PROTEOLYTIC PROCESSING OF LRP6 AND ITS EFFECTS ON CANONICAL WNT SIGNALING

by

Andrew Joseph Connell

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences

Spring 2020

© 2020 Andrew Joseph Connell
All Rights Reserved

PROTEOLYTIC PROCESSING

OF LRP6 AND ITS EFFECTS

ON CANONICAL WNT SIGNALING

by

Andrew Joseph Connell

Approved: _____

Shuo Wei, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____

Velia M. Fowler, Ph.D.
Chair of the Department of Biological Sciences

Approved: _____

John A. Pelesko,
Dean of the College of Arts and Sciences

Approved: _____

Douglas J. Doren, Ph.D.
Interim Vice Provost for Graduate and Professional Education and
Dean of the Graduate College

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Shuo Wei, for his consistent support and encouragement throughout my graduate studies. He has passed on practical and scientific knowledge that has helped shape me into a more confident scientist. He spurred me on without breaking my spirits, which is a delicate balance to achieve in this field. He has been an overly generous mentor and I am grateful to have been one of his students. I would also like to thank my lab members for their guidance, willing helpfulness, senses of humor, and real companionship. We have experienced and endured much over these past few years, and it has forged us together to be more like family members rather than colleagues. I also need to extend thanks to Dr. Jian Sun and Dr. Ira Daar's laboratory for all of the preliminary work that went into crafting this project. I want to extend my gratitude also to my committee members, Dr. Erica Selva and Dr. Jia Song, for being scientific sounding boards. They have tolerated my frequent befuddlements regarding this project and helped steer me in the proper direction. Thanks are also due to Betty Cowgill and the rest of the office staff for their invaluable work to quench logistical headaches and make sure our litany of precious orders made it safely into our hands. I am also grateful to the University of Delaware for affording me this prime opportunity to extend my collegiate career.

I want to also extend gratitude to my parents, Dan and Lisa, for their constant support and encouragement throughout all of my life. They taught me to pursue learning joyfully from a young age and enabled me to pursue my scholastic aspirations. I would not be who I am, nor where I am, apart from their love and guidance.

Thanks would not be complete without acknowledging my brilliant, encouraging, and beautiful wife Devon, who has kept the earth below my feet during this season. In the storm that is graduate school, she has brought calming reassurance and wise perspective. She worked selflessly throughout to provide for our family while I got to "play with science". After asking how my day was, she listened patiently to stories of western blotting blunders and plasmid-prep pitfalls. She is my amazing *complimentary strand*, and I am forever thankful for her.

Finally, how could I fail to acknowledge the reason for life and breath and being. I give thanks to God who made all of the intricate jaw-dropping marvels that science gets the privilege of studying. In this field, we hold a match up to the mysteries within all created things and catch a sliver of wonder. May my ambitions and pursuits culminate in a life that whispers awestruck honor to the Maker.

TABLE OF CONTENTS

LIST OF FIGURES vi
ABSTRACT viii

Chapter

1 INTRODUCTION 1

1.1 Canonical Wnt Signaling 1
1.2 The LRP6 Co-Receptor 5
1.3 Regulated Intramembrane Proteolysis and ADAM Proteases 10
1.4 LRP6 Undergoes RIP 14

2 MATERIALS AND METHODS 18

2.1 Cell Culture and Transfection 18
2.2. Drug Treatments 18
2.3 Plasmids and Antibodies 19
2.4 Dual Luciferase Reporter Assay 19
2.5 Western Blotting 20

3 RESULTS 22

4 DISCUSSION 31

REFERENCES 40

Appendix

A FIGURES 54

LIST OF FIGURES

Figure 1. The canonical Wnt pathway 54

Figure 2. LRP6 structure and predicted cleavage site mutation 55

Figure 3. LRP6 undergoes regulated intramembrane proteolysis 56

Figure 4. Effect of LRP6-WT and LRP6-NC overexpression on canonical Wnt activity in HEK293T cells 57

Figure 5. Canonical Wnt activity in HEK293T cells treated with GI254023 or DAPT 58

Figure 6. Canonical Wnt activity in HEK293T cells with overexpressed LRP6-WT and treated with GI254023 or DAPT compared to overexpressed LRP6-NC 59

Figure 7. Protein expression and cleavage products of overexpressed LRP6-WT and LRP6-NC treated with GI254023 or DAPT in HEK293T cells 60

Figure 8. Effect of Wnt3a overexpression on canonical Wnt activity in HEK293T cells overexpressing LRP6-WT or LRP6-NC 61

Figure 9. Effect of Wnt1 overexpression on canonical Wnt activity in HEK293T cells overexpressing LRP6-WT or LRP6-NC 62

Figure 10. Endogenous canonical Wnt activity in HEK293T cells treated with increasing dosages of WNTC59 63

Figure 11. Canonical Wnt activity in HEK293T cells with overexpressed LRP6-WT and treated with increasing dosages of WNTC59 64

Figure 12. Canonical Wnt activity in HEK293T cells with overexpressed LRP6-NC and treated with increasing dosages of WNTC59 65

Figure 13. Effect of EphrinB2 overexpression on canonical Wnt signaling in HEK293T cells with overexpressed LRP6-WT or LRP6-NC 66

Figure 14. Protein expression and cleavage products of overexpressed LRP6-WT and LRP6-NC with overexpressed EphrinB2 in HEK293T cells... 67

Figure 15. Protein expression and cleavage products of LRP6-WT and LRP6-NC in SW620 cells 68

Figure 16. Hypothesized mechanism of high vs. low Wnt dosage responses..... 69

Figure 17. Hypothesized mechanism of LRP6 cleavage involving EphrinB2 and ADAM10..... 70

ABSTRACT

LRP6 acts as a co-receptor in the canonical Wnt pathway, a key signaling network involved in regulating embryonic development, tissue patterning, cell migration, and homeostasis. Aberrations in the canonical Wnt pathway are often correlated with various types of cancers. Mutants of the LRP6 co-receptor lacking the majority of the extracellular domain are able to constitutively activate the Wnt pathway. Recently, it was discovered that LRP6 undergoes regulated intramembrane proteolysis (RIP). RIP is a two-step process wherein transmembrane proteins are cleaved first in the extracellular region releasing a soluble form of the extracellular domain, followed by cleavage in the transmembrane region that releases a biologically active intracellular domain (ICD). How LRP6 undergoes RIP and its potential implications for the regulation of the canonical Wnt pathway remain unclear. The goal of this thesis was to elucidate the mechanism and conditions in which LRP6 is cleaved and confirm the responsible proteases. The project also analyzed the effects of a novel mutation that renders the co-receptor resistant to cleavage. Together, the data revealed a complex process by which LRP6 is subjected to RIP possibly through the recruitment of ADAM10 by EphrinB2 and subsequent intramembrane cleavage by y-secretase. Additionally, this mechanism may only occur in cellular environments where Wnt ligand concentrations are sufficiently low enough to cause an alternative route of Wnt activation.

Chapter 1

INTRODUCTION

1.1 Canonical Wnt Signaling

The canonical Wnt pathway is a highly conserved signal transduction pathway with vital roles in embryonic development, tissue patterning, cell fate determination, cell proliferation, and cell migration. The pathway is activated by Wnt ligands, a family of glycoproteins first discovered in *Drosophila melanogaster.* Sharma and Chopra utilized mutation studies and noticed a reduction or absence in wing formation, and thus termed the corresponding locus *wingless (Wg)* (Sharma and Chopra, 1976). Shortly thereafter, Nusse and Varmus identified the mammalian homolog in mice, termed *int-1*, which was found to play a role in tumorigenesis (Nusse et al., 1984). In 1991, the two previously distinct gene names were merged to bear the name Wnt (Nusse et al., 1991). These early watershed studies were the first to hint at the importance of the canonical Wnt signaling pathway in embryonic development and cancer pathophysiology. Shortly after its discovery in *Drosophila*, Nobel Prize winners Nusslein-Volhard and Wieschaus used forward genetic screens to identify other loci responsible for embryonic patterning (Nusslein-Volhard and Wieschaus, 1980). Many of the genes they identified would later be revealed as key components in the canonical Wnt pathway. Studies in *Xenopus* embryos showed that canonical Wnt activation leads to the formation of specific tissue organizers, and

injection of *Wnt* mRNAs into blastomeres was able to generate an additional body axis (Sokol et al., 1991). Indeed, overexpression of positive Wnt regulators and/or inhibition of negative Wnt regulators can also induce this secondary body axis.

The Wnt family of proteins are involved in multiple signaling cascades, including the planar cell polarity and Wnt/Ca^{2+} pathways; however, this thesis will focus solely on their role in the canonical Wnt pathway. The canonical pathway functions by prompting the stabilization of β-catenin, which translocates into the nucleus and serves to regulate the transcription of several Wnt target genes. In the absence of Wnt ligand binding to extracellular receptors, newly synthesized cytoplasmic β-catenin is targeted for degradation by a constitutively active group of proteins known as the β-catenin destruction complex (Figure 1). The β-catenin destruction complex is comprised of the scaffolding proteins Axin and adenomatous polyposis coli (APC), serine/threonine kinases glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1), and the E3-ubiquitin ligase β-TrCP (Stamos and Weis, 2013). Axin is responsible for binding and positioning GSK3 and CK1, and the initial sequestering of cytosolic β-catenin (Lee et al., 2003). Research has shown that phosphorylation of a 20-mer repeat on APC preferentially binds β-catenin compared to Axin. This competition transfers β-catenin from Axin to APC, priming it for phosphorylation and simultaneously opens the binding site on Axin for a new molecule of β-catenin to enter (Xing et al., 2003), thus continuing the destruction cycle. Once bound to APC, β-catenin is first phosphorylated at serine 45 by CK1 which in turn promotes the phosphorylation of threonine 41, serine 37, and serine 33

by GSK3 (Liu et al., 2003). The phosphorylations at serine 33 and 37 serve as a destruction motif for the F-box protein β-TrCP to bind and catalyze the polyubiquitination of lysine 48 on β-catenin (Wu et al., 2003). Once phosphorylated and ubiquitinated, β-catenin is brought to the proteasome for degradation. Interestingly, mutations in the phosphorylatable residues on β-catenin are seen in some colorectal cancers and cause increased activation of the Wnt pathway (Morin et al., 1997). Similarly, loss of function mutations in APC correspond to elevated levels of intracellular β-catenin and are found in approximately 80% of colon cancers (Rubinfeld et al., 1997), suggesting a role for unregulated Wnt signaling in tumorigenesis.

Vertebrates contain 19 Wnt ligands, all of which undergo crucial glycosylation and lipid modifications. Wnts are secreted from the endoplasmic reticulum only after being palmitoylated by the acetyltransferase Porcupine (MacDonald et al., 2009). Once inside the Golgi network, the transmembrane protein Wntless oversees Wnt transport to the plasma membrane (Port and Basler, 2010), where it is released into the extracellular space. When a Wnt ligand is presented to the cell surface it binds to co-receptors LRP5/6 and Frizzled to initiate canonical Wnt signaling. Frizzled receptors are seven-pass transmembrane proteins with structural similarity to GPCRs and contain a cysteine-rich domain where Wnts bind (Hsieh et al., 1999). Low-density lipoprotein receptor-related proteins 5/6 (LRP5/6) are single-span transmembrane proteins that are capable of recruiting Axin and other destruction complex members to the plasma membrane (Baig-Lewis et al., 2007). Another key event upon ligand

binding is the phosphorylation and subsequent activation of the cytoplasmic adaptor protein Dishevelled (Dvl). Dvl is capable of binding the intracellular domain of Frizzled and may be involved in the clustering of co-receptors (Schwarz-Romond et al., 2007a). Dvl can also bind Axin and thus may promote its recruitment to the membrane (Schwarz-Romond et al., 2007b). LRP5/6 contain five motifs on their cytoplasmic tail that are phosphorylated by GSK3 and CK1, and serve to effectively sequester the kinases from targeting β-catenin (Wu et al., 2009). Through this series of events, the components of the β-catenin destruction complex are disarmed, allowing for nuclear β-catenin to accumulate. Inside the nucleus, β-catenin functions to displace the co-represser Groucho/transducin-like enhancer of split (TLE) from T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors (Daniels and Weis, 2005). Co-activators also interact with the β-catenin/TCF promoter regions and further promote gene transcription through chromatin remodeling (Cadigan and Waterman, 2012). TCF/LEF transcription factors bind to regions of DNA known as Wnt-responsive elements (WREs) and activate transcription of downstream target genes. Wnt target genes include c-Myc (He et al., 1998) and CyclinD1 (Tetsu and McCormick, 1999), substantiating the role of canonical Wnt signaling in cancer progression via cell cycle control. Axin2 (Yan et al., 2001) is also an established Wnt target gene, suggesting the pathway contains feedback mechanisms for self-regulation.

1.2 The LRP6 Co-Receptor

LRP5/6 are type I single-span transmembrane glycoproteins that share 71% amino acid similarity and are homologs of Arrow, found in *Drosophila*. Studies have shown that *Lrp6*$^{-/-}$ mice are embryonic lethal and exhibit severe embryological defects similar to mice with mutations in *Wnt1, Wnt3a,* and *Wnt7a* (Pinson et al., 2000). Conversely, *Lrp5*$^{-/-}$ mice develop normally but show signs of osteoporosis in adulthood (Kato et al., 2002). These significant differences highlight the unique influence of LRP6 in development and may imply a more crucial role for LRP6 in the canonical Wnt pathway. Accordingly, the scope of this thesis will focus primarily on LRP6. LRP6 is a member of the LDL family of receptors which function collectively in endocytosis, cargo transport, and cell signaling (Strickland et al., 2002). LRP6 consists of 1,613 amino acids and contains a large extracellular domain comprised of four consecutive pairs of distinct YWTD β-propeller (BP) and EGF-like domains followed by three LDLR (low-density lipoprotein receptor) type A repeats (Figure 2). The BP domains serve as ligand binding sites (He et al., 2004), and different classes of Wnt ligands preferentially bind to either BP1/2 or BP3/4 (Bourhis et al., 2010). The secreted Dickkopf (Dkk) proteins act as Wnt antagonists by competing directly for these binding sites on LPR6 (Mao et al., 2001). The C-terminus of Dkk1 binds BP3/4 and its N-terminus binds BP1/2, thus effectively blocking all classes of Wnt ligands (Ahn et al., 2011). Three-D analysis of Dkk1 binding to the LRP6 ectodomain reveals a constricted conformation of the four BP domains that may allosterically inhibit Wnts from binding (Matoba et al., 2017). An alternative theory regarding the function of Dkk1 suggests that it works to induce clathrin-

dependent LRP6 endocytosis, preventing LRP6 phosphorylation thereby blocking Wnt activity (Yamamoto et al., 2008). The chaperone protein Mesd (mesoderm development) is required for the intricate folding and trafficking of LRP6 in the secretory pathway (Hsieh et al., 2003) and is especially necessary for the maturation of the BP domains (Culi et al., 2004). Intriguingly, Mesd can also bind to mature LRP6 present on the cell surface and act as an antagonist for Dkk1 (Li et al., 2005). Other studies reveal that Mesd also inhibits Wnt ligands from binding and activating the pathway, and could serve as a potential therapeutic for different cancers (Lu et al., 2010). The LDLR type A domains have been implied in receptor dimerization, which has been shown to be required for ligand-induced pathway activation (Chen et al., 2014). LRP6 has the ability to aggregate in a Dvl-dependent manner upon treatment with Wnt ligand, and these multimers form complexes known as signalosomes which cluster in lipid rafts (Bilic et al., 2007). The formation of LRP6 signalosomes helps to amplify the phosphorylation of LRP6 and induce the Wnt signaling cascade. The localization of LRP6 to lipid rafts may be due in part to its palmitoylation, a lipid modification mediated by acyl-transferases in the ER which serves to anchor transmembrane proteins. LRP6 palmitoylation is required for its localization to the cell surface and subsequent function in canonical Wnt signaling (Abrami et al., 2008). Receptor-mediated endocytosis may be an additional role of the LDLR type A repeats. A recent study revealed that interactions between the E3 ubiquitin ligase, Itch, and the LDLR repeats of LRP6 are involved in receptor internalization (Vijayakumar et al., 2017). Caveolin-based endocytosis has been a controversial explanation for the

mechanism by which LRP6 activates the canonical Wnt pathway. Studies have shown that Wnt3a binding to LRP6 induces caveolin-mediated internalization (Yamamoto et al., 2006), and that the Rab GTPase RAB8B is required for this endocytic activity (Demir et al., 2013).

Perhaps the most important structural feature of LRP6 is the cytoplasmic tail, which contains five highly conserved phosphorylatable PPPS/TP motifs that are known to play a crucial role in transducing Wnt signal (MacDonald et al., 2008). Studies show that phosphorylation of the PPPS/TP motifs is achieved by both GSK3 and CK1, the kinases is also responsible for targeting β-catenin in the destruction complex (Zeng et al., 2005). Phosphorylated PPPS/TP motifs have a high affinity for Axin and serve as docking sites for the scaffolding protein (Zeng et al., 2008). Thus, cytosolic Axin-GSK3 complexes are recruited to the membrane where GSK3 can further phosphorylate LRP6, effectively generating a positive feedback mechanism for Wnt activation (Baig-Lewis et al., 2007). Tamai et al were the first to show that LRP6 mutants lacking the cytoplasmic domain are unable to initiate Wnt signaling and have dominant negative effects (Tamai et al., 2000). Conversely, LRP6 mutants lacking the majority of the extracellular domain (ΔN) behave in a constitutively active manner and are able to activate Wnt signaling even without Frizzled co-receptor or Wnt ligand (Mao et al., 2001). It is thought that the ability of a ΔN mutant to promote canonical Wnt signaling relies only on the phosphorylation of the PPPS/TP motifs found on the cytoplasmic tail (Brennan et al., 2004). Substitution mutations in the PPPS/TP motifs on ΔN mutants have been shown to hamper Wnt signaling (MacDonald et al., 2008).

Furthermore, the PPPS/TP motifs appear to function in a cooperative manner, whereby the phosphorylation of each motif contributes additional strength to the propagating signal (Wolf et al., 2008). Unsurprisingly, full-length LRP5 and LRP6 both potentiate Wnt activity in the presence of exogenous ligand; however, LRP6 alone is able to increase TCF/LEF transcriptional activity in the absence of ligand. Yet, both LRP5 and LRP6 ΔN mutants are able to activate Wnt signal in a ligand-independent manner. Additionally, these mutants can function in either a membrane-bound or free cytosolic form (Mi and Johnson, 2005). Interestingly, the free LRP6 intracellular domain does not require phosphorylation of its PPPS/TP motifs in order to attenuate GSK3 and increase Wnt activity, implying there may be multiple ways in which LRP6 works to regulate the Wnt pathway (Beagle et al., 2009). This soluble form of the LRP6 cytoplasmic tail has been shown to enter the nucleus where it serves to repress amino terminal enhancer of Split (AES), a positive modulator of TCF/LEF transcriptional activity (Beagle and Johnson, 2010). Adding complexity to the matter, a recent study has shown that nuclear LRP6 can function as an alternative splicing regulator in differentiating myocytes (Yuan et al., 2018). Needless to say, LRP6 plays a complicated role in regulating the canonical Wnt pathway and requires further research.

LRP6 has become an intriguing therapeutic target due to its central function as a co-receptor for the canonical Wnt pathway, for which it has been linked to various disease states. Genomic studies have shown that genetic variants in LRP6 are correlated with atherosclerosis (Sarzani et al., 2011), hyperlipidemia (Go et al., 2014),

and coronary artery disease (Wang et al., 2012). Another group has shown that common LRP6 single nucleotide polymorphisms are associated with late-onset Alzheimer's disease (De Ferrari et al., 2007). Furthermore, an LRP6 alternative splice variant lacking exon 3 is also linked to Alzheimer's disease (Alarcon et al., 2013). Research has shown that LRP6 mRNA and protein levels are downregulated in Alzheimer patient brains and mice devoid of neuronal LRP6 show synaptic abnormalities and exacerbated amyloid pathology (Liu et al., 2014). LRP6 may affect Alzheimer's pathology by protecting amyloid precursor protein (APP) against amyloidogenic proteolytic processing via interactions through their extracellular domains (Buechler and Salinas, 2018). LRP6 may also act as an oncogenic protein. It has been shown that LRP6 is widely expressed in several cancer subtypes and promotes tumorigenesis and proliferation through canonical Wnt induction (Li et al., 2004). LRP6 is overexpressed at the transcript and protein levels in colorectal cancer (CRC) cell lines and is upregulated in human CRC tissues (Rismani et al., 2017). LRP6 phosphorylation is also increased in CRC tissue samples and may be linked to KRAS signaling (Lemieux et al., 2015). Accordingly, upregulated levels of phosphorylated LRP6 correlate with poor prognosis in CRC patients. Overexpression of LRP6 increases CRC cell migration *in vitro* through a mechanism regulating microtubule dynamics and actin remodeling (Yao et al., 2017). Recently, a novel bispecific antibody was generated that blocks both classes of Wnt ligand from binding BP1/2 and BP3/4. The antibody is capable of decreasing Wnt activity *in vivo*, and inhibited tumor growth in CRC xenograft models (Jackson et al., 2016). LRP6

overexpression is also witnessed in several types of breast cancer. Knockdown of LRP6 decreases Wnt signaling as well as cell proliferation and tumor growth (Liu et al., 2010). Transgenic mice expressing promoter-driven LRP6 show hyperplasia in their mammary glands, which is a strong prognosticator of breast cancer (Zhang et al., 2010). LRP6 is upregulated in triple-negative breast cancer, and knockdown of LRP6 or inhibition with recombinant Mesd greatly reduces cell migration and invasion *in vitro* (Ma et al., 2017). Interestingly, one study has seen that knockdown of endogenous LRP6 correlates with breast tumor metastasis *in vitro* and poor prognosis (Ren et al., 2014). This same research group discovered that the LRP6 ectodomain can inhibit metastasis and prolong survival in a mouse model of breast cancer by binding to CXCR4 and preventing its interaction with SDF-1 (Zhang et al., 2019). Due to its complex roles in the development of cancer and other diseases, LRP6 serves as a valuable protein for study.

1.3 Regulated Intramembrane Proteolysis and ADAM Proteases

A recently discovered mechanism for proteolytic cleavage has altered perspectives on protein catalysis. As the name suggests, regulated intramembrane proteolysis (RIP) involves the cleavage of transmembrane protein substrates within their hydrophobic transmembrane domain by a family of enzymes termed intramembrane cleaving proteases (iCLiPs) (Wolfe et al., 1999). iCLiPs consist of four families of integral membrane proteins, including the aspartyl protease-like γ-secretase and signal peptide peptidase (SPP), the zinc metalloproteinase site-2 protease (S2P), and the serine

protease family of rhomboids (McCarthy et al., 2009). Specifically, the γ-secretase complex is responsible for intramembrane cleavage of type I transmembrane proteins and consists of presenilin as its catalytic core, and cofactors nicastrin, anterior pharynx-1 (Aph-1), and presenilin enhancer-2 protein (Pen-2) (Spasic and Annett, 2008). The two isoforms of presenilin (PS1 and PS2) share 67% similarity and share some functional redundancy; however, they are differentially expressed during development (Lai et al., 2003). A typical substrate for γ-secretase first requires the removal of the majority of its extracellular domain (Struhl and Adachi, 2000). This initial shedding step is often carried out by a disintegrin and metalloproteinase (ADAM) protease referred to as an α-secretase. Shedding by an α-secretase is often induced by dimerization or ligand binding and releases a soluble form of the ectodomain which may have unique biological functions (Beel and Sanders, 2008). The initial ectodomain cleavage leaves a short stub of about 30 amino acids that acts as a recognition site for nicastrin and enables the γ-secretase complex to dock to the substrate. Following intramembrane cleavage, the intracellular domain of the substrate will often translocate to the nucleus and modulate gene expression (McCarthy et al., 2009). RIP is highly conserved across species (Brown et al., 2000) and due to its growing number of diverse substrates it is implicated in numerous cellular processes, including embryonic development, signal transduction, immune system functioning, and hematopoiesis (McCarthy et al., 2017). The most famous examples of RIP by γ-secretase include amyloid-β precursor protein (APP), E-Cadherin, and Notch (Lal and Caplan, 2010). In Notch signaling, when a Delta ligand binds to Notch receptor it

prompts a conformational change that allows ADAM-mediated ectodomain shedding. Following RIP, the cytoplasmic tail of Notch translocates into the nucleus where it governs transcriptional regulation of target genes (De Strooper et al., 1999). Interestingly, it is theorized that free γ-secretase may be more promiscuous in its substrate selection, whereas the known localization of γ-secretase to lipid rafts may be a mechanism by which cells ensure the processing of specific substrates (Vetrivel et al., 2005). Remarkably, studies show that PS1 possesses a γ-secretase-independent ability to modulate β-catenin levels and Wnt signaling, either through Wnt-1-mediated suppression or as an alternative scaffold for the destruction complex (Haas et al., 2009).

The α-secretase responsible for the ectodomain-shedding step of RIP is typically an ADAM protease. ADAM proteases were first discovered in the late-20th century in association with the degradation of myelin basic protein (Chantry et al., 1989), and were established as a unique type of metalloprotease through molecular cloning experiments (Howard et al., 1996). They are found in large variety of species, though they have expanded in number in higher vertebrates likely due to gene duplication events (Huxley-Jones et al., 2007). There are 19 *ADAM* genes in humans that encode ADAM proteins, of which only 13 contain catalytic activity. ADAM structure consists of an N-terminal signal peptide that guides the proteins through the secretory pathway, followed by a catalytically inhibitory pro-domain (Gonzales et al., 2004). In the secretory pathway, the pro-domain functions as a chaperone to prompt accurate folding (Milla et al., 2006) and is typically removed by a pro-protein convertase to

render the ADAM functionally active (Endres et al., 2003). The metalloproteinase domain follows the pro-domain, and for those ADAMs possessing catalytic activity it contains the zinc-binding site and Met-turn common to the Metzincin superfamily of enzymes (Bode et al., 1993). The remainder of the ADAM extracellular domain is comprised of a disintegrin domain capable of interacting with integrin receptors on platelets, a cysteine-rich domain, and an EGF-like domain (Giebeler and Zigrino, 2016), although ADAM10 and ADAM17 lack the EGF-like domain (Janes et al., 2005). Following the transmembrane domain lies a cytoplasmic tail that can play roles in signaling, trafficking, and cellular structure (Edwards et al., 2008). It has been shown that several ADAMs undergo alternative splicing whereby proteins of varying structure and function are derived from the same coding region. For instance, alternative splicing yields a secreted form of ADAM9 that possesses the unique capability of cleaving APP (Hotoda et al., 2002). ADAM expression patterns and localization are also influencers of biological function. ADAM9 and ADAM10 are both ubiquitously expressed; however, ADAM9 null mice develop normally (Weskamp et al., 2002), whereas mice lacking ADAM10 die early in embryogenesis and show defects in cardiac and nervous system development (Hartmann et al., 2002). Conversely, some ADAMs are expressed solely in the testis where they may affect fertilization initiation (Cho et al., 1998)

The function of any given proteolytic ADAM is often linked to the role of its substrates. ADAMs are capable of cleaving cytokines, cytokine receptors, growth factors, growth factor receptors, cell-adhesion molecules, and other important proteins

(Seals and Courtneidge, 2018). Due to this diverse impact, ADAMs are often implicated in human diseases, including various cancers. As stimulants of cell proliferation and migration, ADAM expression levels are often correlated with tumor progression and poor clinical diagnosis (Duffy et al., 2009). Accordingly, ADAMs may serve as useful biomarkers for cancer diagnosis; however, they are challenging targets of therapeutics due to the widespread nature of their substrates (Duffy et al., 2011).

1.4 LRP6 Undergoes RIP

Researchers Mi and Johnson have helped elucidate the function of LRP6, specifically regarding the ability of the cytoplasmic tail to promote Wnt activation. They have shown that both membrane-bound and cytosolic intracellular domains of LRP6 can induce Wnt activity in a constitutive manner, and this activity is potentiated by the addition of Wnt3a ligand (Mi and Johnson, 2005). Additionally, they found that the intracellular domain interacts directly with GSK3 in an effort to sequester the kinase from its role in the β-catenin destruction complex (Mi et al., 2005). To determine if these free forms of the LRP6 cytoplasmic tail contribute to Wnt signaling in a physiologically relevant context they set off to investigate whether LRP6 undergoes RIP. LRP6 is a prime candidate for RIP not only for its status as a type I transmembrane protein, but also because it shares transmembrane sequence homology with other RIP substrates, and other low-density lipoprotein receptors are known to undergo RIP (May et al., 2002). Using a Gal4-dependent reporter assay, Mi and

Johnson saw that a Gal4-fused, membrane-tethered mutant of LRP6 lacking the extracellular domain (LRP6C2) underwent cleavage through the release of its cytoplasmic tail. The released ICD was also found in cytosolic fractions through immunoblotting. When immunoblotting for a C-terminal tag on full-length LRP6, two cleavage products occurred, measuring 35kDa and 20kDa. To confirm that the proteolysis was due to RIP, the researchers applied γ-secretase inhibitors and witnessed a decrease in the release of the cytoplasmic tail, increased levels of full-length protein, a reduction of the 20kDa product, and the appearance of a new cleavage product slightly larger than 20kDa (Figure 3a). The 35kDa cleavage product underwent no change with γ-secretase inhibitor treatment, implying that it resulted from another unique type of cleavage. Interestingly, the addition of Wnt3a caused an increase in intracellular domain release of LRP6C2 as measured by a luciferase reporter; however, there was no detectable increase in the ICD via western blotting. Considering the mutant receptor lacks extracellular ligand binding domains the response was likely due to interactions between the transfected mutant and endogenous LRP6, and implies that ligand binding to LRP6 may be a means of inducing RIP. Expectedly, treatment with Dkk1 conditioned media caused an increase in full-length protein retention and a decrease in proteolytic release of overexpressed LRP6 cytoplasmic tail, strengthening the conviction that ligand binding induces cleavage. Probing conditioned media with an N-terminal LRP6 antibody revealed an extracellular fragment that appeared to be 35kDa smaller than full-length LRP6 (Figure 3b). Treatment with Wnt3a caused an increase in the amount of detectable

shed ectodomain (Figure 3c), whereas Dkk1 treatment decreased the levels of the N-terminus found in the media. A final experiment treating with Phorbol 12-myristate 13-acetate (PMA), a protein kinase C activator known for inducing activity of several metalloproteinases involved in transmembrane protein cleavage, showed an increased in the extracellular domain product found in the media (Figure 3d; Mi and Johnson, 2007). While the researchers were able to confidently state that LRP6 does undergo RIP, they were not able to determine which protease was responsible for the initial ectodomain shedding that preceded the secondary γ-secretase-induced transmembrane cleavage. The study also failed to investigate the impact of the shed LRP6 intracellular domain on modulating canonical Wnt activity.

A more recent article from researchers at Nagoya University has shown that LRP5 and LRP6 undergo cleavage by snake venom metalloproteinases (SVMPs) and human ADAMs, which leads to a loss of cell-adhesion and induces hemorrhaging (Seo et al., 2017). Although SVMPs do not belong to the ADAM family, SVMPs exhibit similar domain structure and relatively high homology to certain ADAMs. The researchers have shown that an SVMP termed vascular apoptosis-inducing protein 1 (VAP1) is capable of disrupting cell-cell adhesions *in vitro,* and that this disruption is due in part to relocation of cadherin proteins. It is well known that β-catenin complexes with cadherin at the cell membrane (Nelson, 2008), and so changes in cadherin localization may release β-catenin and impact the canonical Wnt pathway (Hay et al., 2009; Vinyoles et al., 2014). Due to the connection between cadherins and β-catenin, the researchers investigated the effect of VAP1 on Wnt co-receptors and

discovered that VAP1 can cleave both LRP5 and LRP6 at the C-terminus of the fourth β-propeller domain, generating an N-terminal product of 140kDa and a C-terminal product of 60kDa. The researchers were able to determine that the cleavage of LRP6 by VAP1 causes disruptions in cell-cell junctions of cultured endothelial cells and increases hemorrhaging *in vivo*. Of great interest was the discovery that human ADAM8 and ADAM12 (the ADAMs closest resembling VAP1) were both able to cleave LRP6 at the same cleavage site, which may play a role in activating the canonical Wnt pathway (Seo et al., 2017). The researchers focused on cell-adhesion and its impact on hemorrhaging and thus did not analyze the effect of LRP6 cleavage on Wnt activation.

While it is clear that LRP6 can undergo cleavage, the effect that this has on canonical Wnt signaling and downstream cellular processes that may lead to disease states remains unstudied. Additionally, the biological conditions required for such cleavage must be determined in order to better predict when such proteolytic processing occurs. Furthermore, the specific proteases responsible for executing RIP need to be identified. If the proteolysis of LRP6 impacts canonical Wnt signaling then its elucidation will reveal a novel mechanism for signal induction and provide potential therapeutic targets for its regulation.

Chapter 2

MATERIALS AND METHODS

2.1 Cell Culture and Transfection

HEK293T cells were obtained from ATCC and cultured in DMEM (Corning) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin at 37°C with 5% CO_2. SW620 colorectal cancer cells were cultured in Leibovitz's L-15 medium (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C without CO_2. Cells were transfected at ~75% confluency using Lipofectamine3000 (Invitrogen) according to the manufacturer's protocol and grown for an additional 24 hours.

2.2 Drug Treatments

All treatments were performed for 24 hours with DMSO controls. GI254023 (Millipore-Sigma), a selective ADAM10 inhibitor, was added to cells at final a concentration of 20 μM. The γ-secretase inhibitor DAPT (Millipore-Sigma) was used at a final concentration of 25 μM. The general proteasome inhibitor MG132 (Millipore-Sigma) was used at a final concentration of 10 μM and was applied to cells 4-6 hours before harvesting. WNTC59 (Tocris), a potent PORCN inhibitor, was used at concentrations of 100 nM, 200 nM, 500 nM, 1 μM, 2 μM, and 5 μM.

2.3 Plasmids and Antibodies

The following plasmids were used and either generated in-house, purchased from Addgene, or received from Dr. Ira Daar at NCI: pcs2+ (C-terminal HA tag), LRP6 WT, LRP6 WT (C-terminal V5 tag), LRP6 NC (C-terminal V5 tag), LRP6 WT (C-terminal Flag tag), LRP6 NC (C-terminal Flag tag), Wnt3a, Wnt1, EphrinB2 (C-terminal HA tag), and β-catenin (C-terminal GFP).

The following antibodies were used: LRP6 (C5C7) rabbit mAb (Cell Signaling Technologies, 1:1,000), LRP6 (C-10) mouse mAb (Santa Cruz Biotechnology, 1:100), V5-Tag (D3H8Q) rabbit mAb (Cell Signaling Technologies, 1:1,000), V5-Peroxidase (V5-10) mouse mAb (Millipore-Sigma, 1:4,000), DYKDDDDK Tag-HRP (FG4R) mouse mAb (Invitrogen, 1:4,000), β-actin-HRP (AC-15) mouse mAb (Abcam, 1:20,000). HRP-linked anti-mouse or anti-rabbit IgG secondary antibody was used at a concentration of 1:10,000.

2.4 Dual Luciferase Reporter Assay

Cells were transfected with 125 ng of TOP plasmid, which contains 3 copies of the TCF/LEF binding region upstream of a firefly luciferase gene, along with 4 ng of *Renilla* plasmid, as well as plasmids and/or siRNAs of interest. Cells were harvested after 24 hours and washed in ice cold 1x PBS, then lysed in 1x Passive Lysis Buffer (Promega) according to the Dual-Luciferase® Reporter Assay System (Promega) protocol. Then 50 µL of Luciferase Assay Reagent was added to 5 µL of cell lysate in each well of a 96-well white bottom plate. Luminescence of sample triplicates was measured on the Promega GloMax Multidetection plate reader, with an integration

time of 1 second and 3 total reads. 50 µL of Stop & Glo® reagent was added to each well and luminescence was measured again with an integration time of 1 second and 3 total reads. Averages of the 3 reads were collected for both the firefly signal and the *Renilla* signal. Average firefly signal was divided against average *Renilla* signal, and treated samples were normalized against negative controls. The normalized TOP/*Renilla* signal serves as an indirect measurement of Wnt activation. Averages, standard deviation, and SEM were calculated for triplicate groups. Error bars shown are SEM.

2.5 Western Blotting

Cells were transfected with 1 µg of plasmid(s) of interest. Cells were harvested after 24 hours and were treated with 10 µM MG132 (Millipore-Sigma) general proteasome inhibitor at 4 hours prior to harvesting. Cells were washed once in ice cold 1x PBS. Cells were then lysed in 1x Cell Culture Lysis Buffer (Promega) containing 1x HALT Protease Inhibitor Cocktail (Pierce) and 50 µL concentration of 1,10-Phenanthroline (Millipore-Sigma). Cells lysates were shaken on ice for 30 minutes with intermittent vortexing. Protein concentrations of lysates were determined using BCA assay (Peirce). Samples were prepared with 50 µg of protein, 1X SDS loading dye containing DTT, and brought to a final volume of 40 µL with lysis buffer. Samples were then boiled at 95°C for 10 minutes. Samples were loaded onto either 7.5% or 12% Mini-PROTEAN® TGX™ Precast Gels (Bio-Rad) and run at 100-120V in 1X SDS running buffer alongside Precision Plus Dual Color ladder (Bio-Rad). Gels

were transferred to PVDF using the classical wet transfer method at 4°C in 1X transfer buffer for 3 hours at 100V. Membranes were blocked in 5% BSA (Fisher Scientific) dissolved in 1X TBST for 1 hour at room temperature. Primary antibody was applied overnight at 4°C and secondary antibody was applied for 1 hour at room temperature. Membranes underwent three 10-minute washes in 1X TBST between blocking, antibody incubations, and imaging. Membranes were imaged via chemiluminescence on the ChemiDoc (Bio-Rad) using Clarity Max high sensitivity ECL (Bio-Rad) or Immobilon Western Chemiluminescent HRP Substrate (Millipore-Sigma).

Chapter 3

RESULTS

Preliminary work by collaborators at NCI was carried out to elucidate the mechanism of LRP6 cleavage. They postulated that the last LDL domain of LRP6 contains a potential cleavage site for initial ectodomain shedding (Figure 2). Through a series of mutation studies, they discovered that amino acid sequence APQ at site 1367-1369 appears to be required for LRP6 cleavage. Interestingly, this sequence is conserved among higher organisms but is present as a triplet of prolines in *Xenopus* species. Notably, *Xenopus* LRP6 does not appear to undergo cleavage, adding confidence to the hypothesis that this is a site of proteolysis. Furthermore, a human LRP6 mutant containing a transition to the *Xenopus* PPP sequence at site 1367-1369 shows a reduction in cleavage. This APQ-1367-1369PPP mutant will be referred to throughout this thesis as non-cleavable LRP6 (LRP6-NC).

Through the application of several MMP and ADAM specific inhibitors, the research group was able to propose ADAM10 as the responsible protease. Knockdown of ADAM10 with a morpholino brought about a reduction in cleaved LRP6 protein levels. Perhaps the most curious discovery was the implied involvement of EphrinB2 in promoting LRP6 cleavage. Ephrins are membrane-bound ligands for Eph receptors, and together they comprise the largest family of receptor tyrosine kinases. Eph/ephrin signaling can occur bidirectionally and contributes to a vast array of developmental

processes. It is established that crosstalk exists between ephrins and other signaling pathways, cell adhesion molecules, and membrane channels. In addition, it has been shown that Eph-ephrin complexes can serve as substrates for ADAM proteases, and ephrinBs in particular can undergo cleavage by γ-secretase (Arvanitis and Davy, 2008). With this in mind, it was hypothesized that LRP6 undergoes RIP through a mechanism by which interactions with ephrinB2 recruit specific sheddases to induce receptor cleavage. Once cleaved, it is postulated that the released cytoplasmic domain can activate the canonical Wnt pathway.

It is worth noting that the group at NCI only experimented by expressing various forms of human LRP6 in *Xenopus* embryos. Thus, it is vital to confirm the presence of LRP6 cleavage in a human setting and to examine its effects on canonical Wnt signaling in order to elucidate any potential for clinical significance. Accordingly, the first set of experiments performed in Dr. Shuo Wei's laboratory focused on determining the effects of LRP6 cleavage on canonical Wnt activity in mammalian cells. The TOP plasmid, which contains three copies of the TCF/LEF binding region upstream of a firefly luciferase gene, was transfected into HEK293T cells along with a *Renilla* luciferase plasmid to control for transfection efficiency. A dual reporter assay was used to first measure the luminescence output generated from the firefly signal, which serves as a readout of canonical Wnt activity, after the addition of a firefly quencher/*Renilla* activating reagent a second independent luminescent measurement was taken to quantify *Renilla* signal. The firefly signal was then normalized against the stable *Renilla* signal to account for transfection efficiency. This

dual reporter assay was used to analyze the ability of overexpressed LRP6-WT to induce Wnt activation in HEK293T cells compared to the LRP6-NC mutant (Figure 4). Overexpression of LRP6-WT increased canonical Wnt activity nearly 6-fold more than the negative control (an empty vector). Interestingly, LRP6-NC was only able to induce Wnt activity slightly more than the negative control and did not reach levels of activity induced by LRP6-WT overexpression. This implies that under basal conditions, the mutation of LRP6 at amino acid site 1367-1369 is able to impede canonical Wnt signaling in HEK293T cells, possibly by preventing RIP from occurring.

To investigate if RIP is indeed a catalyst for LRP6 to potentiate Wnt signaling, HEK293T cells were treated with an ADAM10 specific inhibitor (GI254023) or a γ-secretase inhibitor (DAPT) and Wnt activity was measured via the dual luciferase assay (Figure 5). Both inhibitors were capable of decreasing endogenous Wnt activity by approximately 50% compared to the vehicle-treated control. This data suggests that ADAM10 and γ-secretase are both involved in regulating Wnt signaling. This concurs with the model established by Mi and Johnson that postulates LRP6 ectodomain shedding and subsequent intramembrane cleavage releases an active form of the intracellular domain. When the same inhibitor treatments were applied to HEK293T cells overexpressing LRP6-WT, a statistical decrease in Wnt signal was witnessed, reaching levels comparable to overexpressed LRP-NC (Figure 6). Although this data does imply that these specific proteases play a role in regulating Wnt signaling, it does not confirm that the effect is correlated to the cleavage of LRP6. In order to observe

the impact of these proteases on LRP6 cleavage, western blotting for the C-terminus of LRP6 was performed on HEK293T lysates overexpressing LRP6-WT or LRP-NC and treated with DMSO, GI254023, or DAPT (Figure 7). Across all conditions, overexpressed LRP6-WT and LRP6-NC were detected at approximately 210 kDa, which corresponds to the mature form of LRP6. Bands at 35 and 45 kDa were also observed in both LRP6-WT and LRP-NC lysates when probing with an endogenous antibody for the C-terminus. However, these bands do not correspond to the predicted molecular weights of products derived from RIP. If LPR6 underwent proteolysis at amino acid 1367-1369, the remaining C-terminus would be approximately 29 kDa. Further intramembranous cleavage by γ-secretase would generate a product with a molecular weight of approximately 27 kDa. Considering the 35 kDa product was observed in both LRP6-WT and LRP6-NC lysates, it is probable that LRP6 is capable of undergoing various upstream cleavage events that result in different C-terminal products. Repeat experiments using either an endogenous LRP6 C-terminal antibody or an antibody for a C-terminal tag continued to detect both the 35 and 45 kDa products in both LRP6-WT and LRP6-NC lysates, but were unable to detect the hypothesized cleavage products. It is possible that phosphorylation contributes to the unexpected increase in molecular weight, although it does not explain why the products are observed in the mutant lysates. Alternatively, the desired cleavage products may be further degraded through other mechanisms, which would explain their perceived absence. Interestingly, the addition of either GI254023 or DAPT showed a reduction in the 35 kDa C-terminal product and a slight increase in full-

length protein for both LRP6-WT and LRP6-NC. This reduction implies that these proteases are capable of interacting with LRP6 to alter its proteolysis.

Although western blotting did not confirm any difference in cleavage products between the wild-type and mutant receptor, the difference in Wnt activation under basal conditions still posed a curiosity. To confirm that LRP6-NC is properly trafficked to the membrane and retains ligand-binding functionality, Wnt3a was overexpressed in HEK293T cells in combination with LRP6-WT or LRP6-NC. Theoretically, if LRP6-NC maintains functionality as a co-receptor in the Wnt pathway, then the addition of Wnt ligand should increase Wnt signal output. The results showed that the overexpression of Wnt3a alone and the overexpression of LRP6-WT alone were able to slightly increase canonical Wnt activity. Again, the overexpression of LRP6-NC alone had little impact on Wnt activity. However, a synergistic impact on activity was observed when Wnt3a and LRP6-WT or LRP6-NC were overexpressed together (Figure 8). Remarkably, upon Wnt3a overexpression the stark difference in pathway activation between wild-type and mutant receptors subsided. Not only does this data confirm that LRP6-NC retains functionality as a co-receptor for Wnt ligands and inducer of downstream signal, but it also suggests that absence of Wnt ligand may promote a RIP-dependent mechanism for pathway activation.

As previously mentioned, it is generally believed that two classes of Wnt ligands exist, each capable of binding to different extracellular domains of LRP6. Through the use of varying LRP6 antibodies scientists have uncovered two distinct

extracellular ligand-binding regions and have categorized Wnts into two classes (Ettenberg et al., 2010). Wnt3 and Wnt3a are known to bind to BP3/4 domains, whereas the remaining majority of human Wnt ligands bind to BP1/2 domains. Considering the impact of Wnt3a on abolishing differences induced by LRP6-WT or LRP6-NC, it was important to determine if this result was dependent on Wnt ligand class. To investigate if the difference in Wnt activation was dependent on ligand type/binding region, Wnt1 was overexpressed in combination with LRP6-WT or LRP6-NC (Figure 9). The overexpression of Wnt1 behaved similarly to that of Wnt3a. Overexpression of Wnt1 alone was able to cause an increase in Wnt activity; however, the combination of Wnt1 and LRP6-WT or LRP6-NC synergistically increased signal. The overall signal output was higher than that induced by Wnt3a which agrees with previous data that showed Wnt1 is capable of causing a greater increase in Wnt activity compared to Wnt3a when overexpressed in HEK293T cells (Najdi et al., 2012). Taken together, these results show that under conditions with abundant Wnt ligand of either class, the mutated region of LRP6 has no effect on ligand-mediated canonical Wnt signaling. However, under basal ligand conditions there remains the possibility of a cleavage-based mechanism for Wnt activation. This difference in signal could be the result of a ligand-independent mechanism of Wnt signaling. Recent studies have revealed a novel ligand-independent model of canonical Wnt signaling related to deficiencies in APC. In this report, it was observed that a loss of APC promotes LRP6 internalization through the clathrin endocytic and constitutive activation of the pathway independent of Wnt ligand (Saito-Diaz et al., 2018). It is

possible that RIP of LRP functions through a similar mechanism wherein absence of ligand triggers a ligand-independent method of proteolytic processing that releases an active ICD of LRP6.

To test this hypothesis, a cellular environment devoid of Wnt ligands was created in HEK293T cells through treatment with a potent Porcupine inhibitor, WNTC59. Recall that Porcupine is the acetyltransferase responsible for palmitoylating Wnts in the ER, a step that is vital for their processing and subsequent secretion. By blocking Porcupine, Wnt production is effectively halted, simulating a ligand-deprived cellular environment. Increasing concentrations of WNTC59 were added to HEK293T cells to observe the effect of ligand inhibition on endogenous canonical Wnt activity (Figure 10). Blocking Wnt production in HEK293T cells with even the lowest concentration of WNTC59 decreased Wnt signal by approximately 50% compared to basal levels. It was difficult to determine if any underlying Wnt activity remained, as a complete negative control (blocking all Wnt activity) was challenging to produce.

Next, the same concentrations of WNTC59 were added to HEK293T cells overexpressing LRP6-WT (Figure 11). There appeared to be a dose-dependent decrease in Wnt activity, which supports the established theory that ligand binding is the central means by which LRP6 initiates Wnt signaling. When WNTC59 was applied to HEK293T cells overexpressing LRP6-NC there did not appear to be any change in Wnt activity (Figure 12). In theory, the mutant receptor should only function through the classical mechanism of ligand binding; however, the continued presence of Wnt activation in the absence of ligand implies that there may be another mechanism by which the mutant receptor initiates signal. Considering the apparent

necessity of Wnt ligands for the wild-type receptor to induce activity it is theorized that RIP may occur when Wnt concentrations are low, whereas high Wnt levels trigger the canonical pathway.

The preliminary data generated by Dr. Jian Sun stressed the importance of EphrinB2 for inducing the cleavage of LRP6. Considering the possibility that HEK293T cells may have low EphrinB2 expression levels, EphrinB2 was overexpressed along with LRP6-WT or LRP6-NC and the effect on canonical Wnt activity was measured (Figure 13). The overexpression of EphrinB2 alone did not appear to have any effect on Wnt activation; however, when EphrinB2 was overexpressed with LRP6-WT there was an increase in signal compared to overexpression of the wild-type receptor alone. This does suggest that the interaction of EphrinB2 and LRP6 is capable of activating the canonical Wnt pathway. Interestingly, the addition of EphrinB2 and LRP6-NC also showed an increase in signal compared to overexpression of the mutant receptor alone, although the overall output was still closer to that of the negative control. To determine if the impact of EphrinB2 is related to LRP6 cleavage, western blotting was performed on HEK293T lysates overexpressing LRP6-WT or LRP6-NC with EphrinB2 (Figure 14). Two cleavage products with molecular weights of 35 and 30 kDa, were visualized in the lanes containing LRP6-WT when using an antibody recognizing the C-terminus of LRP6. Only the 35 kDa product was observed in the lanes containing LRP6-NC. Furthermore, the 30 kDa product appeared to increase when EphrinB2 was

coexpressed with LRP6-WT. Together these data imply that the 30 kDa product is a result of cleavage at amino acid site 1367-1369, which may initiate RIP and activate canonical Wnt signaling through release of the LRP6 cytoplasmic tail. As previously theorized, this cleavage may be promoted through interactions between LRP6 and EphrinB2.

To further elucidate the role of EphrinB2 on LRP6 cleavage, SW620 colorectal cancer cells were used to analyze LRP6 cleavage. SW620 cells are known to have high expression levels of EphrinB2 (Ji et al., 2013) and harbor high levels of LRP6 (Li et al., 2004), making them useful cells for this study. Western blotting was performed on SW620 cell lysates overexpressing LRP6-WT or LRP6-NC (Figure 15). Lysates overexpressing either the wild-type or mutant receptor contained the common 35 kDa C-terminal cleavage product. Two additional cleavage products at 30 and 25 kDa were only observed in cells overexpressing LRP6-WT. These sizes correspond to the predicted molecular weight of a cleavage at amino acid 1367 and an intramembrane proteolysis. This data supports the hypothesis that under physiological contexts where EphrinB2 is present, LRP6 can undergo cleavage.

Chapter 4

DISCUSSION

It is clear that the cleavage of LRP6 and its subsequent impact on downstream canonical Wnt signaling is governed by a complex mechanism that relies on a host of situational factors. This study has revealed that under basal conditions in HEK293T cells, wild-type LRP6 is able to induce Wnt signaling, whereas a mutant receptor containing a triple proline sequence at amino acid site 1367-1369 (as is present in *Xenopus* LRP6) is unable to induce Wnt signaling (Figure 4). This data suggests that mammalian cells may contain a separate mechanism for inducing canonical Wnt signaling that relies on a cleavage within this specific region of the last LDL type-A repeat. It is interesting that the difference in Wnt signal output between wild-type and mutant receptor was only detected under basal ligand conditions; however, when ligand (Wnt3a or Wnt1) was overexpressed, any difference in activity dissipated. In this regard, the LRP6-NC mutant behavior is similar to that of overexpressed LRP5 in HEK293T cells. Studies have shown that overexpression of the LRP5 cannot generate an increase in Wnt activity; however, when combined with overexpressed Wnt ligand it is able to activate signal (MacDonald et al., 2011). It is important to note that LRP5 and LRP6 do not share the same postulated cleavable sequence site at amino acids 1367-1369. LRP5 contains a sequence of SPA at this site. Taken together, the results of this study imply that under basal conditions,

wild-type LRP6, but not LRP5 or LRP6-NC, is able to undergo cleavage which can induce the canonical Wnt pathway.

Considering the stark difference in signal from ligand overexpression compared to basal conditions, it is tempting to conclude that varying Wnt dosages are capable of inducing different mechanistic responses. The work of Nalesso et al. has shown that in articular chondrocytes a high concentration of Wnt3a is capable of inducing the canonical Wnt pathway, whereas low concentrations of Wnt3a activate the non-canonical Wnt pathway (Nalesso et al., 2011). In essence, a Wnt switch can occur under certain physiological conditions wherein the concentration of Wnt ligand dictates which downstream Wnt pathway will proceed. A similar phenomenon may be occurring in HEK293T cells, whereby LRP6 undergoes cleavage-induced activation of canonical Wnt when low concentrations of ligand are present, but switches to the conventional ligand-binding mechanism of induction when Wnt levels are sufficiently high (Figure 16). Data from the Porcupine inhibition experiments add confidence to this theory. When WNTC59 was applied to simulate an environment devoid of Wnt ligands basal Wnt activity was strongly decreased. However, a step-wise decrease in Wnt activity was observed when LRP6-WT was overexpressed with WNTC59. This suggests that some small amount of Wnt ligand may be required to initiate a cleavage-induced Wnt pathway. It could be that the protease responsible for ectodomain shedding interacts more favorably with a ligand-bound receptor compared to unbound receptor. This theory would support data

reported by Mi and Johnson wherein the addition of Wnt3a seemed to increase LRP6 cleavage as seen by decreased full-length LRP6 levels and increased levels of released ectodomain (Mi and Johnson, 2007). It also corresponds with other well-studied examples of RIP, whereby ligand binding serves as a catalyst to initiate ectodomain shedding. One factor that may contribute to the mechanism that went unlooked in this study is the presence and concentration of Frizzled receptor. The amount of Frizzled receptor and the stoichiometry with which it binds Wnt ligand and LRP6 could be a crucial aspect affecting the proposed model. In mammals there are 10 Frizzled receptors (Adler and Lee, 2001), which are divided into four classes. Each class contains specificity for certain Wnt ligands, which then dictate activation of downstream pathways (MacDonald and He, 2012). It may be that Frizzled acts as a rate limiting agent in the proposed model, although considering overexpression of Wnt ligand was able to greatly increase canonical Wnt signal output it would appear that HEK293T cells contain sufficient levels of Frizzled to initiate signal. It may also be that cleavage requires ligand binding to a specific class of Frizzled receptor. These postulations would require further study to determine if there is a precise ligand-receptor combination that regulates LRP6 cleavage.

While this dose-dependent hypothesis may fit the behavior of wild-type LRP6, it does not explain how LRP6-NC overexpression maintained a steady level of Wnt activity when treated with increasing concentrations of Porcupine inhibitor. If the mutant receptor is only capable of inducing activity through the classical ligand-dependent method, then the inhibition of Wnt secretion should have caused a stark decrease in signal. The fact that the signal remained unchanged even with increasing

dosages of WNTC59 may imply that residual Wnt ligand was present to bind the mutant receptor, although this conflicts with the fact that even the lowest WNTC59 concentration produced a strong decrease in endogenous activity. Alternatively, it may be possible that WNTC59 is not capable of blocking the processing of every Wnt ligand. A controversial study recently stated that Porcupine is not required for the processing and subsequent secretion of certain Wnt ligands, although this study was performed in primary astrocytes (Richards et al., 2014). Considering the wild-type receptor responded accordingly to ligand inhibition, it is perplexing as to how the mutant maintained activity.

Although the Wnt signaling luciferase data supported a high vs. low dosage model, the present study was unable to confidently detect the predicted cleavage products for proteolysis at amino acid site 1367-1369. Thus, it is difficult to confirm that the difference in signal activation was due to a difference in cleavage. One might then suggest that the difference in Wnt activity was caused by disruptions in receptor function, trafficking, or localization due to the triple proline mutation. This refutation is unlikely considering the mutant receptor contained the sequence from the fully functional *Xenopus* homolog and performed identically to the wild-type when Wnt ligands were overexpressed. Considering both forms of receptor produced the same C-terminal cleavage products, yet had different impacts on Wnt signal, it is probable that the 35 kDa product is unrelated to signal activation. It is possible that the 35 and 45 kDa products are nonspecific bands; however, inhibitor treatments appeared to affect the signal of these bands. Additionally, Mi and Johnson consistently observed a 35 kDa band and hypothesized that, “other γ-secretase cleavage sites upstream of the 35

kDa fragment may exist" (Mi and Johnson, 2007). Indeed, if these other products are real, this would mean that LRP6 undergoes multiple forms of proteolysis, yielding different sized products which may have varying effects on canonical Wnt signaling.

Regarding the elucidation of responsible proteases, this study showed that treatment with an ADAM10 inhibitor or a γ-secretase inhibitor was capable of decreasing Wnt activity in HEK293T cells. The reduction in Wnt signal indicates that when LRP6 undergoes RIP its products are able to induce downstream activation. It was previously thought that GI254023 was specific only for ADAM10; however, it has been revealed that it also contains specificity for inhibiting ADAM9, with an IC50 of 280 +/- 110 nM (Moss et al., 2010). Previous research in the laboratory of Dr. Shuo Wei has linked ADAM9 to the regulation of canonical Wnt signaling. Therefore, it is difficult to conclude whether the Wnt activity reduction from treatment with GI254023 was due to ADAM10 inhibition, ADAM9 inhibition, or a combinatorial effect. Experiments were performed with validated ADAM10 siRNA in an attempt to confirm specificity, but the data was inconclusive (data not shown). Another caveat to consider is that ADAM10 contains a plethora of substrates, some of which may be linked to the control of canonical Wnt signaling apart from cleavage of LRP6. In order to determine if these drug treatments were effectively altering cleavage, western blotting was utilized. Both the ADAM10 inhibitor and the γ-secretase inhibitor reduced the presence of the 35 kDa cleavage product in lysates overexpressing either the wild-type or mutant LRP6 receptor. Mi and Johnson were able to detect the reduction of a 20 kDa cleavage product by applying DAPT to CHO-K1 cells. They also witnessed the appearance of a slightly larger band upon DAPT treatment which

they believed to be the product from the initial ectodomain shedding (Mi and Johnson, 2007). However, in this study both types of inhibitors were able to reduce the anonymous 35 kDa product. Perhaps the 35 kDa band is a product of RIP and is larger than anticipated due to phosphorylation or other post-translational modifications. Although, that explanation would not account for how inhibition of either the α-secretase or γ-secretase reduced the same product. It is possible that the predicted cleavage products from site 1367-1369 underwent additional degradation by an unknown proteolytic mechanism and hence were absent from the western blots. These results contribute further evidence to the theory that LRP6 undergoes multiple cleavage mechanisms, several of which may be carried out by ADAM10 and/or γ-secretase. In the future, it would be helpful to use mass spectrometry and proteomic methods to analyze the exact position of the cleavage sites observed in this study to better predict the responsible proteases.

It may be that LRP6 undergoes varying forms of cleavage depending on the nature of its environment and through specific interactions with surrounding proteins. For instance, this study assessed the impact of EphrinB2 on the ability of LRP6 to undergo cleavage and discovered that when EphrinB2 is overexpressed in conjunction with wild-type LRP6, there is a synergistic increase in Wnt activity. This increase in activity is not observed when EphrinB2 is overexpressed alone or in combination with the mutant receptor. Interestingly, an increase in a novel 30 kDa cleavage product was observed when EphrinB2 was co-expressed with LRP6-WT, but no such peptide was seen in the samples containing LRP6-NC. Combined with the preliminary data conducted by Jian Sun, this data adds evidence to the theory that EphrinB2 may be

required for the cleavage of LRP6 at amino acid site 1367-1369, yielding a unique C-terminal product capable of inducing the canonical Wnt pathway. Considering that SW620 lysates (which contain high endogenous levels of EphrinB2) also showed a similar 30 kDa cleavage product, it is postulated that when EphrinB2 is present apart from a more favorable Eph receptor, it may interact with LRP6 and recruit ADAM10 to cleave the ectodomain, preparing it for intramembrane proteolysis by γ-secretase (Figure 17). This hypothesis is further supported by the discovery that ADAM10 acts as the major sheddase responsible for cleaving EphrinB2 in fibroblasts (Lagares et al., 2017). Considering the reduction in signal when Wnt ligand production was blocked by Porcupine inhibition, the interaction between EphrinB2, ADAM10 and LRP6 may require the receptor to be in a ligand-bound state.

Ultimately, this project contributed supporting evidence to a potentially novel mechanism for canonical Wnt signaling through the cleavage of LRP6. It was determined that under certain cellular conditions the wild-type LRP6 receptor activates the Wnt pathway, whereas a mutant receptor containing a triple proline sequence at site 1367-1369 does not. While it was hypothesized that the difference in activity was due to cleavage and release of the cytoplasmic tail, direct evidence was not observed to support this theory. The inhibition of ADAM10 and y-secretase did manage to decrease Wnt activity, which supports the hypothesis that cleavage induces the canonical pathway. Western blotting of lysates treated with these inhibitors showed the reduction of some C-terminal cleavage products. Finally, it was observed that in the presence of EphrinB2 wild-type LRP6 yields a novel 30 kDa cleavage product. The size of this cleaved product corresponds to a proteolytic site within the

last LDL type A repeat and is not present in experiments with the mutated receptor. Furthermore, the combined expression of LRP6-WT and EphrinB2 increased Wnt activity more than LRP6-WT or EphrinB2 alone. Also, a similar cleavage product is observed in SW620 cells, which are known to have high endogenous levels of EphrinB2. More studies will need to be performed in order to obtain a clearer picture of the process of LRP6 cleavage and to determine if it contains any physiological relevance. Future work may include the implementation of a CRISPR knock-in of the triple proline mutation at amino acid 1367-1369 into colorectal and breast cancer cell lines known to contain high endogenous levels of EphrinB2 and ADAM10. Reporter assays would be performed on these cells to determine effect on Wnt activation. Western blotting would be used to look for various LRP6 cleavage products. Migration/invasion assays could also be performed to assess the impact of the mutation on the aggressive phenotype of cancerous cells. If these preliminary studies proved hopeful, then the knock-in could be incorporated into the mouse genome and *in vivo* studies could be performed. Mice would be assessed for developmental defects and physiological effects resulting from disruptions in canonical Wnt signaling. Furthermore, the mice could undergo orthotopic implantation of colorectal tumor cells to observe the effect of the mutation on cancer progression and survival rate. Finally, if all of these experiments showed promise, then the cleavage site on LRP6 could serve as a clinically relevant therapeutic target for drug design. Antibodies against that specific region of LRP6 or specific protease inhibitors could be studied for efficacy. It would also be interesting to study high Wnt concentrations compared to low Wnt concentrations as it relates to canonical Wnt activity and more

specifically LRP6 cleavage. One could theoretically build a quantitative model to fit the pattern of high dosage and low dosage Wnt activity. Endogenous Wnt ligands could be eliminated through inhibitor treatment and exogenous Wnt could be applied in a dose-regulated manner while Wnt activity is measured. If effective, a threshold could be determined wherein Wnt concentration dictates a switch in pathway activation.

REFERENCES

Abrami, L., Kunz, B., Iacovache, I., & van der Goot, F. G. (2008, Apr). Palmitoylation and ubiquitination regulate exit of the Wnt signaling protein LRP6 from the endoplasmic reticulum. Proc Natl Acad Sci U S A, 105(14), 5384-5389. https://doi.org/10.1073/pnas.0710389105

Adler, P. N., & Lee, H. (2001, Oct). Frizzled signaling and cell-cell interactions in planar polarity. Curr Opin Cell Biol, 13(5), 635-640. https://doi.org/10.1016/s0955-0674(00)00263-5

Ahn, V. E., Chu, M. L., Choi, H. J., Tran, D., Abo, A., & Weis, W. I. (2011, Nov). Structural basis of Wnt signaling inhibition by Dickkopf binding to LRP5/6. Dev Cell, 21(5), 862-873. https://doi.org/10.1016/j.devcel.2011.09.003

Alarcón, M. A., Medina, M. A., Hu, Q., Avila, M. E., Bustos, B. I., Pérez-Palma, E., Peralta, A., Salazar, P., Ugarte, G. D., Reyes, A. E., Martin, G. M., Opazo, C., Moon, R. T., & De Ferrari, G. V. (2013a, Jun). A novel functional low-density lipoprotein receptor-related protein 6 gene alternative splice variant is associated with Alzheimer's disease. Neurobiol Aging, 34(6), 1709.e1709-1718. https://doi.org/10.1016/j.neurobiolaging.2012.11.004

Arvanitis, D., & Davy, A. (2008, Feb). Eph/ephrin signaling: networks. Genes Dev, 22(4), 416-429. https://doi.org/10.1101/gad.1630408

Baig-Lewis, S., Peterson-Nedry, W., & Wehrli, M. (2007, Jun). Wingless/Wnt signal transduction requires distinct initiation and amplification steps that both depend on Arrow/LRP. Dev Biol, 306(1), 94-111. https://doi.org/10.1016/j.ydbio.2007.03.005

Beagle, B., & Johnson, G. V. (2010, Jul). Differential modulation of TCF/LEF-1 activity by the soluble LRP6-ICD. PLoS One, 5(7), e11821. https://doi.org/10.1371/journal.pone.0011821

Beagle, B., Mi, K., & Johnson, G. V. (2009, Nov). Phosphorylation of PPP(S/T)P motif of the free LRP6 intracellular domain is not required to activate the Wnt/beta-catenin pathway and attenuate GSK3beta activity. J Cell Biochem, 108(4), 886-895. https://doi.org/10.1002/jcb.22318

Beel, A. J., & Sanders, C. R. (2008, May). Substrate specificity of gamma-secretase and other intramembrane proteases. Cell Mol Life Sci, 65(9), 1311-1334. https://doi.org/10.1007/s00018-008-7462-2

Bilic, J., Huang, Y. L., Davidson, G., Zimmermann, T., Cruciat, C. M., Bienz, M., & Niehrs, C. (2007, Jun). Wnt induces LRP6 signalosomes and promotes dishevelled-dependent LRP6 phosphorylation. Science, 316(5831), 1619-1622. https://doi.org/10.1126/science.1137065

Bode, W., Gomis-Rüth, F. X., & Stöckler, W. (1993, Sep). Astacins, serralysins, snake venom and matrix metalloproteinases exhibit identical zinc-binding environments (HEXXHXXGXXH and Met-turn) and topologies and should be grouped into a common family, the 'metzincins'. FEBS Lett, 331(1-2), 134-140. https://doi.org/10.1016/0014-5793(93)80312-i

Brennan, K., Gonzalez-Sancho, J. M., Castelo-Soccio, L. A., Howe, L. R., & Brown, A. M. (2004, Jun). Truncated mutants of the putative Wnt receptor LRP6/Arrow can stabilize beta-catenin independently of Frizzled proteins. Oncogene, 23(28), 4873-4884. https://doi.org/10.1038/sj.onc.1207642

Brown, M. S., Ye, J., Rawson, R. B., & Goldstein, J. L. (2000, Feb). Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans. Cell, 100(4), 391-398. https://doi.org/10.1016/s0092-8674(00)80675-3

Buechler, J., & Salinas, P. C. (2018). Deficient Wnt Signaling and Synaptic Vulnerability in Alzheimer's Disease: Emerging Roles for the LRP6 Receptor. Front Synaptic Neurosci, 10, 38. https://doi.org/10.3389/fnsyn.2018.00038

Cadigan, K. M., & Waterman, M. L. (2012, Nov). TCF/LEFs and Wnt signaling in the nucleus. Cold Spring Harb Perspect Biol, 4(11). https://doi.org/10.1101/cshperspect.a007906

Chantry, A., Gregson, N. A., & Glynn, P. (1989, Dec). A novel metalloproteinase associated with brain myelin membranes. Isolation and characterization. J Biol Chem, 264(36), 21603-21607.

Chen, J., Yan, H., Ren, D. N., Yin, Y., Li, Z., He, Q., Wo, D., Ho, M. S., Chen, Y., Liu, Z., Yang, J., Liu, S., & Zhu, W. (2014, May). LRP6 dimerization through its LDLR domain is required for robust canonical Wnt pathway activation. Cell Signal, 26(5), 1068-1074. https://doi.org/10.1016/j.cellsig.2013.12.020

Cho, C., Bunch, D. O., Faure, J. E., Goulding, E. H., Eddy, E. M., Primakoff, P., & Myles, D. G. (1998, Sep). Fertilization defects in sperm from mice lacking fertilin beta. Science, 281(5384), 1857-1859. https://doi.org/10.1126/science.281.5384.1857

Culi, J., Springer, T. A., & Mann, R. S. (2004, Mar). Boca-dependent maturation of beta-propeller/EGF modules in low-density lipoprotein receptor proteins. EMBO J, 23(6), 1372-1380. https://doi.org/10.1038/sj.emboj.7600132

Daniels, D. L., & Weis, W. I. (2005, Apr). Beta-catenin directly displaces Groucho/TLE repressors from Tcf/Lef in Wnt-mediated transcription activation. Nat Struct Mol Biol, 12(4), 364-371. https://doi.org/10.1038/nsmb912

De Ferrari, G. V., Papassotiropoulos, A., Biechele, T., Wavrant De-Vrieze, F., Avila, M. E., Major, M. B., Myers, A., Sáez, K., Henríquez, J. P., Zhao, A., Wollmer, M. A., Nitsch, R. M., Hock, C., Morris, C. M., Hardy, J., & Moon, R. T. (2007, May). Common genetic variation within the low-density lipoprotein receptor-related protein 6 and late-onset Alzheimer's disease. Proc Natl Acad Sci U S A, 104(22), 9434-9439. https://doi.org/10.1073/pnas.0603523104

De Strooper, B., Annaert, W., Cupers, P., Saftig, P., Craessaerts, K., Mumm, J. S., Schroeter, E. H., Schrijvers, V., Wolfe, M. S., Ray, W. J., Goate, A., & Kopan, R. (1999, Apr). A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain. Nature, 398(6727), 518-522. https://doi.org/10.1038/19083

Demir, K., Kirsch, N., Beretta, C. A., Erdmann, G., Ingelfinger, D., Moro, E., Argenton, F., Carl, M., Niehrs, C., & Boutros, M. (2013, Sep). RAB8B is required for activity and caveolar endocytosis of LRP6. Cell Rep, 4(6), 1224-1234. https://doi.org/10.1016/j.celrep.2013.08.008

Duffy, M. J., McKiernan, E., O'Donovan, N., & McGowan, P. M. (2009a, Feb). Role of ADAMs in cancer formation and progression. Clin Cancer Res, 15(4), 1140-1144. https://doi.org/10.1158/1078-0432.CCR-08-1585

Duffy, M. J., Mullooly, M., O'Donovan, N., Sukor, S., Crown, J., Pierce, A., & McGowan, P. M. (2011, Jun). The ADAMs family of proteases: new biomarkers and therapeutic targets for cancer? Clin Proteomics, 8(1), 9. https://doi.org/10.1186/1559-0275-8-9

Edwards, D. R., Handsley, M. M., & Pennington, C. J. (2008, Oct). The ADAM metalloproteinases. Mol Aspects Med, 29(5), 258-289. https://doi.org/10.1016/j.mam.2008.08.001

Ettenberg, S. A., Charlat, O., Daley, M. P., Liu, S., Vincent, K. J., Stuart, D. D., Schuller, A. G., Yuan, J., Ospina, B., Green, J., Yu, Q., Walsh, R., Li, S., Schmitz, R., Heine, H., Bilic, S., Ostrom, L., Mosher, R., Hartlepp, K. F., Zhu, Z., Fawell,

S., Yao, Y. M., Stover, D., Finan, P. M., Porter, J. A., Sellers, W. R., Klagge, I. M., & Cong, F. (2010, Aug). Inhibition of tumorigenesis driven by different Wnt proteins requires blockade of distinct ligand-binding regions by LRP6 antibodies. *Proc Natl Acad Sci U S A, 107*(35), 15473-15478. https://doi.org/10.1073/pnas.1007428107

Giebeler, N., & Zigrino, P. (2016, Apr). A Disintegrin and Metalloprotease (ADAM): Historical Overview of Their Functions. Toxins (Basel), 8(4), 122. https://doi.org/10.3390/toxins8040122

Go, G. W., Srivastava, R., Hernandez-Ono, A., Gang, G., Smith, S. B., Booth, C. J., Ginsberg, H. N., & Mani, A. (2014, Feb). The combined hyperlipidemia caused by impaired Wnt-LRP6 signaling is reversed by Wnt3a rescue. Cell Metab, 19(2), 209-220. https://doi.org/10.1016/j.cmet.2013.11.023

Gonzales, P. E., Solomon, A., Miller, A. B., Leesnitzer, M. A., Sagi, I., & Milla, M. E. (2004, Jul). Inhibition of the tumor necrosis factor-alpha-converting enzyme by its pro domain. J Biol Chem, 279(30), 31638-31645. https://doi.org/10.1074/jbc.M401311200

Groden, J., Joslyn, G., Samowitz, W., Jones, D., Bhattacharyya, N., Spirio, L., Thliveris, A., Robertson, M., Egan, S., & Meuth, M. (1995, Apr). Response of colon cancer cell lines to the introduction of APC, a colon-specific tumor suppressor gene. Cancer Res, 55(7), 1531-1539.

Hartmann, D., de Strooper, B., Serneels, L., Craessaerts, K., Herreman, A., Annaert, W., Umans, L., Lübke, T., Lena Illert, A., von Figura, K., & Saftig, P. (2002, Oct). The disintegrin/metalloprotease ADAM 10 is essential for Notch signalling but not for alpha-secretase activity in fibroblasts. Hum Mol Genet, 11(21), 2615-2624. https://doi.org/10.1093/hmg/11.21.2615

Haÿ, E., Laplantine, E., Geoffroy, V., Frain, M., Kohler, T., Müller, R., & Marie, P. J. (2009, Feb). N-cadherin interacts with axin and LRP5 to negatively regulate Wnt/beta-catenin signaling, osteoblast function, and bone formation. Mol Cell Biol, 29(4), 953-964. https://doi.org/10.1128/MCB.00349-08

He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B., & Kinzler, K. W. (1998, Sep). Identification of c-MYC as a target of the APC pathway. Science, 281(5382), 1509-1512. https://doi.org/10.1126/science.281.5382.1509

He, X., Semenov, M., Tamai, K., & Zeng, X. (2004, Apr). LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signaling: arrows point the way. Development, 131(8), 1663-1677. https://doi.org/10.1242/dev.01117

Hotoda, N., Koike, H., Sasagawa, N., & Ishiura, S. (2002, May). A secreted form of human ADAM9 has an alpha-secretase activity for APP. Biochem Biophys Res Commun, 293(2), 800-805. https://doi.org/10.1016/S0006-291X(02)00302-9

Howard, L., Lu, X., Mitchell, S., Griffiths, S., & Glynn, P. (1996, Jul). Molecular cloning of MADM: a catalytically active mammalian disintegrin-metalloprotease expressed in various cell types. Biochem J, 317 (Pt 1), 45-50. https://doi.org/10.1042/bj3170045

Hsieh, J. C., Lee, L., Zhang, L., Wefer, S., Brown, K., DeRossi, C., Wines, M. E., Rosenquist, T., & Holdener, B. C. (2003, Feb). Mesd encodes an LRP5/6 chaperone essential for specification of mouse embryonic polarity. Cell, 112(3), 355-367. https://doi.org/10.1016/s0092-8674(03)00045-x

Hsieh, J. C., Rattner, A., Smallwood, P. M., & Nathans, J. (1999, Mar). Biochemical characterization of Wnt-frizzled interactions using a soluble, biologically active vertebrate Wnt protein. Proc Natl Acad Sci U S A, 96(7), 3546-3551. https://doi.org/10.1073/pnas.96.7.3546

Huxley-Jones, J., Clarke, T. K., Beck, C., Toubaris, G., Robertson, D. L., & Boot-Handford, R. P. (2007, Apr). The evolution of the vertebrate metzincins; insights from Ciona intestinalis and Danio rerio. BMC Evol Biol, 7, 63. https://doi.org/10.1186/1471-2148-7-63

Jackson, H., Granger, D., Jones, G., Anderson, L., Friel, S., Rycroft, D., Fieles, W., Tunstead, J., Steward, M., Wattam, T., Walker, A., Griggs, J., Al-Hajj, M., & Shelton, C. (2016, 09). Novel Bispecific Domain Antibody to LRP6 Inhibits Wnt and R-spondin Ligand-Induced Wnt Signaling and Tumor Growth. Mol Cancer Res, 14(9), 859-868. https://doi.org/10.1158/1541-7786.MCR-16-0088

Janes, P. W., Saha, N., Barton, W. A., Kolev, M. V., Wimmer-Kleikamp, S. H., Nievergall, E., Blobel, C. P., Himanen, J. P., Lackmann, M., & Nikolov, D. B. (2005, Oct). Adam meets Eph: an ADAM substrate recognition module acts as a molecular switch for ephrin cleavage in trans. Cell, 123(2), 291-304. https://doi.org/10.1016/j.cell.2005.08.014

Ji, H., Greening, D. W., Barnes, T. W., Lim, J. W., Tauro, B. J., Rai, A., Xu, R., Adda, C., Mathivanan, S., Zhao, W., Xue, Y., Xu, T., Zhu, H. J., & Simpson, R. J. (2013, May). Proteome profiling of exosomes derived from human primary and metastatic colorectal cancer cells reveal differential expression of key metastatic factors and signal transduction components. Proteomics, 13(10-11), 1672-1686. https://doi.org/10.1002/pmic.201200562

Kato, M., Patel, M. S., Levasseur, R., Lobov, I., Chang, B. H., Glass, D. A., Hartmann, C., Li, L., Hwang, T. H., Brayton, C. F., Lang, R. A., Karsenty, G., & Chan, L. (2002, Apr). Cbfa1-independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in Lrp5, a Wnt coreceptor. J Cell Biol, 157(2), 303-314. https://doi.org/10.1083/jcb.200201089

Lagares, D., Ghassemi-Kakroodi, P., Tremblay, C., Santos, A., Probst, C. K., Franklin, A., Santos, D. M., Grasberger, P., Ahluwalia, N., Montesi, S. B., Shea, B. S., Black, K. E., Knipe, R., Blati, M., Baron, M., Wu, B., Fahmi, H., Gandhi, R., Pardo, A., Selman, M., Wu, J., Pelletier, J. P., Martel-Pelletier, J., Tager, A. M., & Kapoor, M. (2017, Dec). ADAM10-mediated ephrin-B2 shedding promotes myofibroblast activation and organ fibrosis. Nat Med, 23(12), 1405-1415. https://doi.org/10.1038/nm.4419

Lai, M. T., Chen, E., Crouthamel, M. C., DiMuzio-Mower, J., Xu, M., Huang, Q., Price, E., Register, R. B., Shi, X. P., Donoviel, D. B., Bernstein, A., Hazuda, D., Gardell, S. J., & Li, Y. M. (2003, Jun). Presenilin-1 and presenilin-2 exhibit distinct yet overlapping gamma-secretase activities. J Biol Chem, 278(25), 22475-22481. https://doi.org/10.1074/jbc.M300974200

Lal, M., & Caplan, M. (2011, Feb). Regulated intramembrane proteolysis: signaling pathways and biological functions. Physiology (Bethesda), 26(1), 34-44. https://doi.org/10.1152/physiol.00028.2010

Lee, E., Salic, A., Krüger, R., Heinrich, R., & Kirschner, M. W. (2003, Oct). The roles of APC and Axin derived from experimental and theoretical analysis of the Wnt pathway. PLoS Biol, 1(1), E10. https://doi.org/10.1371/journal.pbio.0000010

Lemieux, E., Cagnol, S., Beaudry, K., Carrier, J., & Rivard, N. (2015, Sep). Oncogenic KRAS signalling promotes the Wnt/β-catenin pathway through LRP6 in colorectal cancer. Oncogene, 34(38), 4914-4927. https://doi.org/10.1038/onc.2014.416

Li, Y., Chen, J., Lu, W., McCormick, L. M., Wang, J., & Bu, G. (2005, Nov). Mesd binds to mature LDL-receptor-related protein-6 and antagonizes ligand binding. J Cell Sci, 118(Pt 22), 5305-5314. https://doi.org/10.1242/jcs.02651

Li, Y., Lu, W., He, X., Schwartz, A. L., & Bu, G. (2004, Dec). LRP6 expression promotes cancer cell proliferation and tumorigenesis by altering beta-catenin subcellular distribution. Oncogene, 23(56), 9129-9135. https://doi.org/10.1038/sj.onc.1208123

Liu, C. C., Prior, J., Piwnica-Worms, D., & Bu, G. (2010, Mar). LRP6 overexpression defines a class of breast cancer subtype and is a target for therapy. Proc Natl Acad Sci U S A, 107(11), 5136-5141. https://doi.org/10.1073/pnas.0911220107

Liu, C. C., Tsai, C. W., Deak, F., Rogers, J., Penuliar, M., Sung, Y. M., Maher, J. N., Fu, Y., Li, X., Xu, H., Estus, S., Hoe, H. S., Fryer, J. D., Kanekiyo, T., & Bu, G. (2014, Oct). Deficiency in LRP6-mediated Wnt signaling contributes to synaptic abnormalities and amyloid pathology in Alzheimer's disease. Neuron, 84(1), 63-77. https://doi.org/10.1016/j.neuron.2014.08.048

Lu, W., Lin, C., Roberts, M. J., Waud, W. R., Piazza, G. A., & Li, Y. (2011). Niclosamide suppresses cancer cell growth by inducing Wnt co-receptor LRP6 degradation and inhibiting the Wnt/β-catenin pathway. PLoS One, 6(12), e29290. https://doi.org/10.1371/journal.pone.0029290

Ma, J., Lu, W., Chen, D., Xu, B., & Li, Y. (2017, 09). Role of Wnt Co-Receptor LRP6 in Triple Negative Breast Cancer Cell Migration and Invasion. J Cell Biochem, 118(9), 2968-2976. https://doi.org/10.1002/jcb.25956

MacDonald, B. T., & He, X. (2012, Dec). Frizzled and LRP5/6 receptors for Wnt/β-catenin signaling. Cold Spring Harb Perspect Biol, 4(12). https://doi.org/10.1101/cshperspect.a007880

MacDonald, B. T., Tamai, K., & He, X. (2009, Jul). Wnt/beta-catenin signaling: components, mechanisms, and diseases. Dev Cell, 17(1), 9-26. https://doi.org/10.1016/j.devcel.2009.06.016

MacDonald, B. T., Yokota, C., Tamai, K., Zeng, X., & He, X. (2008, Jun). Wnt signal amplification via activity, cooperativity, and regulation of multiple intracellular PPPSP motifs in the Wnt co-receptor LRP6. J Biol Chem, 283(23), 16115-16123. https://doi.org/10.1074/jbc.M800327200

MacDonald, B. T., Semenov, M. V., Huang, H., & He, X. (2011). Dissecting molecular differences between Wnt coreceptors LRP5 and LRP6. PLoS One, 6(8), e23537. https://doi.org/10.1371/journal.pone.0023537

Mao, B., Wu, W., Li, Y., Hoppe, D., Stannek, P., Glinka, A., & Niehrs, C. (2001, May). LDL-receptor-related protein 6 is a receptor for Dickkopf proteins. Nature, 411(6835), 321-325. https://doi.org/10.1038/35077108

Matoba, K., Mihara, E., Tamura-Kawakami, K., Miyazaki, N., Maeda, S., Hirai, H., Thompson, S., Iwasaki, K., & Takagi, J. (2017, 01). Conformational Freedom of the LRP6 Ectodomain Is Regulated by N-glycosylation and the Binding of the Wnt Antagonist Dkk1. Cell Rep, 18(1), 32-40. https://doi.org/10.1016/j.celrep.2016.12.017

May, P., Reddy, Y. K., & Herz, J. (2002, May). Proteolytic processing of low density lipoprotein receptor-related protein mediates regulated release of its intracellular domain. J Biol Chem, 277(21), 18736-18743. https://doi.org/10.1074/jbc.M201979200

McCarthy, A. J., Coleman-Vaughan, C., & McCarthy, J. V. (2017, Dec). Regulated intramembrane proteolysis: emergent role in cell signalling pathways. Biochem Soc Trans, 45(6), 1185-1202. https://doi.org/10.1042/BST20170002

McCarthy, J. V., Twomey, C., & Wujek, P. (2009, May). Presenilin-dependent regulated intramembrane proteolysis and gamma-secretase activity. Cell Mol Life Sci, 66(9), 1534-1555. https://doi.org/10.1007/s00018-009-8435-9

Mi, K., Dolan, P. J., & Johnson, G. V. (2006, Feb). The low density lipoprotein receptor-related protein 6 interacts with glycogen synthase kinase 3 and attenuates activity. J Biol Chem, 281(8), 4787-4794. https://doi.org/10.1074/jbc.M508657200

Mi, K., & Johnson, G. V. (2005, May). Role of the intracellular domains of LRP5 and LRP6 in activating the Wnt canonical pathway. J Cell Biochem, 95(2), 328-338. https://doi.org/10.1002/jcb.20400

Mi, K., & Johnson, G. V. (2007, Apr). Regulated proteolytic processing of LRP6 results in release of its intracellular domain. J Neurochem, 101(2), 517-529. https://doi.org/10.1111/j.1471-4159.2007.04447.x

Milla, M. E., Gonzales, P. E., & Leonard, J. D. (2006). The TACE zymogen: re-examining the role of the cysteine switch. Cell Biochem Biophys, 44(3), 342-348. https://doi.org/10.1385/CBB:44:3:342

Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., & Kinzler, K. W. (1997, Mar). Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science, 275(5307), 1787-1790. https://doi.org/10.1126/science.275.5307.1787

Moss, M. L., Rasmussen, F. H., Nudelman, R., Dempsey, P. J., & Williams, J. (2010, May). Fluorescent substrates useful as high-throughput screening tools for ADAM9. Comb Chem High Throughput Screen, 13(4), 358-365. https://doi.org/10.2174/138620710791054259

Najdi, R., Proffitt, K., Sprowl, S., Kaur, S., Yu, J., Covey, T. M., Virshup, D. M., & Waterman, M. L. (2012, Sep). A uniform human Wnt expression library reveals a shared secretory pathway and unique signaling activities. Differentiation, 84(2), 203-213. https://doi.org/10.1016/j.diff.2012.06.004

Nalesso, G., Sherwood, J., Bertrand, J., Pap, T., Ramachandran, M., De Bari, C., Pitzalis, C., & Dell'accio, F. (2011, May). WNT-3A modulates articular chondrocyte phenotype by activating both canonical and noncanonical pathways. J Cell Biol, 193(3), 551-564. https://doi.org/10.1083/jcb.201011051

Nelson, W. J. (2008, Apr). Regulation of cell-cell adhesion by the cadherin-catenin complex. Biochem Soc Trans, 36(Pt 2), 149-155. https://doi.org/10.1042/BST0360149

Nusse, R., Brown, A., Papkoff, J., Scambler, P., Shackleford, G., McMahon, A., Moon, R., & Varmus, H. (1991a, Jan). A new nomenclature for int-1 and related genes: the Wnt gene family. Cell, 64(2), 231. https://doi.org/10.1016/0092-8674(91)90633-a

Nusse, R., van Ooyen, A., Cox, D., Fung, Y. K., & Varmus, H. (1984, 1984 Jan 12-18). Mode of proviral activation of a putative mammary oncogene (int-1) on mouse chromosome 15. Nature, 307(5947), 131-136. https://doi.org/10.1038/307131a0

Nüsslein-Volhard, C., & Wieschaus, E. (1980, Oct). Mutations affecting segment number and polarity in Drosophila. Nature, 287(5785), 795-801. https://doi.org/10.1038/287795a0

Pinson, K. I., Brennan, J., Monkley, S., Avery, B. J., & Skarnes, W. C. (2000, Sep). An LDL-receptor-related protein mediates Wnt signalling in mice. Nature, 407(6803), 535-538. https://doi.org/10.1038/35035124

Port, F., & Basler, K. (2010, Oct). Wnt trafficking: new insights into Wnt maturation, secretion and spreading. Traffic, 11(10), 1265-1271. https://doi.org/10.1111/j.1600-0854.2010.01076.x

Ren, D. N., Chen, J., Li, Z., Yan, H., Yin, Y., Wo, D., Zhang, J., Ao, L., Chen, B., Ito, T. K., Chen, Y., Liu, Z., Li, Y., Yang, J., Lu, X., Peng, Y., Pan, L., Zhao, Y., Liu, S., & Zhu, W. (2015, Apr). LRP5/6 directly bind to Frizzled and prevent Frizzled-regulated tumour metastasis. Nat Commun, 6, 6906. https://doi.org/10.1038/ncomms7906

Richards, M. H., Seaton, M. S., Wallace, J., & Al-Harthi, L. (2014). Porcupine is not required for the production of the majority of Wnts from primary human astrocytes and CD8+ T cells. PLoS One, 9(3), e92159. https://doi.org/10.1371/journal.pone.0092159

Rismani, E., Fazeli, M. S., Mahmoodzadeh, H., Movassagh, A., Azami, S., Karimipoor, M., & Teimoori-Toolabi, L. (2017). Pattern of LRP6 gene expression in tumoral tissues of colorectal cancer. Cancer Biomark, 19(2), 151-159. https://doi.org/10.3233/CBM-160175

Rubinfeld, B., Albert, I., Porfiri, E., Munemitsu, S., & Polakis, P. (1997, Oct). Loss of beta-catenin regulation by the APC tumor suppressor protein correlates with loss of structure due to common somatic mutations of the gene. Cancer Res, 57(20), 4624-4630.

Saito-Diaz, K., Benchabane, H., Tiwari, A., Tian, A., Li, B., Thompson, J. J., Hyde, A. S., Sawyer, L. M., Jodoin, J. N., Santos, E., Lee, L. A., Coffey, R. J., Beauchamp, R. D., Williams, C. S., Kenworthy, A. K., Robbins, D. J., Ahmed, Y., & Lee, E. (2018, 03). APC Inhibits Ligand-Independent Wnt Signaling by the Clathrin Endocytic Pathway. Dev Cell, 44(5), 566-581.e568. https://doi.org/10.1016/j.devcel.2018.02.013

Sarzani, R., Salvi, F., Bordicchia, M., Guerra, F., Battistoni, I., Pagliariccio, G., Carbonari, L., Dessì-Fulgheri, P., & Rappelli, A. (2011, Feb). Carotid artery atherosclerosis in hypertensive patients with a functional LDL receptor-related protein 6 gene variant. Nutr Metab Cardiovasc Dis, 21(2), 150-156. https://doi.org/10.1016/j.numecd.2009.08.004

Schwarz-Romond, T., Fiedler, M., Shibata, N., Butler, P. J., Kikuchi, A., Higuchi, Y., & Bienz, M. (2007a, Jun). The DIX domain of Dishevelled confers Wnt signaling by dynamic polymerization. Nat Struct Mol Biol, 14(6), 484-492. https://doi.org/10.1038/nsmb1247

Schwarz-Romond, T., Metcalfe, C., & Bienz, M. (2007b, Jul). Dynamic recruitment of axin by Dishevelled protein assemblies. J Cell Sci, 120(Pt 14), 2402-2412. https://doi.org/10.1242/jcs.002956

Seals, D. F., & Courtneidge, S. A. (2003, Jan). The ADAMs family of metalloproteases: multidomain proteins with multiple functions. Genes Dev, 17(1), 7-30. https://doi.org/10.1101/gad.1039703

Seo, T., Sakon, T., Nakazawa, S., Nishioka, A., Watanabe, K., Matsumoto, K., Akasaka, M., Shioi, N., Sawada, H., & Araki, S. (2017a, 06). Haemorrhagic snake

venom metalloproteases and human ADAMs cleave LRP5/6, which disrupts cell-cell adhesions in vitro and induces haemorrhage in vivo. FEBS J, 284(11), 1657-1671. https://doi.org/10.1111/febs.14066

Seth, C., & Ruiz I Altaba, A. (2016, 2016). Correction: Metastases and Colon Cancer Tumor Growth Display Divergent Responses to Modulation of Canonical WNT Signaling. PLoS One, 11(8), e0160904. https://doi.org/10.1371/journal.pone.0160904

Sharma, R. P., & Chopra, V. L. (1976, Feb). Effect of the Wingless (wg1) mutation on wing and haltere development in Drosophila melanogaster. Dev Biol, 48(2), 461-465. https://doi.org/10.1016/0012-1606(76)90108-1

Sokol, S., Christian, J. L., Moon, R. T., & Melton, D. A. (1991, Nov). Injected Wnt RNA induces a complete body axis in Xenopus embryos. Cell, 67(4), 741-752. https://doi.org/10.1016/0092-8674(91)90069-b

Spasic, D., & Annaert, W. (2008, Feb). Building gamma-secretase: the bits and pieces. J Cell Sci, 121(Pt 4), 413-420. https://doi.org/10.1242/jcs.015255

Stamos, J. L., & Weis, W. I. (2013, Jan). The β-catenin destruction complex. Cold Spring Harb Perspect Biol, 5(1), a007898. https://doi.org/10.1101/cshperspect.a007898

Strickland, D. K., Gonias, S. L., & Argraves, W. S. (2002, Mar). Diverse roles for the LDL receptor family. Trends Endocrinol Metab, 13(2), 66-74. https://doi.org/10.1016/s1043-2760(01)00526-4

Struhl, G., & Adachi, A. (2000, Sep). Requirements for presenilin-dependent cleavage of notch and other transmembrane proteins. Mol Cell, 6(3), 625-636. https://doi.org/10.1016/s1097-2765(00)00061-7

Tamai, K., Semenov, M., Kato, Y., Spokony, R., Liu, C., Katsuyama, Y., Hess, F., Saint-Jeannet, J. P., & He, X. (2000, Sep). LDL-receptor-related proteins in Wnt signal transduction. Nature, 407(6803), 530-535. https://doi.org/10.1038/35035117

Tetsu, O., & McCormick, F. (1999, Apr). Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature, 398(6726), 422-426. https://doi.org/10.1038/18884

Vetrivel, K. S., Cheng, H., Kim, S. H., Chen, Y., Barnes, N. Y., Parent, A. T., Sisodia, S. S., & Thinakaran, G. (2005, Jul). Spatial segregation of gamma-secretase and

substrates in distinct membrane domains. J Biol Chem, 280(27), 25892-25900. https://doi.org/10.1074/jbc.M503570200

Vijayakumar, S., Liu, G., Wen, H. C., Abu, Y., Chong, R., Nastri, H., Bornstein, G. G., Pan, Z. Q., & Aaronson, S. A. (2017, Jul). Extracellular LDLR repeats modulate Wnt signaling activity by promoting LRP6 receptor endocytosis mediated by the Itch E3 ubiquitin ligase. Genes Cancer, 8(7-8), 613-627. https://doi.org/10.18632/genesandcancer.146

Vinyoles, M., Del Valle-Pérez, B., Curto, J., Viñas-Castells, R., Alba-Castellón, L., García de Herreros, A., & Duñach, M. (2014, Feb). Multivesicular GSK3 sequestration upon Wnt signaling is controlled by p120-catenin/cadherin interaction with LRP5/6. Mol Cell, 53(3), 444-457. https://doi.org/10.1016/j.molcel.2013.12.010

Wang, H., Liu, Q. J., Chen, M. Z., Li, L., Zhang, K., Cheng, G. H., Ma, L., & Gong, Y. Q. (2012, Feb). Association of common polymorphisms in the LRP6 gene with sporadic coronary artery disease in a Chinese population. Chin Med J (Engl), 125(3), 444-449.

Weskamp, G., Cai, H., Brodie, T. A., Higashyama, S., Manova, K., Ludwig, T., & Blobel, C. P. (2002, Mar). Mice lacking the metalloprotease-disintegrin MDC9 (ADAM9) have no evident major abnormalities during development or adult life. Mol Cell Biol, 22(5), 1537-1544. https://doi.org/10.1128/mcb.22.5.1537-1544.2002

Wolf, J., Palmby, T. R., Gavard, J., Williams, B. O., & Gutkind, J. S. (2008, Jan). Multiple PPPS/TP motifs act in a combinatorial fashion to transduce Wnt signaling through LRP6. FEBS Lett, 582(2), 255-261. https://doi.org/10.1016/j.febslet.2007.12.013

Wolfe, M. S., De Los Angeles, J., Miller, D. D., Xia, W., & Selkoe, D. J. (1999, Aug). Are presenilins intramembrane-cleaving proteases? Implications for the molecular mechanism of Alzheimer's disease. Biochemistry, 38(35), 11223-11230. https://doi.org/10.1021/bi991080q

Wu, G., Huang, H., Garcia Abreu, J., & He, X. (2009). Inhibition of GSK3 phosphorylation of beta-catenin via phosphorylated PPPSPXS motifs of Wnt coreceptor LRP6. PLoS One, 4(3), e4926. https://doi.org/10.1371/journal.pone.0004926

Wu, G., Xu, G., Schulman, B. A., Jeffrey, P. D., Harper, J. W., & Pavletich, N. P. (2003, Jun). Structure of a beta-TrCP1-Skp1-beta-catenin complex: destruction

motif binding and lysine specificity of the SCF(beta-TrCP1) ubiquitin ligase. Mol Cell, 11(6), 1445-1456. https://doi.org/10.1016/s1097-2765(03)00234-x

Wu, Z. Q., Brabletz, T., Fearon, E., Willis, A. L., Hu, C. Y., Li, X. Y., & Weiss, S. J. (2012, Jul). Canonical Wnt suppressor, Axin2, promotes colon carcinoma oncogenic activity. Proc Natl Acad Sci U S A, 109(28), 11312-11317. https://doi.org/10.1073/pnas.1203015109

Xing, Y., Clements, W. K., Kimelman, D., & Xu, W. (2003, Nov). Crystal structure of a beta-catenin/axin complex suggests a mechanism for the beta-catenin destruction complex. Genes Dev, 17(22), 2753-2764. https://doi.org/10.1101/gad.1142603

Yamamoto, H., Komekado, H., & Kikuchi, A. (2006, Aug). Caveolin is necessary for Wnt-3a-dependent internalization of LRP6 and accumulation of beta-catenin. Dev Cell, 11(2), 213-223. https://doi.org/10.1016/j.devcel.2006.07.003

Yamamoto, H., Sakane, H., Michiue, T., & Kikuchi, A. (2008, Jul). Wnt3a and Dkk1 regulate distinct internalization pathways of LRP6 to tune the activation of beta-catenin signaling. Dev Cell, 15(1), 37-48. https://doi.org/10.1016/j.devcel.2008.04.015

Yan, D., Wiesmann, M., Rohan, M., Chan, V., Jefferson, A. B., Guo, L., Sakamoto, D., Caothien, R. H., Fuller, J. H., Reinhard, C., Garcia, P. D., Randazzo, F. M., Escobedo, J., Fantl, W. J., & Williams, L. T. (2001, Dec). Elevated expression of axin2 and hnkd mRNA provides evidence that Wnt/beta -catenin signaling is activated in human colon tumors. Proc Natl Acad Sci U S A, 98(26), 14973-14978. https://doi.org/10.1073/pnas.261574498

Yao, Q., An, Y., Hou, W., Cao, Y. N., Yao, M. F., Ma, N. N., Hou, L., Zhang, H., Liu, H. J., & Zhang, B. (2017, Dec). LRP6 promotes invasion and metastasis of colorectal cancer through cytoskeleton dynamics. Oncotarget, 8(65), 109632-109645. https://doi.org/10.18632/oncotarget.22759

Yuan, T., Wang, S., Hu, C., Wu, Y., Liang, D., Li, L., Liu, Y., Li, J., & Chen, Y. H. (2018, Oct). Low-density lipoprotein receptor-related protein 6 regulates alternative pre-mRNA splicing. J Cell Mol Med, 22(10), 4653-4663. https://doi.org/10.1111/jcmm.13682

Zeng, X., Huang, H., Tamai, K., Zhang, X., Harada, Y., Yokota, C., Almeida, K., Wang, J., Doble, B., Woodgett, J., Wynshaw-Boris, A., Hsieh, J. C., & He, X. (2008, Jan). Initiation of Wnt signaling: control of Wnt coreceptor Lrp6 phosphorylation/activation via frizzled, dishevelled and axin functions. Development, 135(2), 367-375. https://doi.org/10.1242/dev.013540

Zeng, X., Tamai, K., Doble, B., Li, S., Huang, H., Habas, R., Okamura, H., Woodgett, J., & He, X. (2005, Dec). A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation. Nature, 438(7069), 873-877. https://doi.org/10.1038/nature04185

Zhang, J., Chen, J., Wo, D., Yan, H., Liu, P., Ma, E., Li, L., Zheng, L., Chen, D., Yu, Z., Liang, C., Peng, J., Ren, D. N., & Zhu, W. (2019, Aug). LRP6 Ectodomain Prevents SDF-1/CXCR4-Induced Breast Cancer Metastasis to Lung. Clin Cancer Res, 25(15), 4832-4845. https://doi.org/10.1158/1078-0432.CCR-18-3557

Zhang, J., Li, Y., Liu, Q., Lu, W., & Bu, G. (2010, Jan). Wnt signaling activation and mammary gland hyperplasia in MMTV-LRP6 transgenic mice: implication for breast cancer tumorigenesis. Oncogene, 29(4), 539-549. https://doi.org/10.1038/onc.2009.339

Appendix A

FIGURES

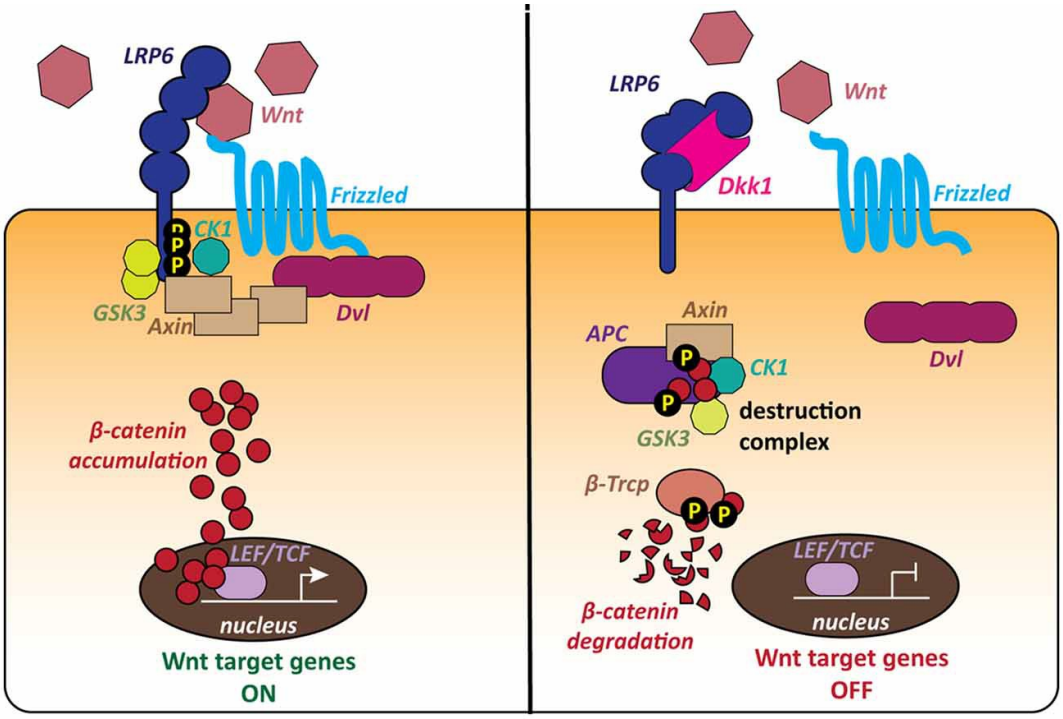

Figure 1. The canonical Wnt pathway. In the on state, a Wnt ligand binds co-receptors LRP6 and Frizzled, triggering members of the β-catenin destruction complex to be recruited to the membrane. This allows β-catenin to accumulate in the cytosol and eventually translocate into the nucleus where it helps activate TCF/LEF transcription factors that initiate transcription of several Wnt target genes. When a Wnt ligand is not present, or when membrane co-receptors are blocked, the β-catenin destruction complex (composed of Axin, APC, GSK3, and CK1) sequester and phosphorylate β-catenin, priming it for ubiquitination by β-TrCP and subsequent proteasomal degradation. Under these circumstances, Wnt target genes remain inactivated by co-repressors bound to TCF/LEF transcription factors. Image reprinted with permission from Frontiers in Synaptic Neuroscience (Buechler and Salinas, 2018).

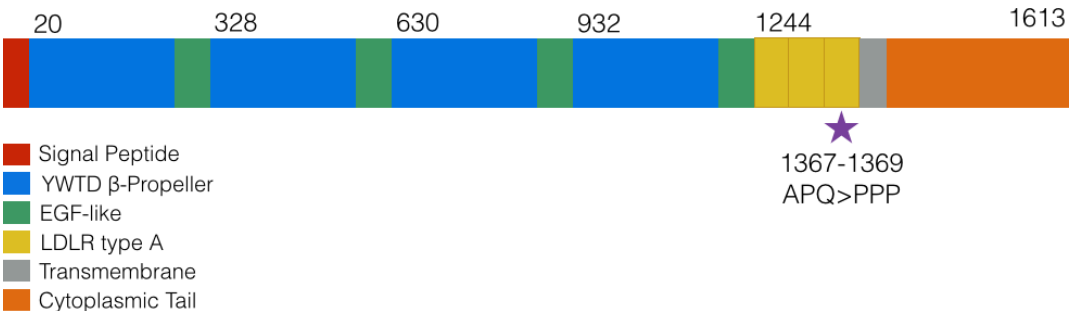

Figure 2. LRP6 structure and predicted cleavage site mutation. Schematic representation of LRP6 structure, including the amino acid site that indicates the start of each domain. LRP6 contains a signal peptide followed by four β-propeller/EGF-like repeats, then three LDLR type A repeats, a transmembrane domain, and a cytoplasmic tail. The purple star indicates the predicted cleavage site that was mutated from an amino acid sequence of APQ to PPP.

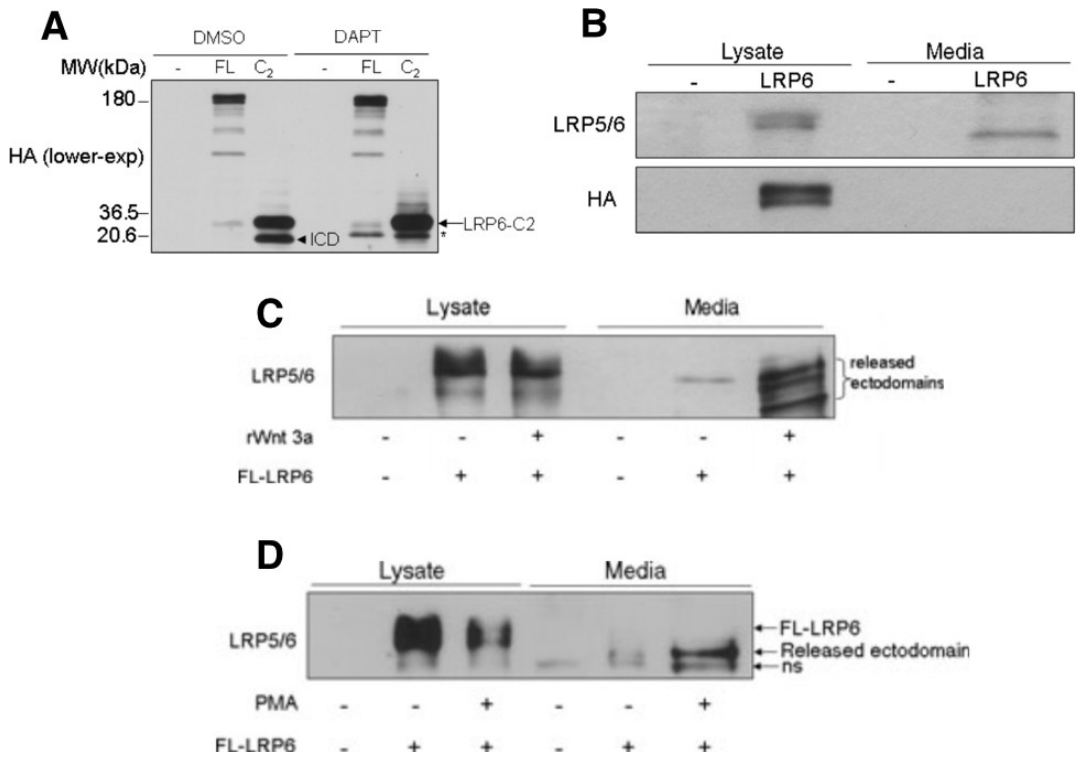

Figure 3. LRP6 undergoes regulated intramembrane proteolysis. A) Overexpression of LRP6 in CHO-K1 cells exhibits two cleavage products at 35 and 20 kDa. The cleavage product at 20 kDa is increased through the inhibition of γ-secretase by DAPT. B) An N-terminal released ectodomain of LRP6 is observed in conditioned media from HEK293T cells and is approximately 35 kDa smaller than full-length LRP6. C) The addition of Wnt3a to HEK293T cells causes an increase in shed LRP6 ectodomain observed in conditioned media. D) Treatment with PMA causes a decrease in full-length LRP6 observed in lysate from HEK293T cells and an increase in shed LRP6 ectodomain observed in conditioned media. Figures reprinted with permission from Journal of Neurochemistry (Mi and Johnson, 2007).

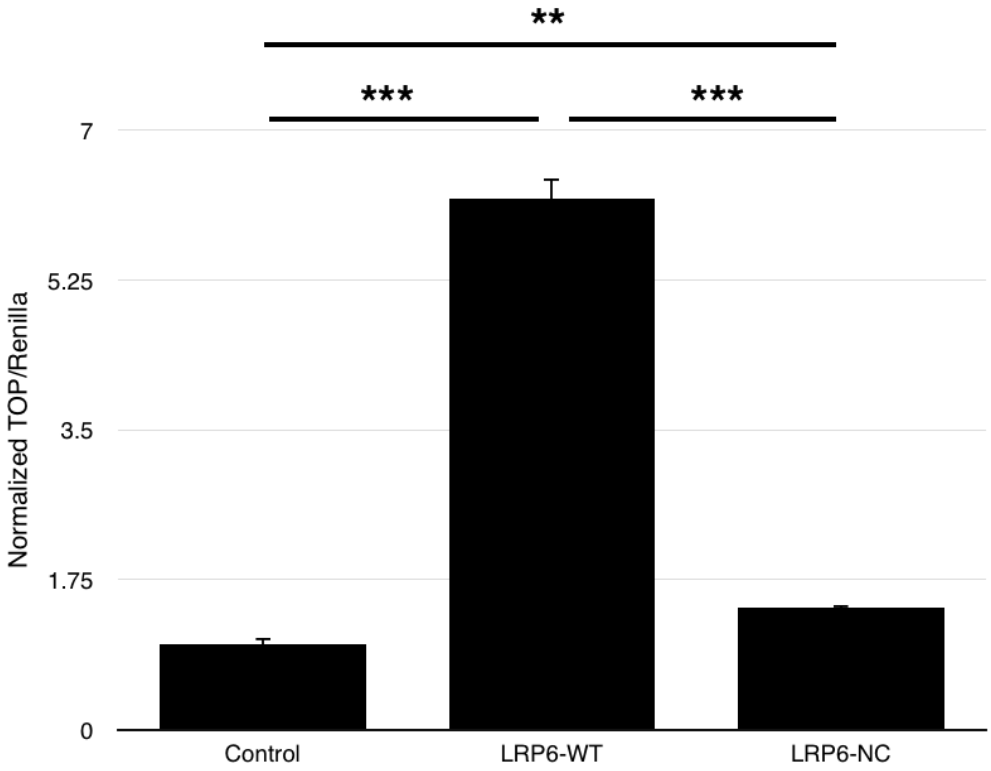

Figure 4. Effect of LRP6-WT and LRP6-NC overexpression on canonical Wnt activity in HEK293T cells. HEK293T cells were transfected with TOP plasmid *Renilla* plasmid, and plasmid of interest. Luminescent measurements were taken for firefly and *Renilla* signal. TOP firefly signal was normalized to *Renilla* signal and serves as an indirect measurement of canonical Wnt activity. Overexpression of LRP6-WT was capable of increasing canonical Wnt activity compared to control. Overexpression of LRP6-NC did not increase Wnt activity compared to control. Error bars shown are SEM. Asterisks correspond to unpaired t-test p-values less than 0.05 (*), 0.01 (**), and 0.001(***).

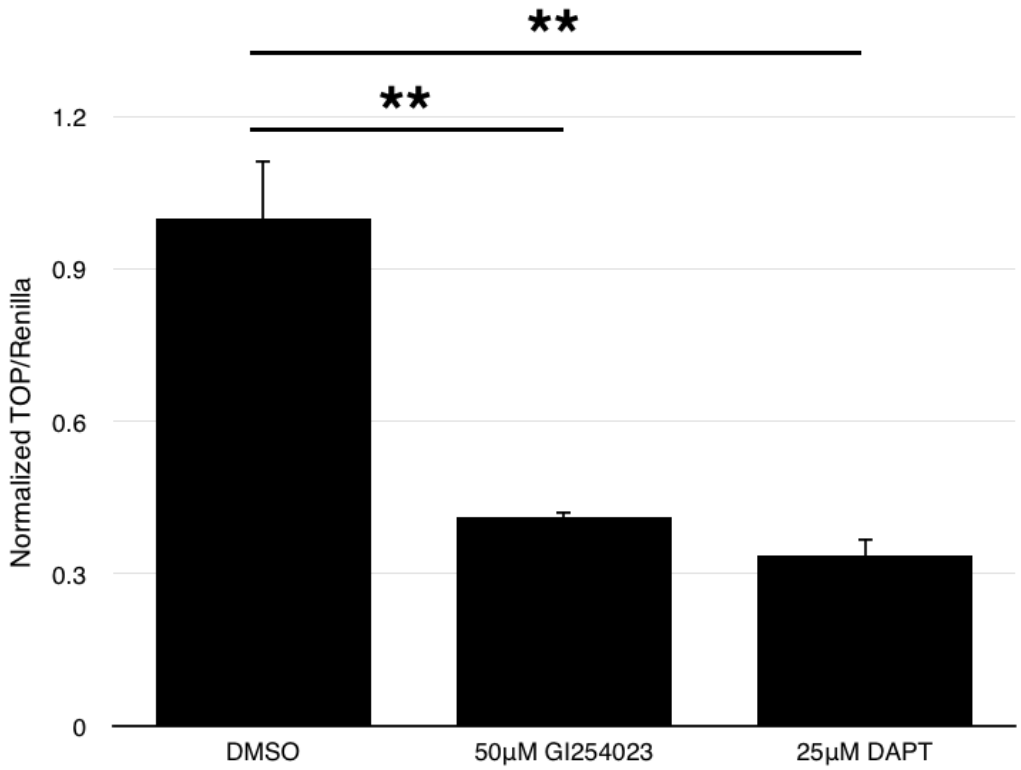

Figure 5. Canonical Wnt activity in HEK293T cells treated with GI254023 or DAPT. Treatment of HEK293T cells with either ADAM10 inhibitor (GI254023) or γ-secretase inhibitor (DAPT) decreases Wnt activity by over 50%. This implies that under basal conditions HEK293T cells rely on ADAM10 and γ-secretase to induce a portion of canonical Wnt activity. Error bars shown are SEM. Asterisks correspond to unpaired t-test p-values less than 0.05 (*), 0.01 (**), and 0.001(***).

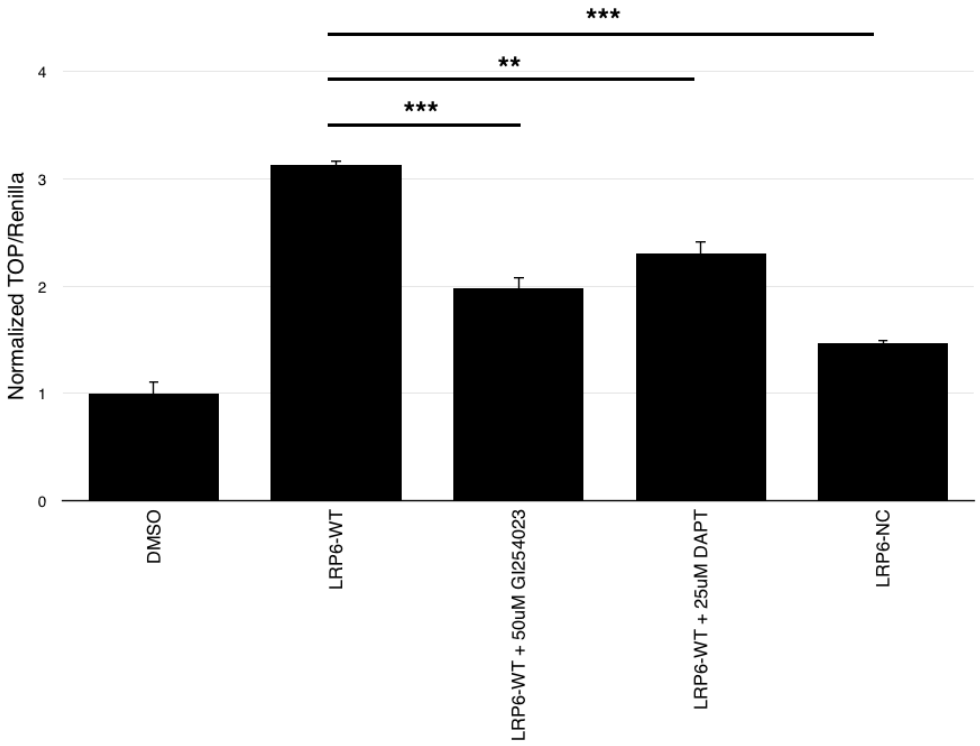

Figure 6. Canonical Wnt activity in HEK293T cells with overexpressed LRP6-WT and treated with GI254023 or DAPT compared to overexpressed LRP6-NC. Treatment of HEK293T cells overexpressing LRP6-WT with either ADAM10 inhibitor (GI254023) or γ-secretase inhibitor (DAPT) decreases Wnt activity nearly to the level LRP6-NC. This may mean that a portion of canonical Wnt signaling in HEK293T cells occurs through a cleavage-induced mechanism. Error bars shown are SEM. Asterisks correspond to unpaired t-test p-values less than 0.05 (*), 0.01 (**), and 0.001(***).

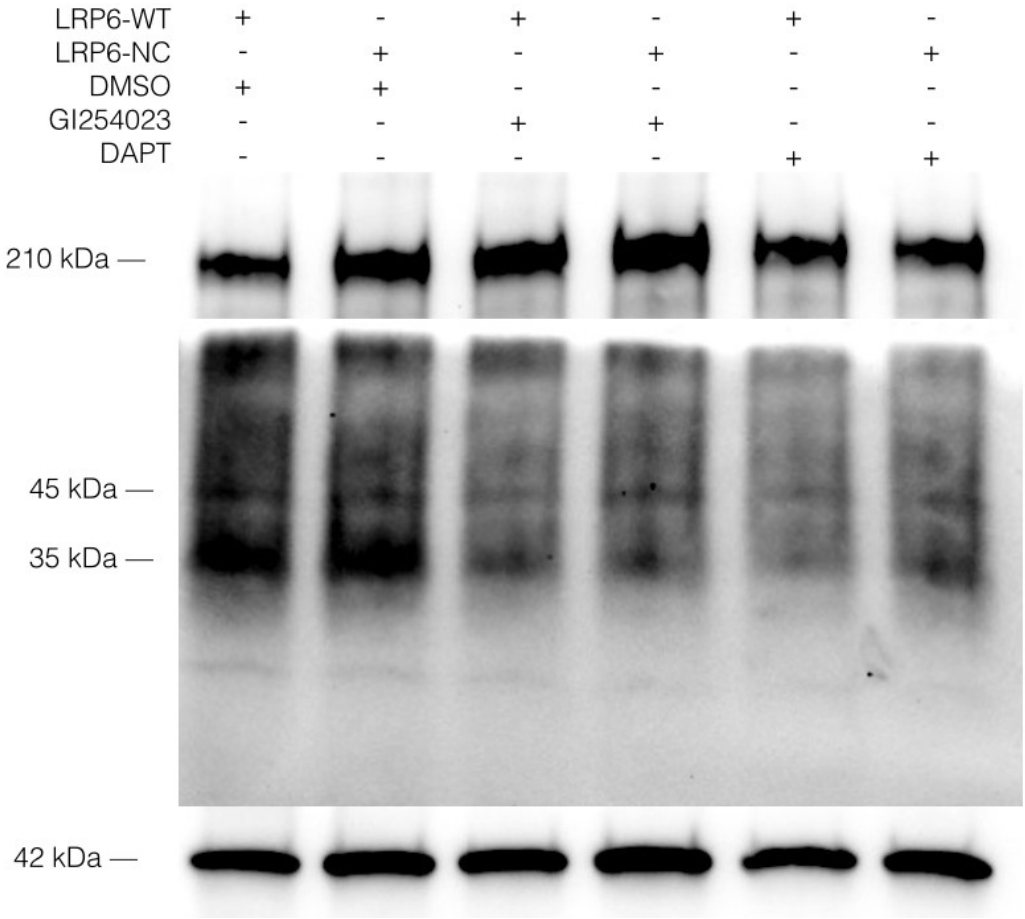

Figure 7. Protein expression and cleavage products of overexpressed LRP6-WT and LRP6-NC treated with GI254023 or DAPT in HEK293T cells. Overexpressing both forms of receptor showed a strong amount of full-length LRP6 at 210 kDa. Cells overexpressing either form of receptor alone showed cleavage products at 45 and 35 kDa. Treatment with either ADAM10 inhibitor (GI254023) or γ-secretase inhibitor (DAPT) decreased the amount of both cleavage products but did not appear to increase the amount of full-length protein. β-actin is shown at a molecular weight of 42 kDa and served as a loading control.

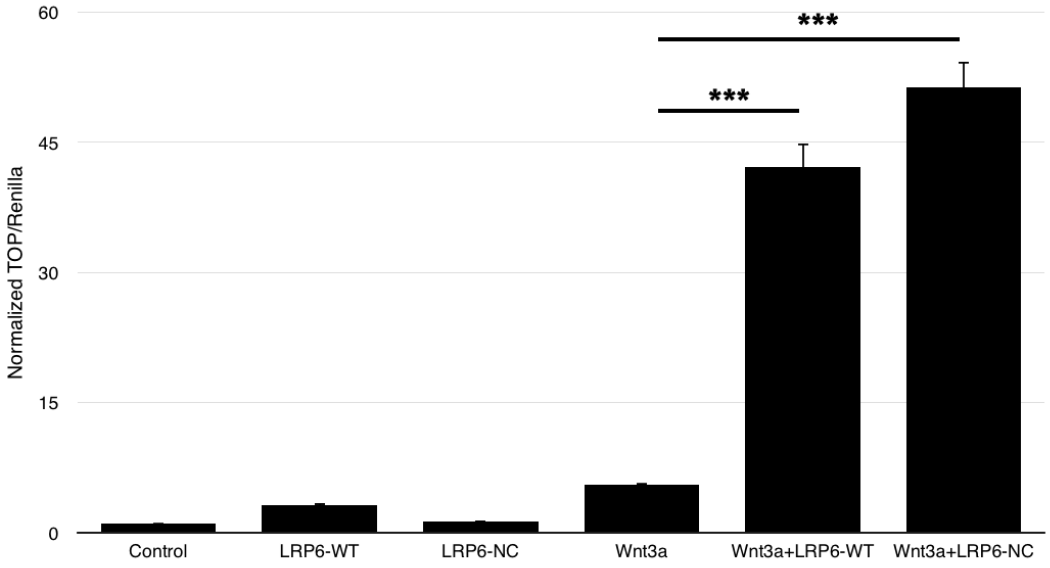

Figure 8. Effect of Wnt3a overexpression on canonical Wnt activity in HEK293T cells overexpressing LRP6-WT or LRP6-NC. Overexpression of Wnt3a was capable of increasing canonical Wnt activity only slightly compared to control, LRP6-WT, and LRP6-NC. Overexpression of both Wnt3a and either form of receptor had a synergist effect on Wnt activation. This implies that the wild-type and mutant receptor function similarly in high concentrations of Wnt3a. Error bars shown are SEM. Asterisks correspond to unpaired t-test p-values less than 0.05 (*), 0.01 (**), and 0.001(***).

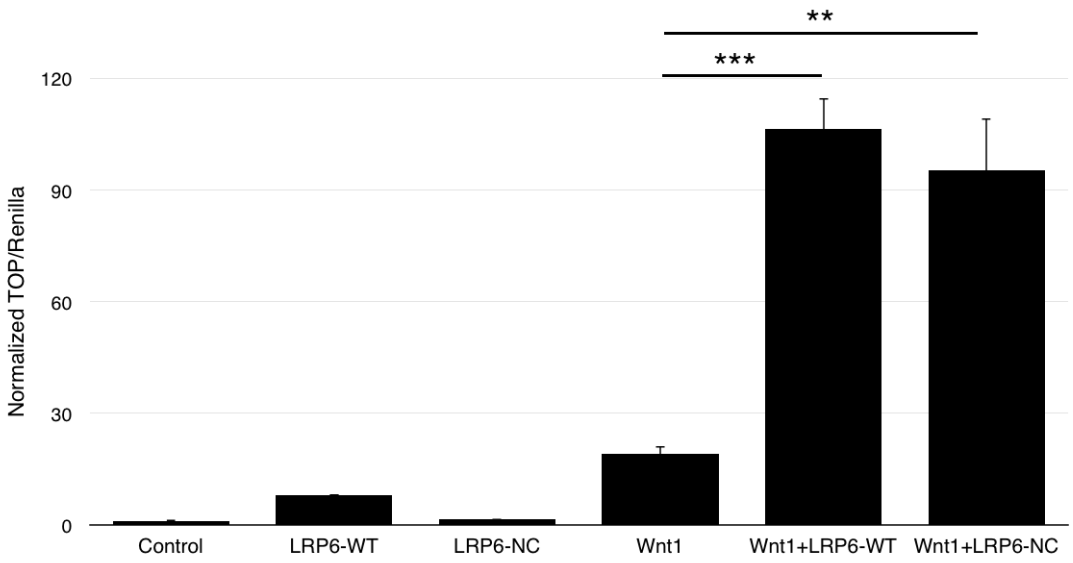

Figure 9. Effect of Wnt1 overexpression on canonical Wnt activity in HEK293T cells overexpressing LRP6-WT or LRP6-NC. Overexpression of Wnt1 was capable of increasing canonical Wnt activity compared to control, LRP6-WT, and LRP6-NC. Overexpression of both Wnt1 and either form of receptor had a synergist effect on Wnt activation. This implies that the wild-type and mutant receptor function similarly in high concentrations of Wnt1. Error bars shown are SEM. Asterisks correspond to unpaired t-test p-values less than 0.05 (*), 0.01 (**), and 0.001(***).

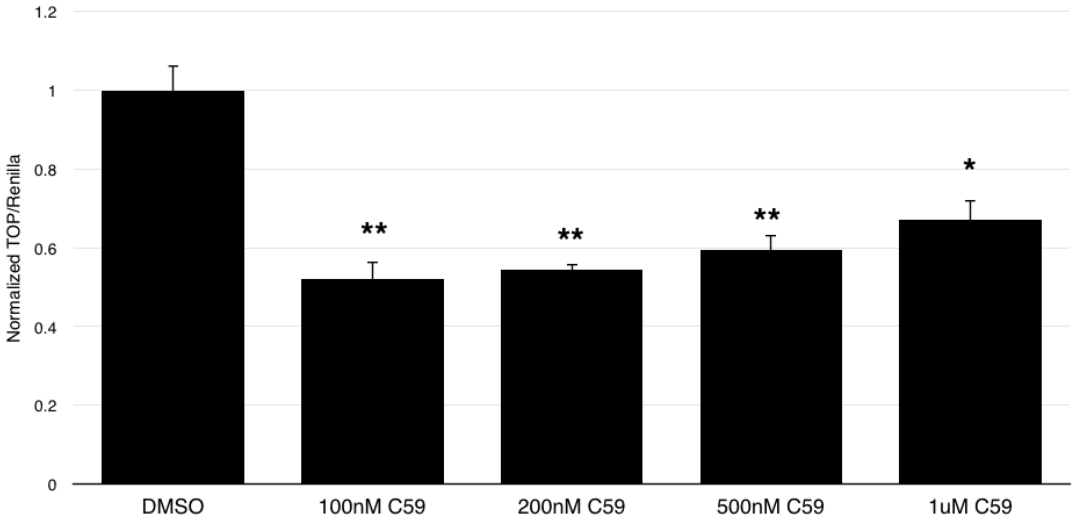

Figure 10. Canonical Wnt activity in HEK293T cells with increasing dosages of WNTC59. Treatment with increasing doses of Porcupine inhibitor (WNTC59) decreased canonical Wnt activation compared to vehicle treatment. Error bars shown are SEM. Unpaired t-tests were calculated for each treatment group against DMSO control. Asterisks correspond to unpaired t-test p-values less than 0.05 (*), 0.01 (**), and 0.001(***).

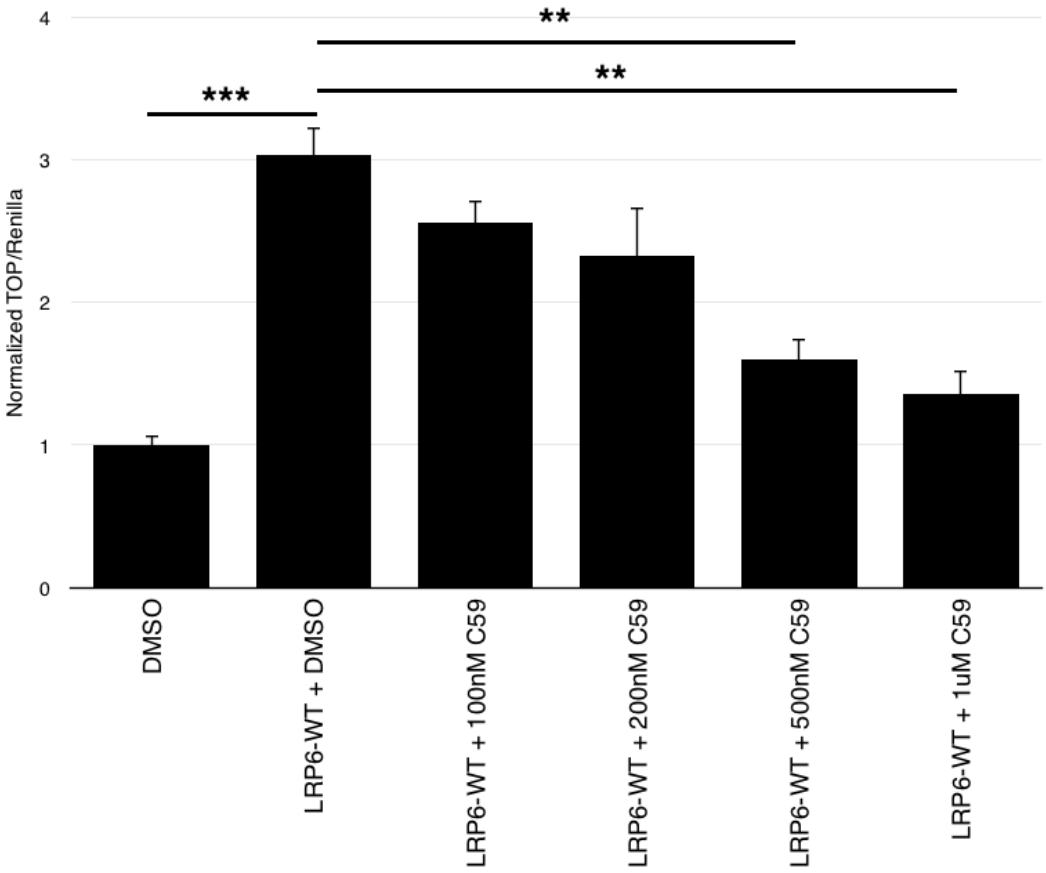

Figure 11. Canonical Wnt activity in HEK293T cells with overexpressed LRP6-WT and increasing dosages of WNTC59. Treatment of cells overexpressing LRP6-WT with increasing doses of Porcupine inhibitor (WNTC59) decreased canonical Wnt activation in a step-wise fashion. This implies that Wnt ligands still play a role in canonical Wnt induction, either through a classical ligand-binding mechanism or through interactions resulting in cleavage. Error bars shown are SEM. Unpaired t-tests were calculated for each treatment group against DMSO control. Asterisks correspond to unpaired t-test p-values less than 0.05 (*), 0.01 (**), and 0.001(***).

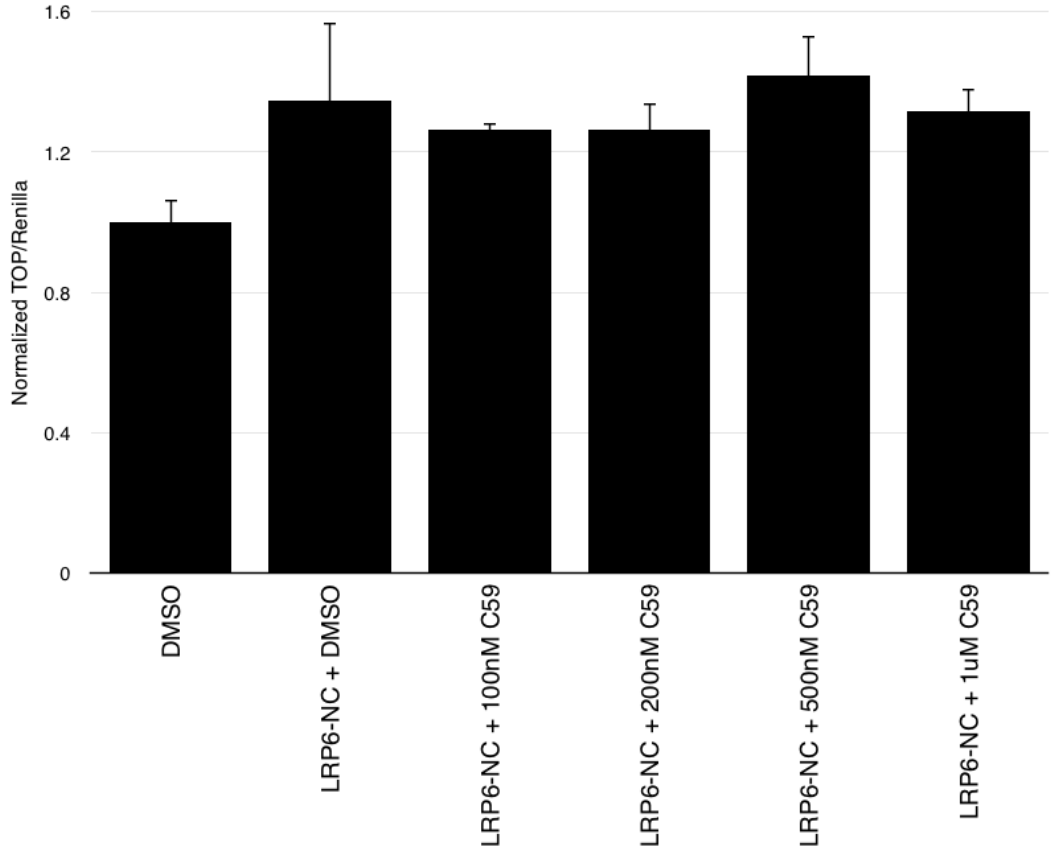

Figure 12. Canonical Wnt activity in HEK293T cells with overexpressed LRP6-NC and increasing dosages of WNTC59. Treatment of cells overexpressing LRP6-NC with increasing doses of Porcupine inhibitor (WNTC59) appeared to have no effect on canonical Wnt activation. This implies that the mutant receptor is capable of inducing a small amount of canonical Wnt activity through an unknown mechanism. Error bars shown are SEM. Unpaired t-tests were calculated for each treatment group against DMSO control. Asterisks correspond to unpaired t-test p-values less than 0.05 (*), 0.01 (**), and 0.001(***).

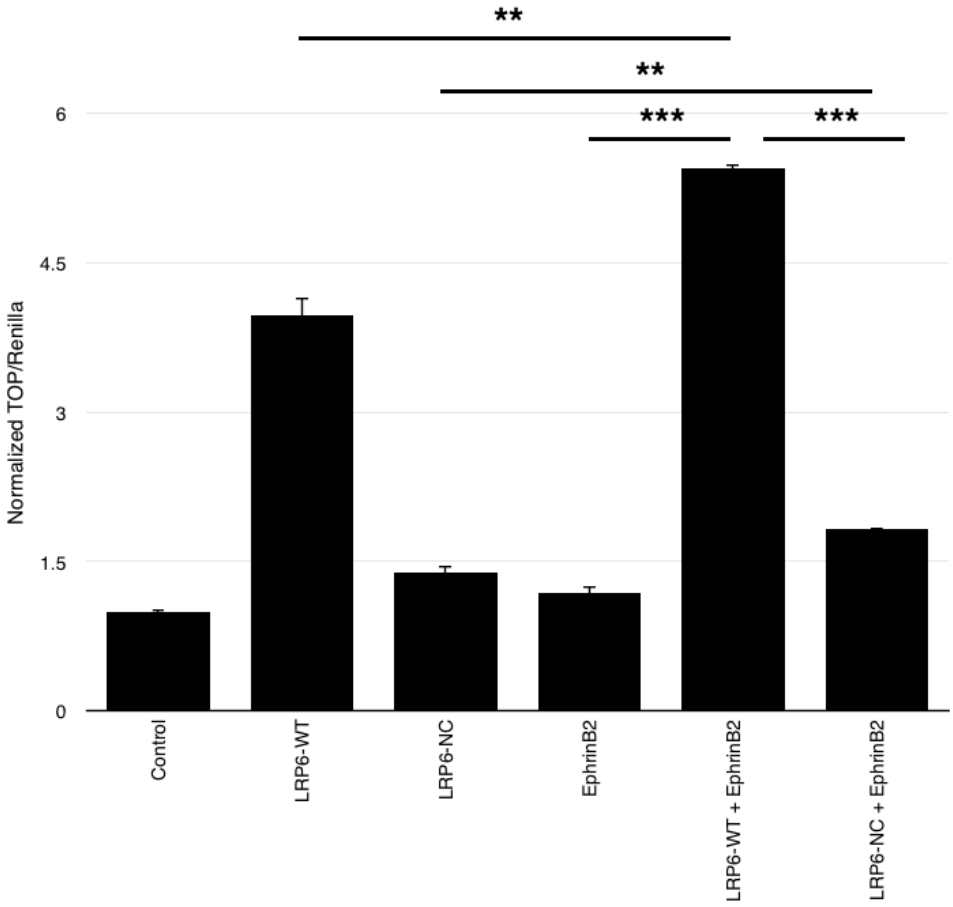

Figure 13. Effect of EphrinB2 overexpression on canonical Wnt signaling in HEK293T cells with overexpressed LRP6-WT or LRP6-NC. Overexpression of EphrinB2 alone did not have any effect on canonical Wnt signaling in HEK293T cells. When EphrinB2 was overexpressed along with LRP6-WT there was a greater increase in activity than in cells overexpressing LRP6-WT alone. This implies that EprhinB2 is capable of causing an activating effect on canonical Wnt when sufficient LRP6 is present. Overexpressing EphrinB2 along with LRP6-NC increased signal slightly more than LRP6-NC alone, but this may be due to EphrinB2 acting on endogenous wild-type LRP6. Error bars shown are SEM. Asterisks correspond to unpaired t-test p-values less than 0.05 (*), 0.01 (**), and 0.001(***).

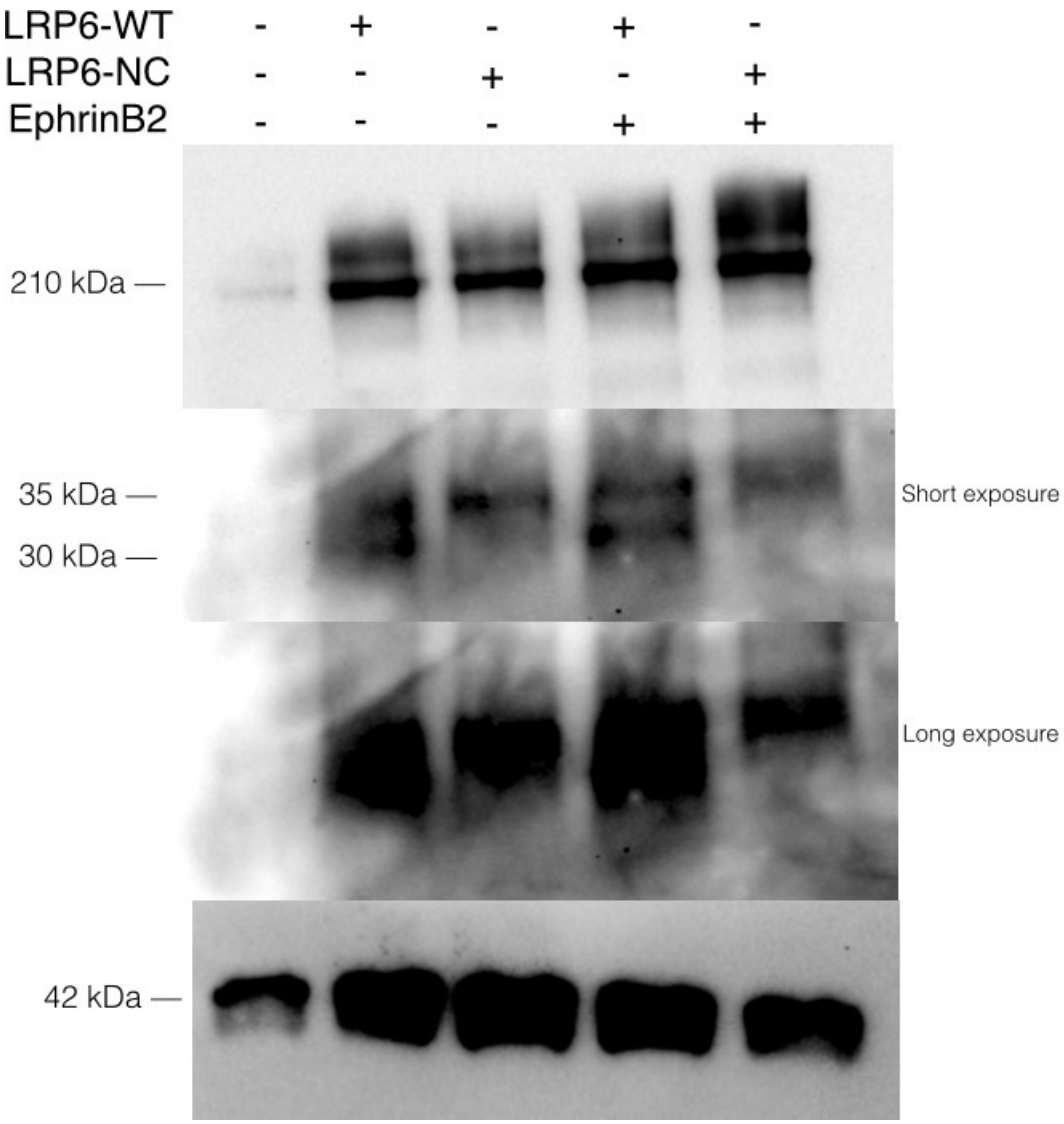

Figure 14. Protein expression and cleavage products of overexpressed LRP6-WT and LRP6-NC with overexpressed EphrinB2 in HEK293T cells. Overexpressing both forms of receptor increased the amount of full-length LRP6 at 210 kDa. Cells overexpressing LRP6-WT contained cleavage products of 35 and 30 kDa. Cells overexpressing LRP6-NC only contained the 35 kDa cleavage product. There appears to be an increase in the 30 kDa cleavage product in cells overexpressing LRP6-WT along with EphrinB2. This cleavage product corresponds to a proteolysis site near the predicted amino acid site 1367-1369. β-actin is shown at a molecular weight of 42 kDa and served as a loading control.

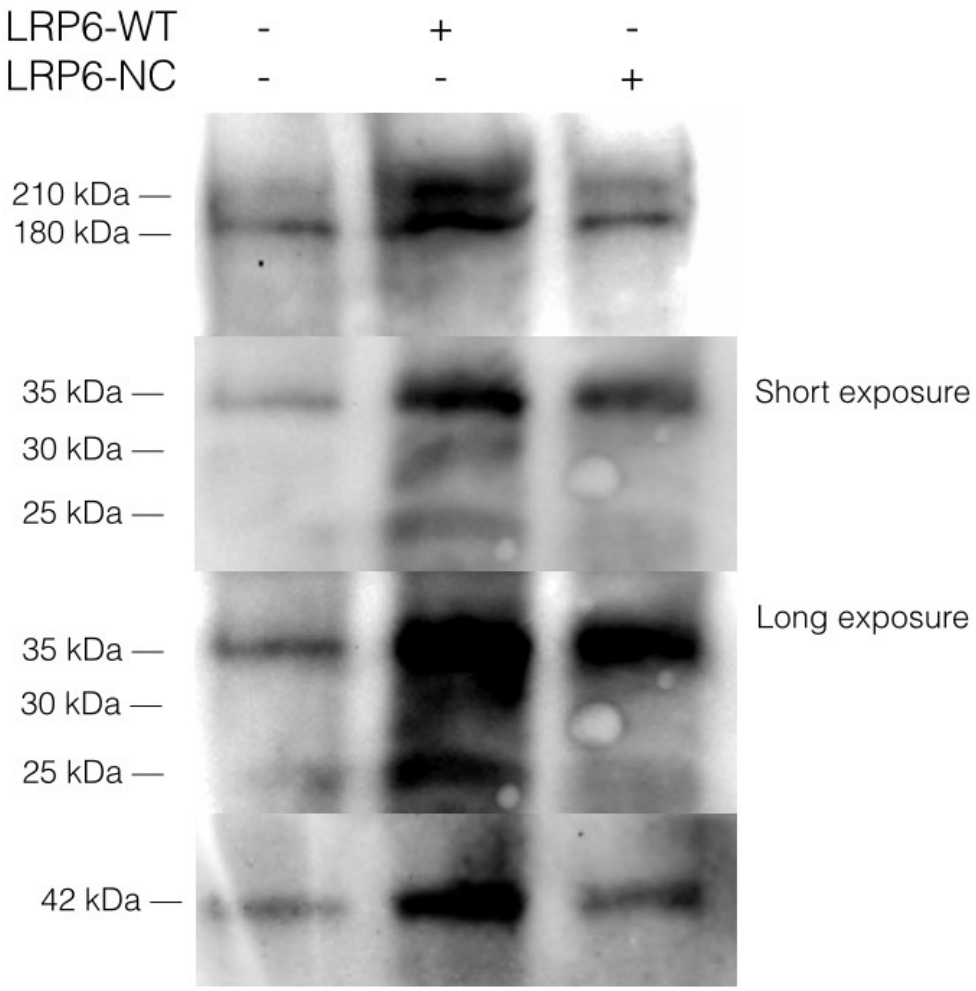

Figure 15. Protein expression and cleavage products of LRP6-WT and LRP6-NC in SW620 cells. SW620 cells appear to have a greater proportion of ER-form LRP6 compared to mature full-length protein. SW620 cells overexpressing LRP6-WT contained cleavage products of 35, 30, and 25 kDa. SW620 cells overexpressing LRP6-NC only contained the 35 kDa cleavage product. It is likely that the 30 kDa peptide seen in the wild-type lane is a product of cleavage occurring at amino acid site 1367-1369. β-actin is shown at a molecular weight of 42 kDa and served as a loading control.

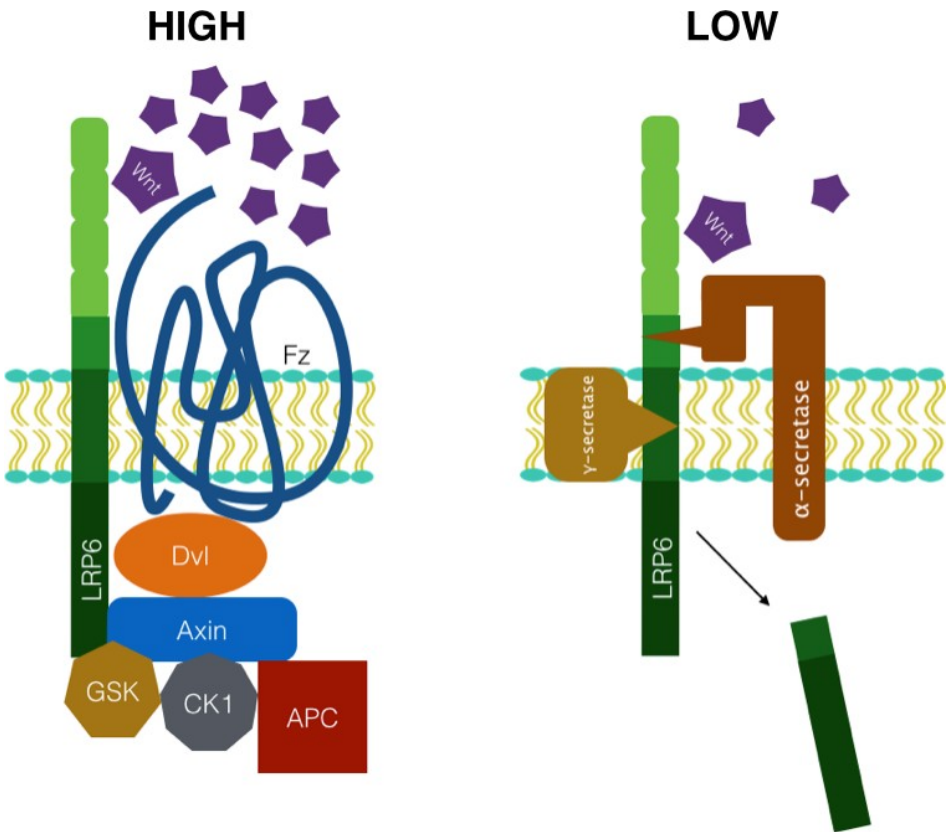

Figure 16. Hypothesized mechanism of high vs. low Wnt dosage responses. In cellular conditions when Wnt ligand concentrations are high the canonical Wnt pathway proceeds down the classical ligand-induced method of activation, involving scaffolding proteins Axin and APC, and kinases GSK3 and CK1. When Wnt concentrations are sufficiently low, another method of Wnt activation may occur where LRP6 is cleaved to release a biologically active form of the cytoplasmic tail.

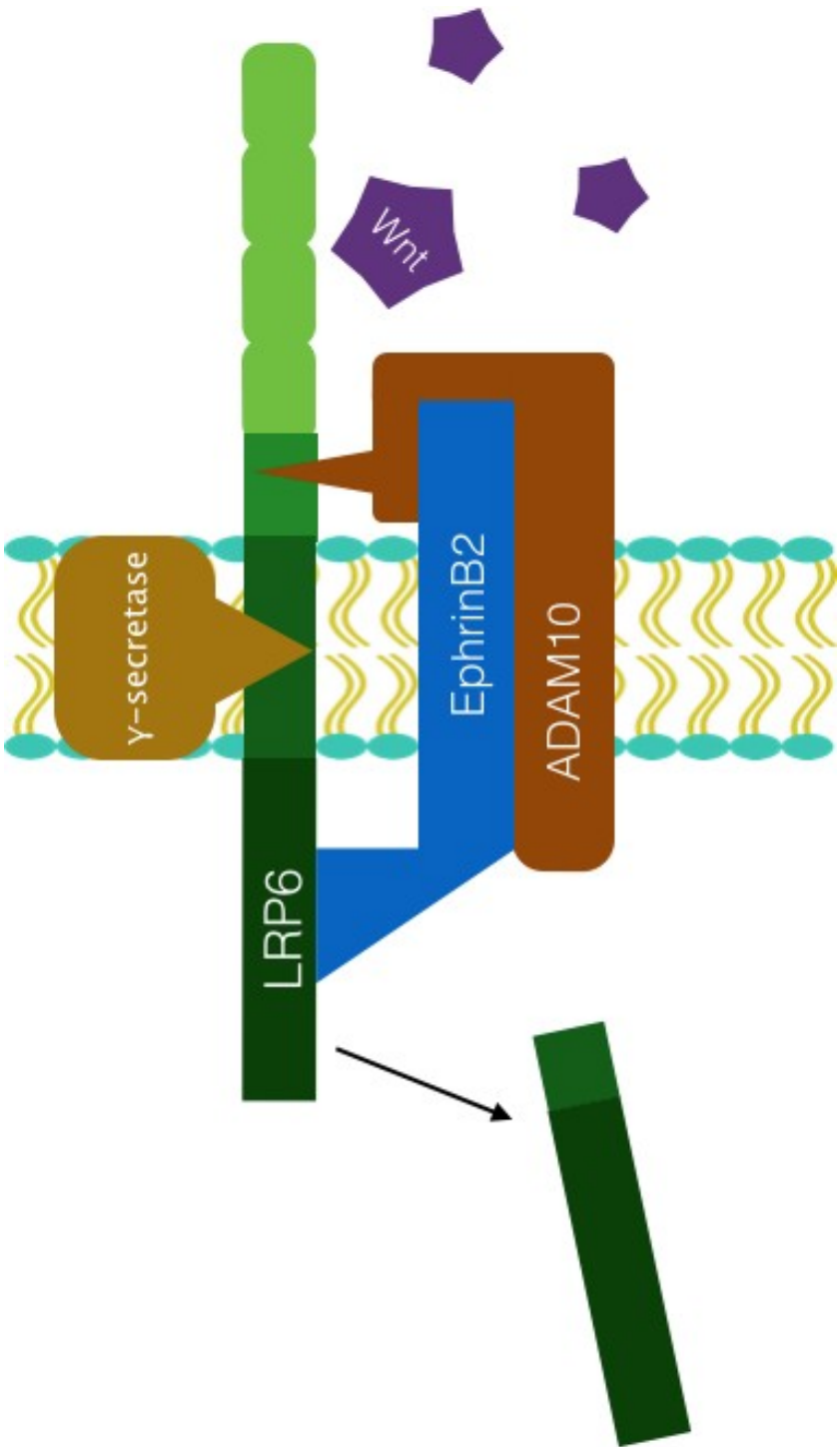

Figure 17. Hypothesized mechanism of LRP6 cleavage involving EphrinB2 and ADAM10. Through interactions involving its cytoplasmic tail, EphrinB2 may recruit ADAM10 to LRP6 to initiate RIP and release a biologically active form of the LRP6 ICD.