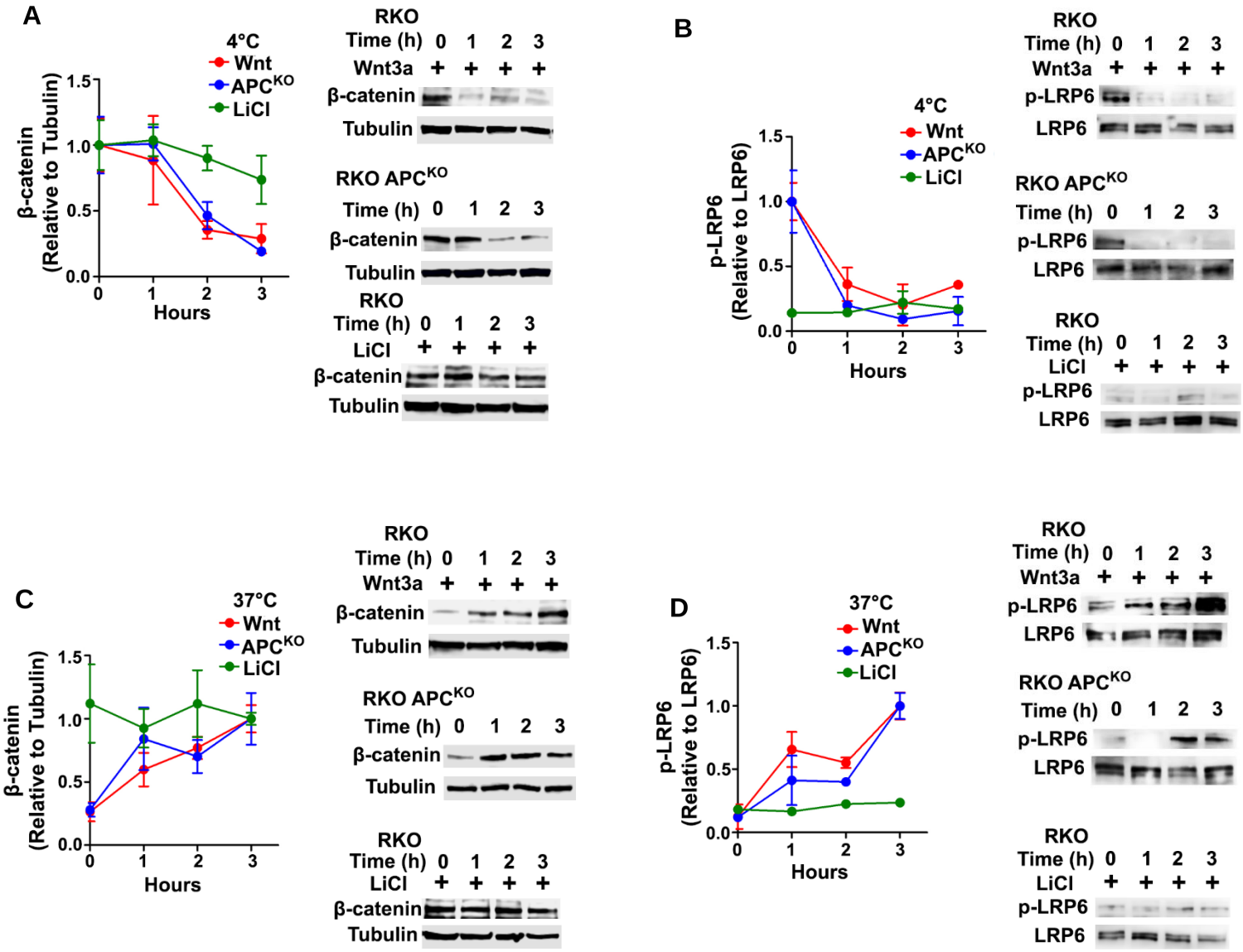
TCF4 (dnTCF4) (van de Wetering et al. 2002) that prevents TCF-mediated expression of Wnt target genes. We found that dnTCF4 inhibited reporter activation, but not accumulation of either β-catenin or phospho-LRP6, upon APC knockdown (Figure 4.10B and C). Thus, stabilization of β-catenin and activation of Wnt target genes are not sufficient to activate Wnt receptors upon APC loss.

APC exhibits multiple activities in addition to its role in Wnt signaling (Saito-Diaz et al., 2012). The aberrant activation of Wnt signaling in APC mutant cells is rescued by an APC fragment (APC^T) containing its β-catenin and Axin binding domains (Li et al. 2012) (Figure 4.10A). We found that APC^T suppresses LRP6 phosphorylation in APC-deficient cells (Figure 4.10B-D), suggesting that LRP6 activation in APC mutant cells is due to loss of the region of APC associated with its Wnt pathway function. If formation of an active Wnt receptor complex is not due to a transcriptional/translational feedback mechanism in APC-deficient cells, LRP6 phosphorylation and β-catenin stabilization upon APC loss should be temporally correlated. To test this hypothesis, we investigated the timing of LRP6 phosphorylation and β-catenin accumulation following APC loss. We fused the APC^T fragment to an auxin-inducible degron (AID) that allows for auxin-induced turnover (Holland et al. 2012) by the plant E3 TIR1 protein (Figure 4.10A). Transfection of AID-Myc-mCherry-APC^T into RKO APC^{KO} cells stably expressing E3 TIR1 suppresses Wnt signaling to a similar extent as the APC^T fragment (Figure 4.10E, compare left and right panels). The addition of auxin resulted in a rapid loss of AID-Myc-mCherry-APC^T (within 30 minutes) that paralleled the stabilization of β-catenin. Significantly, the accumulation of β-catenin levels paralleled the appearance of phospho-LRP6 (Figure 4.10E, right panel).

Endocytosis is required for Wnt signaling upon loss of APC

Endocytosis is required for receptor-mediated activation of the Wnt pathway (Blitzer and Nusse 2006; Yamamoto et al. 2006; Gagliardi et al. 2014). As expected, we found that blocking endocytosis by shifting Wnt3a-treated RKO or RKO APC^{KO} cells to 4˚C decreased cytoplasmic β-catenin and phospho-LRP6 levels; this effect was reversed by shifting cells back to 37˚C. In contrast, activating RKO cells with lithium had no effect on cytoplasmic β-catenin levels when incubated at 4˚C (Figure 4.11A-D). Similarly, we found that shifting SW480 (APC mutant), but not HC116 (β-catenin mutant) cells, to 4°C decreased cytoplasmic β-catenin levels (Figure 4.12). To test the requirement for endocytosis further, we used a dominant-negative Dynamin ($Dynamin^{K44A}$), which inhibits both clathrin and caveolin-mediated endocytosis and has been shown to inhibit Wnt signaling (Blitzer and Nusse 2006; Yamamoto et al. 2008; Dutta and Donaldson 2012). $Dynamin^{K44A}$ decreased β-catenin levels in all APC-deficient cell lines tested (Figure 4.13A-D).

Given that we found that Dynamin is required for Wnt pathway activation in APC-deficient cells, we asked whether LRP6 is internalized upon loss of APC. Consistent with previous reports (Yamamoto et al., 2006), we found that LRP6 was internalized within two hours after addition of Wnt3a and could be blocked with mAb7E5 (Figure 4.14A). As expected, lithium had no effect on LRP6 internalization. Internalization of LRP6 induced by APC knockdown was blocked with mAb7E5 but not with PORCN inhibitors (Figure 4.14B and C). In RPE cells, fluorescence intensity profiles from an axial scan showed two distinct peaks of LRP6-eYFP signal cells that corresponded to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LRP6

Figure 4.11. Endocytosis inhibition induced by temperature shift prevents β-catenin accumulation and LRP6 phosphorylation.

(A-D) RKO cells (treated with Wnt3a or 30 mM LiCl), and RKO APCKO cells were incubated at 4°C (A and B) and shifted back to 37°C (C and D). The average of three independent replicates were plotted.

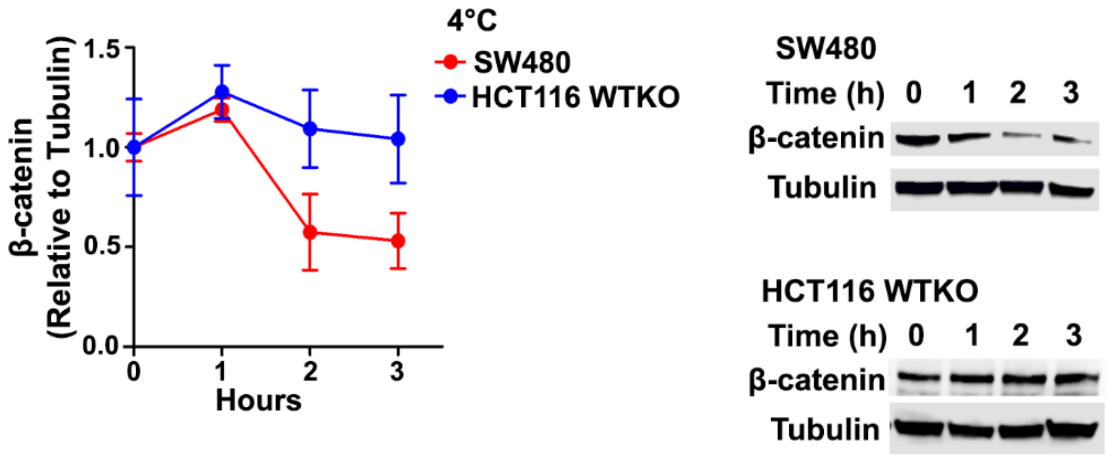


Figure 4.12. Endocytosis inhibition induced by temperature shift prevents β-catenin accumulation in APC-mutant CRC cells.

CRC cells were incubated at 4°C. Lysates were obtained at specified times and analyzed by immunoblotting. The average of three independent replicates were plotted. Blot are representative experiments.

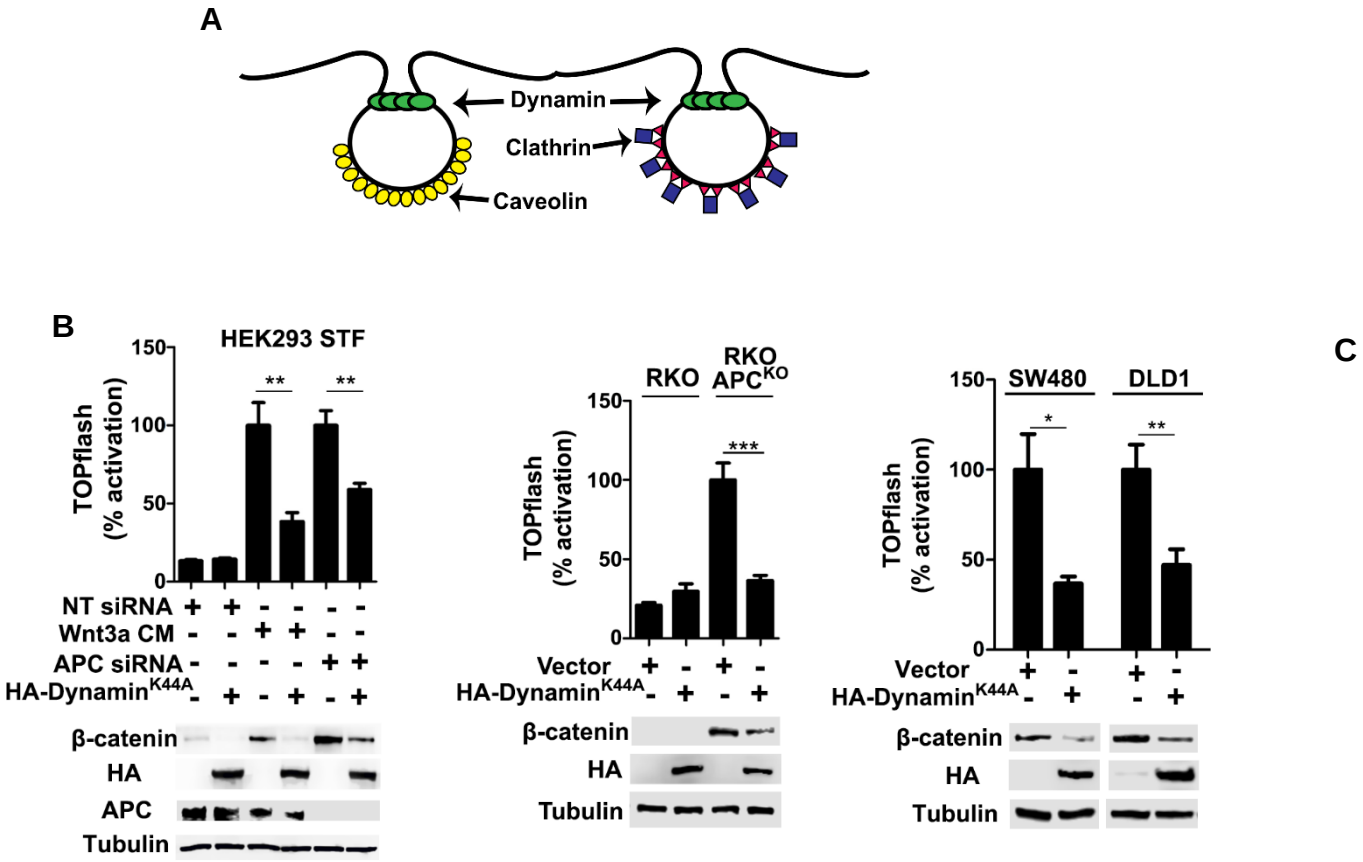


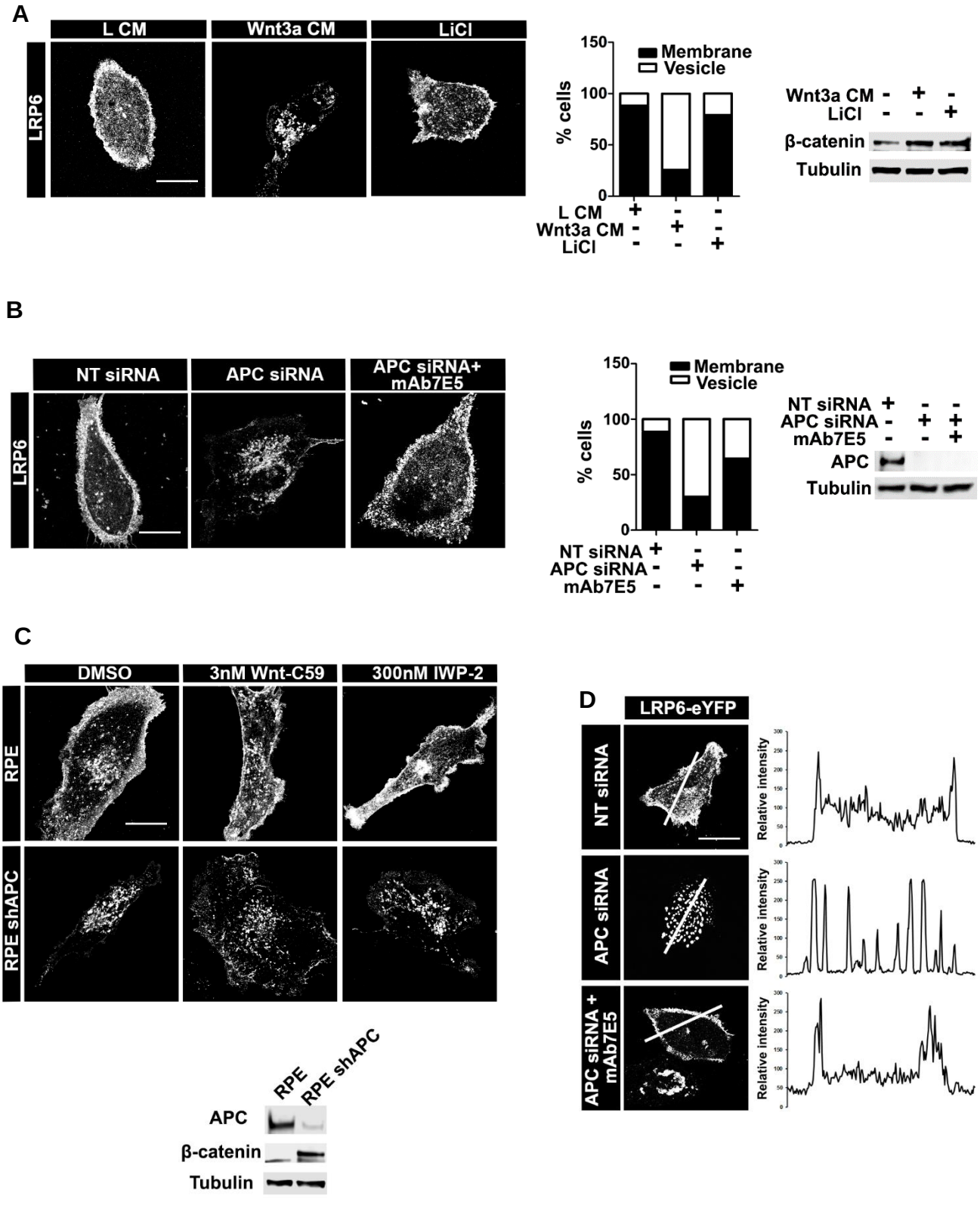
Figure 4.13. Dynamin is required for Wnt signaling in the absence of functional APC.

(A) Schematics of Dynamin function in endocytosis.

(B) Dynamins is required for Wnt activation. HEK293 STF cells incubated with Wnt3a or APC siRNA and transfected with HA-Dynamin^{K44A}

(C and D) Dynamin is required for Wnt signaling upon loss of APC function. (C) RKO APC^{KO} cells and (D) CRC cells were transfected with HA-Dynamin^{K44A}.

Graphs show mean ± s.e.m., n.s. non-significant, *p<0.05, **p<0.01, ***p<0.001.


A
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Wnt3a CM - + -
LiCl - - +
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Tubulin
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NT siRNA
APC siRNA
APC siRNA+ mAb7E5
LRP6
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APC siRNA - + -
mAb7E5 - - +
NT siRNA + - -
APC siRNA - + +
mAb7E5 - - +
APC
Tubulin
C
DMSO
3nM Wnt-C59
300nM IWP-2
RPE
RPE shAPC
RPE
RPE shAPC
APC
β-catenin
Tubulin
D
LRP6-eYFP
NT siRNA
APC siRNA
APC siRNA + mAb7E5
Relative intensity
Relative intensity
Relative intensity

Figure 4.14. LRP6 is internalized in APC-deficient cells.

(A and B) LRP6 is internalized in Wnt3a treated and APC deficient cells. RPE cells were transfected with LRP6-eYFP and incubated with Wnt3a or 30mM LiCl or (B) APC siRNA and mAb7E5. Cells (>100/condition) demonstrating membrane or internalized LRP6 were quantified.

(C) PORCN inhibition does not block LRP6 internalization upon loss of APC. RPE shAPC cells were transfected with LRP6-eYFP and incubated with Wnt-C59 or IWP-2.

(D) Loss of APC promotes LRP6 internalization. RPE cells were transfected with LRP6-eYFP and APC siRNA in the absence or presence of mAb7E5. Line scans were performed to quantify changes in LRP6 intensity across cells.

Scale bars: 20 μm.

high concentrations of LRP6 at the plasma membrane. In contrast, APC-depleted cells, we observed multiple peaks corresponding to previously reported large, punctate, LRP6-containing structures postulated to represent intracellular vesicles (Bilić et al., 2007). Treatment of APC-depleted cells with mAb7E5 re-established the formation of two predominant peaks of fluorescence signal (Figure 4.14D). These studies provide further evidence that the endocytic pathway is required to activate the Wnt pathway in APC-deficient cells.

Wnt receptor activation in APC-deficient cells occurs via a clathrin-dependent mechanism.

Clathrin- and caveolin-mediated endocytosis have both been implicated in Wnt signaling (Blitzer and Nusse 2006; Yamamoto et al. 2006; Bilić et al. 2007; Hanson et al. 2013). Chloroquine and nystatin are small molecule inhibitors of clathrin- and caveolin-mediated endocytosis, respectively (Yamamoto et al. 2006; Chen et al. 2009; Dutta and Donaldson 2012). Consistent with a previous study (Yamamoto et al., 2006), nystatin, but not chloroquine, inhibited Wnt3a-mediated accumulation of β-catenin in RKO cells (Figure 4.15A and B). In RKO APC^{KO} cells, however, chloroquine and pitstop-2 (another inhibitor of clathrin-mediated endocytosis) (Dutta et al. 2012) decreased β-catenin, whereas nystatin had no observable effect (Figure 4.15C-F). To confirm these results, we knocked down clathrin and caveolin (Figure 4.16A and B). Knocking down caveolin, but not clathrin, inhibited Wnt3a signaling in RKO cells (Figure 4.16C). In contrast, knocking down clathrin, but not caveolin, inhibited Wnt signaling in RKO

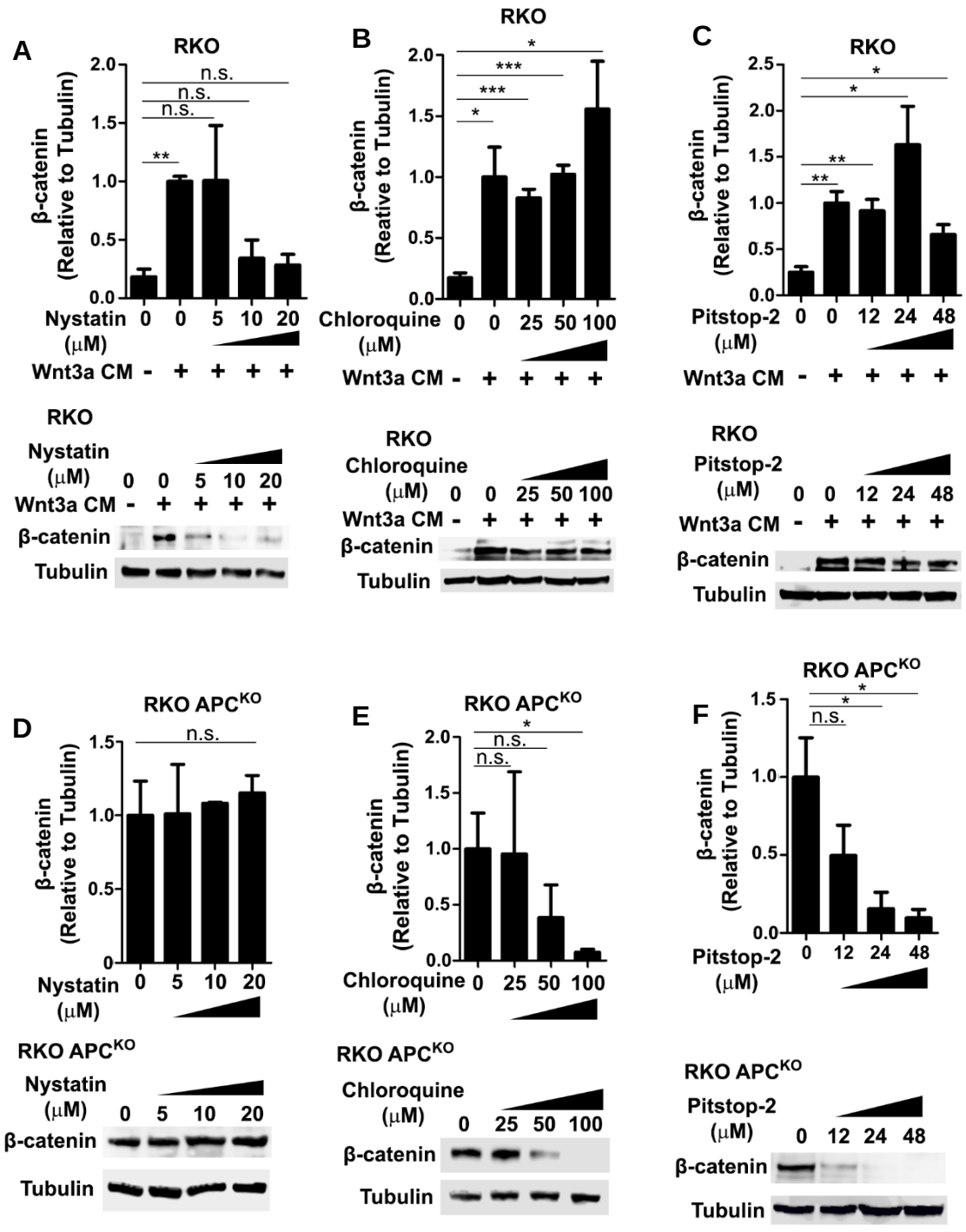


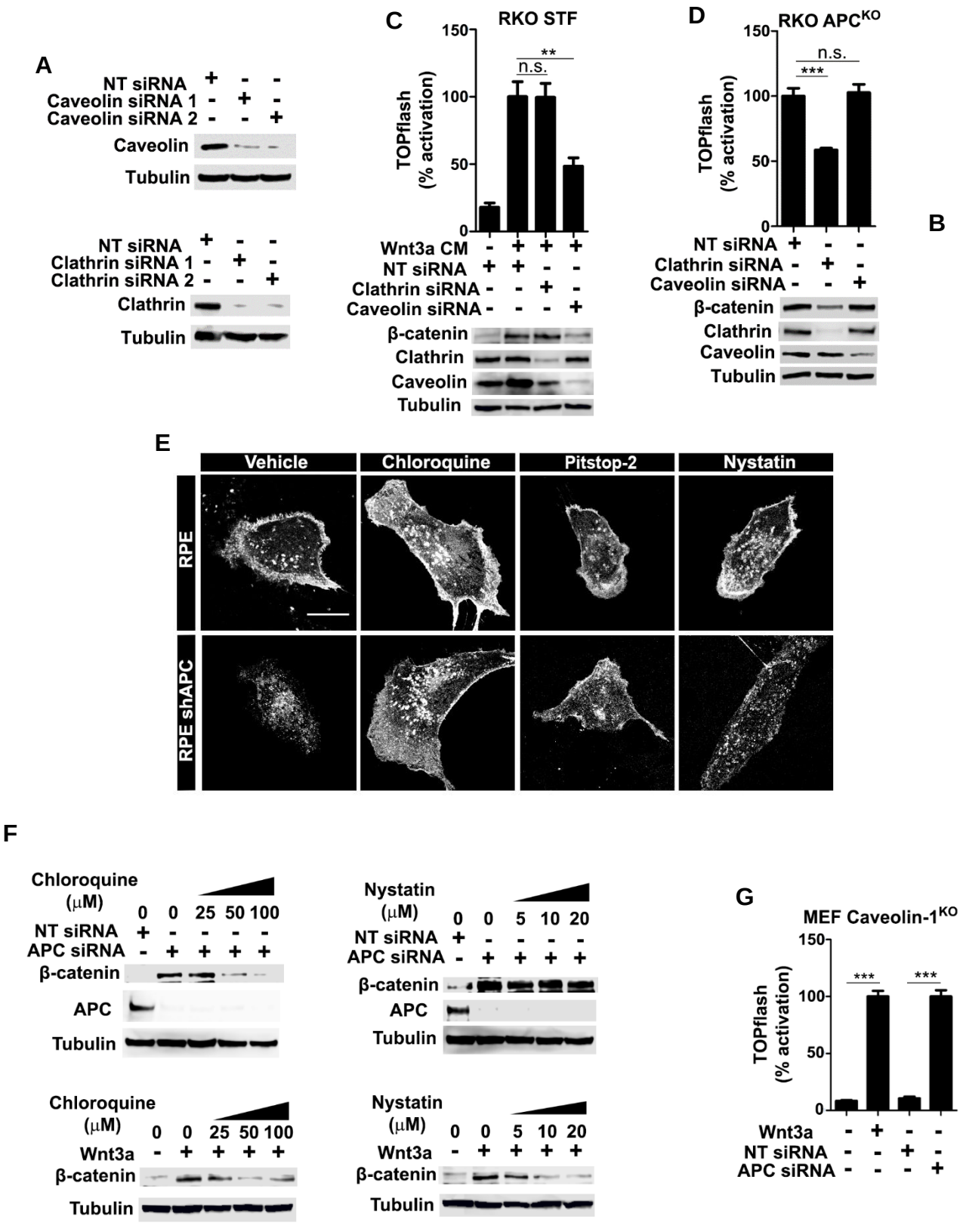
Figure 4.15. Ligand-dependent and -independent Wnt activation requires distinct endocytic pathways.

(A-C) RKO cells incubated with Wnt3a were treated with indicated concentrations of nystatin, chloroquine, or Pitstop-2. Followed by immunoblotting.

(D-F) RKO APC^{KO} cells were treated with indicated concentrations of nystatin, chloroquine, or Pitstop-2. Followed by immunoblotting.

The average of three independent replicates were plotted. Blot are representative experiments.

Graphs show mean ± s.e.m., n.s. non-significant, *p<0.05, **p<0.01, ***p<0.001.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Clathrin siRNA 1 - + -
Clathrin siRNA 2 - - +
Clathrin
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TOPflash (% activation)
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Wnt3a CM - + + +
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Clathrin siRNA - - + -
Caveolin siRNA - - - +
β-catenin
Clathrin
Caveolin
Tubulin
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NT siRNA + - -
Clathrin siRNA - + -
Caveolin siRNA - - +
β-catenin
Clathrin
Caveolin
Tubulin
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Vehicle
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Pitstop-2
Nystatin
RPE
RPE shAPC
F
Chloroquine (μM)
0 0 25 50 100
NT siRNA + - - - -
APC siRNA - + + + +
β-catenin
APC
Tubulin
Chloroquine (μM)
0 0 25 50 100
Wnt3a - + + + +
β-catenin
Tubulin
Nystatin (μM)
0 0 5 10 20
NT siRNA + - - - -
APC siRNA - + + + +
β-catenin
APC
Tubulin
Nystatin (μM)
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TOPflash (% activation)
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Wnt3a - + - -
NT siRNA - - + -
APC siRNA - - - +

Figure 4.16. Clathrin-mediated endocytosis is required for Wnt activation upon loss of APC.

(A) Downregulation of clathrin by siRNAs. RKO cells were incubated with two different siRNAs.

(B) Two distinct siRNAs decrease caveolin levels. RKO cells were incubated with two siRNAs.

(C) RKO cells incubated with Wnt3a were transfected with caveolin-1 or clathrin siRNA.

(D) RKO APCKO cells were transfected with clathrin or caveolin-1 siRNA.

(E) Inhibition of clathrin-mediated endocytosis blocks LRP6 endocytosis in APC-depleted cells. RPE and RPE shAPC were incubated with chloroquine, Pitstop-2, and nystatin, and live cells were imaged by confocal microscopy.

(F) Clathrin requirement for Wnt activation upon loss of APC is conserved. L-cells were incubated with Wnt3a or APC siRNA and treated with chloroquine or nystatin.

(G) Wnt activation upon loss of APC is caveolin-1-independent. Caveolin-1^{KO} MEFs were incubated with Wnt3a or APC siRNA.

Graphs show mean ± s.e.m., *p<0.05, **p<0.01, ***p<0.001.

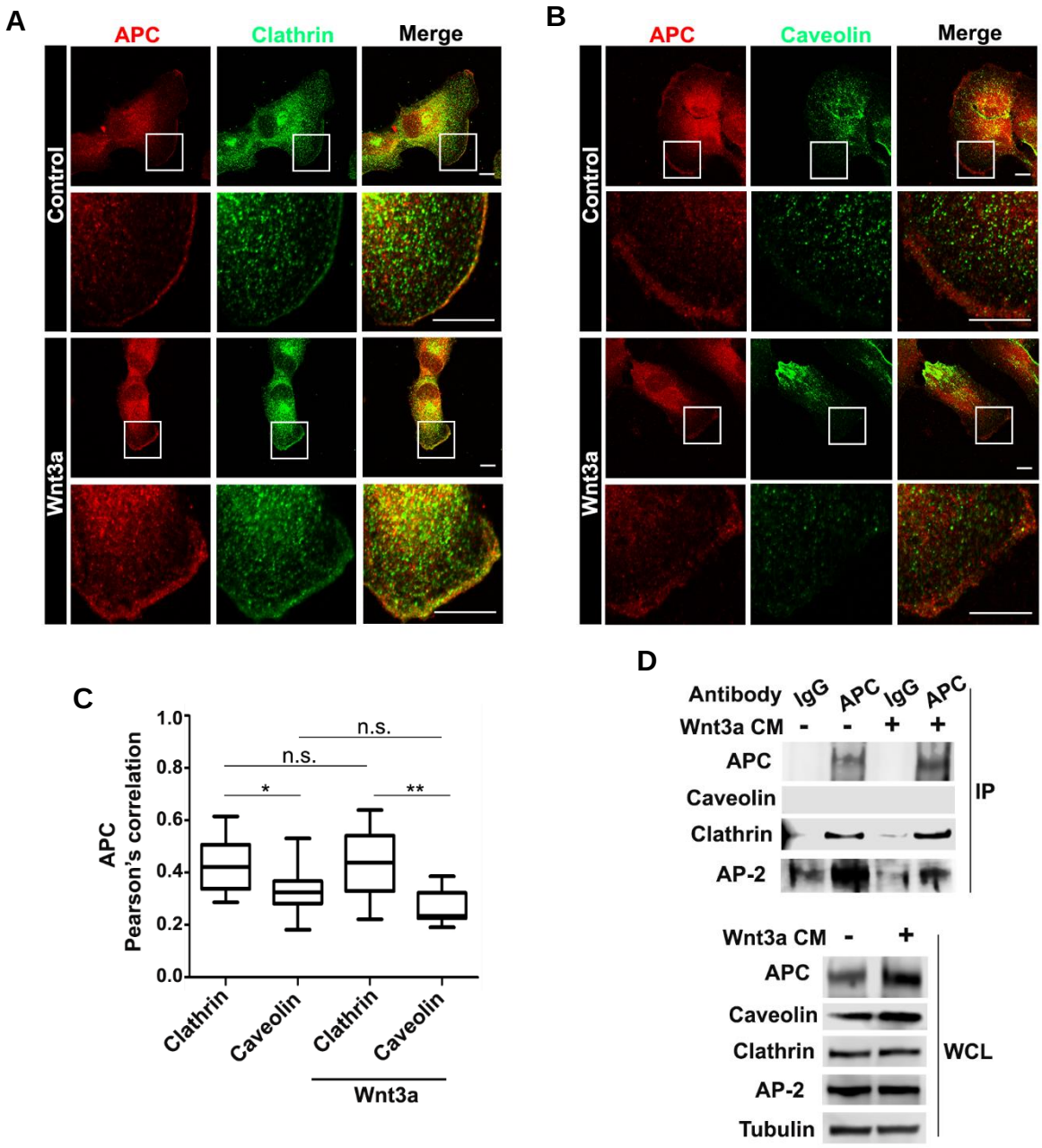

Figure 4.17. APC forms a complex with clathrin.

(A-C) APC partially colocalizes with clathrin but not caveolin-1. RPE cells were incubated with or without Wnt3a, fixed, and stained for APC and (A) Clathrin or (B) caveolin-1. Scale bar: 10μm. (C) Intensity correlation analysis of APC-clathrin/caveolin-1 signal.

(D) APC co-immunoprecipitates with clathrin and its adaptor protein, AP2

Graph show mean ± s.e.m., n.s. non-significant, *p<0.05, **p<0.01.

APC^{KO} cells (Figure 4.16D). We next assessed whether treatment with chloroquine, pitstop-2, or nystatin altered LRP6-eYFP accumulation at the plasma membrane, whereas nystatin treatment had no observable effect (Figure 4.16E). These studies provide further evidence that Wnt signaling in APC-depleted cells occurs via a clathrin-mediated mechanism.

A previous study showed that disruption of clathrin-mediated endocytosis in mouse fibroblasts L-cells inhibits Wnt ligand-mediated activation (Blitzer and Nusse, 2006). We found that Wnt3a signaling in L-cells is mediated by both clathrin and caveolin-pathways (Figure 4.16F). Wnt pathway activation upon APC knockdown, however, is a clathrin-dependent process in both L-cells (Figure 4.16F), and in a MEF cell line deficient for caveolin (Hanson et al., 2013) (Figure 4.16G). Thus, the requirement for caveolae versus clathrin for Wnt ligand activation appears to be cell-type dependent, whereas clathrin is specifically required for constitutive Wnt pathway activation in APC-deficient cells regardless of cell type.

Finally, we tested whether endogenous APC forms a complex with endogenous clathrin. Immunofluorescence microscopy studies revealed significant overlap of APC with clathrin, but not caveolin, at the plasma membrane in RPE cells (Figure 4.17A-C). To further test these results using a biochemical approach, we performed co-immunoprecipitation studies and found that endogenous APC was pulled down with clathrin and its adaptor protein, AP-2, but not caveolin (Figure 4.17D).

Discussion

Despite extensive investigation, the mechanistic basis for APC function in Wnt signaling has remained an enigma (Saito-Diaz et al., 2012). Based on our studies, we propose that a major role of APC is to prevent constitutive, ligand-independent, clathrin-dependent signalosome formation and pathway activation (Figure 4.18). Our findings are not inconsistent with the role for APC in β-catenin phosphorylation and degradation (Clevers and Nusse 2012). APC and APC2 are functionally redundant in some contexts (Ahmed et al. 2002; Daly et al. 2016). Thus, in the absence of APC, APC2 could act as the primary scaffold protein in the β-catenin degradation complex. This could explain why β-catenin can still be degraded in cells upon loss of APC when the Wnt receptor complex is disrupted; APC2 is able to associate with the degradation complex to promote β-catenin turnover

Previous studies provide evidence for both caveolin and clathrin-based mechanisms in the activation of the Wnt pathway (Bilić et al., 2007; Blitzer and Nusse, 2006; Hanson et al., 2013; Yamamoto et al., 2006). Consistent with this possibility, we observed that epithelial cells (e.g., HEK293, RKO and RPE cells) mediate Wnt signaling via the caveolin pathway, whereas fibroblasts (e.g., L-cells and MEFs) mediate Wnt signaling via the clathrin pathway. Because caveolin- and clathrin-dependent endocytotic pathways differ in their dynamic association with the plasma membrane, Wnt signaling could potentially be qualitatively different depending on the particular pathway utilized. In addition, clathrin-dependent endocytosis has been linked to an early response to stimuli, whereas caveolae play a later role in maintaining responses (Zhang et al. 2014). The clathrin-mediated pathway can be promiscuous: a well-known example

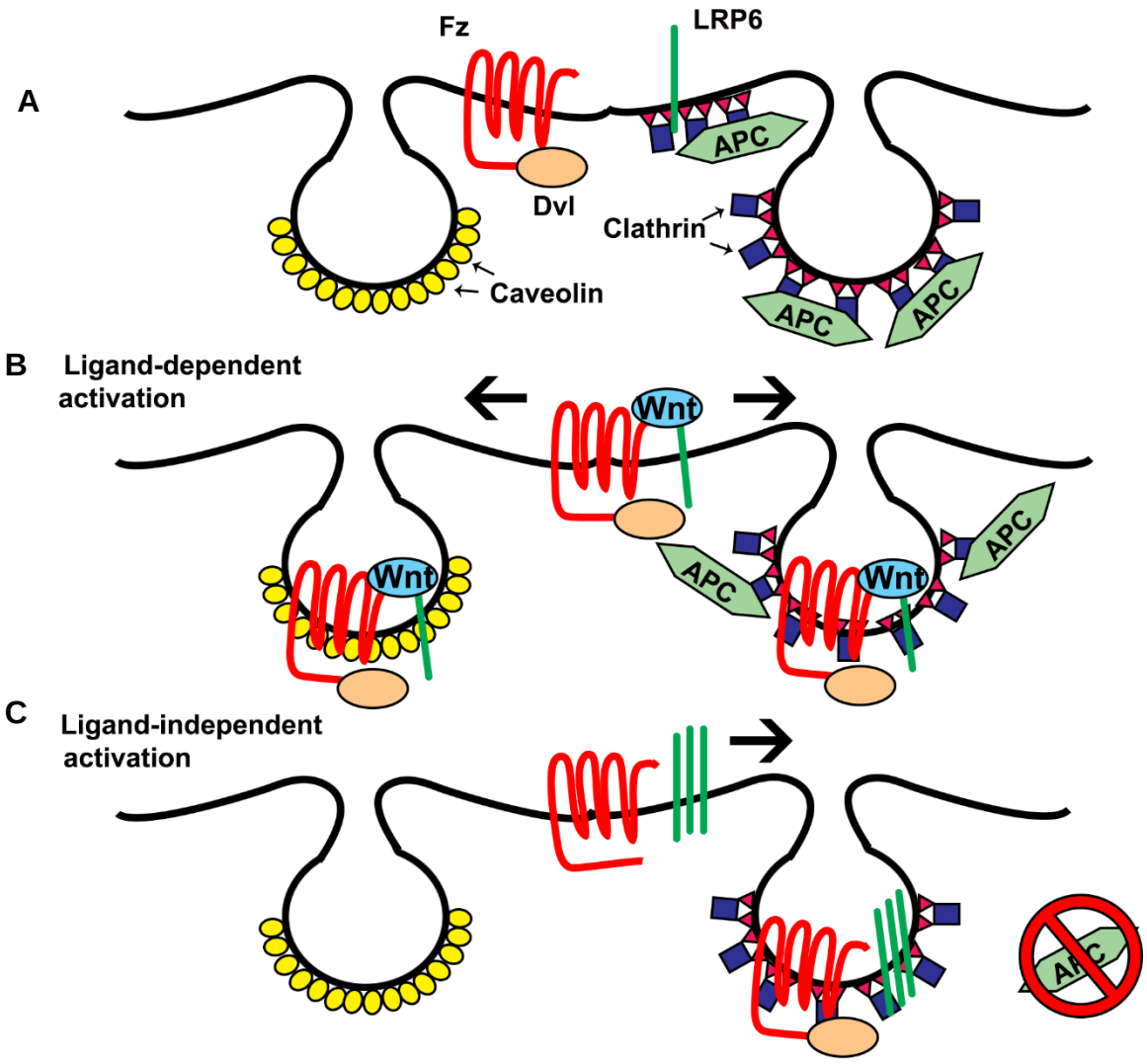

Figure 4.18. Proposed model for APC functioning as a negative regulator of clathrin-mediated endocytosis of Wnt pathway membrane components.

(A) In the absence of a Wnt ligand, LRP6 localizes in nascent Clathrin-coated pits. APC forms a complex with clathrin to block spontaneous Wnt receptor aggregation and activation.

(B) Wnt binds to its co-receptors, Fz and LRP6, to promote signalosome formation. The Wnt signalosome is internalized via the caveolae or clathrin pathways.

(C) Loss of APC results in ligand-independent LRP6 oligomerization, Wnt signalosome formation, and pathway activation via the clathrin endocytic pathway. This event drives constitutive activation of the Wnt pathway.

is the constitutive internalization of the transferrin receptor. Thus, it is possible that APC acts as a “brake” on receptor activation via the clathrin endocytic pathway in the absence of Wnt ligand. Future studies will focus on the detailed mechanism by which APC regulates this process.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Since the discovery of the Wnt signaling pathway, much of the work has focused on understanding the regulatory steps in the pathway as well as identifying targets for developing therapeutics against Wnt-driven diseases. The work presented herein provides insight into both areas of investigation. In this Chapter, I will briefly summarize my findings and describe important questions that arose from my work.

Biochemical characterization of mAb7E5

We identified the monoclonal antibody mAb7E5 that targets the extracellular domain of LRP6. Using biochemical analysis, we identified that mAb7E5 inhibits ligand-dependent and independent Wnt activation. We found that mAb7E5 binds to the LRP6-2 and LRP6-3 fragments of human LRP6. Our working hypothesis is that mAb7E5 blocks the LRP6 conformational change required to effectively transduce the Wnt signal from the plasma membrane to downstream, cytoplasmic effectors. Further studies are needed to more accurately identify the region(s) where mAb7E5 binds to LRP6. This may be readily accomplished by performing binding assays with a series of LRP6 truncations and point mutations to further narrow the mAb7E5 binding domain on LRP6. Ideally, these results will ultimately be confirmed by analyzing the X-ray crystal structure of mAb7E5 bound to LRP6. Such a structure may provide important insight into the

mechanism by which Wnt ligand can induces the conformation of LRP6 to promote its activation.

Regulation of endocytosis by APC

Loss of APC results in constitutive activation of the Wnt pathway. This was thought to occur primarily by disrupting the assembly of the β-catenin degradation complex, thereby bypassing the need for activation of membrane receptors components. Based on my studies, a new role for APC function in the Wnt pathway is revealed that does not involve its role in the degradation complex. My studies indicate that APC prevents inappropriate activation of the membrane components of the Wnt pathway via the clathrin endocytic pathway. To my knowledge, this is the first time such a phenomenon has been demonstrated to occur in the Wnt pathway. Significantly, loss of APC resulting in Wnt pathway activation via the clathrin endocytic pathway is ligand-independent and is, thus, cell autonomous. Although p53 and Ras mutant cancer cells have been shown to exhibit high rates of receptor endocytosis (Mellman and Yarden 2013), the process that links the endocytic pathway and cell signaling remains poorly understood.

Signaling pathways rely on exquisite mechanisms to effectively transduce external cues to downstream effectors. Consistent with this, a growing body of evidence links endocytosis to activation of the Wnt pathway however the mechanism is not clear. Both clathrin and caveolin have been reported to be required for LRP6 endocytosis and Wnt pathway activation (Blitzer and Nusse 2006; Yamamoto et al. 2006). I found that whether the clathrin or the caveolin endocytic machinery is required for Wnt ligand-mediated signaling is highly dependent on the particular cell type tested. I found,

however, that activation of the Wnt pathway upon APC loss is clathrin-mediated and is independent of cell type. Why would a cell require two different endocytic pathways to regulate Wnt signaling? It is possible that Wnt activation mediated by clathrin and caveolin-dependent endocytosis is qualitatively different and may be required in different biological contexts (e.g. during development). Future studies should focus on understanding whether there are qualitative differences in Wnt signaling that occur via the clathrin versus the caveolin pathway.

The evolutionary conservation between Drosophila and vertebrate in the regulation of Wnt signaling by APC suggests that this mechanism may play a critical role in early animal development. Interestingly, *Drosophila* do not express caveolin suggesting that caveolin-mediated Wnt activation may be a more evolutionarily recent development in vertebrates (Fischer et al. 2006). Although the Wnt pathway is highly conserved, some differences in how Wnt signaling is regulated between *Drosophila* and vertebrates are not unexpected, particularly when it involves regulation of Wnt receptor activity. For example, secreted Wnt inhibitors (e.g. Dkk1 and Sfrp-1) and the Wnt agonist, R-Spondin, are present in vertebrates but not in *Drosophila*. Wnt signaling has been proposed to be critical for the evolution of epithelial morphogenesis (Lapébie et al. 2009). Interestingly, Wnt signaling via the caveolin pathway occur primarily in mesodermal cells and may reflect an evolutionary requirement in vertebrate for Wnt signaling in patterning mesenchymal tissues.

We found that APC associates with clathrin by coimmunoprecipitation. One possible model is that LRP6 is present in nascent clathrin coated pits, and APC acts as a brake to block the activity of the LRP6-bound clathrin. Upon Wnt stimulation, LRP6

subsequently translocates to mature clathrin vesicles where it is ultimately activated upon internalization of the clathrin vesicles. Careful colocalization studies of APC, clathrin, and LRP6 by electron microscopy may provide a better (high resolution) understanding of their spatial and temporal regulation upon Wnt ligand activation.

My findings demonstrate that inhibiting the activities of LRP6, Fz, or Dvl result in β-catenin downregulation (and inhibition of Wnt signaling) in APC-deficient cells. These studies suggest that the β-catenin destruction complex remains fully functional upon loss of APC. APC and APC2 have been proposed to exhibit redundant functions (Ahmed et al. 2002; Daly et al. 2016). In *Drosophila*, overactivation of Wg signaling due to inactivation of one of the APC proteins can be inhibited by overexpression of the other Apc (Ahmed et al. 2002). Loss of both APC and APC2 disrupts mammary tissue homeostasis and promotes mammary tumor formation in mice (Daly et al. 2016). This study suggests that APC and APC2 may act in a redundant fashion to promote tumorigenesis in certain tissues.

Despite their functional redundancy in human cells, APC2 shows lower affinity to β-catenin and is less efficient promoting its turnover compared to APC (Schneikert et al. 2013). Thus, I hypothesize that in the absence of APC, APC2 functions as the predominant scaffold (along with Axin) in the β-catenin degradation complex. One would predict that inhibiting the function of LRP6, Fz, or Dvl would no longer be able to promote β-catenin downregulation in APC-APC2 double knockdowns. The vast majority of human cancers occur due to disruption of APC (not APC2) function. Also, APC mutant CRC cells express APC2. One possibility is that only APC can block Wnt-ligand independent activation of the Wnt signaling by the clathrin-endocytic pathway. Thus,

loss of APC cannot be fully compensated by APC2. Further experiments should be performed to test this possibility.

My work supports a role for APC regulating the function of the β-catenin destruction complex and the activation of plasma membrane components. It is unclear which of these two activities represent the “the main function” of APC in the Wnt pathway. I have shown that a fragment of APC encoding the β-catenin and Axin-binding domains (APC^T) is sufficient to prevent LRP6 phosphorylation and β-catenin turnover in APC-depleted cells, suggesting that both events are dependent on APC function. One possible explanation is that independent pools of APC regulate each event. However, if this were true, APC^T degradation would only affect β-catenin degradation and not LRP6 activation because it only binds to Axin and β-catenin. Another explanation is that both functions are not mutually exclusive and APC regulates both the β-catenin destruction complex function and LRP6 activation. The multiple roles of APC in the Wnt pathway is reminiscent of the role of GSK3 in the destruction complex and receptor activation. Further experiments, are necessary to understand the precise mechanism of APC action, particularly at the membrane.

Tight regulation of Wnt signal transduction is necessary for proper development of all metazoans. In adults, APC inhibition is involved in early development of colorectal adenocarcinomas. Because homozygous deletion of APC is embryonic lethal (Dow et al. 2015), the effects caused by APC loss are typically studied in the context of disease in fully developed organisms in which conditional knockouts can be performed. Indeed, loss of APC function in adult mice deregulates the Wnt pathway and recapitulates the molecular features of CRC. Furthermore, experimental APC restoration reestablishes

intestinal crypt homeostasis in APC-deficient mice (Chartier et al. 2016). Given the seemingly elaborate mechanism by which APC regulates Wnt receptor activation, one could hypothesize that regulating APC levels may represent another way by which the pathway can be activated in a cell autonomous fashion (independent of Wnt ligands) during certain normal developmental processes. This is not without precedence. Albeit, via a distinct mechanism, Notch signaling can also occur via a ligand-independent manner involving endocytosis of the Notch receptor (Palmer and Deng 2015). In *Drosophila*, the hematopoietic crystal cells exhibit ligand-independent Notch pathway activation at the endosome that promotes their survival during development (Mukherjee et al. 2011). Thus, ligand-independent activation during development might be advantageous in a context where there is no guaranteed ligand source. Further experiments will be required to test our hypothesis that downregulating APC levels represents a normal developmental mechanism to activate the Wnt pathway in a cell autonomous manner.

Targeting LRP6 in TNBC and CRC

We found that mAb7E5 inhibits the Wnt pathway in triple negative breast cancer (TNBC) and colorectal cancer (CRC) cells, suggesting that mAb7E5 could be developed further as a therapeutic agent to treat Wnt-driven tumors. Our preliminary study indicated that mAb7E5 inhibit Wnt signaling in the TNBC cell line, MDA-MB-231. Previous reports have shown that LRP6 expression is upregulated in TNBC, and RNAi knockdown of LRP6 results in inhibition of the Wnt pathway and decreased cellular growth/proliferation (Liu et al. 2010).

Receptor activation in APC-mutant colorectal cancer cells has not been previously studied in detail. Past studies suggested that constitutive activation of the Wnt pathway in CRC may occur via an autocrine signal loop (Voloshanenko et al. 2013). In the current study, I found that inhibition of Porcupine (PORCN), the protein required for Wnt secretion and function, was not sufficient to inhibit β-catenin accumulation and loss of APC in PORCN null cells did not inhibit transcriptional activation of a Wnt reporter. These results suggest that an autocrine Wnt signal is unlikely to play a major role in Wnt pathway activation upon loss of APC. Thus, future therapeutic approaches to target APC mutant tumors should focus on disrupting the receptors and other Wnt signaling components necessary for formation of active receptor complexes (e.g., signalosomes) other than Wnt ligands. Our own studies with mAb7E5 suggest that it may represent an attractive candidate to target CRC.

If the utility of mAb7E for treating TNBC and APC mutant CRC is to be fully realized, further studies are required to test whether mAb7E5 is effective in i*n vivo* tumor models (either cancer cell line xenografts or patient derived xenografts). Other tasks that will be required for its development as a therapeutic agent include the development and testing of a humanized version of the antibody (mAb7E5 is a mouse monoclonal) as well as assessing its bioavailability, *in vivo* half-life, and pharmacodynamics/pharmacokinetics.

Concluding remarks

The work presented herein combines multiple approaches to understand the mechanism of Wnt signaling transduction. We identified a new function of the tumor suppressor, APC, that provides a link between a basic cell biological process (endocytosis) with the Wnt signaling pathway. The current work highlights the importance of studying signaling pathways as part of a complex system that is regulated in a temporal and spatial fashion and not simply as an isolated biochemical cascade of events. It is likely that every signaling pathway is ultimately interconnected to form complex signaling networks that are tightly regulated by the cell in a similar manner. Thus, to understand how cells respond to their environment, we must take into account this level of complexity, which represents an exciting challenge for scientists studying how diverse chemically encoded information is organized to regulate behavior at the cellular and organismal level.

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