Defining Wnt activity and Nkd1 function in mammalian cell lines IEC-18 and HEK293

by

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ABSTRACT

DEFINING WNT ACTIVITY AND FUNCTION OF NKD1 IN MAMMALIAN CELL LINES IEC-18 AND HEK293

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University of Guelph, 2016

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Wnt signaling is an evolutionarily conserved pathway involved in many aspects of development and stem cell homeostasis. Activating mutations within the Wnt pathway are key in the initiation of colorectal cancers. Nkd1 is a negative feedback regulator of Wnt signaling with a unique method of antagonism that could potentially block the effect of the activating mutations. However, our understanding of the molecular function of Nkd1 has been derived mainly through zebrafish studies. To ultimately better understand the role of Nkd1 in human disease, I first employed the Intestinal Epithelial Cells line 18 (IEC-18) and Human Embryonic Kidney cells (HEK293) as mammalian models for the investigation of Nkd1 function. Using these two cell lines, I characterized the biochemical and genetic status of Wnt signaling in IEC-18 and HEK293 cells, followed by an analysis of Nkd1 function as it relates to previous work performed in zebrafish. My results indicate that while both cells respond biochemically and genetically to Wnt signaling, there are significant differences in the level of response at both levels that are not congruent with the current dogma. In addition, I found that while Nkd1 activity in these mammalian cell types shares some similarities with our previous findings in zebrafish, there exists cell-dependent differences in Nkd1 function.
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DECLARATION OF WORK PERFORMED

All of the work in this thesis was performed by Alexander Weiss, with the exception of the confocal imaging by Terry Van Raay. Summer students Esther Matus and Jacqueline Farr assisted with western blotting and RT-qPCR respectively under the supervision of Alexander Weiss.
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<tr>
<td>ABC</td>
<td>Active β-catenin</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous Polyposis Coli</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>BIO</td>
<td>(Z′Z,3′E)-6-Bromoindirubin-3′-oxime</td>
</tr>
<tr>
<td>CK1</td>
<td>Casein Kinase 1</td>
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<tr>
<td>CRC</td>
<td>Colorectal Cancer</td>
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<tr>
<td>CM</td>
<td>Conditioned Media</td>
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<td>Dvl/Dsh</td>
<td>Disheveled</td>
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<td>EMT</td>
<td>Epithelial to Mesenchymal Transition</td>
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<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<td>Glycogen Synthase Kinase 3</td>
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<td>IEC-18</td>
<td>Intestinal Epithelial Cell line 18</td>
</tr>
<tr>
<td>LEF</td>
<td>Lymphoid Enhancer-Binding Factor</td>
</tr>
<tr>
<td>LRP6</td>
<td>Lipoprotein Receptor Related Protein 6</td>
</tr>
<tr>
<td>NHR1/2</td>
<td>Naked Homology Region 1/2</td>
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<tr>
<td>Nkd1</td>
<td>Naked Cuticle Homologue 1</td>
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<tr>
<td>PBC</td>
<td>Phosphorylated β-catenin</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<td>Planar Cell Polarity</td>
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<td>Protein phosphatase holoenzyme</td>
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<td>PP2A subunit</td>
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<td>TCF</td>
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Chapter 1 - Introduction

1.1 Wnt Signaling, overview

Cellular signaling pathways are integral to life, coordinating cellular fate, proliferation, and organization during development as well as adaptive or regulatory responses in adult organisms. The Notch, Hedgehog, Fibroblast growth factor, Wnt, and other pathways are all examples of such critical signaling cascades required for the existence of multicellular organisms (ten Berge et al. 2008; Borday et al. 2012; Collu, Hidalgo-Sastre, and Brennan 2014). As these signaling systems coordinate complex events spatially and temporally, they have evolved to be tightly regulated on many levels. In addition to being internally regulated, many signaling pathways are networked together in a complex web of protein and gene interactions. Understanding the function of these pathways and how they are controlled is important not just for general knowledge, but also for treatment and diagnosis of cancers or developmental diseases.

Evolutionarily conserved across nearly all metazoans, the Wnt signaling pathway, like others major pathways such as Notch or Hedgehog, controls a myriad of processes in the adult and developing organism (Murat, Hopfen, and McGregor 2010; Nichols et al. 2006; Zhao, Reynolds, and Gaucher 2011). It is important to note that Wnt signaling is divided into two broad categories, canonical Wnt/β-catenin signaling and the non-canonical PCP or Ca^{2+} pathways, defined by their different intracellular signaling pathways (Yang 2012). The focus of this project is based on the canonical aspects of this pathway, hereby referred to simply as “Wnt Signaling”.

The first major discoveries of Wnt signaling were elucidated in fruit flies, revealing a gene that controlled the patterning and definition of segmental regions
during development. Loss of this gene function resulted in a wingless phenotype, and was named accordingly (Nüsslein-Volhard and Wieschaus 1980). Shortly thereafter, a study in mice examining the oncogenic activation of genes using a mammary tumor virus revealed a proto oncogene, in fact a homolog of Wingless, they dubbed Integration-1 (Nusse and Varmus 1982). The amalgamation of the names Wingless and Integration-1 lead to the name Wnt and the discovery of this evolutionarily conserved pathway was the beginning of decades of research, creating the field of Wnt signaling. Humans, as well as most mammals, contain 19 Wnt genes within their genome.

While Wnt signaling is a complex and versatile pathway, loss of function experiments in animal models have provided insight into what roles Wnt plays during development. Knocking out the various Wnt components in mice, including the receptor Frizzled, Wnt ligands, or other components, results in a wide range of defects including but not limited to complications with haematopoiesis, neurological development, embryogenesis, osteogenesis, and angiogenesis (Day et al. 2005; Ishikawa, Tamai, and Zorn 2001; Ranheim and Kwan 2005; Zhao et al. 2005). In Xenopus, Wnt is important in establishing dorsal-ventral identity during development, promoting limb regeneration, neural differentiation, and more (Heeg-Truesdell and LaBonne 2006; Medina and Steinbeisser 2000; Yokoyama et al. 2011). Further studies in Zebrafish have shown similar findings, with Wnt activity regulating development in the neural crest, heart, organelle development, and other important processes (Lewis et al. 2004; Sumoy, Kiefer, and Kimelman 1999; Ueno et al. 2007; Yin et al. 2011). In chicks these developmental trends continue, with the Wnt signaling pathway managing bone development, limb initiation, axis formation, and organ development (Day et al. 2005; Y.
Kawakami et al. 2001; Moura et al. 2014; Schneider et al. 2015). The high degree of conservation within the components and phenotypes of the Wnt signaling pathway allows for correlated research across a number of different animal models.

As with other vertebrate species, Wnt signaling plays a role during human development and in the adult organism, with evidence of its function found in various Wnt associated diseases. Normally acting to regulate bone growth by affecting osteogenic stem cells, mutations in the Wnt pathway are a large contributing factor to many bone diseases (Glass et al. 2005; Kramer et al. 2010). These mutations in Wnt components frequently result in undesired alterations of bone density, fragility, and formation (Chang et al. 2014; Gong et al. 2001; Jenkins et al. 2009). Recent research also reveals a role for Wnt signaling in developing and maintaining the blood brain barrier (Paolinelli et al. 2013), with mutations in this pathway contributing to the breakdown seen in Alzheimer’s disease (Liu et al. 2014). During early heart development, and later maintenance, Wnt signaling is one of the main active pathways guiding cardiac processes (Gessert and Kühl 2010; Pahnke et al. 2015). Perturbations to this pathway are highly correlated with heart complication and disease later in life (Askevold et al. 2012; Goliasch et al. 2012). With an important role in the developing eye, mutations in this pathway are responsible for a variety of familial retinal diseases (Lad, Cheshier, and Kalani 2009). Beyond development, research also reveals a relationship between WNT5B and type 2 diabetes through a metabolic pathway, as this particular ligand regulates adipocyte function and development (Grant et al. 2006; Mitchell et al. 2014). There are many more examples of the Wnt signaling pathway in human disease and development (as reviewed in Clevers 2006), but the small sample
reviewed highlights the diversity of tissues and biological processes this integral pathway plays a part in.

Diversity of the Wnt pathway is cell type dependent, the expression of Wnt genes is affected at several levels by the action of different Wnt ligands and receptors, as well as by cross regulation through other signaling pathways (Clevers 2006). Accordingly, outcome of activated or inhibited Wnt signaling can sometimes provide conflicting results. Differences in the cell context of Wnt gene expression can promote proliferation and maintain populations of stem cells (Aubert et al. 2002; Hoffmeyer et al. 2012; Katoh and Katoh 2007; Yoshikawa et al. 1997) or induce differentiation and halt proliferation instead (Davidson et al. 2012; Ling, Nurcombe, and Cool 2009; Yang et al. 2011). Despite these differences, one common theme within Wnt signaling is autoregulation. Several components of the pathway including the receptors or antagonists are differentially regulated upon active Wnt signaling, frequently in an inhibitory manner. Discussed in further detail later, much of this autoregulation occurs at the level of the receptor or the destruction complex. Common mutations within the Wnt pathway occur below the level of the destruction complex, and thus bypass these regulations.

1.2 Pathway Mechanics

The Wnt family of glycoprotein ligands share several conserved and defining characteristics: 22 spatially conserved cysteine residues, a 20 amino acid signal peptide, and a serine residue that is acylated in the complete 350-400 amino acid protein (Willert and Nusse 2012). This acylation, or addition of a lipid palmitoleic acid, is essential for the signaling properties of Wnt ligands and imparts the protein with hydrophobic properties (Takada et al. 2006). The Frizzled (Fz) family of receptor
proteins are defined by containing seven hydrophobic transmembrane domains with conserved intracellular loops, as well as an extracellular cysteine rich domain responsible for interacting with Wnt ligands or antagonists (Dann et al. 2001). The interaction of the Wnt ligand with Fz and coreceptor LDL related protein 6 (LRP6, Arrow in *Drosophila*) initiates the events leading to activated Wnt signaling (Zeng et al. 2008).

The previously mentioned lipid moiety of the Wnt ligand fits into a groove within the cysteine rich domain of Fz, and allows for secondary contacts between the ligand and receptor, also leading to recruitment of the LRP6 coreceptor (Dann et al. 2001). LRP6, a single-pass membrane protein, binds Wnt ligands through three conserved domains, bringing it in close contact with Fz (Cheng et al. 2011; Tamai et al. 2000). When the pathway is activated, the adaptor protein Disheveled (Dvl), in combination with LRP6, mediates the initial recruitment of the scaffold protein Axin1, and thus the destruction complex, to the membrane initiating the signaling cascade. The third intracellular loop and C-terminal of Fz interacts with Dvl through two of Dvl’s three well conserved domains, Dvl Egl-10 (DEP) and PSD95/Dlg1/Zo-1 (PDZ) (Tauriello et al. 2012; Wallingford and Habas 2005). Upon interacting with Fz at the receptor complex, Dvl can associate with Axin1 along its third conserved domain, Disheveled and Axin (DIX), and this may be responsible for the initial recruitment of Axin1 to the membrane signaling site. Axin1 also contains a DIX domain, and the polymerization of these domains between Axin1/Dvl along a large number of signaling complexes may promote receptor clustering, thus increasing signaling sensitivity through the formation of large signalsomes (Bilic et al. 2007; Schwarz-Romond et al. 2007). Two kinases, Glycogen Synthase Kinase 3 (GSK-3) and Casein Kinase 1 (CK1) are Axin1 binding partners,
recruited with the scaffolding protein to the receptor complex. GSK-3 primes the tail end of LRP6 by phosphorylating a critical PPPSP motif, preparing it for further phosphorylation by CK1 (MacDonald et al. 2008). The phosphorylated tail of LRP6 is able to strongly bind Axin1, thus sequestering the destruction complex at the plasma membrane. This leads to further LRP6 phosphorylation as the GSK-3 and CK1 bound to destruction complexes acts upon the intracellular portion of the receptor (MacDonald et al. 2008). The initial Dvl and LRP6 mediated recruitment of the destruction complex, combined with the feed forward system of LRP6 phosphorylation and receptor clustering inactivates the ability of the destruction complex to degrade β-catenin. This recruitment event is likely mediated by the dephosphorylation of Axin1 residues S497/S600 as Kim et al., found that Wnt signaling induced dephosphorylation of Axin1 resulted in an ‘inactive’ form of the protein (Kim et al. 2013).

β-catenin serves as the central signal transducer of Wnt signaling. This 90kd protein contains several conserved elements: 12 Armadillo repeats, as well as distinct N/C terminal domains (NTD/CTD) (Valenta, Hausmann, and Basler 2012). These 12 Armadillo repeats form a superhelical structure resulting in a positively charged groove. It is within this groove, specifically repeats 3-9, where β-catenin interacts with its main binding partners including Adenomatous Polyposis Coli, E-Cadherin, and TCF/LEF proteins (Graham et al. 2000; Huber and Weis 2001). These partners bind β-catenin competitively, vying for the same or overlapping regions along the groove. This competition, combined with conformational changes within β-catenin that result in altered binding partner preferences likely plays a complex role in Wnt signaling regulation (Gottardi and Gumbiner 2004; Solanas et al. 2004).
constantly being transcribed and translated within cells, but is normally degraded by the destruction complex in the absence of a Wnt ligand (Ikeda et al. 1998). The destruction complex is an assembly of several proteins; Axin1, APC, kinases GSK-3 and CKy-1, as well as the β-transducin repeat containing proteins (β-TrCP) ubiquitin E3 ligase. Axin1 serves as the central scaffold, bringing all the components together to act on β-catenin. The relatively low supply of Axin1 acts as the rate limiting step, regulating the levels of β-catenin degradation (and thus pathway inhibition), as proper function of the destruction complex relies on whole assembly of the component proteins (Lee et al. 2003). APC binds to Axin1 at 3 distinct sites, and is responsible for binding to β-catenin, holding it within the complex and allowing the kinases to act upon it sequentially. CK1 and GSK-3 work in tandem to phosphorylate β-catenin, CK1 first priming β-catenin on Ser45, followed by GSK-3 mediated phosphorylation of Thr41/Ser37/Ser33 (Liu et al. 2002). This phosphorylation targets β-catenin for ubiquitination by β-TrCP, leading to degradation of β-catenin by the proteasome (Kitagawa et al. 1999). Beyond its role as a transcription factor, β-catenin also has an important role as a cell-cell adhesion molecule. At the membrane, β-catenin binds to the intracellular portion of transmembrane E-cadherin as well as α-catenin, and this complex is capable of interacting with elements of the actin-cytoskeletal network (Baum and Georgiou 2011). This pool of membrane bound β-catenin is separate but not independent of the cytoplasmic signaling pool; disrupting the E-cadherin, β-catenin, α-catenin complex through various methods has been shown to increase Wnt signaling by increasing the amount of cytoplasmic β-catenin (Kim, Kim, and Jho 2013).
During activated Wnt signaling, the inactivated destruction complex allows β-catenin to accumulate in the cytoplasm, where it reaches a sufficient concentration and begins to translocate into the nucleus. β–catenin enters the nucleus and displaces the transcriptional repressor Groucho from transcription factors of the TCF/LEF family. These transcription factors provide the DNA binding specificity that target β-catenin to Wnt target genes, while β-catenin itself provides the transcriptional activation portion of the transcription complex (Novak and Dedhar 1999). Interestingly, the nuclear import of β-catenin functions independently of the classical importin pathway, instead interacting directly with the nuclear pore complex through the Armadillo repeats and hydrophobic CTD/NTD (Sharma et al. 2012). Upon entering the nucleus, it activates transcription of Wnt target genes as previously described. For a summary of the “On” and “Off” state of Wnt signaling, see Fig. 1.1 below.
Figure 1.1 The Off and On of Wnt signaling. A) Without a Wnt ligand, the destruction complex is uninhibited. APC binds β-catenin in the cytoplasm, permitting CK1 and GSK-3 mediated phosphorylation and targeting it for ubiquitination by E3 Ubiquitin ligase. This leads to the degradation of β-catenin leaving Groucho bound to TCF/LEF, preventing the activation of Wnt target genes. B) Activation of Wnt signaling begins with the binding of the ligand to Frizzled and LRP6. This leads to the localization of Disheveled to the receptor complex, recruiting Axin1. CK1 and GSK-3, brought by Axin1, phosphorylate five motifs on the intracellular domain of LRP6. This allows for further recruitment of Axin1 in a positive feedback manner. This prevents the destruction complex from phosphorylating β-catenin and targeting it for destruction. β-catenin can then accumulate in the cytoplasm and translocate to the nucleus, associating with TCF/LEF and activating transcription of Wnt target genes.
1.3 Wnt Signaling in Cancer

Of particular interest to my project is the role of Wnt signaling in colorectal cancer, originating in the colon of the gastrointestinal tract. This tract is made up of four layers. From exterior to interior these are the adventitia/serosa (structural, connective tissues), muscularis externa (smooth muscle), submucosa (vasculature, lymphatic elements, nerves), and the mucosa (secretion, epithelium, vasculature and lymphatic elements) (Reed and Wickham 2009). As digested matter is constantly being moved through the gastrointestinal tract, the epithelium layer of the mucosa must be carefully maintained and replenished. New intestinal epithelial cells are generated in the intestinal crypt. At the base of these crypts, specialized stem cells are kept in a constant proliferative state by Wnt signaling (Barker et al. 2009). These cells undergo asymmetric division, creating new cells that will begin to migrate up and out of the crypt, as well as maintaining the original stem cell niche (Pinto et al. 2003). These new migrating cells transitioning out of the intestinal crypt form what is known as the transit amplifying region, with each new cell “pushing” the older cells out of the crypt. In the small intestine, these cells migrate up the villi where Wnt signaling is antagonized by bone morphogenic proteins (BMPs). Combined with increased distance from the intestinal crypt signaling environment and the Wnt morphogen, the new signaling environment promotes cellular differentiation into the epithelium proper (He et al. 2004). At the apex of the villi, these cells are sloughed off and passed into the intestinal tract, and the cycle continues. In the large intestine or colon, the function of crypts are largely the same, though cells migrate out into a surface epithelium layer as opposed to villi. This process is shown in Figure 1.2. Abolishing Wnt signaling by transgenic expression
of Dickkopf-1 in mice results in a loss of these crypts (Kuhnert et al. 2004), signifying the critical importance of Wnt signaling in maintaining this structure.

Figure 1.2 Replenishment of intestinal epithelium in the small intestine (A) and colon (B). Stem cells located at the base of the crypts are maintained in a proliferative state by Wnt signaling. The proliferative progenitors generated by the asymmetric cell division migrate out of the crypt, leaving the original stem cell niche intact. Upon being removed the signaling environment of the intestinal crypt and subject to Wnt antagonism, cells begin to differentiate into proper epithelium. Figures adapted from Reya and Clevers, 2005.
As a pathway that promotes cellular division within the intestinal crypt, loss of Wnt pathway regulation is a major step in oncogenic development, with the majority of colorectal cancers having a Wnt signaling mutation (Muzny et al. 2012). The most common mutation found within colorectal cancers occurs in the APC gene (Muzny et al. 2012). As mentioned previously, APC forms an integral part of the destruction complex, mediating the interaction of the other components with β-catenin. Complete loss of APC function results in β-catenin no longer being degraded, mimicking activation of the pathway in the absence of a ligand. Less frequent, but equally oncogenic, is mutation of the β-catenin gene itself, disrupting the motif that would normally be phosphorylated by GSK-3 and CK1 of the destruction complex (Morin et al. 1997). Without this phosphorylation, β-catenin is insensitive to the ubiquitin ligase associated with the destruction complex and continues to accumulate (Barker et al. 2009). Within the context of the intestinal crypt, constitutive activation of the Wnt pathway leads to increased cellular proliferation of colonic crypt stem cells and elevated susceptibility to new mutations. Familial adenomatous polyposis (FAP) is a hereditary condition in which an individual inherits a mutant APC allele from one parent, putting them at high risk of developing colorectal cancer later in life (Groden et al. 1991). FAP positive individuals who have taken a hit to their second wild type allele develop polyps along their colonic tracts, though the severity and rate of polyp formation can vary depending on the specific mutation inherited from the parent (Christie et al. 2013). As this common mutation occurs below the level of the receptor and targets the critically important destruction complex, many normal regulators of Wnt signaling are unable to manage this ectopic activation. Further mutations leading to activation of oncogenes or affecting
tumour suppressor function, such as KRAS activation or P53 mutation, promote adenoma growth and maturation **Figure 1.3** (Davies, Miller, and Coleman 2005).

![Figure 1.3 Key mutational events in the formation of colorectal cancers. Figure adapted from Davies et al., 2005.](image)

Beyond its major role in colorectal cancers, mutations within the Wnt pathway are common in many types of oncogenic disease. Loss of Wnt antagonism by mutation in Wnt regulators (covered below) have been found in a number of kidney cancers (Kawakami et al. 2009). Overexpression of receptors or mutations within β-catenin are common within ovarian cancers and serve as markers of poor prognosis (Dai et al. 2013). Stabilization of β-catenin through pathway crosstalk is also common in pancreatic cancers (Wang et al. 2009). There are many excellent reviews covering the role of Wnt signaling in a variety of cancers (Anastas and Moon 2012; Arend et al. 2013;
Kim, Kim, and Jho 2013; MacDonald, Tamai, and He 2009; McDonald and Silver 2011; Reya and Clevers 2005).

1.4 **Wnt regulation and Nkd1**

Under normal conditions, the Wnt pathway is heavily regulated at the level of the receptor. Two groups of proteins, Wnt inhibitory factor (WIFs) and Secreted Frizzled-related proteins (sFRPs) antagonize Wnt signaling by binding the ligand and competing with the cell surface receptors (Kawano and Kypta 2003). These inhibitors are greatly involved during early development. For example, in both mice and chickens, WIFs and sFRPs restrict the activation of the Wnt, regulating such processes as cell differentiation, migration, and limb initiation (Enomoto-Iwamoto et al. 2002; Jin, Burrus, and Erickson 2002; Witte et al. 2009). Again acting at the level of the receptor, Dickkopf-1 is an antagonist that binds to LRP6 and prevents its interaction with Fz, thus inhibiting inactivation of the destruction complex and halting β-catenin accumulation. Dickkopf-1 can also be a transcriptional target of Wnt signaling acting as a negative feedback regulator, with decreased Dickkopf-1 levels found in colorectal cancers suggesting a role as a tumor suppressor (González-Sancho et al. 2005). In addition, cell cycle regulator p53 has also been found to regulate Dickkopf-1 transcription, allowing for cell-cycle dependent control of the Wnt pathway (Semēnov et al. 2001). Wise/Sost proteins are another class of regulators with a function similar to that of Dickkopf-1, and loss of Wise/Sost function leads to complications with tooth development and the inability to remove certain vestigial tissues during development (Ahn et al. 2010).

The Protein Phosphatase 2A (PP2A) holoenzyme is a regulator of the Wnt pathway, exerting both positive and negative effects. This holoenzyme is responsible for
reversing the action of kinases from major signaling pathways, and its specificity is likely regulated through subunit composition. Subunits PR61α/β/γ interact with Axin, while subunits PR61α/δ interact with APC, with both of these interactions resulting in a decrease of cytoplasmic pool of β–catenin (Eichhorn, Creyghton, and Bernards 2009). Interestingly, the function of the Wnt negative feedback regulator Nkd1 requires subunit PR72 for its antagonistic function, though the mechanism of which remains undefined (Creyghton et al. 2005). On the opposite spectrum PR130, an alternative transcript product of PR72, inhibits Nkd1 function (Creyghton et al. 2006). Of interest is the fact that despite these interactions between Wnt components and the PP2A holoenzyme, there is no evidence of PP2A dephosphorylating these Wnt pathway proteins (Eichhorn, Creyghton, and Bernards 2009). This suggests that the PP2A complex plays multiple but indirect roles throughout Wnt signaling, interacting with components of the pathway but not acting upon them, perhaps being targeting to other proteins instead. These studies reveal that control of the Wnt signaling pathway is tightly regulated at both the receptor and the destruction complex. As mutations frequently affect the pathway below the level of the destruction complex and thus avoid the activity of various Wnt antagonists, negative feedback regulators such as Nkd1 with its unique method of antagonism could have an important role in suppressing ectopic Wnt signals.

As Wnt target genes, both Axin2 and Nkd1 function as negative feedback regulators (Jho et al. 2002; W. Zeng et al. 2000). Axin2 (also known as Conductin and Axil) is structurally and functionally similar to Axin1, able to replace Axin1 function within the destruction complex. This was revealed by an experiment in mice where Axin1 was replaced with Axin2, indicating that the two are interchangeable under most
circumstances (Chia and Costantini 2005). While playing a similar role within the destruction complex, Axin2 has been shown to be less sensitive to recruitment by Dvl2 during pathway activation (Bernkopf, Hadjihannas, and Behrens 2015). While Axin1 and the destruction complex would be inactivated by the receptor complex during ligand binding, Axin2 destruction complexes would be able to continue to antagonize the pathway. As a Wnt antagonist and presumed tumour suppressor, therapeutics targeting the stability of Axin1 to increase their levels have been pursued (Sheikh et al. 2014). Interestingly, Wu et al., found that the elevated levels of Axin2 in various colorectal cancer lines were actually responsible for promoting an epithelial to mesenchymal transition (EMT) through interaction with the Snail1 pathway, and failed to antagonize overactive Wnt signaling (Wu et al. 2012). A similar finding was reported earlier where breast cancer cells underwent an EMT transition promoted by the Axin2-Snail1 dependent interaction (Yook et al. 2006). While Axin2 is a capable Wnt antagonist, its other functions within the cell suggest a more complex role in addition to the regulation of Wnt signaling.

Nkd1 is an enigmatic and evolutionarily conserved Wnt antagonist Figure 1.4, first discovered in Drosophila when mutation of this gene disrupted Wnt mediated patterning and yielded an embryonic lethal naked cuticle phenotype (Zeng et al. 2000). After this initial discovery, Nkd1 orthologs were found in many different species, including humans. Nkd1 contains four conserved domains within vertebrates, an N-terminal myristoylation sequence, a Naked Homology Region 1 (NHR1), a second NHR2 domain, and lastly a C-terminal histidine rich tail. Investigation by our lab utilizing zebrafish as a vertebrate model has revealed several key mechanics of Nkd1 function.
Figure 1.4 Conservation of Nkd1 amino acid sequence identity across species. Multalin.toulouse.inra.fr/multalin/ was used to generate an amino acid alignment of confirmed Nkd1 sequences across a number of different species. In descending order, the species shown are Zebrafish, Mouse, Human, Chicken, and Xenopus.
The myristoylation of Nkd1 at the N-terminus is critical for membrane localization and function, as a mutation interrupting this lipid modification prevents Wnt antagonism by Nkd1 (Van Raay et al. 2011). While *Drosophila* Nkd does not contain a myristoylation sequence at its N-terminal, it instead has an alternative amino acid sequence that still permits membrane localization (Chan et al. 2007). To antagonize the pathway, Nkd1 prevents the nuclear accumulation of β-catenin rather than promoting its degradation through the destruction complex, and has been shown through immunoprecipitation experiments to interact with β-catenin (Larraguibel et al. 2015; Van Raay et al. 2011). In addition, Nkd1 requires the presence of the Wnt ligand to function, as Nkd1 cannot antagonize constitutively active LRP6, but is fully capable of rescuing over-activation by the Wnt ligand. Importantly, when combining constitutively active LRP6 and the Wnt ligand, Nkd1 is able to rescue LRP6 overactivation, emphasizing the significance of the ligand interaction. The EF-hand region of the NHR1 domain has been shown to interact with Dvl2, and research indicates that Nkd1 may antagonize Wnt signaling via degradation of Dvl2, though two independent studies performed in zebrafish have had conflicting results (Lum, Robertson, and Van Raay 2011; I. Schneider et al. 2010b). While Nkd1 colocalizes with Dvl2 in zebrafish at the plasma membrane (Van Raay et al. 2011), experiments in *Drosophila* have demonstrated that the interaction between Nkd and Dsh (Nkd1/Dvl orthologs, respectively) is unnecessary for the antagonism of Wnt signaling (Waldrop et al. 2006). In addition, Dvl2 competes with β-catenin for Nkd1 binding along the same domain that is phosphorylated during active Wnt signaling (Van Raay et al. 2011). During activation of the pathway, Nkd1 undergoes a redistribution within the cell, resulting in less membrane bound Nkd1 and a reduction in Nkd1 punctae
size found within the cytoplasm (Larraguibel et al. 2015). Taking into consideration these aspects of Nkd1 function, the Van Raay lab has proposed a model for Nkd1 regulation where active Wnt signaling produces Nkd1 protein, which then requires membrane localization via its myristoylation moiety, assisted by its interaction with Dvl2. At the membrane, Nkd1 is potentially being ‘activated’, but only in the presence of the Wnt ligand. Upon activation, Nkd1 is released from the membrane to interact with β-catenin in the cytoplasm to prevent its nuclear accumulation, and thus inhibits Wnt transcriptional activation. A summary of this model in a disease context is provided in Figure 1.5. Nkd1 has also been shown to interact with Axin1/2 through its histidine tail, and this interaction is required for efficient Nkd1 antagonism of Wnt signaling (Miller et al. 2009). While the basis of this interaction has not been investigated, Nkd1 is not supporting the destruction complex in degrading β-catenin, since Nkd1 expression and function does not alter the levels of this central signal transducer. (Van Raay et al. 2011). However, the interaction between Nkd1 and Axin1/2 may help recruit Nkd1 to the signalsomes for activation. Earlier, components of the PP2A holoenzyme were discussed in regards to their role in Nkd1 antagonism. Interestingly, no gross changes in the phosphorylation of Nkd1 were observed upon experimentation with the components of the holoenzyme (Creyghton et al. 2006; Eichhorn, Creyghton, and Bernards 2009). This suggests that Nkd1 may be recruiting the PP2A holoenzyme to act upon β-catenin or Dvl2, though further investigation is required.

Knockouts of Nkd1/Nkd2 in mice have very little effect on physical development aside from minor skeletal defects in the skull and spermatogenesis (Zhang et al. 2007). This is in stark contrast to the lethal phenotype seen in Drosophila when Nkd1 is
knocked out (Zeng et al. 2000). However, it is important to note that while embryonic lethal, rescue of Nkd past embryonic lethality in *Nkd Drosophila* mutants results in normal growth and development, suggesting Nkd is not required to antagonize all Wnt signaling events at larval stages and beyond (Zeng et al. 2000). Overexpression of Nkd in *Drosophila* results in a weak Wnt over-activation phenotype, rather than complete antagonism (Zeng et al. 2000). This suggests that in vertebrate systems, Nkd1 may be functioning as a threshold regulator, keeping normal Wnt ligand mediated signaling within appropriate boundaries. This is supported by our work in zebrafish, where overexpression of Nkd1 can rescue Wnt8 mediated over-activation, but does not result in a Wnt loss of function phenotype (Van Raay et al. 2011).
Figure 1.5 Proposed model for Nkd1 activation and antagonism in a disease state. Note the destruction complex has been simplified for this image. A) Cells containing mutant APC (mt APC) are no longer able to degrade β-catenin, allowing it to accumulate in the cytoplasm and translocate into the nucleus where it activates Wnt target genes such as Nkd1. In the absence of the Wnt ligand, Nkd1 associates with Dvl but is unable to antagonize Wnt signaling. B) Upon stimulation of the pathway with a Wnt ligand, Nkd1 is recruited to the membrane receptor complex through its interaction with Dvl2 and the myristoylation sequence. At the receptor complex, Nkd1 is freed from its interaction with Dvl and may be activated through a currently unknown mechanism. C) Upon ligand mediated activation, Nkd1 is able to physically interact with β-catenin and prevent its nuclear accumulation, halting transcription of Wnt target genes.
1.5 Conclusion

Since its discovery, the Wnt signaling pathway has been revealed to play an important role in many processes during development and in tissue maintenance in mature organisms. These findings have been confirmed and characterized in a number of different animal models, signifying the depth of evolutionary conservation across species. To maintain fine control of this pathway, regulation frequently occurs at the level of the receptor and the destruction complex, with mutations affecting the degradation of β-catenin playing a critical role in oncogenesis. Negative feedback regulators of the Wnt pathway such as Nkd1 are not well characterized, yet play an important part in the control of this pathway.

Very little is known about how Nkd1 functions within mammalian cells. As previously discussed, Nkd1/2 knockout in mice yielded no significant phenotype. What is known about Nkd1 function in mammalian cells, such as the requirement for Axin2 or the PP2A holoenzyme, or its interaction with Dvl, are not well understood. Our previous studies have shown that in zebrafish, Nkd1 functions as a ligand dependent negative feedback regulator, working independently of β-catenin degradation and instead affecting its accumulation in the nucleus (Larraguibel et al. 2015; Van Raay et al. 2011). Since oncogenic activation of Wnt signaling disrupts β-catenin degradation, characterizing how Nkd1 uniquely regulates this pathway independently of the destruction complex could provide insight into oncogenesis and offer a potential therapeutic target.

I hypothesize that Nkd1 retains its function as a negative feedback regulator in mammalian cells, requiring localization to the membrane, dependence on a Wnt ligand for activation, and working independently of β-catenin degradation. To test this, I will
employ two mammalian cell lines; human embryonic kidney cells line 293 (HEK293) and intestinal epithelial cells line 18 (IEC-18 cells). HEK293 cells are a robust line widely used for biochemical studies, and most investigations into molecular mechanisms of Wnt signaling utilize this line, so we will be able to build upon the knowledge of others for my investigation. These HEK293 cells will be modified to express Nkd1-FLAG under the control of a tetracycline inducible promoter to better evaluate how Nkd1 is affecting the Wnt pathway. IEC-18 cells are an immortalized and non-oncogenic cell line isolated from the intestinal crypts of rats. IEC-18 cells could prove to be a valuable mammalian cell model for the study of Nkd1 in an intestinal context, though there is little research on Wnt signaling in this line. I will investigate how and if the Wnt pathway is functioning within these cells.
Chapter 2 – Methods

2.1 Cell Culture

2.1a General

All cell lines were cultured in HyClone Dulbecco’s Modified Eagle’s Medium (High Glucose), supplemented with 10% heat inactivated FBS, (100 U/ml penicillin, 100ug/mL Streptomycin), 2mM L-Glutamine at 37°C and 5% CO₂. For conditioned media, mouse L-cells (control) or Wnt3a expressing mouse L-cells (Wnt3a media) were grown to 80% confluence before the media was replenished and allowed to condition for 48 hours before collection. This conditioning was repeated twice more, and each collection of media was syringe filtered through a 0.22 um filter pad. Conditioned media was stored at 4°C for no more than three months. For experimental treatments, unconditioned media was replaced with conditioned media for the stated duration. For IEC-18 treatments, XAV939 and BIO (function described later) were added to control media at a final concentration of 1um and 3um respectively. If cells were to undergo tetracycline induction, it was added to the media at a final concentration of 1 ug/ml at least 24 hours prior to treatment, as well as to the conditioned media during treatment. IEC-18 cells were generously provided by Dr. Emma Allen-Vercoe (University of Guelph).

2.1b Transfections and Constructs

Transfections for transient or stable expression were done using PolyPlus JetPrime™ (Polyplus, 114-01) transfection reagent according to the manufacturer’s protocol. Flp-In™ T-Rex™ vectors and HEK293 cells carrying the Flp-In T-Rex cassette
were generously provided by Dr. Stephane Angers (University of Toronto). These HEK293 cells were used to generate tetracycline inducible HEK293 cells expressing human Nkd1-FLAG (hNkd1-FLAG). This was done by restriction enzyme digest utilizing HindIII and NotI on both the hNkd1-FLAG pcDNA3 (generated by R. Kondra, 2014), and the pcDNA5/FRT/TO FLAG-Gli3 vector (provided). Digestion of the hNkd1-FLAG pcDNA3 vector was performed to isolate the full length sequence for human Nkd1, which is modified with a triple flag tag on the carboxy terminal end. The pcDNA5/FRT/TO FLAG-Gli3 vector contains the Flp Recombinase Target site (FRT), the CMV/TetO2 promoter region, and FLAG-tagged Gli3. Digestion of this vector was performed to remove FLAG-Gli3 to test for successful digestion and to isolate the pcDNA5/FRT/TO backbone. The hNkd1-FLAG insert and pcDNA5/FRT/TO backbone were then gel purified before ligation at 0:1, 1:1, 3:1 insert:backbone ratios. HindIII and NotI digest allowed for compatible ends and integration of hNkd1-FLAG into the pcDNA5/FRT/TO backbone. One Shot® TOP10 chemically competent E. coli (Thermo Fisher, C404010) were then transformed with the ligated pcDNA5/FRT/TO hNkd1-FLAG product, and selected colonies isolated for a patch plate. Vectors were isolated from 6 colonies by midiprep and sent off for sequencing to confirm in-frame integration.

pcDNA5/FRT/TO hNkd1-FLAG and pOG44 (containing the Flp recombinase sequence) were then used to transform HEK293 cells carrying only one pFRT/LacZeo target site and multiple pcDNA6/TR (Tet repressor) elements. Expression of Flp recombinase mediates integration of the gene of interest, in this case hNkd1-FLAG and the CMV/TetO2 promoter, into the pFRT/LacZeo target site by catalyzing a homologous recombination event between FRT sites in the host cells and pcDNA5/FRT/TO vector.
The end result is HEK293 cells carrying hNkd1-FLAG under control of a CMV/TetO2 promoter kept inactive by constitutive expression of the Tet repressor. Addition of tetracycline removes the repressor and allows the CMV promoter to transcribe hNkd1-FLAG. Colonies then underwent selection with Blasticidin (10ug/ml) and Hygromycin (200 ug/ml) for approximately 9 days. Individual colonies were isolated from a 10cm plate with sterile trypsin-soaked filter paper by gently rubbing the cells free from the base before transplanting to a 6 well plate. Seven clones were tested for “leaky” expression of Nkd1-FLAG in the absence of tetracycline, with two showing variable expression of Nkd1-FLAG in the absence of induction. Out of the remaining five, one was chosen at random for further experiments.

2.2 Western Blotting

2.2a Cell Lysis

Approximately 3x10^6 cells were washed twice with 5 ml of Dulbecco’s Phosphate Buffered saline (dPBS) while on ice, collected in 1ml dPBS with a disposable cell scraper, and pelleted at 5,000 revolutions per minute for 5 minutes at 4°C before decanting the supernatant and freezing at -20°C. When needed, cells were resuspended in 1X Laemmli buffer without β-mercaptoethanol (Tris-HCl 0.05M, 10% Glycerol, 2% SDS, pH 6.8), supplemented with 100X protease/phosphatase inhibitor cocktail (Cell Signaling, #5872, proprietary mix of Aprotinin, Bestatin, E64, Leupeptin, sodium fluoride, sodium pyrophosphate, β -glycerophosphate, sodium orthovanadate) to 1x, with a total lysis volume dependent on the pellet size (60ul-150ul). Cell
suspensions were boiled at 95°C for 5 minutes before being sonicated (10 x 1 second pulses) and placed back on ice. Protein concentrations were determined by utilizing the Pierce™ BCA Protein Assay Kit (Thermo Fisher, 23225) as per manufacturer's instructions. Protein concentrations were adjusted to 1.5 ug/ul using 5x and 1x Laemmli buffer with β-mercaptoethanol (Tris-HCL 0.05M, 10% Glycerol, 2% SDS, 5% BME) and bromophenol blue and stored at -20°C until needed.

2.2b Blotting

15ug of protein per sample (as determined by BCA assay) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), bis-acrylamide (Bio-rad, #1610156) concentration at 7-8 % depending on desired separation of bands (running buffer, 0.25M Tris-Base, 1.92M Glycine, 0.1% SDS) for 50 minutes at constant 200V (Electrophoretic Wet Transfer cell, Bio-Rad Laboratories). Gels were washed in transfer buffer (0.25M Tris-Base, 1.92M Glycine) for 10 minutes before continuing. Proteins were transferred to a methanol permeabilized polyvinylidene fluoride membrane by wet transfer over 60 minutes at constant 400mA, with the transfer buffer cooled by ice packs. Following transfer, membranes were immersed in blocking solution (5% non-fat dry milk powder in Tris Buffered Saline (20mM Tris Base, 150mM NaCl, pH 7.4) 0.1% Tween solution (TBST)) at room temperature for 60 minutes. Membranes were incubated in primary antibody solutions (antibody dilution dependent on supplier’s guidelines, antibody in blocking solution supplemented with 0.02% sodium azide) overnight at 4°C with agitation. The following day, membranes were washed for 5 minutes in TBST before incubation in secondary HRP antibody (1:10000 dilution in
blocking solution) for one hour at RT. Membranes were washed four times in 15 minute intervals with TBST before applying Pierce™ ECL (Thermo Fisher, 32106) for visualization with photosensitive film. Quantification of band intensities was performed using ImageJ. Active β-catenin levels were normalized to actin, with the control being arbitrarily set = 1.

2.2c Immunoprecipitation

Cells were harvested as per the cell lysis protocol listed above, though were not boiled. A 50ul dPBS Protein G (GE Life Sciences, 17-0618-01) or M2 Flag Affinity gel (Sigma-Aldrich, A2220) slurry was added to the equivalent of approximately 10^6 cells, and processed according to the manufacturer’s protocol. Several different lysis and wash buffers were used as listed in the manufacturer’s protocol including TRITON X-100 (50mM Tris HCL, pH 7.4, 150mM NaCl, 1mM EDTA, 1% Triton X-100) and NP-40 (as Triton X-100 buffer, but 1% NP-40) based buffers.

2.2d Antibodies

Primary antibodies included monoclonal mouse anti-β–catenin 1:1000 (BD Transduction Laboratories, 610154), polyclonal rabbit anti-phospho-β–catenin 1:1000 (Thermo Fisher PA5-17915), monoclonal anti-active-β–catenin 1:2000 (Millipore, 05-665), monoclonal mouse anti-β-actin 1:5000 (Sigma, A5441), polyclonal rabbit anti-phospho-LRP6 1:1000 (Millipore, 07-2187), monoclonal mouse anti-total-LRP5/6 1:1000 (Millipore, 489475), monoclonal rabbit anti-Nkd1 1:1000 (Cell Signaling Technology, 2201), polyclonal rabbit anti-FLAG 1:1000 (Thermo Scientific, PA1-984B). Secondary antibodies included Alexa Fluor 594 goat anti-mouse IgG (Life Technologies, A11005),
Alexa Fluor 488 donkey anti-rabbit IgG (Life Technologies, A21206), Peroxidase conjugated donkey anti-mouse 1:10000 (Jackson Labs, 715-035-150), Peroxidase conjugated donkey anti-rabbit 1:10000 (Jackson Labs, 715-035-152).

2.3 QPCR

2.3a RNA Extraction

Cells were washed twice with 5 ml of dPBS while on ice, and collected in 1 ml dPBS with a disposable cell scraper. If a portion of the experiment was needed for protein analysis, 200 μl of cell PBS solution was taken and treated as per Western Blotting. Otherwise, cells were pelleted at 5,000 RPM for 5 minutes at 4°C before being resuspended in 1 ml TRIzol® Reagent (Thermo Fisher, 15596026) and flash frozen in liquid nitrogen until needed. RNA was then extracted and isolated following manufacturer’s protocol for adherent cells, and quantified on a DV-8100 Nanodrop.

2.3b cDNA Synthesis

cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814). A total of 500 ng of total RNA was added to a master mix consisting of 1X Reverse Transcription Buffer, 1X dNTP mix, 1X random primers, 1X of MultiScribe Reverse Transcriptase and water up to 20 μL. Contents were gently mixed and spun down. Transcription took place in a thermocycler with the following protocol: 25°C for 10 mins, 37°C for 120 mins, 85°C for 5 mins and a final hold at 4°C. Samples were then diluted with 80 μL of ultrapure water and stored at -20°C.
2.3c RT-qPCR

Target genes used for RT-qPCR analysis were Axin2, Nkd1, CyclinD1 as targets of Wnt signaling, and GAPDH as a reference gene. Primer sequences were generated using coding sequence data taken from the NCBI database and algorithmic selection by PrimerBlast, or from previous publications. These primers efficiency were tested by serial dilution of cDNA, with a primer efficiency >90% deemed acceptable. Individual reactions consisted of 1X PerfeCTa SYBR Green Fast Mix with ROX (Quanta Biosciences, 95073-05K), 10um of both forward and reverse primer, 5ul of cDNA template, with water to adjust the total reaction volume to 15 ul. Each treatment was loaded in triplicate onto a 96 well plate and covered with clear adhesive film before RT-qPCR analysis by a CFX-96 real-time PCR machine (Bio-Rad). The protocol used is as follows: 40 Cycles of 95°C for 15s, 57°C for for human primers, 58°C for rat at 1 minute, followed by a melt curve analysis at 95°C held for 2 minutes. Data was then analyzed through CFX Manager™ Software and Microsoft Excel 2013. Non-template reactions were used as controls.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’-3’)</th>
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<tr>
<td>Rat GAPDH</td>
<td>Forward: ATGCTGGTGCTGAGTATGTC</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGTTGTCATATTTTCTCGTGG</td>
</tr>
<tr>
<td>Rat Axin2</td>
<td>Forward: CAGGACCCACATCCTTCT</td>
</tr>
<tr>
<td></td>
<td>Reverse: ACGCGGAGGTGCACGC GG</td>
</tr>
<tr>
<td>Human GAPDH</td>
<td>Forward: GTCAAGGCTGAGAACGGGAA</td>
</tr>
<tr>
<td></td>
<td>Reverse: TCGCCCCACTTGATTTTGGA</td>
</tr>
<tr>
<td>Human Nkd1</td>
<td>Forward: ATCGAGGAGTGGATCGGGAG</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGCGTGCTCTCAACACGTC</td>
</tr>
<tr>
<td>Human Axin2</td>
<td>Forward: TGGCTATCTCCCCACCTTGA</td>
</tr>
<tr>
<td></td>
<td>Reverse: CAGTTTCGTGGACCTCACA</td>
</tr>
</tbody>
</table>
2.4 Growth Curve Analysis

IEC-18 cells were split from a single 10cm plate into a 96 well plate at a seeding density of 5000 cells per well, determined by a dilution series, with each treatment performed in triplicate. Resazurin (Sigma, R7017) working solution (0.5mM) was diluted into conditioned media at 1/10th of volume. At 0, 24, and 48 hour timepoints, conditioned media was replaced with Resazurin conditioned media, with an immediate absorbance reading taken at Ex560/Em590 to account for background. Following incubation over 4 hours to allow for resorufin conversion, another reading at Ex560/Em590 was recorded. Background values were subtracted and technical replicates were averaged.

2.5 Immunofluorescence

Cells were grown atop sterile coverslips placed within a 6-well plate until a desired density had been reached. Following a 2 hour treatment with Wnt or control conditioned media, coverslips were washed quickly 3 times with 1ml of PBS before fixation with 4% paraformaldehyde for 10 minutes at RT with gentle agitation. Coverslips were removed from the wells and washed 2x with PBS before permeabilization with 0.1% Triton X-100 PBS for 10 minutes. After permeabilization, cells were washed at five minute intervals three times with 1ml PBS 0.1% Tween (PBST) before incubation with primary antibody for 1 hour at RT (antibody dilution dependent on supplier's guidelines, 5% BSA in PBST). The 3x5 minute wash step was repeated before incubation in secondary antibody (1:500 dilution, 5% BSA in PBST) for 1 hour at RT in the dark. Following another 3x5 minute wash step, coverslips were incubated in 1ml of a 1:3600
DAPI (4’, 6-diamidino-2-phenylindole) solution for 15 minutes followed by one rinse in dH2O, then allowed to dry prior to mounting on glass slides. Images were taken with a modified DMIRE2 microscope system or with a Lieca SP5 confocal microscope.
Chapter 3 – Results

3.1 Wnt3a stimulation activates the Wnt pathway in IEC-18

To determine how the Wnt pathway is functioning within IEC-18 cells, different treatments that either activate or inhibit the Wnt signaling pathway were employed. To mimic the expected ligand mediated activation of the pathway, Wnt3a conditioned media was used. This treatment was generated by conditioning media for 48 hours with Wnt3a expressing L-cells and filtering, with the ligand present in this media activating the pathway at the level of the receptor. BIO ((2’Z,3’E)-6-Bromoindirubin-3′-oxime) functions as a GSK-3 inhibitor, preventing the phosphorylation of β-catenin in the cytoplasm by the destruction complex and thus its subsequent degradation. This allows β-catenin to accumulate in the cytoplasm, mimicking an activating mutation in the pathway in the absence of a Wnt ligand (Meijer et al. 2003). XAV939 is a tankyrase inhibitor, inhibiting a protein that would normally degrade Axin (Huang et al. 2009). This promotes the formation of destruction complexes, and accelerates the degradation of β-catenin. These treatments were performed over 1, 4, and 24 hours to capture the events of Wnt signaling in a temporal manner, as autoregulatory responses may change pathway dynamics during later signaling (Kim et al. 2013). The status of two key Wnt signaling proteins, β-catenin and LRP6, were examined through Western Blotting in order to determine how the pathway was functioning at a biochemical level. Activated Wnt signaling is predicted to result in an accumulation of cytoplasmic or activated β-catenin (ABC), marked by an absence of phosphorylation on residues S33/S37/T41/S45. Total β-catenin (TBC) levels were also evaluated. Ligand mediated activation of the pathway results in phosphorylation of LRP6, which can be identified by
phospho-specific antibodies (P-LRP6) in combination with antibodies targeting total LRP6 (T-LRP6).
Figure 3.1 Biochemical response of IEC-18 cells during varying Wnt treatments. (A) IEC-18 cells were treated with various Wnt compounds including Wnt3a conditioned media (WNT), Wnt antagonist compound XAV939 (XAV), and Wnt agonist compound BIO. These treatments were repeated at 1, 4, and 24 hour timepoints, with control conditioned media used for comparison (CTRL). Levels of β-catenin and the phosphorylation of LRP6 were examined. (B) Intensity of ABC and Actin bands were measured by J-image and used to create an ABC/Actin ratio for each treatment. Double asterisks denote significance by unpaired Student’s t test, relative to the control for the time point (p < 0.05). Standard error bars shown, N=3. T/P-LRP6 = Total/Phospho LRP6, A/TBC = Active/Total β-catenin, Actin = β-Actin.
As predicted, Wnt ligand mediated stimulation of the pathway with Wnt3a conditioned media was the only treatment resulting in phosphorylation of LRP6 at all three time points Fig 3.1a. The accumulation of ABC had a more delayed onset, the largest increase occurring at the 24 hour time point Fig 3.1a. This difference in ABC levels between treatments was quantified in Fig 3.1b. Ligand independent activation of the pathway through treatment with BIO followed a similar though somewhat reduced trend of increased ABC. XAV did reduce the levels of ABC, with the strongest effect occurring at 4 hours (X4 lane, Fig 3.1b). The reduced XAV response at 24 hours may be due to the half-life of the compound in the media, the age of the reagent, or compensation by the signaling pathway. The total levels of β-catenin (TBC) did not appear to change significantly regardless of the treatment. There was no major variance in the control sample over 24 hours (data not shown).

Upon determining that various Wnt stimuli were activating the pathway as expected, the next step was to confirm a change in transcriptional activity that corresponded with these events. The 24 hour time point displaying the largest change in ABC was chosen for Reverse Transcription quantitative PCR (RT-qPCR) analysis of Axin2 mRNA, a well-documented target of Wnt signaling. Data was normalized using the Livak method (Livak and Schmittgen 2001) to determine the fold difference relative to the control experiment. Interestingly, all three samples showed some increase in transcription of Axin2, though the ligand mediated event resulted in the most intense increase by far with over a 100 fold increase compared to the control Fig. 3.2.
Figure 3.2 Quantification of Axin2 transcript levels through qRT-PCR in response to different Wnt treatments. IEC-18 cells underwent control, XAV939, Wnt3a, or BIO treatments for a total of 24 hours before harvesting and RNA collection. Fold difference was quantified via the Livak method and normalized to the control (CTRL=1), shown in log scale. Error bars represent standard error of the mean, N=3. Double asterisks denote significance by unpaired Student’s t test, relative to the control (p < 0.05).

3.2 Wnt3a results in a redistribution of Nkd1-FLAG in IEC-18 cells

Previous work from our lab and others indicates that Nkd1 requires membrane localization to function, and undergoes a cytoplasmic redistribution upon stimulation with the Wnt ligand (Larraguibel et al. 2015). To determine if this function is conserved, IEC-18 cells were transfected with a Nkd1-FLAG vector and treated with Wnt3a or control conditioned media for two hours before fixation and staining. Interestingly, Nkd1 does not localize to the membrane in IEC-18 cells even weakly, a stark contrast to the
robust membrane localization seen in zebrafish blastula cells (Larraguibel et al. 2015; Van Raay et al. 2011). Despite this, activation of the Wnt pathway does result in a more perinuclear distribution of Nkd1-FLAG within these cells Fig 3.3. It is important to note that over the course of this project I found that IEC-18 cells are sensitive to transfection, and run into complications when overexpressing exogenous protein. While this effect might be specific to Nkd1 overexpression, similar results were found when testing a GFP control vector. Nkd1-FLAG may not be properly processed post-translationally in these cells, with large amounts of the protein remaining in what appears to be the rough endoplasmic reticulum contributing to the perinuclear distribution. Despite this, the redistribution appears to be consistent between cells in the treatments in response to the Wnt3a ligand. This would suggest that Nkd1 is being altered, or potentially ‘activated’ in response to the Wnt ligand similar to what is seen in zebrafish. In zebrafish, this activation of Nkd1 by the Wnt ligand not only redistributes Nkd1, but also increases the colocalization between Nkd1 and β-catenin. While not quantified, the observed shift of Nkd1 does not appear to increase colocalization with β-catenin, nor reduce the amount of nuclear β-catenin in IEC-18 cells.
Figure 3.3 Redistribution of Nkd1-FLAG in response to the Wnt3a ligand in IEC-18 cells. IEC-18 cells were treated with control (top) or Wnt3a conditioned (bottom) media for 2 hours before fixation and immunocytochemistry with anti FLAG (red) and anti β-catenin (green) antibodies. Positive colocalization (yellow) indicates colocalization of β-catenin and Nkd1-FLAG.
3.3 Wnt3a does not increase proliferation of IEC-18 cells over 48 hours

Given the changes observed in the activation of the Wnt pathway with Wnt3a treatment (Fig 3.1, 3.2), I hypothesized that this would translate into an increase in IEC-18 cell proliferation, consistent with Wnts role in the intestinal crypt. Therefore, I performed a proliferation assay using the Alamar Blue metabolic readout to determine if altering the activity of the Wnt pathway would affect cellular proliferation in IEC-18 cells. Utilizing the same treatments as the western panel (BIO, Wnt3a, XAV, Control) it was revealed that altering the Wnt pathway in this way had no significant effect on the rate of proliferation for IEC-18 cells over 48 hours (Fig 3.4).

Figure 3.4 Wnt treatments do not alter the proliferation of IEC-18 cells over a 48 hour period. IEC-18 cells were treated with either control, XAV939, Wnt3a, or BIO for 48 hours. At 0, 24, and 48 hours, treatment media was replaced with treatment+resazurin media and incubated for an additional 4 hours before fluorometric readings were taken. Conversion of Resazurin to Resofluorin by the metabolic action of live cells increases fluorescence of a sample and is proportional to cell population, N=3.
3.4 IEC-18 Summary

- IEC-18 cells have an active Wnt signaling pathway. Activation or inhibition of this pathway through Wnt3a conditioned media, BIO, or XAV results in expected changes in the levels of ABC and LRP6 phosphorylation.

- This molecular activation coincides with an expected increase in Axin2 transcription for both BIO and WNT, as well as an unexpectedly variable but nonsignificant increase in Axin2 transcription under XAV treatment.

- Overexpression of Nkd1-FLAG and GFP control protein in IEC-18 cells results in increased cell death and abnormal physiology for a percentage of the population.

- Similar to how Nkd1 functions in zebrafish, treatment with a Wnt ligand results in a more perinuclear redistribution of Nkd1-FLAG, though this may be in part due to complications with post-translational processing.

- Activating or inhibiting the Wnt pathway does not alter growth parameters over 48 hours.

While IEC-18 cells would appear to be strong model for the study of Nkd1 function, difficulties in transient transfections for Nkd1-FLAG expression prompted the use of another cell line during the investigation. HEK293 cells are a stable human embryonic kidney cell line that has been widely used to study biochemical interactions and the Wnt signaling pathway, and so should perform well in the evaluation of Nkd1 function.
3.5 Nkd1 does not alter β–catenin levels during pathway activation in HEK293

As discussed, our current model derived from zebrafish studies suggests that Nkd1 functions by inhibiting the nuclear accumulation of β–catenin, rather than affecting the activity of the destruction complex. To test this model, I generated a tetracycline inducible Nkd1-FLAG line in HEK293 cells to observe how the presence or absence of Nkd1 can affect the Wnt signal, and if activation by a Wnt ligand would affect Nkd1. Accordingly, these cells were treated with Wnt3a or control conditioned media in the presence or absence of tetracycline induced Nkd1. As expected, stimulation with the Wnt3a ligand results in proper activation of the pathway leading to phosphorylation of LRP6 and the accumulation of ABC Fig 3.5a. There is a slight increase in TBC that is likely due to the increased stability of β–catenin, as seen through ABC. Lastly, there is a slight increase in PBC during activated Wnt signaling. In line with our model, Nkd1 overexpression does not affect the levels of active, phosphorylated, or of total β–catenin, nor does it affect phosphorylation of the LRP6 coreceptor. ABC levels were quantified relative to Actin Fig 3.5b.
Figure 3.5 Effect of Nkd1 on the biochemistry of Wnt signaling in HEK293 cells. (A) HEK293 cells were treated with either Wnt3a or control media in the presence or absence of tetracycline induced Nkd1-FLAG over 1, 4, and 24 hours before harvest and western blotting. (B) Intensity of ABC and Actin bands were measured by J-image and used to create an ABC/Actin ratio for each treatment. Double asterisks denote significance by unpaired Student's t test, relative to the control for the time point (p < 0.05). Standard error bars shown, N=3. Abbreviations as per Fig 3.1.
3.6 Nkd1 expression alters Dvl2 levels in HEK293

Nkd1 interacts with Dvl2, though the function of this interaction is currently unclear. Some groups suggest that this interaction results in the degradation of Dvl leading to reactivation of the destruction complex as an explanation for Nkd1 antagonism (Schneider et al. 2010a; Wharton et al. 2001). One study tested this hypothesis and found no evidence of this phenomenon (Lum, Robertson, and Van Raay 2011). Therefore, I wanted to examine how Dvl is functioning within my system in response to Nkd1 induction. To do this, HEK293 cells were treated with or without Wnt in the presence or absence of Nkd1 (induced for 24 before treatment), and status of Dvl2 evaluated Fig 3.6. In the control treatment (no Wnt3a, No Nkd1-FLAG), the majority of Dvl2 is found in the fast migrating form, with some in the slow migrating form. The difference in the migration of these forms is associated with Dvl phosphorylation, discussed later. Treatment with Wnt3a results in an increase of the slower migrating form. Tetracycline induced expression of Nkd1 reduces the amount of the slower migrating form with or without the presence of Wnt3a. Activation under the Wnt3a ligand appears to increase Nkd1 mediated degradation of both fast and slow migrating forms.
Figure 3.6 Nkd1 overexpression alters the levels of Dvl2 fast and slow migrating forms. Over 1, 4, or 24 hours, HEK293 cells were treated with Wnt3a or control conditioned media in combination with tetracycline induced Nkd1-FLAG expression before harvesting and western blotting. Note that these blots were performed using the same samples as the HEK293 cell Wnt panel, and the same representative Nkd1 blot is used.

3.7 The effect of Nkd1-FLAG overexpression on Wnt target mRNA levels in HEk293 cells

As Nkd1 expression was not altering the levels of β-catenin in HEK293 cells, the next step was to determine its effect on gene transcription. As with IEC-18 cells, Axin2 was chosen as the Wnt target gene, in addition to Nkd1 and CyclinD1, with GAPDH as the reference Fig 3.7. In contrast to the Wnt3a stimulation of IEC-18 cells, activating the pathway utilizing the ligand did not result in any significant changes of Axin2 mRNA levels, and induction of Nkd1-FLAG did not affect this outcome. When induced by tetracycline, Nkd1 expression increased approximately 35 fold in a consistent manner. Under Wnt3a treatment, endogenous Nkd1 increased approximately 1.5 fold, consistent with what other groups have found and signifying activation of the pathway (Li et al.)
CycinD1 is a Wnt target gene, but not an obligate target of the pathway (Tetsu and McCormick 1999). Wnt3a/Nkd1 treatment did not alter CyclinD1 expression for three of the treatments, but interestingly resulted in a modest but significant increase when utilized together.

**Figure 3.7** Quantification of Axin2, Nkd1, and CyclinD1 transcript levels through qRT-PCR in combination with Nkd1-FLAG/Wnt3a treatments. HEK293 cells were treated with Wnt3a or control conditioned media in the presence or absence of induced Nkd1-FLAG for a total of 24 hours before harvesting and RNA collection. Fold difference was quantified via the Livak method and normalized to the control (CTRL=1). Error bars represent standard error of the mean, N=5 for Axin2/Nkd1, N=3 for CyclinD1. Double asterisks denote significance by unpaired Student’s t test, relative to the control (p < 0.05). Hash sign denotes Nkd1-FLAG induction by tetracycline.
3.8 Wnt3a stimulation does not result in a redistribution of Nkd1 in HEK293

IEC-18 cells exhibited some redistribution of Nkd1 upon stimulation with a Wnt ligand. To see if this is conserved in HEK293 cells, cells were treated with or without Wnt3a conditioned media and tetracycline induction of Nkd1-FLAG for two hours, fixed and immunostained. The size of these cells is significantly less than that of IEC-18 cells, making it difficult to analyze differences in the cytoplasmic distribution of Nkd1. Nkd1-FLAG expressed in HEK293 cells maintains a diffuse punctate distribution throughout the cytoplasm, and localizes more aptly to the membrane when compared to IEC-18 cells, though still significantly less than Nkd1 observed in zebrafish cells (Van Raay et al. 2011) Fig 3.8. Stimulation of the pathway with the Wnt3a ligand does not appear to result in a redistribution of Nkd1-FLAG.
Figure 3.8 Nkd1-FLAG does not undergo a redistribution during Wnt3a stimulation in HEK293 cells. HEK293 cells underwent tetracycline induced Nkd1-FLAG expression for 24 hours prior to a 2 hour treatment with Wnt3a or control conditioned media before fixation and immunostaining. Cells were stained for Nkd1-FLAG (red) and β–catenin (green), with nuclear staining by DAPI (blue).
3.9 HEK293 Summary

- HEK293 cells have an intact Wnt pathway with β–catenin and LRP6 responding as expected to Wnt3a conditioned media.
- Overexpression of Nkd1 does not alter the levels of β–catenin or affect the phosphorylation of LRP6 in the presence or absence of the Wnt ligand, in line with our model of predicted Nkd1 function.
- Overexpression of Nkd1 does alter Dvl2 phosphorylation, resulting in reduced levels of both slow and fast migrating forms.
- The addition of the Wnt3a ligand and Nkd1 results in a further reduction of Dvl2 compared to Nkd1 alone.
- Treatment of HEK293 cells with Wnt3a conditioned media results in the predicted increase of Nkd1, but does not significantly alter Axin2 levels. A combination of Wnt3a treatment and Nkd1-FLAG expression causes a modest and unexpected increase in CyclinD1 expression.
- In contrast to IEC-18 cells, treatment of HEK293 cells with Wnt3a did not appear to affect the distribution of Nkd1-FLAG.
Chapter 4 – Discussion

4.1 IEC-18 cells

4.1a IEC-18 cells are sensitive to Wnt signaling at the biochemical and genetic level

As an evolutionarily conserved signaling pathway, Wnt signaling has major roles during development and in stem cell homeostasis. In particular, oncogenic activation of Wnt signaling plays a significant part in the development of colorectal cancers. Most of these mutations affect the ability of the destruction complex to degrade β–catenin, allowing it to accumulate in the nucleus and activate transcription of Wnt target genes that promote proliferation. Our lab has previously interrogated the function of Nkd1 in zebrafish, characterizing it’s unique method of antagonism that instead prevents the nuclear accumulation of β–catenin rather than acting through the destruction complex (Larraguibel et al. 2015; Van Raay et al. 2011). In this thesis, I first investigated the potential for IEC-18 to serve as a model mammalian cell to study Nkd1 in an intestinal context. Derived from the intestinal crypt of rats and non-oncogenic, IEC-18 cells could provide a unique physiological environment for the study of Nkd1 and Wnt signaling, though there is currently little research on this cell line. Results indicated that IEC-18 cells are responsive to Wnt signaling on a molecular and transcriptional level, responding to both ligand mediated and ligand-independent treatments in a predictable way. Interestingly, despite the rapid phosphorylation of LRP6, β–catenin did not accumulate significantly until after the 4 hour time point. This suggests that it may take time for the destruction complexes to be recruited to the receptors and inactivated, despite the initial phosphorylation of LRP6 at one hour. Alternatively, the existing pool of
\(\beta\)-catenin that is being rapidly turned over may be quickly stabilized, but take time to accumulate. To test this, future studies could examine the levels of \textit{Axin2} transcription at earlier time points. It was determined that both Wnt3a conditioned media and BIO significantly increased \textit{Axin2} mRNA levels at 24 hours, while XAV939 had produced a variable but nonsignificant increase. Though unexpected, one group found that overexpressed \textit{Axin2} did not antagonize Wnt signaling in colorectal cancer cell lines with many mesenchymal traits (Wu et al. 2012). Despite the name, IEC-18 cells are simply a population of cells derived from the crypt, and may not be fully differentiated epithelial cells. This is supported by the fact that IEC-18 cells can be differentiated into a more epithelial like state in response to certain treatments (Ma et al. 1992; Shintani et al. 1989). Thus, the function of \textit{Axin2} in these cells may be different if untreated IEC-18 cells are a more mesenchymal cell type, as the cellular signaling environment within these cells could be altered compared to a more differentiated cell. Examining mesenchymal or epithelial markers such as N/E-cadherin through Western Blotting would provide insight into the tissue identity of IEC-18, as it may influence Wnt and Nkd1 function. Despite the effect of XAV939 on \textit{Axin2} transcription, results indicate that IEC-18 cells maintain an intact and responsive Wnt signaling pathway.

\textit{4.1b Nkd1-FLAG redistribution upon Wnt3a ligand activation in IEC-18 cells}

Our current model derived from experiments in zebrafish suggests that Nkd1 is activated at the receptor complex during ligand mediated Wnt signaling and undergoes a shift in cellular distribution (Larraguiibel et al. 2015). To test this in a mammalian cell model, I examined the redistribution of Nkd1 in response to Wnt ligand stimulation.
within IEC-18 cells, and found a more perinuclear distribution of Nkd1-FLAG upon activating the pathway, matching our previous findings (Kondra, 2014). This is comparable to the shift seen in developing zebrafish blastula cells, though membrane localization was essentially non-existent within IEC-18 cells (Larraguibel et al. 2015). In our zebrafish experiments where Nkd1 strongly localizes to the membrane and is able to efficiently antagonize Wnt signaling, these cells are in a developmental context, maintaining a basal-apical cellular polarity. The literature currently identifies IEC-18 cells as possible undifferentiated intestinal crypt cells, which may affect Nkd1 function (Shintani et al. 1989). One group found IEC-18 cells grown on a microporous substrate formed what they also deemed a differentiated enterocyte epithelium, and this synthetic substrate may provide some form of basal-apical polarity for future studies (Ma et al. 1992). Assaying for the ability to form colonospheres, spherical colonies that develop in anchorage-independent conditions, is a relatively recent development for in vitro analysis of colorectal cancer lines (Ricci-Vitiani et al. 2007; Sukach and Ivanov 2007). Human colorectal cancers contain a heterogeneous mixture of cells (Perez et al. 2013), with a minor sub population exhibiting poor differentiation and gene expression patterns that are reminiscent of stem cells (Visvader and Lindeman 2008). These cancer stem-like cells, commonly referred to as CSCs, are thought to make up the core proliferating and drug resistant cell population of tumors (Anderson et al. 2011), and are the cell population responsible for forming colonospheres (Shaheen et al. 2016). These colonospheres provide a unique 3D environment that promotes proliferation of these CSCs. While well characterized colorectal cancer cell lines such as HCT116 and SW480 form colonospheres in vitro, interestingly, IEC-18 cells could not (Farias 2013).
This suggests that IEC-18 cells may not contain a CSC subpopulation, or that these cells are sufficiently differentiated and do not contain the stem cell-like expression profile needed for colonosphere formation. As Wnt signaling has a key role in maintaining the intestinal crypt and regulating colonosphere formation (Kanwar et al. 2010), characterizing the activity of Nkd1 in a CSC population could provide valuable insight. Lgr5, a G-protein coupled receptor commonly found within adult stem cells, was able to promote the growth of crypt-villus structures from single isolated crypt stem cells (Sato et al. 2009). Expression of Lgr5 within IEC-18 cells may potentially promote colonosphere formation, and would be an interesting endeavor as an alternative to colorectal cancer lines.

During my thesis, this cell line was revealed to be highly sensitive to transient transfection and overexpression of exogenous protein. Transfection rates were approximately 5-10% at the minimum four hour incubation time recommended for sensitive cells. When tested with Nkd1-GFP, many positive cells expressed large amounts of the protein that resulted in the formation of large punctae and cell death. These transient transfections may be partially responsible for the more perinuclear distribution of Nkd1-FLAG in some cells, as the protein appears to be caught within the rough endoplasmic reticulum during post-translational processing. To compensate for this, I attempted to generate a stable tetracycline inducible cre recombinase IEC-18 cell line to study Nkd1, though found that the method of action for the Zeocin selection reagent was an obstacle in the isolation of colonies. Despite these difficulties I managed to isolate clones of Zeocin resistant colonies, likely containing the recombinase target site. Future work on this project would screen these clones for single site integrants.
using RT-qPCR before working towards the integration of tetracycline repressors and eventually Nkd1-FLAG. A single integration event within the host cell line in combination with a tetracycline promoter allows for tighter control of expression of the gene of interest.

4.1c Wnt3a, BIO, and XAV939 treatment does not alter IEC-18 proliferation over 48 hours

Above, I have shown that IEC-18 cells maintain a poised Wnt signaling pathway, undergoing the expected molecular events leading to transcription of Axin2 and presumably other Wnt target genes when the Wnt pathway is stimulated. Given the role of Wnt signaling in promoting proliferation of intestinal crypt stem cells, I hypothesized that IEC-18 cells would show an increase in proliferation under Wnt3a or BIO stimulation, with a corresponding decrease in proliferation under XAV treatment. To examine this, the proliferative response of IEC-18 cells was measured in response to several Wnt treatments using an Alamar Blue assay. Over a 48 hour period, activating the pathway using Wnt3a conditioned media/BIO, or antagonizing it with XAV939 did not result in a significant change in the rate of IEC-18 proliferation. In contrast to my findings, Wei et al., found that Wnt3a could promote IEC-18 growth and survival, though the experimental setup differed significantly (Wei et al. 2012). Wei et al., also found that inhibiting the pathway through receptor antagonists such as sFRPs promoted cell death and blocked the pro survival/proliferation effects of the Wnt3a ligand. In this thesis, the effect of Wnt3a was determined through use of media conditioned by Wnt3a expressing L-cells, while Wei et al., used purified recombinant mouse Wnt3a at various
concentrations. In addition, the Wei et al (2012) Alamar Blue growth assay took place in low-serum (0.1%) over 6 days, and the survival assay in serum-free conditions for 16 hours. Comparatively, complete media (10% serum) was conditioned by Wnt3a or control L-cells for a period of 48 hours in this thesis. While conditioning the media for 2 days prior to the assay undoubtedly depletes some of the nutrients and growth factors, there is no accurate way to gauge serum depletion. Thus, residual growth factors and nutrients within the conditioned media may have IEC-18 cells proliferating at their maximum rate regardless of Wnt stimulation. In future experiments, attempts could be made to generate low-serum conditioned media, though this might affect Wnt3a expression of the Wnt3a expressing L-cells. While 48 hours seems to be a common timepoint for growth curve analysis, IEC-18 proliferation may need to be monitored over a longer period of time as well. The transit rate for epithelial cells to be shed from the villus is approximately 3-5 days (Savidge, Walker-Smith, and Phillips 1995), and could assumed to be tied to the proliferation at the base of the crypt. Thus, the effect of altered Wnt signaling on proliferation might be too small to observe at 48 hours through an Alamar Blue assay. XAV939 functions as a Wnt antagonist, and should have a comparable result to the sFRPs utilized by Wei et al. despite the different modes of action. In this thesis, Western Blotting revealed that the effect of XAV939s on the pathway was more subtle after four hours, and when combined with the transcription data indicates that it did not antagonize Wnt signaling. The current results indicate that stimulating or antagonizing the pathway over 48 hours in conditioned media has no effect on the proliferation of IEC-18 cells.
4.2 HEK293 cells

4.2a Nkd1 does not alter the levels of β–catenin, but does affect Dvl2

Our lab has shown that in the developing zebrafish, ligand activated Nkd1 blocks overactive Wnt signaling by preventing the nuclear accumulation of β–catenin, rather than assisting with its degradation through the destruction complex. To confirm that Nkd1 is not degrading β–catenin in a mammalian cell line, I examined the effect of Nkd1 overexpression on activation of the Wnt pathway over 1, 4, and 24 hours in HEK293 cells. The pathway responded as predicted to Wnt treatments, and at all timepoints Nkd1 did not have an effect on the levels of total, active, or phosphorylated β–catenin, nor did it affect the phosphorylation of the coreceptor, consistent with our analysis in zebrafish blastula cells (Larraguibel et al. 2015; Van Raay et al. 2011).

Interestingly, Nkd1 did have a ligand dependent effect on the levels of Dvl2, leading to decreased amounts of both the slow and fast migrating forms. These slow and fast migrating forms are correlated to phosphorylated and dephosphorylated Dvl2 respectively (Hino et al. 2003; Klimowski et al. 2006; Peters et al. 1999). Without Wnt stimulation, HEK293 cells seem to maintain a basal level of phosphorylated Dvl2, and as predicted Wnt stimulation increases the amount of phosphorylated Dvl2 protein. Nkd1 overexpression strongly reduces this basal level of Dvl2 phosphorylation in the absence of a ligand. During ligand mediated activation of the pathway, overexpression of Nkd1 reduces Wnt induced phosphorylation of Dvl2, and also results in a reduction of the non-phosphorylated form. This suggests that Nkd1 is targeting Dvl2 for degradation. Within the Wnt pathway, the current model is that Wnt-ligand activated Dvl2 relays the signal from the receptor to the destruction complex in order to stop its function. Allowing
for β–catenin accumulation and translocation into the nucleus. If Nkd1's main method of antagonism was to promote the degradation of Dvl2, this would allow the destruction complex to regain its function in the presence of a Wnt ligand and Nkd1, resulting in decreased levels of total or activated β–catenin and thus Wnt gene inactivation. My results show that despite the overexpression of Nkd1, the reduction of Dvl2 is not enough to affect overall β–catenin levels. It is important to note that while phosphorylation of Dvl2 occurs in response to Wnt stimulation, it is not necessarily sufficient to activate the pathway, as non-canonical Wnts also induce phosphorylation (González-Sancho et al. 2004). Despite this phenomenon, one group found that a Dvl1 mutant (Ser 139/142) unable to be phosphorylated by CK1 led to a decreased Wnt signal using a TCF/LEF reporter when compared to wildtype, suggesting a positive effect on Wnt signaling for Dvl phosphorylation (Klimowski et al. 2006). In contrast, a Dvl2 CK1 phosphorylation defective mutant (Ser594/597, Thr595) resulted in increased transcription of Wnt target genes (González-Sancho et al. 2013). This phosphorylation defective Dvl2 also inhibited neurite outgrowth, a marker of non-canonical Wnt signaling (González-Sancho et al. 2013). A third group examined another Dvl2 phosphorylation mutant (Ser298/380), and found that phosphorylation of Dvl2 by kinase RIPK4 was required for “maximal” Wnt signaling, and that this phosphorylation was mediated by the Wnt ligand at the receptor level (Huang et al. 2013). Given the multitude of phosphorylation sites on the Dvl protein and the number of interacting partners, it is hard to determine what effect phosphorylated Dvl may be having within a cell. In this thesis, a partial decrease in Dvl2 phosphorylation by Nkd1 did not coincide with an increase or decrease in total or active β–catenin. Nkd1 mediated dephosphorylated or
degradation of Dvl2 may help sensitize the pathway to further regulation however, as the aforementioned groups found that Dvl phosphorylation at certain residues was not essential, but rather amplified Wnt signaling. Indeed, a rare subset of colorectal cancers that did not harbor APC or β–catenin mutations had mutant Nkd1 that was unable to bind and alter Dvl proteins (Guo et al. 2009). Overexpression of this mutant Nkd1 resulted in an increased proliferation of colorectal cancer derived cells (Guo et al. 2009).

In zebrafish, our previous work revealed that Nkd1 is able to physically interact with β–catenin, and that this association was increased by stimulation with a Wnt ligand and required for Wnt antagonism (Larraguibel et al. 2015; Van Raay et al. 2011). I attempted to capture this interaction in HEK293 cells by immunoprecipitating FLAG tagged Nkd1 with and without Wnt3a stimulation. One trial managed to capture an interaction between active β–catenin and Nkd1-FLAG that was similar to what was found in zebrafish (Appendix). We have previously demonstrated that Nkd1 interacts with both the fast and slow migrating forms of Dvl2, and that both β–catenin and Dvl2 proteins compete for binding with Nkd1 (Van Raay et al. 2011). Thus, degradation of Dvl2 under the presence of a Wnt ligand may work to enhance Nkd1 function in a feed forward system by allowing more frequent interactions between Nkd1 and β-catenin. In zebrafish blastula cells, Nkd1 overexpression by injected mRNA does not alter Dvl2 levels. Again, the cellular environment of an in vivo blastula cell compared to an in vitro cell model may be affecting Nkd1 function. Once immunoprecipitation experiments have been optimized, examining how phosphorylation defective Dvl2 mutants interact with Nkd1 and its ability to antagonize the pathway/bind β–catenin could provide some insight on Nkd1s method of antagonism.
4.2b Nkd1 does not undergo a cytoplasmic redistribution upon Wnt3a stimulation in HEK293 cells

After observing the redistribution of Nkd1-FLAG within IEC-18 cells, it was important to determine if the same phenomenon was conserved in HEK293 cells. A previous member of our lab attempted to generate a HEK293 Nkd1-FLAG line, but had a mixed population of Nkd1-FLAG expressing and non-expressing cells (Kondra, 2014). This experiment was repeated using the tetracycline inducible Nkd1-FLAG HEK293 cell line, and found that Wnt3a conditioned media did not result in a redistribution of Nkd1-FLAG within these cells, supporting our previous findings in HEK293 cells (Kondra 2014). The tissue origin of HEK293 cells have not been well characterized, said to have been derived from epithelial, endothelial, and fibroblastic cells of the kidney, but have also been found to maintain several markers of neuronal cells (Shaw et al. 2002). As such, the signaling environment within these cells may not be ideal for the examination of Wnt signaling and Nkd1 activity. In addition, the small cytoplasm of these cells makes it hard to quantify the distribution of Nkd1 under confocal microscopy. It is also important to note that the Nkd1-FLAG is being heavily induced in these cells, and thus the high levels of Nkd1 protein may be affecting the results through a cellular stress response, or non-specific interactions of the expressed protein with other proteins. Still, our lab and others have shown that HEK293 cells do maintain an intact and responsive Wnt signaling pathway, and can serve as a useful tool to examine the biochemistry of the Wnt pathway.
4.2c Wnt target mRNA levels in HEK293 cells under Wnt3a/Nkd1-FLAG treatments

As Nkd1 overexpression was not altering active β–catenin, I next investigated if Nkd1 could antagonize Wnt signaling in HEK293 cells, and if the ligand dependence for this antagonism observed in zebrafish was conserved. Nkd1-FLAG overexpression or Wnt3a stimulation did not have any effect on Axin2 mRNA levels at 24 hours. Investigating the literature reveals that Axin2 mRNA levels can be elevated at lower time points with Wnt3a conditioned media. One group found stimulating HEK293 cells with Wnt3a conditioned media for 7 hours resulted in an approximately 2.5 fold increase in Axin2 mRNA levels compared to control (Li et al. 2011). Another study using purified Wnt3a protein over 6 hours found a similar 2 fold increase (Callow et al. 2011). Lee et al., also found that treating HEK293 cells with Wnt3a conditioned media for 6 hours results in an approximate 2 fold increase compared to control (Lee et al. 2013). This suggests that the high levels of ABC present at 24 hours are not sufficient to activate the pathway, or that extended activation of the Wnt pathway can affect Axin2 mRNA levels. There is a slight increase in Axin2 levels under Wnt3a treatment, but is nonsignificant at p < 0.05. Interestingly, Nkd1 mRNA levels increased approximately 1.5 fold under Wnt3a stimulation, suggesting that the at least some transcriptional targets are active or stable at 24 hours. CyclinD1 mRNA levels follow a trend similar to that of Axin2 in the literature, an increase of ~2 fold under Wnt3a stimulation for 6-7 hours (Lee et al. 2013). The modest 1.1 fold increase observed when Nkd1-FLAG is present during Wnt3a treatment may be a result of a low sample size (N=3, compared to N=5 for Axin2/Nkd1), or could be a result of non-activated Nkd1 interacting with β–catenin, but there is currently little explanation for this phenomenon. While there is no obvious effect
of a Wnt ligand dependent interaction in HEK293 cells from this study, Koch et al., found that Nkd1 antagonizes Wnt3a induced signaling, but not signaling resulting from constitutively active β-catenin in liver tumor cells (Koch et al. 2005). This experiment was very similar to our protocol in zebrafish and shows identical results (Van Raay et al. 2011), suggesting a conservation of function. Future experiments should focus on earlier time points, and explore different cell types for the study of Nkd1 function. My data suggests that at 24 hours the Wnt3a ligand may be losing some efficacy, or that auto-regulatory mechanisms are affecting the levels of Axin2 mRNA on a transcriptional or transcript level. In contrast, Nkd1 mRNA levels are increasing as expected in response to Wnt stimulation, and may be under alternative regulation.

4.3 Concluding Remarks and Future Directions

In this thesis I investigated the ability of IEC-18 cells to serve as a mammalian intestinal cell model for the study of Wnt signaling/Nkd1 function. I also evaluated the function of Nkd1 in a biochemical and genetic context within HEK293 cells. It is revealed that IEC-18 cells maintain an active Wnt signaling pathway, responded as predicted to various Wnt treatments on a biochemical and genetic level. In addition, Nkd1 shows a cytoplasmic redistribution during Wnt3a mediated activation, mimicking the activity of its zebrafish counterpart despite the lower membrane localization. While the results of my growth assay did not indicate any significant change over 48 hours, work by another group revealed a Wnt signaling dependent growth and survival response in IEC-18 cells over a longer period of time than the one examined in this thesis. This evidence suggests that IEC-18 cells may be a good model for the study of Wnt signaling and
Nkd1. Continuing my work in generating a tetracycline inducible cre recombinase targeted line of IEC-18 cells would allow for conditional expression of Nkd1 (and Nkd1 constructs) in a more controlled manner. Once this line is generated, the effect of Nkd1 on the Wnt signaling pathway and any physiological responses could be easily tested. Exploration into Nkd1 mutants, such as the G2A membrane localization mutant could be performed easily and provide a mammalian counterpart to our labs zebrafish studies.

HEK293 cells were found to have a responsive Wnt signaling pathway, confirming ours and others previous findings. In addition, the expression of Nkd1 was not found to alter the levels of β–catenin, synonymous with our findings in zebrafish. While Nkd1 in IEC-18 cells underwent a redistribution in response to Wnt stimulation, the same phenomenon did not occur in HEK293 cells, possibly due to heterogenous tissue identity of these transformed cells, or the difficulty in quantifying the small space in which Nkd1 has to maneuver. Interestingly, it was shown that Nkd1 decreases the levels of fast and slow migrating Dvl2, and this alteration can be shifted depending on the presence of the Wnt ligand. This reduction in Dvl2 protein did not result in a corresponding increase of total/active β–catenin, which was expected given the known role of Dvl2 in activating the canonical Wnt pathway. The purpose of Nkd1's interaction with Dvl is not well characterized, and further investigation into how this might affect Wnt signaling sensitivity should be investigated.

In the Wnt pathway nearly all activity is regulated at the level of the receptor and the destruction complex, leading to the accumulation or degradation of β–catenin. The majority of oncogenic mutations in different genes prevent β–catenin degradation by inactivating or avoiding the destruction complex, bypassing most Wnt antagonists. Our
previous studies in zebrafish reveal that Nkd1 utilizes a unique method of antagonism that instead prevents the nuclear import of β–catenin rather than acting through the destruction complex (Larraguibel et al. 2015; van Raay et al. 2011). As such, it may be a valuable therapeutic target for the treatment of Wnt based cancers to properly antagonize β–catenin accumulation in the presence of a compromised destruction complex. Characterizing how Nkd1 is activated and what effect it has on the Wnt pathway in a suitable mammalian cell line would provide valuable insight for clinical application.
Chapter 5 – References


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Appendix

Appendix Figure 1. Coimmunoprecipitation of Nkd1-myc in and immunoblotting for β-catenin in Zebrafish (Van Raay et al. 2011), left. Coimmunoprecipitation of Nkd1-FLAG and immunoblotting for total and active β-catenin in HEK293 cells (own work), right. Nkd1-FLAG was successfully immunoprecipitated from HEK293 cells, though β-catenin binds non-specifically to the Protein G agarose. Despite this, the amount of β-catenin increases substantially when Nkd1-FLAG is present, suggesting an interaction. While total β-catenin levels are fairly similar between treatments, the amount of ABC appears to increase under Wnt stimulation.
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**T+ notes tetracycline induced Nkd1-FLAG expression. W+ notes treatment with Wnt3a conditioned media for 24 hours.**
Appendix Figure 2. Sample calculations for Axin2/Nkd1 RT-qPCR set and example amplification curve. Each experimental treatment is performed in triplicate with extreme values (CT difference compared to other technical replicates > 0.6) being removed. The fold difference is then calculated relative to the control (T-/W-) for each set, using GAPDH as a reference. Sets were then pooled.