Analysis of Naked in the Canonical Wnt Pathway

by

Garnet Jean Lau

A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Biochemistry
University of Toronto

© Copyright by Garnet Jean Lau 2008
Analysis of Naked in the Canonical Wnt Pathway

Garnet Jean Lau

Master of Science

Department of Biochemistry
University of Toronto

2008

Abstract

Wnt signalling is involved throughout development and is inappropriately activated in a variety of human cancers. In the canonical pathway, secreted Wnt proteins induce the stabilization of β-catenin. *Drosophila* Naked (Nkd), or Nkd1 and Nkd2 in vertebrates, is believed to antagonize canonical Wnt signalling through an interaction with Dishevelled (Dvl). Analysis of a high-throughput protein-protein interaction screen conducted in our lab led to the identification of novel Nkd1 interacting proteins, including Nkd1/2 and Axin1. Mapping of Nkd1 regions required for these novel interactions and functional studies, including transcriptional and siRNA mediated knockdown assays, were performed to examine the role of Nkd1 in canonical Wnt signalling. Previous work suggests that Nkd1 functions only through Dvl, but this work suggests that Nkd1 acts via a more complex mechanism. In addition to serving as an antagonist to regulate the canonical Wnt pathway, we propose that Nkd1 may also act positively to promote signalling.
Acknowledgments

First and foremost, I would like to thank my supervisor, Dr. Liliana Attisano. She has been a great source of scientific knowledge and expertise and I am grateful for the amazing opportunity she has given me to work and study in her lab. As a member of the Attisano lab, I was surrounded by a tremendous group of scientists, many of whom were always willing to go the extra mile to help a colleague, whether or not the situation was science-related. Luisa Izzi, Cristoforo Silvestri, Stephen Perusini, Monika Podkowa, Bryan Miller, Etienne Labbé, Peter Poliszczuk, Xin Zhao, and Siyuan Song – I thank each of you for your time, efforts and friendship.

Next, I thank my parents, Florence and Larry Lau, for their continued love, support and encouragement, regardless of my chosen path of study. I would like to acknowledge my brother Garrick and my sister Cara for being the motivating role models that every little sister needs. I would also like to thank Uncle John and Auntie Mei for their advice, encouragement and generosity. Lastly, I would like to thank my fiancé Edward, my constant source of love and strength, who never ceases to believe in me.

Thank you!
Data Attributions

All the experiments, data and figures presented in this thesis were performed and compiled by myself, under the guidance of my supervisor L. Attisano, with the exception of the following:

Bryan Miller completed the interaction and functional high throughput screens that are described and provided the associated screen data presented in Figure 4 and the mLIR values shown in Fig. 7B and C.
# Table of Contents

Acknowledgments.......................................................................................................................... iii

Data Attributions.......................................................................................................................... iv

Table of Contents.......................................................................................................................... v

List of Figures .............................................................................................................................. viii

List of Abbreviations .................................................................................................................. x

1 Introduction................................................................................................................................. 1

1.1 The Wnt signalling pathway ............................................................................................... 1

1.1.1 Wnt Signalling overview ........................................................................................ 1

1.1.2 Wnt genes and proteins ......................................................................................... 3

1.1.3 Wnt receptors ........................................................................................................ 4

1.1.4 Intracellular signal transduction ........................................................................... 5

1.2 Wnt signalling in development and disease ...................................................................... 13

1.2.1 Drosophila development ....................................................................................... 13

1.2.2 Xenopus development ........................................................................................... 14

1.2.3 Zebrafish development .......................................................................................... 15

1.2.4 Mammalian development ...................................................................................... 16

1.2.5 Disease and cancer ................................................................................................ 19

1.3 Naked in the canonical Wnt Pathway ............................................................................ 21

1.3.1 Protein function and interactions .......................................................................... 21

1.3.2 Expression and regulation ..................................................................................... 27

1.4 Thesis perspective .......................................................................................................... 28

2 Materials and Methods ............................................................................................................. 29

2.1 Mammalian expression constructs ................................................................................. 29

2.2 Cell culture and transfection .......................................................................................... 31
2.3 Production of Wnt3a conditioned medium .............................................................. 32
2.4 LUMIER assay ........................................................................................................... 32
2.5 Topflash reporter assay ............................................................................................ 33
2.6 siRNA assays ............................................................................................................. 35
2.7 Real time quantitative reverse transcription PCR (QPCR) ........................................ 35
2.8 Immunoprecipitation and immunoblotting ............................................................... 36
2.9 Immunofluorescence ............................................................................................... 37

3 Results .......................................................................................................................... 38

3.1 The hNKD1 and mNkd2 interactomes ...................................................................... 38
3.1.1 Manual verification of PPI identified by LUMIER ............................................... 43
3.2 Overexpressed hNKD1 blocks Wnt3a-dependent activation of the Topflash reporter. ... 49
3.3 Determination of regions on hNKD1 required for blocking Wnt3a-dependent activation of the Topflash reporter ................................................................. 52
3.4 Substitution of the histidine-rich tail with that of hNKD1 confers repressive activity to mNkd2 on the Topflash reporter ................................................................. 57
3.5 siRNA mediated knockdown of hNKD1 blocks Wnt3a-dependent activation of the Topflash reporter ................................................................. 59
3.6 siRNA mediated knockdown of hNKD1 represses the expression of Axin2, a Wnt3a-target gene ........... 66
3.7 Characterizing the Nkd-Nkd interaction .................................................................. 68
3.8 Characterizing the Dishevelled-Nkd interaction ...................................................... 75
3.9 Characterizing the Axin-Nkd interaction ................................................................ 80
3.10 Nkd localization at the plasma membrane .............................................................. 84

4 Discussion ...................................................................................................................... 88

4.1 General discussion .................................................................................................... 88
4.2 Physical and functional high throughput screens involving hNKD1 and mNkd2 ......... 89
4.3 The differential effects of Nkd1 and Nkd2 ............................................................... 91
4.4 hNKD1 as a positive regulator in canonical Wnt signalling ..................................... 92
4.5 hNKD1 homodimerization ................................................................. 96
4.6 hNKD1 interacts with DVL2 ............................................................. 97
4.7 hNKD1 interacts with Axin1 ............................................................... 99
4.8 hNKD1 as a mediator between Axin and Dvl ....................................... 100
4.9 General conclusions ........................................................................ 103
References .......................................................................................... 105
List of Figures

Figure 1: The canonical Wnt pathway................................................................. 2

Figure 2: Invertebrate and vertebrate Naked................................................. 24

Figure 3: Schematic of methodology.............................................................. 34

Figure 4: The hNKD1 and mNkd2 interactomes as defined by LUMIER........ 41

Figure 5: Validation of Nkd binding partners by LUMIER............................ 44

Figure 6: Validation of Nkd binding partners by LUMIER............................. 45

Figure 7: Validation of Nkd binding partners by LUMIER............................. 47

Figure 8: Overexpressed hNKD1 blocks Wnt3a-dependent activation of the Topflash reporter. 51

Figure 9: Naked schematic........................................................................... 53

Figure 10: The N-terminal and C-terminal regions of hNKD1 are not sufficient to block Wnt3a-dependent activation of the Topflash reporter to the extent of wild type hNKD1. ............... 54

Figure 11: A mNkd2 chimera expressing the poly-His domain from hNKD1 blocks Wnt3a-dependent activation of the Topflash reporter.......................................................... 58

Figure 12: Knocking down hNKD1 expression by pooled siRNA transfection blocks Wnt3a-dependent activation of the Topflash reporter................................................. 61

Figure 13: Knocking down hNKD1 expression by single siRNA duplex transfection blocks Wnt3a-dependent activation of the Topflash reporter................................................. 63

Figure 14: Knocking down hNKD1 expression by siRNA transfection in represses the expression of Axin2, a Wnt3a-target gene................................................................. 67

Figure 15: Mapping the hNKD1 homodimerization domain to the internal and C-terminal region.......................................................... 71
Figure 16: Mapping the Nkd dimerization domain................................................................. 72

Figure 17: Naked domains and interactions........................................................................ 74

Figure 18: Mapping the DVL2 binding domain to the hNKD1 internal region. ................ 76

Figure 19: Mapping the DVL3 binding domain to the hNKD1 internal region. ................ 78

Figure 20: Mapping the Axin1 binding domain to the hNKD1 C-terminal region. .......... 81

Figure 21: Mapping the Axin1 binding domain to the mNkd2 C-terminal region............. 83

Figure 22: hNKD1 cellular localization at the membrane.................................................. 86

Figure 23: Proposed dual role for hNKD1 in canonical Wnt signalling......................... 101
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>Axin</td>
<td>Axis Inhibitor</td>
</tr>
<tr>
<td>Boz</td>
<td>Bozozok</td>
</tr>
<tr>
<td>CaM</td>
<td>Calcium/calmodulin-dependent</td>
</tr>
<tr>
<td>CaMK</td>
<td>Calcium/calmodulin-dependent kinase</td>
</tr>
<tr>
<td>CBP</td>
<td>Cyclic AMP response element-binding protein</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CE</td>
<td>Convergent extension</td>
</tr>
<tr>
<td>Chd</td>
<td>Chordin</td>
</tr>
<tr>
<td>CKI</td>
<td>Casein kinase I</td>
</tr>
<tr>
<td>cMyc</td>
<td>Cellular myelocytomatosis oncogene</td>
</tr>
<tr>
<td>CPS</td>
<td>Combined Pathway Score</td>
</tr>
<tr>
<td>CRD</td>
<td>Cysteine-rich domain</td>
</tr>
<tr>
<td>d.p.c.</td>
<td>days postcoitum</td>
</tr>
<tr>
<td>DAAM</td>
<td>Dishevelled associated activator of morphogenesis</td>
</tr>
<tr>
<td>DAG</td>
<td>di-acyl glycerol</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DEP</td>
<td>Dishevelled EGL-10 Pleckstrin</td>
</tr>
<tr>
<td>Dfz</td>
<td>Drosophila Frizzled</td>
</tr>
<tr>
<td>DGO</td>
<td>Diego</td>
</tr>
<tr>
<td>DIX</td>
<td>Dishevelled-Axin</td>
</tr>
<tr>
<td>Dkk1</td>
<td>Dickkopf 1</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>Dsh</td>
<td>Dishevelled</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double stranded RNA</td>
</tr>
<tr>
<td>Dvl</td>
<td>Dishevelled (vertebrate)</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>En</td>
<td>engrailed</td>
</tr>
<tr>
<td>ES</td>
<td>embryonic stem</td>
</tr>
<tr>
<td>Evi</td>
<td>Evenness interrupted</td>
</tr>
<tr>
<td>FAP</td>
<td>Familial Adenomatous Polyposis</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FFLuc</td>
<td>Firefly Luciferase</td>
</tr>
<tr>
<td>FMI</td>
<td>Flamingo</td>
</tr>
<tr>
<td>Fz</td>
<td>Frizzled</td>
</tr>
<tr>
<td>GNGT</td>
<td>guanine nucleotide binding protein</td>
</tr>
<tr>
<td>Gsc</td>
<td>Goosecoid</td>
</tr>
<tr>
<td>GSK-3</td>
<td>Glycogen synthase kinase-3</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylases</td>
</tr>
<tr>
<td>HEK</td>
<td>Human Embryonic Kidney</td>
</tr>
<tr>
<td>Hh</td>
<td>Hedgehog</td>
</tr>
<tr>
<td>Hpf</td>
<td>hour post fertilization</td>
</tr>
</tbody>
</table>
HTP: High throughput
IB: Immunoblotting
Int: Integration
IP: Immunoprecipitation
IP3: inositol 1,4,5-triphosphate
JNK: c-Jun N-terminal kinase
LDL: Low-density lipoprotein
LEF1: Lymphoid enhancer binding factor-1
Lgs: Legless
LRP: LDL-receptor related protein
LUMIER: LUminescence-based Mammalian IntERactome
MAPK: Mitogen Activated Protein Kinase
MBT: Mid-blastula transition
MDCK: Madin Darby Canine Kidney
mLIR: median-based Luminescence Intensity Ratio
MMTV: Mouse Mammary Tumour Virus
MO: morpholino oligonucleotide
mRNA: Messenger Ribonucleic acid
Msn: Misshapen
Nkd: Naked, Naked Cuticle
NLS: Nuclear localization sequence
NMuMG: Normal Murine Mammary Gland
PAK: p21-activated kinase
PAR: Partitioning defective
PCP: Planar cell polarity pathway
PCR: Polymerase chain reaction
PDZ: Post-synaptic density-95 Disc-large-Zonula occludens-1
PI3K: Phosphatidylinositol 3-kinase
PK: Prickle
PKC: Protein kinase C
PP2: Protein Phosphatase 2
PPI: Protein-protein interaction
Pygo: Pygopus
Rho: Ras homology
RLuc: Renilla Luciferase
RNA: Ribonucleic acid
RNAi: RNA interference
ROK: Rho-associated kinase
SD: Standard Deviation
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM: Standard Error of the Mean
Ser: Serine
SERCA: Sarco(Endo)plasmic Reticulum Calcium-ATPase
sFRPs: Soluble Frizzled related proteins
siCTL: control siRNA
sihNKD1: human NKD1 siRNA
siRNA: Small interfering RNA
Smurf: MAD specific E3 ubiquitin protein ligase
STBM: Strabismus
SWI/SNF: Switching-defective, sucrose nonfermenting
TCF: T-cell specific factor
TGF: Transforming Growth Factor
Thr: Threonine

β-TrCP: β-transducin repeat-containing protein
VEGF: Vascular endothelial growth factor
Wg: Wingless
WIF: Wnt inhibitory factor
Wls: Wntless
Wnt: Wingless/int
Y2H: Yeast two-hybrid
1 Introduction

1.1 The Wnt signalling pathway

1.1.1 Wnt Signalling overview

Signalling pathways are crucial throughout an organism’s life and ensure properly coordinated activities in events such as development, tissue homeostasis and tissue regeneration. Moreover, aberrantly regulated signalling pathways have been shown to result in developmental defects and diseases into adulthood (Clevers, 2006; Logan and Nusse, 2004; Wodarz and Nusse, 1998).

The Wnt signalling pathway was first characterized in 1982, when the mouse mammary tumour virus (MMTV) was reported to induce mammary gland tumours in mice by activating a gene, which was named Int1 (integration 1) (Nusse and Varmus, 1982). The term ‘Wnt’ was derived five years later when Int1 was found to be similar to the Wingless (wg) segment polarity gene in Drosophila (Rijsewijk et al., 1987). Mutations in wg caused segmentation defects in Drosophila embryos and studies had already identified various related segment polarity gene mutations (Nusslein-Volhard and Wieschaus, 1980). Collective observations have shown that Wnt signalling is highly conserved from invertebrates to vertebrates (Clevers, 2006). Secreted Wnts, or Wg proteins in Drosophila, are thought to signal through either non-canonical or canonical pathways. In the less studied non-canonical Wnt pathway, mediators such as intracellular Ca\textsuperscript{2+} release and cytoskeletal regulators are involved in cellular processes including gastrulation movements and planar cell polarity (Kohn and Moon, 2005).
(A) In the absence of Wnt ligand, β-catenin is constitutively marked for proteosomal degradation by a destruction complex composed of Axin, APC, CK1α, and GSK3β. (B) Upon Wnt stimulation, this destruction complex is disrupted and leads to β-catenin accumulation. β-catenin then translocates to the nucleus and associates with TCF/LEF binding partners to initiate transcription of Wnt target genes.
The canonical Wnt pathway (Fig.1), also referred to as the β–catenin pathway, involves Wnt-induced stabilization of β–catenin. In the absence of Wnt ligand, β–Catenin is constitutively marked for proteasomal degradation by a complex that includes Axin, casein kinase 1α (CK1α), glycogen synthase kinase 3β (GSK3β) and adenomatous polyposis coli (APC). Upon Wnt stimulation, this destruction complex is disrupted by Dishevelled (Dvl), which allows cytoplasmic β–catenin to accumulate and translocate to the nucleus, where β–catenin can associate with TCF/LEF transcription factors to initiate transcription of specific target genes (Logan and Nusse, 2004).

### 1.1.2 Wnt genes and proteins

In addition to functional properties, Wnt proteins are principally defined by amino acid sequence similarity. Wnt proteins range in length from 350 to 400 amino acids. In mammals, a family of 19 Wnt genes has been described (Miller, 2002). Each member is characterized by an amino terminal signal for secretion, several sites for glycosylation and a cysteine-rich stretch (Miller, 2002). Glycosylation is crucial for Wnt protein folding, secretion and biological activity (Smolich et al., 1993). While Wnt is secreted, it is relatively insoluble, which may be a result of cysteine palmitoylation (Willert et al., 2003). In *Drosophila*, palmitoylation is thought to be effected by *porcupine*, a gene required in Wnt-secreting cells that displays homology to acyltransferases (Zhai et al., 2004). In addition to *porcupine*, *wntless/evenness interrupted* (*wls/evi*) has been found to be required for proper Wnt secretion. The *wls/evi* gene encodes a seven-pass transmembrane protein that is found in the Golgi apparatus that interacts with Wnt proteins and may regulate a Wnt gradient (Banziger et al., 2006; Bartscherer et al., 2006; Coudreuse et al., 2006). Once properly secreted and modified, Wnt proteins act as morphogens, whereby the long
range gradient and concentration-dependent activity may be possible due to tethering to intercellular transport vesicles or lipoprotein particles (Logan and Nusse, 2004; Panakova et al., 2005). *Drosophila* Wg proteins may also associate with extracellular heparin sulfate proteoglycans (HSPG) for transport or stabilization (Lin, 2004).

### 1.1.3 Wnt receptors

At the membrane, Wnt proteins bind with the Frizzled (Fz) family of seven-pass transmembrane receptors, which have an extracellular amino terminal cysteine-rich domain (CRD) and a carboxy terminal cytoplasmic tail. Ten members of the Fz receptor family have been identified and via the CRD, each Fz receptor can bind to a number of distinct Wnt proteins to elicit signals and vice versa (Bhanot et al., 1996). Different Wnt/Fz pairings are thought to dictate the specificity of downstream signalling events (Weeraratna, 2005). In *Drosophila*, overexpression of dFz2 is unable to activate Wnt signalling in the absence of Wg, which suggests that canonical signalling, at least, is ligand dependent (Bhanot et al., 1996; Rulifson et al., 2000). In addition to the Wnt/Fz interaction, Wnt signalling also engages the single-pass transmembrane molecule low-density lipoprotein (LDL)-receptor related proteins (LRP) 5 and LRP6 (Tamai et al., 2004), or *arrow* in *Drosophila* (Wehrli et al., 2000). Indeed, it is proposed that Wnt may induce the formation of a trimeric complex of Wnt, Fz and LRP/5/6 to trigger cytoplasmic activities (Tamai et al., 2000). However in *Drosophila*, Wg has not been shown to bind to Arrow (Wu and Nusse, 2002). In any event, Fz and LRP5/6 are believed to be co-receptors for canonical Wnt signalling since surface expression of both receptors is required for intracellular signal transduction (Clevers, 2006). In addition to Fz and LRP5/6, Wnt proteins have also been shown to bind to the Derailed receptor, a transmembrane tyrosine kinase from the RYK subfamily. In *Drosophila*,
DWnt-5 binds to Derailed and may signal to affect neuronal projections (Yoshikawa et al., 2003), however downstream signalling events remain unclear.

In the extracellular environment, Wnt protein access to receptors appears to be highly regulated. The molecules, secreted frizzled related proteins (sFRPs), Wnt inhibitory factor (WIF-1) and Cereberus (Cer-1) bind directly to Wnt proteins to inhibit Fz/LRP5-Wnt association (Kawano and Kypta, 2003; Patthy, 2000). Similar to Fz, sFRPs contain a CRD, that binds Wnts, but a recent study suggests that the association may not only be antagonistic to signalling and may also promote Wnt signalling by stabilizing Wnt proteins or facilitating secretion (Uren et al., 2000).

Proteins unrelated to Wnt have also been shown to associate with Fz and LRP5/6 co-receptors to elicit agonistic or antagonistic effects. For instance, the cysteine-knot protein Norrin binds with Fz4 to activate canonical signalling in the presence of LRP5/6 (Xu et al., 2004). In addition, R-spondins, which contain thrombospondin domains, may synergize with Wnt proteins and bind to the extracellular domains of LRP6 and Fz8 to activate the Wnt pathway (Kazanskaya et al., 2004; Nam et al., 2006). On the other hand, the antagonist, Dickkopf (Dkk), a secreted protein, inhibits the Wnt pathway by binding to LRP5/6 (Glinka et al., 1998); (Mao et al., 2001). This interaction links LRP5/6 to the Kremens, another class of transmembrane proteins, and leads to LRP5/6 internalization and inactivation (Mao et al., 2002).

1.1.4 Intracellular signal transduction

Intracellular pathway activation is dependent on Wnt and Fz interactions. While Fz proteins, such as Fz7, have been harder to classify as either non-canonical or canonical (Medina et al., 2000; Weeraratna, 2005), Wnt proteins have been more distinguishable. In fact, it has been suggested that certain canonical Wnt proteins may promote specific Fz-LRP interactions that
activate canonical signalling (Liu et al., 2005). It is now believed that certain Wnt proteins have a tendency to activate non-canonical signalling while other Wnt proteins are known to preferentially activate canonical signalling (Cadigan and Liu, 2006).

1.1.4.1 Non-canonical

Non-canonical Wnt signalling is further classified into two β-catenin independent signalling pathways. The first being the Wnt/Ca\(^{2+}\) pathway, which activates protein kinase C (PKC), and the second being the planar cell polarity (PCP) pathway, which guides cell movements during gastrulation. The Wnt protein family members, Wnt5a, Wnt11 and Wnt4, have been involved in non-canonical signalling through modulating movements and cell adhesion in *Xenopus* embryos (Du et al., 1995; Moon et al., 1993).

In the calcium pathway, Wnt5a and Wnt11 have been implicated in activating PKC and Ca\(^{2+}\)/calmodulin-dependent (CaM) protein kinase (CaMK) (Kuhl et al., 2000; Sheldahl et al., 1999). Frizzled is thought to activate heterotrimeric G-proteins (Malbon et al., 2001) to activate phospholipase C, which then translocates to the membrane. At the membrane, phospholipase C hydrolyzes membrane phospholipids and initiates phosphatidylinositol signalling (Slusarski et al., 1997). Consequently, the second messengers di-acyl glycerol (DAG) and inositol 1,4,5-triphosphate (IP3) are released, the former of which directly activates PKC. IP3, however induces the release of Ca\(^{2+}\) from intracellular stores through an interaction with the SERCA-ATPase pump at the endoplasmic reticulum membrane (Jafri and Keizer, 1995) and ultimately also activates PKC. Effects downstream of PKC activation include cell motility, morphogenesis, apoptosis, cytoskeletal changes and differentiation (Dissanayake et al., 2007; Weeraratna, 2005). PKC may also act in a positive feedback loop with Wnt5a (Jonsson et al., 1998).
Proper orientation and polarity of cells are crucial features that involve coordinated movements throughout development. Planar cell polarity is the polarization of fields of cells within a plane perpendicular to the apical-basal axis (Klein and Mlodzik, 2005). In Drosophila PCP may mediate the arrangement of photoreceptor cells and cuticular hairs by coordinating cytoskeletal changes while in vertebrates, convergent extension (CE), which is homologous to PCP, is implicated in gastrulation to elongate columns of mesodermal and neuroectodermal cells (Prunier et al., 2004). CE is also shown to be involved in mammalian skin and inner ear epithelia arrangement (Prunier et al., 2004). In PCP, intracellular signalling downstream of Fz includes cytoplasmic components that associate to the membrane during signalling (Seifert and Mlodzik, 2007). Core components encompass the membrane spanning receptors Fz, Flamingo (Fmi) and Strabismus (Stbm) along with Dishevelled (Dsh), Diego (Dgo), and Prickle (Pk) (Strutt, 2003). In Drosophila, PCP signalling leads to the asymmetric rearrangement of the core components. While Fmi and Dgo localize proximally and distally, Fz and Dsh localize distally, and Pk and Stbm localize proximally (Widelitz, 2005). In other words, Fz-Dsh-Dgo and Stbm-Pk complexes antagonize each other, in which Fz-Dsh-Dgo act to positively promote Dsh downstream effectors and Stbm-Pk limit Dsh activation (Seifert and Mlodzik, 2007). As Fmi is found both proximally and distally, it is thought to stabilize the complexes through homophilic cell-adhesion behaviour (Usui et al., 1999). While emerging evidence indicates that PCP components in vertebrates are also asymmetrically distributed, the precise patterns appear to differ (Wang and Nathans, 2007). It remains to be clarified whether Wnt proteins are instructive or permissive for PCP signalling (Klein and Mlodzik, 2005). In addition, as Dsh is also a member of the canonical signalling cascade, it remains unclear whether the manner through which Fz and Dsh interact is pathway specific (Klein and Mlodzik, 2005). Interestingly, the Formin homology domain protein Daam1 is proposed to bridge Dsh and Rho GTPase and Daam1 has been shown to be required for
Xenopus Fz-mediated Rho activation (Habas et al., 2001). In any case, the cascade downstream of Dsh is thought to involve small GTPases of the Rho subfamily, the Rho-associated kinase (dROK), the STE20-like kinase Misshapen (Msn), and the JNK-type MAPK cascade (Mlodzik, 2002). Depending on the context, the readouts for PCP differ. For example, in the fly eye, JNK/p38-type MAPK modules and Jun-Fos (AP-1) are activated downstream of Dsh and Rho GTPases (Boutros et al., 1998; Weber et al., 2000). On the other hand, in Drosophila, dROK activation in the wing has been shown to affect cytoskeletal rearrangements in hair formation (Winter et al., 2001).

### 1.1.4.2 Canonical

In response to Wnt proteins such as Wnt1, Wnt3a and Wnt8 (Weeraratna, 2005), canonical signalling focuses largely on the stabilization of the protein β-catenin. β-catenin, or armadillo in Drosophila, was originally identified as a segment polarity gene (Perrimon and Mahowald, 1987) and is characterized by multiple armadillo repeats, each of which comprises 42 amino acids that arrange into a dense alpha helical array (Huber et al., 1997). In addition to possibly facilitating nuclear localization, the armadillo repeats allow β-catenin to functionally associate with various proteins, such as Axin, APC, TCF and cadherins (Funayama et al., 1995; Huber et al., 1997; von Kries et al., 2000). Both the N- and C-terminals contain transcriptional activation domains and the N-terminus also carries Ser/Thr residues, which regulate degradation (Hsu et al., 1998; Lustig and Behrens, 2003; van de Wetering et al., 1997; van Es et al., 2003). β-catenin is a component of the adherens junctions as an essential binding partner to cadherins (Peifer et al., 1993). As a result, β-catenin is dynamically involved in both cell adhesion and transcription. It is suggested that the function of β-catenin in transcriptional activation may be promoted at the cost of its role in the adherens junctions (Brembeck et al., 2006). The balance between the functions
may be mediated by several mechanisms, such as tyrosine phosphorylation and protein conformation (Brembeck et al., 2006).

1.1.4.2.1 Cytoplasmic β-catenin destruction complex

In the absence of canonical Fz-LRP5/6 signalling, cytoplasmic β-catenin levels are regulated by a destruction complex (Fig. 1). Within the destruction complex, β-catenin is phosphorylated by the Ser/Thr kinases, glycogen synthase kinase 3β (GSK3β) (Yost et al., 1998) and casein kinase Iα (CKIα) (Amit et al., 2002; Liu et al., 2002), whose interactions are made possible through the scaffolding proteins Axin and APC (Hart et al., 1998; Kishida et al., 1998). Phosphorylated β-catenin is then recognized by β-TrCP, a component of the E3 ubiquitin ligase protein, and targeted for ubiquitination and subsequently degraded by the proteosome (Aberle et al., 1997; Liu et al., 1999a). The phosphorylation events by GSK3 and CK1α in the destruction complex are essential for efficient β-catenin regulation. It has been shown that β-catenin mutants lacking the phosphorylation sites lack Wnt responsiveness and induce constitutive Wnt signalling (Munemitsu et al., 1996).

Many recent studies have sought to clarify the mechanism by which Fz-LRP5/6 co-receptor activation leads to β-catenin destruction complex deactivation. Dsh, or Dvl in vertebrates, a component of non-canonical signalling, is also a crucial component of canonical signalling. Upstream of the destruction complex, Dvl is a cytosolic phosphoprotein that can associate with the Fz receptor and is phosphorylated in a Wnt-dependent manner (Sun et al., 2001; Wong et al., 2003). Upon ligand binding (Fig. 1B), Dvl is recruited to the Fz receptor, which then leads to the disruption of the β-catenin destruction complex and ultimately promotes Wnt signalling (Logan and Nusse, 2004). Dvl is thought to be regulated by Naked (Nkd) (Rousset et al., 2001; Wharton
et al., 2001), a segment polarity gene originally characterized in Drosophila (Jürgens et al., 1984; Zeng et al., 2000). Another protein whose function seems critical to canonical signalling activation is Axin. Studies suggest that like Dvl, Axin may also be recruited to the membrane, to associate with the LRP5/6 receptor upon Wnt ligand binding (Cliffe et al., 2003; Tamai et al., 2004). Axin was originally described as an inhibitor of Wnt signalling (Zeng et al., 1997) and indeed Axin overexpression results in an increase in β-catenin degradation (Behrens et al., 1998; Hart et al., 1998). It is suggested that Axin may be a limiting component of the Wnt signalling cascade, presumably because its cellular levels are lower than those of the other proteins involved (Lee et al., 2003).

The current model mechanism by which Wnt protein binding to Fz-LRP5/6 receptors initiates and propagates canonical signalling involves a series of events at the membrane. The Wnt-induced Fz-LRP5/6 co-receptor complex recruits Dvl (Cadigan and Liu, 2006; Wong et al., 2003) and in turn, Fz is thought to promote Dvl phosphorylation by the kinase PAR-1 (Sun et al., 2001; Takada et al., 2005). The highly related Casein kinase I family members, CKIδ and CKIε, are also thought to phosphorylate Dvl in response to Wnt signal (Gao et al., 2002; Price, 2006). Concurrently, the LRP5/6 co-receptor is phosphorylated by the kinases GSK3 and CK1γ (Davidson et al., 2005; Zeng et al., 2005). The phosphorylation events at the LRP5/6 cytoplasmic tail then facilitate recruitment of Axin towards the membrane, which thus sequesters Axin away from the destruction complex (Clevers, 2006). At the plasma membrane, Dvl and Axin may associate and cooperate to propagate signalling by inhibiting proper assembly of the β-catenin destruction complex. Dvl may also dephosphorylate and destabilize Axin through an associated protein phosphatase 2C (PP2C) (Strovel et al., 2000). Dvl-Axin associations have been reported (Cliffe et al., 2003; Smalley et al., 1999) but because of varying
immunoprecipitation results, it has been suggested that the interaction may require additional partners (Schwarz-Romond et al., 2007).

### 1.1.4.2.2 Nuclear activities

The Wnt-induced disruption of the destruction complex leads to β-catenin stabilization and within 2 to 3 hours of ligand addition, cytoplasmic β-catenin levels can dramatically increase (Labbe et al., 2007; Papkoff, 1997; Peifer et al., 1994). Through interactions with the nuclear pore machinery, β-catenin freely diffuses into the nucleus (Fagotto et al., 1998) to interact with lymphoid enhancer binding factor1/T-cell specific factor (LEF1/TCF) DNA-binding proteins (Korinek et al., 1997; Molenaar et al., 1996). Additionally, β-catenin nuclear accumulation may be facilitated through interactions with Rac1, a member of the Rho family of small GTPases that contains a nuclear localization signal sequence (Esufali and Bapat, 2004; Lanning et al., 2003). In the absence of nuclear β-catenin, LEF1/TCF proteins act as Wnt target gene repressors by complexing with Groucho (Brannon et al., 1997; Cavallo et al., 1998), which mediates interactions with histone deacetylases (HDAC) to render DNA unavailable for transcriptional activation (Chen et al., 1999). Nuclear β-catenin displaces Groucho (Daniels and Weis, 2005) from interacting with LEF1/TCF protein and initiates transcriptional activation by recruiting the histone acetylase cyclic AMP response element-binding protein (CBP/p300) (Hecht et al., 2000) and Brg-1, the switching-defective and sucrose nonfermenting (SWI/SNF) chromatin remodelling component (Barker et al., 2001). Transcriptional activation has also been shown to require the proteins Legless (Lgs), Pygopus (Pygo) and hyrax/parafibromin (Kramps et al., 2002; Mosimann et al., 2006). While in the nucleus, β-catenin may be inhibited from activating Wnt target genes by direct interactions with proteins such as Chibby (Takemaru et al., 2003) and ICAT (Tago et al., 2000). LEF1/TCF may additionally be regulated by the mitogen-activated...
protein (MAP) kinase-related protein kinase NLK/Nemo, which itself is regulated by the MAP kinase kinase, TAK1 (Ishitani et al., 1999).

1.1.4.2.3  Wnt target genes and feedback loops

The loss of a Wnt gene can lead to diverse phenotypes ranging from truncated limbs to delayed maturation of synapses in the cerebellum (Logan and Nusse, 2004). Even within the same structure, Wnt signalling may elicit different biological processes, such as proliferation and differentiation processes within the intestinal crypt (Reya and Clevers, 2005). Rather than being signal specific, the distinct transcriptional outputs in Wnt signalling are cell specific. Studies, most of which were conducted with cancer cell lines, have identified a wide array of Wnt target genes such as Cyclin D1, cMyc, VEGF and engrailed (Vlad et al., 2008). Efforts towards consolidating findings to generate a Wnt targetome (Vlad et al., 2008) have emphasized the diversity of events downstream of Wnt signalling.

While a universal Wnt target gene has not been identified, classes of target genes may exist (Logan and Nusse, 2004). Of these, one that has garnered much interest is the class of Wnt-induced Wnt pathway components. In vertebrates, a well described Wnt target gene is Axin2 (Jho et al., 2002), a member of the β-catenin destruction complex. In Drosophila, the Dvl antagonist Nkd has been described as a Wnt-target gene (Rousset et al., 2001). The E3 ubiquitin ligase, β-TrCP has also been found to be upregulated in response to Wnt signalling (Spiegelman et al., 2000). As Wnt antagonists, Axin2, Nkd, β-TrCP and others are described as upregulated genes that serve in a negative feedback loop in canonical Wnt signalling. On the other hand, Drosophila DFz2 and Fz receptor genes have been reported to be downregulated in response to Wnt signalling, to effectively inactivate further signalling (Cadigan et al., 1998; Muller et al.,
Together, the controlled expression of these genes highlights the sophisticated autoregulatory system that has evolved in Wnt signalling.

1.2 Wnt signalling in development and disease

1.2.1 Drosophila development

The function of Wnt, or Wg in *Drosophila*, as a segment polarity gene was first characterized by genetic screening for zygotic lethal mutations that affected larval cuticle patterning (Nusslein-Volhard and Wieschaus, 1980). Through a series of epistasis experiments, the upstream and downstream relationships between major Wg signalling components have been uncovered. *Drosophila* studies have also enabled the discrimination between inhibitory and stimulatory activities (Noordermeer et al., 1994; Siegfried et al., 1994).

After cellularization of the blastoderm embryo (stages 5-7), gap and pair rule transcription factors sequentially act to initiate activity of segment polarity genes, which spatially and temporally specify body segmentation (Nusslein-Volhard and Wieschaus, 1980). Segment polarity genes encode components of Wg and Hedgehog (Hh) signals, while pair rule genes act to impart competence to alternating cell territories to initiate *wg* and *hh* transcription and produce Wg or Hh (Cadigan et al., 1994; Martinez Arias et al., 1988). Consequently, Wg and Hh are expressed in alternating segments of narrow stripes of cells along the embryo (DiNardo et al., 1994).

During ectodermal patterning, Wg serves in two distinct processes. From stages 8-11, Wg maintains the transcription of *hh* and *engrailed* (*en*) in the two to three cells posterior to the Wg-producing cells (Hatini and DiNardo, 2001). Therefore, the width of these bands of cells is
indicative of a Wg signalling gradient. The posterior border of each band of cells serves as a segmentation boundary, which then serves as a marker for neuronal and muscular processes (Larsen et al., 2003). Beyond stage 11, ventral Wg producing cells suppress the synthesis of cell protrusions, termed denticles, and appear naked instead. Concurrently, ventral cells distant from Wg producing cells secrete a belt-like array of denticles, which facilitate larval movement. As a result, definitive segmentation is marked by alternating ventral territories of denticle synthesis and naked cuticle secretion (Bejsovec and Martinez Arias, 1991; Dougan and DiNardo, 1992). Larval cuticle patterning thus serves as a readout for Wg signalling in Drosophila, in which defective signalling results in a ‘lawn of denticles’ while enhanced signalling, due to mutations in negative regulators such as Axin and GSK3, gives rise to an increased secretion of ‘naked cuticle’ (Hamada et al., 1999; Nusslein-Volhard and Wieschaus, 1980; Siegfried et al., 1992; Siegfried et al., 1994). It should be noted that in addition to Wg, dynamic combinations of Hh, Notch, and EGF signals also interplay to influence the fate of each cell (Bejsovec and Wieschaus, 1993; Hatini and DiNardo, 2001; Payre et al., 1999).

1.2.2 Xenopus development

Studies in Xenopus uncovered contributions of Wnt signalling during the earliest stages of development. During vertebrate development, including that of Xenopus, canonical signalling is implicated in establishing the dorsoventral axis and later, in patterning the anteroposterior axis (Wodarz and Nusse, 1998). In the developing embryo, a dorsal region termed the Spemann organizer works to coordinate major axis and body organization (De Robertis et al., 2000). The Spemann organizer is comprised of cells that secrete growth-factor signalling antagonists into extracellular space to block signalling through cognate receptors (De Robertis et al., 2000). Stabilized β-catenin is involved in activating the homeobox genes Siamois and Twin, which
induce the activation of signalling molecules and transcription factors in the Spemann organizer (Laurent et al., 1997; Lemaire et al., 1995). During the early *Xenopus* blastula stage as the organizer develops, β-catenin accumulates in the dorsal cytoplasm and nuclei of the blastula. The nuclear accumulation of β-catenin sends signals to neighbouring cells to establish dorsoventral identity (Wylie et al., 1996).

Interestingly, injecting Wnt-1 into ventral blastomeres of early embryos results in duplication of the body axis (McMahon and Moon, 1989), while depleting β-catenin from frog oocytes using antisense morpholinos results in embryos lacking dorsal structures, such as the notochord, somites and nervous system (Heasman et al., 2000). The axis duplication assay in *Xenopus* has become a well versed tool that has aided in classifying Wnt proteins into groups that might employ different signal transduction mechanisms. For example, Wnt-1 was soon categorized into the XWnt-8 class, which induce axis duplication while members of the XWnt-5A class were those that were unable to induce a secondary axis (Wodarz and Nusse, 1998). When downstream components, such as Dvl and a dominant negative GSK3, were overexpressed, embryonic axis duplication was also induced (Dominguez et al., 1995; Sokol, 1996). Further studies in frog oocytes have shown that canonical components are required for proper axis formation and gene expression. Together, the studies of *Drosophila* and vertebrate components in the *Xenopus* system have highlighted the level of conservation in Wnt signalling from flies to mammals (Wodarz and Nusse, 1998).

### 1.2.3 Zebrafish development

In Zebrafish, both maternal and embryonic Wnt signalling are implicated in dorsal axis specification. From ferialization to 3 hour post fertilization (hpf), maternal canonical signalling
serves to establish the dorsal organizer and is active up to when the embryo first becomes transcriptionally active, a period termed mid-blastula transition (MBT) (Kimmel et al., 1995). The maternal to zygotic transition is quite drawn out, which suggests that gene products are provided maternally as well as zygotically (Kane and Kimmel, 1993). While maternal canonical signalling is active, the homeodomain transcriptional repressor protein Bozozok (Boz) and the FGF antagonist MKP3 protein are induced (Koos and Ho, 1998; Tsang et al., 2004; Yamanaka et al., 1998). Boz is then required to induce the expression of *goosecoid* (*gsc*) and *chordin* (*chd*), markers of the dorsal organizer (Fekany et al., 1999; Miller-Bertoglio et al., 1997; Schulte-Merker et al., 1994; Shimizu et al., 2000).

At 5 hpf, or 30% epiboly, zygotic canonical signalling commences and results in ventrolateral expression of *wnt8* (Ramel and Lekven, 2004). The zygotically expressed Wnt8 in the ventrolateral margin then restricts lateral *gsc* and *chd* expression in the dorsal organizer (Erter et al., 2001; Fekany-Lee et al., 2000; Ramel and Lekven, 2004). As a result, at 5.5 hpf (50% epiboly), dorsal expression of *gsc* and *chd* are a product of balancing positive influences from maternal signalling and negative influences from zygotic signalling (Bellipanni et al., 2006). Later at 7 hpf (60% epiboly), ventrolateral zygotic Wnt-8 expression is implicated in anterior-posterior neural patterning (Erter et al., 2001; Lekven et al., 2001). Through manipulating maternal and zygotic signalling and studying resulting dorsal organizer expression patterns, it is thus possible to use Zebrafish embryos to investigate canonical signalling (Van Raay et al., 2007).

### 1.2.4 Mammalian development

Studies concerning canonical signalling in the mammalian system have been largely conducted in the mouse system. In mammals, body axis and germ layer specifications are secondary to
implantation, in contrast to non-mammalians, in which the processes are primary events (Marikawa, 2006). Mammalian developmental studies suggest that canonical Wnt signalling is not essential until after implantation (Kemler et al., 2004), despite the pre-implantation expression of Wnt signalling components (Hamatani et al., 2004). However, it has been suggested that maintenance of embryonic stem (ES) cells derived from the blastocyst inner cell mass may involve the activation of canonical signalling, as evidenced by enhanced ES cell renewal in the presence of a GSK3 inhibitor (Sato et al., 2004), but it remains unclear whether β-catenin is involved (Huelsken et al., 2000).

Studies suggest that β-catenin is essential in the epiblast for the development of the distal visceral endoderm along the proximal-distal and anterior-posterior axes and its subsequent anterior migration. This is thought to be effected through regulating the expression of target molecules, such as the Nodal co-receptor Cripto, to control anterior movements of the visceral endoderm (Ding et al., 1998; Huelsken et al., 2000; Varlet et al., 1997). Studies have also suggested that inhibition of the canonical pathway is required for directional movement of the visceral endoderm (Kimura-Yoshida et al., 2005).

At the onset of gastrulation, cytoplasmic β-catenin is enriched and accumulates in spatially distinct distributions coincident with where the primitive streak emerges (Mohamed et al., 2004). The cells emigrating through the primitive streak ultimately give rise to the definitive endoderm and mesoderm (Marikawa, 2006). During gastrulation, many Wnt proteins including Wnt3, Wnt5a and Wnt8a, are expressed similarly in the primitive streak, posterior visceral endoderm and migrating mesoderm (Kemp et al., 2005; Kispert et al., 1996; Liu et al., 1999b; Yamaguchi et al., 1999). Loss of function studies reveal that canonical signalling is essential to primitive streak formation as evidenced by null mutant embryos for Wnt3 or β-catenin that fail to form the
primitive streak or undergo gastrulation (Huelsken et al., 2000; Liu et al., 1999b). Gain of function studies that mimicked excessive canonical signalling, on the other hand, resulted in expansion and disorganization of primitive streak derivatives and premature mesoderm formation (Kepler et al., 2004).

Following gastrulation, the anterior visceral endoderm and mesoderm, which derive from the primitive streak, are implicated in the formation of anterior structures. Studies suggest that inhibition of the canonical Wnt pathway is essential for the formation of the anterior neural ectoderm (Marikawa, 2006). Interestingly, the anterior visceral endoderm and mesoderm express several secreted Wnt antagonists such as Dkk and sFRPs (Glinka et al., 1998; Kemp et al., 2005), suggesting a requirement for proper regulation. Ectopic activation of canonical signalling has been shown to consistently suppress the formation of anterior structures in addition to inducing partial axis duplication. Indeed, axial duplications were observed in mutant embryos harbouring mutations in the canonical negative regulator, Axin (Zeng et al., 1997). Embryos in which canonical signalling was mildly activated, such as APC hypomorphic mutants or TCF3 mutants, also resulted in expansion or duplication of axial structures (Ishikawa et al., 2003; Merrill et al., 2004).

Mouse studies using transgenic and knockout mice have enabled the study of previous observations from lower organisms in a more complex system. Indeed, the mouse system has proven more complex with the existence of functional redundancy between components, as illustrated by comparing double knockout to single knockout mice phenotypes. For example, while single knockout animals for the LRP5 and LRP6 receptors are viable and exhibit defects in bone and eye development or other development deficiencies (Kato et al., 2002; Pinson et al., 2000), LRP5-LRP6 double knockout animals fail to form a primitive streak and die during
gastrulation (Kelly et al., 2004). With the advent of new approaches, including spatial and temporal control over gene expression and new imaging technologies, the mouse model offers an opportunity to study Wnt signalling at later stages of development through to adult life (van Amerongen and Berns, 2006).

1.2.5 Disease and cancer

Unregulated Wnt signalling has been implicated in a number of diseases and cancers in the adult system (Logan and Nusse, 2004). Loss of function mutations in Wnt components, $FZD4$ and $WNT3$ for example, have been associated with diseases and abnormalities such as retinal angiogenesis defects (Robitaille et al., 2002) and tetra-amelia, a human genetic disorder characterized by the absence of limbs (Niemann et al., 2004). Interestingly, activating mutations in $LRP5$, which renders insensitivity to the Dkk inhibitor and thus enhances canonical signalling, leads to increased bone density (Boyden et al., 2002) while deactivating mutations in $LRP5$ results in decreased bone density (Gong et al., 2001). Together, these effects illustrate the necessity of properly regulated Wnt signalling.

Adenomatous polyposis coli, or APC, was originally characterized for its role in the hereditary cancer syndrome termed Familial Adenomatous Polyposis (FAP), in which numerous polyps form in the epithelium of the large intestine (Kinzler and Vogelstein, 1996; Nishisho et al., 1991). When APC was later found to be a scaffolding member of the $\beta$-catenin destruction complex, the overlap between Wnt signalling and human cancer research began (Rubinfeld et al., 1993; Su et al., 1993). Studies uncovered that FAP patients inherited one defective $APC$ allele and developed polyps in early adulthood. The polyps, of which some would progress into malignant adenocarcinoma, were a result of epithelial cells in which the second $APC$ allele was inactivated. The inherited defective $APC$ allele was found to give rise to truncated forms of APC,
whose interactions with β-catenin is abrogated. The loss of interaction leads to inappropriate stabilization of β-catenin, which ultimately results in aberrant canonical Wnt pathway activation (Kinzler and Vogelstein, 1996). It is suggested that in APC mutant cancer cells, β-catenin may be constitutively active and associated with the intestinal TCF family member Tcf4, as evidenced by TCF reporter assays (Korinek et al., 1997). Although rare, colorectal cancer cases have also been reported in which mutations in APC are not present, but instead mutations in AXIN1, AXIN2 or β-catenin are described (Liu et al., 2000; Morin et al., 1997; Satoh et al., 2000). Notably, patients with hereditary AXIN2 mutations seem to have a predisposition to colorectal cancer in addition to tooth agenesis (Lammi et al., 2004). Aberrant Wnt pathway activation in cancers outside of the intestine has also been reported. Loss of function mutations in Axin have been reported in hepatocellular carcinomas while β-catenin mutations have been found in a variety of solid tumours, such as desmoid tumours (Reya and Clevers, 2005; Tejpar et al., 1999).

Generally, tumour-causing mutations in APC, AXIN1, AXIN2 and β-catenin are reported to result in β-catenin stabilization and inappropriate activation of Wnt target genes, such as myc and cyclin D1 (He et al., 1998; Rubinfeld et al., 1997; Shtutman et al., 1999). In further support of the correlation between tumourogenesis and activated canonical signalling, inactivating mutations in canonical pathway inhibitors, such as sFRP or Dkk, have also been reported in tumours (Caldwell et al., 2004; Sato et al., 2007; Suzuki et al., 2004). Additionally, epigenetic inactivation of the pathway inhibitors Dkk and WIF-1 by promoter hypermethylation have also been correlated with cancers (Aguilera et al., 2006; Taniguchi et al., 2005).

Not surprisingly, diagnostics and therapeutics for conditions related to aberrant Wnt signalling have garnered much attention since the link with cancer was uncovered. For instance, studies citing a correlation between Wnt antagonist expression and tumours have emerged. Specifically,
human colon cancer cell lines and tissues were shown to have an increased expression of *AXIN2* and *hNKD1* that corresponded to APC truncation mutation occurrence (Yan et al., 2001b). Another group also reports elevated levels of *hNKD1* and *β-TrCP* in malignant liver tumors (Koch et al., 2005). Taken together, it is suggested that these genes may serve as possible diagnostic markers for activated Wnt signalling (Koch et al., 2005; Yan et al., 2001b). Therapeutically, much interest has been invested in developing Wnt signalling inhibitors to target the pathway directly or indirectly (Klaus and Birchmeier, 2008). In 2004, a high-throughput screen reported that fungal derivatives were found to suppress the transcriptional activity of β-catenin by targeting the interaction between TCF4 and β-catenin (Lepourcelet et al., 2004). Another group characterized a compound from a screen that was capable of inhibiting Wnt transcriptional activity by binding to a β-catenin transcriptional partner (Emami KH, 2004). A structure-based screen looked at compounds capable of binding Dvl, which also disrupted the Dvl-Fz interaction needed for pathway activation (Shan et al., 2005). Similarly, antibody-based therapies to target molecules, such as Wnt2 and Fzd10, that are overexpressed in disease have been studied (Nagayama et al., 2005; You et al., 2004). The use of Wnt inhibitors, like sFRPs, is also being tested in tumour models (DeAlmeida et al., 2007). Moreover, oncolytic viruses to restrict TCF-β-catenin association are being developed and studied (Chen and McCormick, 2001; Malerba et al., 2003).

1.3 Naked in the canonical Wnt Pathway

1.3.1 Protein function and interactions

The segment polarity gene *Naked (Nkd)*, or *Naked cuticle (dNkd)* in *Drosophila*, was first characterized during a large-scale screen developed to assess zygotic mutations that altered the
morphology of *Drosophila* embryonic cuticle patterning (Jürgens et al., 1984). Early studies with Nkd were performed in the *Drosophila* and the *nkd* phenotype was described as an embryonic lethal recessive zygotic mutation that resulted in multiple segmentation defects, including the replacement of denticles by excess secreted naked cuticle (Zeng et al., 2000). It is worth noting that *nkd* mutants in these studies were generated via P-element-mediated transformation to result in truncated, but not total loss of, *nkd* transcript. The strongest phenotype was presented by *nkd* mutants that expressed a residual Nkd protein of the first 59 amino acids and secreted cuticles with a fully exteriorized head skeleton, displayed residual denticle belts and were less than 75% of wild-type length (Zeng et al., 2000). Because the *nkd* phenotype resembled that of embryos exposed to excess Wg (Noordermeer et al., 1992), Nkd was presumed to act as an antagonist in Wg signalling (Zeng et al., 2000). Indeed the *nkd* phenotype could be rescued to wild-type by *nkd* cDNA overexpression (Zeng et al., 2000) or injection of GSK3 mRNA, a Wg antagonist (Rousset et al., 2001). While the method by which Nkd antagonizes canonical Wnt signalling is not clear, inferences have been made based on binding partners and functional assays.

In *Drosophila*, Nkd function was found to be cell autonomous and Nkd overexpression suppressed the stimulatory effects of excess Wg or Dsh, but not those of β-catenin overexpression (Rousset et al., 2001). Yeast two-hybrid (Y2H) and GST pull-down experiments revealed that Nkd interacts with Dsh (Rousset et al., 2001), an interaction that was recapitulated using vertebrate orthologs (Wharton et al., 2001; Yan et al., 2001a).

Originally identified in the fly, mutations in the *dishevelled* (*dsh*) gene were observed to yield improper wing and body hair orientation (Perrimon and Mahowald, 1987). Studies in *Drosophila* propose that Dsh may act at the decisive branchpoint between canonical and non-canonical Wnt signalling (Axelrod et al., 1998; Boutros et al., 2000). In canonical signalling,
hyperphosphorylated dishevelled leads to the disruption of the destruction complex through an
unknown mechanism and ultimately activates signalling (Clevers, 2006). While there is one Dsh
form in the *Drosophila*, there are three vertebrate Dishevelled isoforms termed Dvl1, Dvl2 and
Dvl3 (Lee et al., 2008; Malbon and Wang, 2006; Wharton, 2003). All Dsh/Dvl proteins share
three regions of homology: an N-terminal Dishevelled and Axin (DIX) binding domain (Kishida
et al., 1999b), an internal Post-synaptic density-95 Discs-large and Zonula occludens-1 (PDZ)
domain (Wong et al., 2003) and a downstream Dishevelled EGL-10 Pleckstrin (DEP) domain
(Wong et al., 2000) (Fig. 15B). The PDZ domain and short adjacent basic region on Dishevelled
have been shown to interact with Nkd. Studies suggest that Nkd can bind to inactivate
Dishevelled in canonical signalling (Rousset et al., 2001; Rousset et al., 2002; Yan et al., 2001a).

Two *Nkd* genes have been identified in vertebrates. *Nkd1* and *Nkd2* share sequence similarity
with *Drosophila dNkd* and alignments show two major regions of homology: an internal single
EF-hand motif and a C-terminal histidine-rich region (Fig. 2) (Katoh, 2001; Wharton et al.,
2001). The EF-hand is a common calcium-binding motif characterized by a helix-loop-helix
secondary structure. The opening of the EF-hand exposes a hydrophobic surface, which may
bind to target sequences to achieve tasks such as cytosolic metal buffering or signal transduction
between cellular compartments (Lewit-Bentley and Rety, 2000). Most often, EF-hands occur in
even numbered clusters, whereby pairings may functionally lead to positive cooperation between
components (Gifford et al., 2007).

A function has not been characterized for the histidine-rich C-terminal region on Nkd, but the
EF-hand and an adjacent downstream sequence, together termed EFX, is thought to bind
Dsh/Dvl, as shown by binding assays (Rousset et al., 2002; Wharton et al., 2001). When the
Figure 2: Invertebrate and vertebrate Naked.

A schematic representation of Naked isoforms is shown. The myristoylation (M), EF-hand (EF) and histidine-rich (H) domains are marked. Numbering refers to corresponding amino acids in indicated isoforms.
dNkd EFX region is deleted, the interaction with Dsh is abrogated and the ability for dNkd to antagonize Wg signalling is lost (Rousset et al., 2002). Interestingly, the dNkd-Dvl interaction was found to be Zn^{2+} dependent, and not Ca^{2+} dependent, as would be expected (Rousset et al., 2002). Immunoprecipitation assays in mammalian cells, however, suggest that the EF-hand is not required to interact with Dvl1, Dvl2 or Dvl3 (Yan et al., 2001a). Furthermore, it was shown that while EF-hand point mutations in mNkd1 impair the ability to antagonize a canonical Wnt-responsive reporter, the mutations do not affect the mNkd1-mDvl interaction (Yan et al., 2001a). This suggests that the Nkd-Dvl interaction alone is not sufficient for Nkd to inhibit canonical signalling.

Nkd1 and Nkd2 are thought to be functionally equivalent, as suggested by overexpression and morpholino experiments in Zebrafish (Van Raay et al., 2007), and indeed both Nkd1 and Nkd2 can interact with Dvl (Wharton et al., 2001; Yan et al., 2001a). However most functional studies presented have focused on Nkd1. Vertebrate Nkd1 and Nkd2 contain an N-terminal consensus sequence that directs the post-translational addition of a myristate moiety (Fig. 2) (Chan et al., 2007; Wharton et al., 2001; Yan et al., 2001a). Lipid modifications, such as myristoylation, are important for regulating membrane targeting, trafficking, and signalling (Resh, 2006). Nkd2 has been shown to be myristoylated (Li et al., 2004) and this myristoylation is believed to be required for the proper delivery and fusion of transforming growth factor alpha (TGFα) containing vesicles to the lower lateral membrane of polarized cells (Li et al., 2007). Nkd2, and not Nkd1, can recognize TGFα (Li et al., 2004) but whether Nkd1 is myristoylated and what role this modification could play has yet to be formally shown. Interestingly, it has been shown that while dNkd does not contain a myristoylation site, the N-terminus can confer membrane association different from that of myristoylated proteins (Chan et al., 2007). In the Drosophila,
dNkd staining is predominantly cytoplasmic and weakly nuclear (Waldrop et al., 2006). Further studies reveal that dNkd contains two nuclear localization sequences (NLS), one of which interacts with Importin-α3, a nuclear import adaptor (Chan et al., 2008; Waldrop et al., 2006). The interaction promotes nuclear localization, but it is not clear what role dNkd may have in nucleus (Chan et al., 2008). A similar NLS is predicted in vertebrate Nkd (Waldrop et al., 2006), but whether vertebrate Nkd enters and acts in the nucleus is not known. To date, the subcellular distribution of endogenous and overexpressed Nkd1 has not been reported.

Nkd1 has been shown to interact with PR72 and PR130, which are regulatory B subunits of protein phosphatase type 2A (PP2A). As evidenced by *Xenopus* second body axis assays, PR72 is required for the inhibitory action of Nkd1 (Creighton et al., 2005) while PR130 overexpression counters Nkd1 activity and activates canonical signalling (Creighton et al., 2006). The mechanisms by which the PP2A subunits regulate Nkd and canonical signalling are unclear.

Early experiments in the fly demonstrate that dNkd overexpression can abolish Dsh activity in planar cell polarity (non-canonical signalling), as shown through wing hair polarity defects (Rousset et al., 2001). In the vertebrate, however, it is suggested that mNkd acts as a switch between canonical and non-canonical signalling by regulating Dvl activities (Yan et al., 2001a). This is further supported by observations in which c-Jun-N-terminal kinase activity, a downstream readout of PCP signalling, was enhanced in response to mNkd overexpression (Yan et al., 2001a). On the other hand, a recent study in Zebrafish suggests that Nkd1 and Nkd2 antagonize both canonical and non-canonical signalling (Van Raay et al., 2007).

Mouse studies suggest that Nkd may be dispensable for embryonic development (Li et al., 2005; Zhang et al., 2007). In one study, mice homozygous for an internal mutation in *Nkd1*, in which
the EFX domain was deleted, had decreased fertility, reduced testis size and a defect in sperm maturation (Li et al., 2005). In contrast, another study performed with mice homozygous for a Nkd1 allele in which the residual protein was truncated upstream of the EFX domain reported reduced litter sizes, shortened nasal bones and no obvious changes in spermatogenesis (Zhang et al., 2007). Together, these studies suggest that the sperm maturation defect may be due to regions outside of the EFX domain. When mice were homozygous for truncating mutations in both Nkd1 and Nkd2, it was observed that the mice exhibited altered cranial bone morphology, in addition to reduced fertility and increased cannibalization of litters (Zhang et al., 2007). Interestingly, the cranial phenotype is similar to that of mice with mutations in Axin2, an inducible antagonist of canonical signalling (Yu et al., 2005).

1.3.2 Expression and regulation

In normal human tissues, Northern blot analyses suggest that hNKD1 is highly expressed in fetal kidney, fetal lung, placenta, adult liver, kidney, pancreas, spleen, testis, heart, and brain (Katoh, 2001; Wharton et al., 2001; Yan et al., 2001a). Human NKD2 is upregulated in fetal kidney, fetal lung, and adult lung (Katoh, 2001). In 8.5 days postcoitum (d.p.c.) mouse embryos, mNkd1 is expressed in the neural folds and by 9.5 d.p.c., gene expression is detected in the forelimb buds and somite boundaries (Wharton et al., 2001). At 10.5 d.p.c., mNkd1 is expressed in the branchial arches and later, in the endocardial cushion, pulmonary epithelium, renal mesenchyme and hair follicles (Wharton et al., 2001).

Studies in cultured cells and vertebrate embryonic node and somites suggest that Nkd1 expression can be induced by canonical signalling (Schmidt et al., 2006; Yan et al., 2001a). In fact, elevated Nkd1 expression is reported in tumours in which canonical Wnt signalling may be
constitutively active (Koch et al., 2005; Yan et al., 2001b). Together, these findings support a role for Nkd1 as an inducible regulator in canonical signalling through a feedback loop.

1.4 Thesis perspective

Protein-protein interactions (PPIs) are essential components of cellular signalling. Screens conducted with the aim to detect interactions have provided a wealth of insight into signalling pathways. Originally, such interaction screens were conducted using yeast two-hybrid (Y2H) assays (Stelzl and Wanker, 2006). While Y2H screens have proven successful in detecting interactions, unless yeast proteins are studied, they are limited to detecting PPIs outside normal cellular environments. As such, approaches to study PPIs in mammalian systems have a greater potential to gain functional knowledge and insight into cellular signalling (Stelzl and Wanker, 2006). In addition, it is now possible to perform other high throughput (HTP) screens, such as RNA interference (RNAi) screens (Moffat and Sabatini, 2006; Perrimon and Mathey-Prevot, 2007) to gain further insight into signal transduction. To study Wnt signalling, Dr. Bryan Miller from our lab recently conducted a series of HTP screens in mammalian cells including PPI, RNAi and protein overexpression. The work presented in this thesis pertains to the data obtained from this screen, integration of results and subsequent follow up studies. Specifically, the work presented focuses largely on the protein Nkd1, which was included in the HTP screens. Previous work with Nkd has been predominantly performed in Drosophila and has characterized Nkd as an antagonist in canonical Wnt signalling (Chan et al., 2007; Chan et al., 2008; Rousset et al., 2001; Zeng et al., 2000). We thus analyzed vertebrate Nkd1 through functional studies and examined Nkd1 interacting partners, both previously described and novel. Overall, the objective of the work presented in this thesis is to gain insight into the role that Nkd1 may have in canonical Wnt signalling in mammalian cells.
2 Materials and Methods

2.1 Mammalian expression constructs

Dr. Bryan Miller generously provided the following constructs from the screen library: hNKD1-FFLuc, mNkd2-FFLuc, DVL2-FFLuc, DVL3-FFLuc, Axin1-FFLuc, β-catenin-FFLuc, 3xFlag-hNKD1, 3xFlag-mNkd2, 3xFlag-hNKD2short, 3xFlag-DVL1, 3xFlag-DVL2, 3xFlag-DVL3, 3xFlag-Axin1, 3xFlag-SMURF1 CA, 3xFlag-SMURF1 WT, 3xFlag-SMURF2 CA, 3xFlag-SMURF2 WT, 3xFlag-β-catenin, 3xFlag-GNGT1, 3xFlag-GNGT2, Renilla luciferase (RLuc) – SMURF1, RLuc-SMURF2, 3xFlag-PLEKHA2, 3xFlag-PLEKHB1, 3xFlag-PARD6. The 3xFlag-PAK1 construct was obtained from Ms. Monika Podkowa. Constructs hNKD1-3xFlag, mNkd2-3xFlag and hNKD2short-3xFlag were provided by Dr. Ainhoa Letamendia. All constructs were in a pcMV5c backbone. The Topflash and Fopflash reporters in pGL3 (pOT) were obtained from B. Vogelstein.

To generate 3xFlag-hNKD2, RNA was extracted from MCF7 cells and reverse transcribed using random hexamers (Fermentas) and RervertAid™ H Minus M-MuLV Reverse Transcriptase (Fermentas). From the obtained cDNA, hNKD2 encoding nucleotides 775-1356 were then generated by PCR to include a stop codon followed by a BamHI restriction site. Nucleotides 1-790 were amplified from 3xFlag-hNKD2short and included an N-terminal HindIII restriction site. There is an internal HinfI restriction site in hNKD2 beginning at nucleotide 776. PCR product containing nucleotides 1-790 was digested with HindIII (Fermentas) and HinfI (Fermentas) while product containing nucleotides 775-1356 was digested with BamHI (Fermentas) and HinfI (Fermentas). The digested products were then subjected to a three-way ligation with T4 DNA Ligase (Fermentas) into the HindIII and BamHI sites of the pcMV5c
vector containing the 3xFlag epitope at the N-terminus. The hNKD1ΔH-FFLuc construct was generated using primers to amplify sequence encoding amino acids 1-454 of hNKD1 from hNKD1-3xFlag and inserted with MluI and NotI restriction sites into a pCMV5c backbone containing a C-terminal FFLuc encoding region.

Fly and vertebrate Nkd were aligned and vertebrate Nkd1 and Nkd2 domains were characterized based on previously outlined domains in *Drosophila* (Rousset et al., 2002). Corresponding hNKD1 and mNkd2 deletion mutants were generated via PCR with primers at corresponding amino acids using 3xFlag-hNKD1 and 3xFlag-mNkd2 as templates and inserted into a pCMV5c backbone containing an N-terminal 3xFlag via HindIII and BamHI restriction sites.

Untagged hNKD1 was generated by inserting full length hNKD1 as amplified by PCR from 3xFlag-hNKD1 into empty pCMV5c backbone via HindIII and BamHI restriction sites. A start codon was added. The hNKD1-HA construct was generated by amplifying hNKD1 from hNKD1-3xFlag and inserting into the pCMV5b backbone containing a C-terminal HA tag. A consensus start sequence was added and subcloning used restriction sites EcoRI and HindIII. The mNkd1-3xFlag construct was generated by amplifying mNkd1 from MGC clone 40995 (Accession No. BC034838) and cloned into the pCMV5c backbone containing a C-terminal 3xFlag tag. A consensus start sequence was added and insertion was done via restriction sites MluI and NotI. The 3xFlag-mNkd2 chimera was generated by PCR using the 3xFlag-mNkd2 template. The forward primer included a HindIII restriction site. The reverse primer (GGC GGA TCC CTA TGT CTG GTA GAA GTG GTG GTA ATG GTG GTG GTG GTG CT) included a BamHI restriction site, a stop codon and the last 21 nucleotides from hNKD1, designed to replace the last 27 nucleotides from the mNkd2 template. The PCR product was then digested
and inserted into the pCMV5c backbone containing an N-terminal 3xFlag tag with restriction sites BamHI and HindIII.

### 2.2 Cell culture and transfection

Human Embryonic Kidney (HEK) 293T cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Hyclone) and transfected by calcium phosphate DNA precipitation method, as previously described (Wang et al., 2006) or by using 1.5 µl Lipofectamine2000 (Invitrogen) per 0.8 µg of cDNA per well of a 24-well dish, according to manufacturer’s recommendations. Briefly, for transfecting one well of a 24-well dish using calcium phosphate DNA precipitation method, 0.67 µg of DNA (including expression and control vectors) was resuspended in 22.5 µl of water, to which 2.5 µl of 2.5 M CaCl$_2$ was added. Next, 25 µl of 2x HeBs buffer (16.4 g NaCl, 11.9 g HEPES acid, 0.21 g Na$_2$HPO$_4$ in 1000 ml distilled H$_2$O) was added with bubbling and vortexed. After incubation at room temperature for 15-20 minutes, 50 µl of complexes were applied to 30-40% confluent cells containing 500 µl of growth medium. Medium was changed 24 hours post-transfection. MDA MB 231 cells were cultured in RPMI supplemented with 5% FBS and siRNA was transfected using 2 µl of Dharmafect reagent #4 (Dharmacon) per 25 pmol of siRNA per well of a 12-well dish, according to manufacturer’s recommendations. MCF7 cells were maintained in DMEM supplemented with 10% FBS and 1% non-essential amino acids (NEAA, Gibco). Wnt3a expressing L-cells (Labbe et al., 2007) were maintained in DMEM containing 10% FBS and 0.4 mg/ml G418 (Gibco). RKO human colon carcinoma cells stably expressing a firefly-luciferase based β-catenin-activated reporter and *Renilla* luciferase under the control of a constitutively active thymidine kinase were generously provided by S. Angers (Major et al., 2007). RKO cells were grown in DMEM supplemented with
10% FBS and 2 µg/ml puromycin (Sigma) and transfected by using 0.75 µl Lipofectamine2000 (Invitrogen) per 20 nM of siRNA per well of a 24-well dish according to manufacturer’s recommendations. All cells were cultured at 37°C in a humidified 5% CO₂ incubator.

2.3 Production of Wnt3a conditioned medium

Using pPGK-neo/Wnt3a (Shibamoto et al., 1998), mouse L-cells that stably expressed mouse Wnt3a were generated (Labbe et al., 2007). Control or Wnt3a conditioned medium was collected from confluent cells that were cultured at 37°C in a humidified 5% CO₂ incubator for 3 days in DMEM supplemented with 0.2% FBS, 1% penicillin/streptomycin and 0.2% Amphotericin B. Collected medium was centrifuged to remove cell debris and stored at 4°C.

2.4 LUMIER assay

The LUminescence-based Mammalian IntERactome (LUMIER) assay (Fig. 3A) was used to detect protein-protein interactions (Barrios-Rodiles et al., 2005). HEK293T cells were transiently transfected by calcium phosphate DNA precipitation method with 0.14 µg ‘bait’ firefly luciferase- (FFLuc-) tagged protein and 0.14 µg 3xFlag-tagged ‘prey’ protein along with 0.025 µg pCMV5/β-galactosidase reporter and pCMV5 empty vector to a total of 0.67 µg DNA per well in a 24-well dish. Medium was changed 24 hours post-transfection and incubated overnight. Cells were washed with 0.2% FBS DMEM and lysed in cell lysis buffer (250 µl for each well, 50 nM Tris-HCl, 150 nM NaCl, 1 mM EDTA, 0.1% Triton X-100, 10% glycerol) containing protease inhibitors. Lysates were immunoprecipitated (150 µl) with anti-Flag M2 monoclonal antibody (1:1000, Sigma) and collected using Protein G Sepharose 4 Fast Flow (GE Healthcare). Associated FFLuc was detected by luciferase assay. To quantify luciferase activity, the luciferase
assay system (Promega) was used. In this system, luciferase substrate (25 µl) was added to an aliquot (10 µl) of total or purified cell lysate. The resulting reaction with luciferase yielded light, which could be measured by the EG&G Berthold microplate luminometer. Pre-immune total lysates and immunoprecipitates were separated by SDS-PAGE and prey protein expression was verified by anti-Flag (1:3000, Sigma) immunoblotting. Luciferase activity was normalized to β-galactosidase levels from pre-immune total lysates.

2.5 Topflash reporter assay

The Topflash reporter (Korinek et al., 1997) contains three LEF1/TCF binding sites upstream of a promoter that drives the firefly luciferase gene, the activity of which can be quantified (Fig. 3B). The Fopflash reporter, which is used as a control, is identical to the Topflash reporter except for mutated LEF1/TCF binding sites (Labbe et al., 2007). The Topflash reporter is dependent on β-catenin levels and is thus Wnt responsive. HEK293T cells were typically transiently-transfected with 0.05 µg Topflash/Fopflash reporter, 0.02 µg pCMV5/β-galactosidase reporter, 0.1 µg of indicated pCMV5/3xFlag constructs, 20 nM of siRNA (if indicated) and pCMV5 empty vector to a total of 0.67 µg DNA per well in a 24-well dish by calcium phosphate DNA precipitation method. After 24 hours, cells were starved with 0.2% FBS DMEM and treated overnight (16 hours) with control or Wnt3a conditioned medium. Cells were lysed in Lux lysis buffer (25 mM Tris-phosphate, 2 mM DTT, 2 mM DCTA, 10% glycerol and 1% Triton X-100) 40 hours post-transfection. Luciferase activity was quantified and normalized as previously described. M2 anti-Flag (1:3000, Sigma) anti-hNKD1 (1:2500, #2262, Cell Signalling) immunoblotting was performed to verify construct expression.
Figure 3: Schematic of methodology.

(A) In the LUMIER assay, a firefly luciferase-tagged protein (bait) is co-expressed with a 3xFlag-tagged partner (prey). After stimulation and lysis, the prey is immunoprecipitated using anti-Flag antibodies and the presence of bait is determined by luciferase assay. Barios-Rodiles, M et al (2005). (B) The Wnt-responsive Topflash luciferase reporter contains three LEF1/TCF optimal promoter sites upstream of the Firefly luciferase gene, the activity of which can be quantified. Nuclear β-catenin associates with LEF1/TCF to promote luciferase transcription. Luciferase activity can be used to gage relative amounts of β-catenin, and thus activation of canonical Wnt signalling.
Where indicated, anti-actin (1:3000, Sigma) immunoblotting was also performed as a loading control. Figures show representative data that has been repeated 2-3 times with indicated ‘n’ individual samples.

2.6 siRNA assays

siRNA oligonucleotides designed to target internal hNKD1 sequences were purchased from Dharmacon as a pool of four duplexes and as four individual duplexes (Dharmacon product number MU-018984). For HEK293T cells, the duplexes were transfected at a concentration of 20 nM by calcium phosphate DNA precipitation method. After 24 hours, cells were washed with DMEM supplemented with 0.2% FBS and treated overnight with control or Wnt3a conditioned medium. For MDA MB 231 cells, the duplexes were transfected with Dharmafect reagent #4 (Dharmacon) at 25 nM. After 48 hours, cells were washed with DMEM supplemented with 0.2% FBS and treated with control or Wnt3a conditioned medium for the indicated time. For control, the following siCTL 21mer was designed and ordered from Dharmacon: GGGAAGACGGGAGGdTdT.

2.7 Real time quantitative reverse transcription PCR (QPCR)

HEK293T cells plated in 12-well dishes were transiently-transfected with 20 nM siRNA by calcium phosphate DNA precipitation method. After 24 hours, cells were disrupted and RNA was isolated using the RNeasy Mini Kit (Qiagen) according to manufacturer’s instructions. RNA samples were quantified on a spectrophotometer (Eppendorf) and 1 µg of each was treated with treated with 1 unit of DNaseI (Fermentas), primed with Random Hexamers (1 µl of 100 µM stock, Fermentas) and reverse transcribed using 200 units of RevertAid™ H Minus M-MuLV Reverse Transcriptase (Fermentas). Following reverse transcription, quantitative PCR was
performed using SYBR Green PCR Master Mix (Applied Biosystems) on the ABI Prism 7000 or 7900 sequence detection system (Applied Biosystems) and primers at 100 nM for \textit{hNKD1} (forward: TGA GAA GAA GAT GGA GAG AG T GAG C GA, reverse: GGT GAC CTT GCC GTT GTT GTC AAA) and \textit{GAPDH} (forward: ACA TCA AGA AGG TGG TGA AGC AGG, reverse: ACG AAT TTG GCT ACA GCA ACA GGG, (Labbe et al., 2007)). MDA MB 231 cells plated in 12-well dishes were transiently transfected with 25nM siRNA with Dharmafect #4 (Dharmacon) according to manufacturer’s recommendations. After 48 hours, cells were rinsed with DMEM supplemented with 0.2% FBS and treated with control or Wnt3a conditioned medium for 2 or 4 hours, as indicated. RNA was collected and treated as described above. Quantitative PCR was performed using primers for \textit{hNKD1}, \textit{GAPDH} and \textit{Axin2} (forward: AGA AAT GCA TCG CAG TGT GAA G, reverse: GGG TTC TCG GGA AAT GAG GTA, (Labbe et al., 2007)). Relative quantitation was calculated by the $\Delta\Delta C_t$ method normalized to GAPDH levels (docs.appliedbiosystems.com/pebiodocs/04303859.pdf). Figures show representative data that has been repeated 2-3 times, with the exception of Fig. 13D.

2.8 Immunoprecipitation and immunoblotting

HEK293T cells were transfected by calcium phosphate DNA precipitation method. Briefly, for a 100 mm dish, 10 µg of DNA (2.25 µg hNKD1-HA, 1.5 µg 3xFlag-Axin1 topped off with empty vector pCMV5) complexes were applied to 30-40% confluent cells containing 7 ml of growth medium. Medium was changed 24 hours post-transfection with prewarmed fresh medium. Cells were lysed in cell lysis buffer 40 hours post transfection. For each 100 mm dish, 1 ml of lysis buffer was applied to cells. Cell lysates were then subjected to immunoprecipitation with M2 anti-Flag antibodies (1:1000, Sigma) for one hour, followed by incubation with Protein G Sepharose 4 Fast Flow (GE Healthcare) an additional hour. Protein complexes were washed 3
times and resuspended with TNTE lysis buffer (0.5% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl). The beads were then boiled in SDS loading buffer and samples were run on 8% SDS-PAGE gel and transferred to nitrocellulose membrane. After blocking the membranes with 5% milk in TBST, immunoblotting was carried out using polyclonal goat anti-HA (Y11) antibodies (1:1000, Santa Cruz) and monoclonal M2 anti-Flag (1:3000, Sigma) antibodies.

2.9 Immunofluorescence

HEK293T cells were plated onto poly-L-Lysine (PLL) coated plates and transfected by Lipofectamine 2000 (Invitrogen) according to manufacturer’s recommendation. Forty hours post transfection, cells were washed once with pre-warmed PBS, fixed with 4% paraformaldehyde for 10 minutes, permeabilized with 0.25% Triton X-100 for 2 minutes, and blocked for 10% heat-inactivated goat serum for 1 hour. Cells were then incubated with M2 anti-Flag (1:350, Sigma) or anti-hNKD1 (1:150, Cell Signalling) antibodies at room temperature. After 5 washes, cells were stained with Alexa Fluor 488 goat anti-mouse or anti-rabbit antibodies (1:1000, Molecular Probes) for 1 hour at room temperature, protected from light. Concurrently, cells were stained for F-actin with rhodamine-conjugated phalloidin (1:250, Molecular Probes). Following another 5 washes, cells were stained briefly with DAPI for 5 minutes and washed 5 additional times. All antibodies and fluorescent stains were prepared in 10% heat-inactivated goat serum in PBS. All washes were done with PBS. Epifluorescent images were captured with a Zeiss Axiovert 200 inverted microscope and analyzed with Volocity software (Improvision).
3 Results

3.1 The hNKD1 and mNkd2 interactomes

While canonical Wnt signalling has been heavily studied in recent years, many aspects of the pathway remain unknown. For example, it is unclear how phosphorylated Dvl leads to the disruption of the destruction complex and how, in turn, Dvl may be regulated by Nkd (Clevers, 2006; Wharton, 2003). To this end, Dr. Bryan Miller from our lab recently conducted a series of high throughput (HTP) screens to investigate Wnt signalling. The screens were conducted in mammalian cells and included protein-protein interaction (PPI) mapping along with systematic functional analysis through cDNA overexpression and RNA interference (RNAi). The PPI screen sought to identify novel interactions in Wnt signalling while the functional screens sought to identify novel pathway mediators. Of particular relevance to this thesis, human Nkd1 (hNKD1) and mouse Nkd2 (mNkd2) were included in these screens.

For the PPI screen, 11 Firefly Luciferase (FFLuc) -tagged bait proteins were co-transfected with a library of 640 3xFlag-tagged prey proteins. The baits were comprised of known Wnt signalling components, including Nkd1 and Nkd2, while the library of preys consisted of proteins enriched in cellular signalling. The LUMIER (LUminescence-based Mammalian IntERactome) assay (Fig. 3A) was performed to determine interactions (Barrios-Rodiles et al., 2005). In LUMIER, HEK293T cells are co-transfected with a FFLuc-tagged bait and an individual 3xFlag-tagged prey in each well of a 96-well dish. Lysates are subjected to anti-Flag immunoprecipitation and the presence of FFLuc is detected by Luciferase assay. To define a Wnt interactome, interactions were identified as combinations in which the fold change over plate median, or mLIR (median-based Luminescence Intensity Ratio), was equal to or exceeded 2. The subinteractomes for
human Nkd1 (hNKD1) and mouse Nkd2 (mNkd2), extracted from the LUMIER-derived full Wnt interactome, are shown in figures 4A and 4C, respectively. It is interesting to note the difference in number of interacting partners between the interactomes. Specifically, the interactome for Nkd1 displays a much greater number of interacting partners than does the interactome for Nkd2.

As integration of diverse data sets can enhance selection of novel signalling components, the PPI data was complemented by functional screens. In parallel to the PPI screens, Dr. Miller also performed HTP functional screens, in which the effect of modulating the expression of specific genes by cDNA overexpression or siRNA mediated knockdown on Wnt signalling was examined. For this, signalling was measured using the Topflash transcriptional reporter, which contains 3 multimerized LEF1/TCF binding sites upstream of a promoter that drives luciferase expression in response to Wnt3a (Fig. 3B) (Korinek et al., 1997; Labbe et al., 2007). HEK293T cells were transiently-transfected with the Topflash reporter with 3xFlag-tagged constructs or siRNAs directed to endogenous genes. Following treatment with control or Wnt3a conditioned medium, cells were lysed. Cell lysates were then subjected to the luciferase assay to measure Topflash activity. In all screens, luciferase activity was normalized for transfection efficiency with a coexpressed β-galactosidase reporter gene. Normalized fold-over-median Topflash screen data for hNKD1 and mNkd2 are shown in figure 4E.

The PPI and functional screens were next integrated by first converting raw experimental results into normalized log intensities. A rigorous Z-score transformation was thus applied to raw data sets from replicate runs from each screen. For each Z-score, a numerical P-value was derived for each gene. P-values for each gene from each replicate run were then combined using Fisher’s method. The combined P-values were then thresholded at the lower limit of $10^{-15}$. For each
screen, a confidence score between 0 and 15 was calculated by taking the negative log\(_{10}\) of the combined P-value. The final P-value is a measure of confidence that the experimentally observed effect deviated significantly from control. In the case of the screen, the plate median served as the control. For LUMIER, the P-values were calculated for observed effects above plate medians, while the cDNA overexpression and siRNA screen P-values considered effects above and below plate medians. For the functional screens, the largest value achieved in the absence or presence of Wnt ligand was considered. Finally, confidence scores for each protein in each screen were added to give a final combined pathway score (CPS), a theoretical measure of likelihood that a certain protein was a component of canonical signalling. As such, if a gene achieved the maximal score of 15 in all three screens, then the maximal CPS of 45 could be achieved and would indicate that the gene is very likely a component of canonical Wnt signalling. Since the maximal score of each screen is 15, then CPS close to 15 may reflect a very strong hit in a single screen. The tables in figures 4B and 4C list the top 15 proteins according to hNKD1 or mNkd2 LUMIER P-values. Corresponding P-values for the signalling assays are listed, as well as CPS values. With the final integration of the PPI and functional screen data sets, previously described and novel signalling components and regulatory networks could be confidently identified. For instance, strong CPS values were obtained for known canonical pathway members such as DVL2 (CPS = 21.31) and Axin1 (CPS = 12.91). In addition, hNKD1 LUMIER P-Values for previously published interactions (Yan et al., 2001a) were also strong, as seen with DVL1 (6.58), DVL2 (4.42) and DVL3 (8.96).
Figure 4: The hNKD1 and mNkd2 interactomes as defined by LUMIER.

HEK293T cells were transiently-transfected with (A) hNKD1- or (C) mNkd2-FFLuc baits and a library of 3xFlag-tagged preys. The LUMIER assay was performed on cell lysates to identify binding partners. The 15 top hits as determined by (B) hNKD1 and (D) mNkd2 LUMIER P-Values. Corresponding P-values for RNAi and Topflash screen results and Combined Pathway Scores (CPS) are listed. (E) Averages of normalized Topflash reporter data from the RNAi and cDNA overexpression HTP screens are indicated. Normalized data represents fold over plate median values. A value of 1 indicates that no change was observed. Subinteractomes and data were provided by Dr. Bryan Miller.
Figure 4
3.1.1 Manual verification of PPI identified by LUMIER

In order to verify whether PPIs identified in the screen were experimentally reliable, the interactions of hNKD1 and mNkd2 with protein partners displaying a range of mLIR values were verified by manual LUMIER (Fig. 5, 6 and 7). In all cases, lysates from HEK293T cells transiently-transfected with FFLuc- or Renilla Luciferase (RLuc)-tagged baits with the indicated 3xFlag-tagged proteins were subjected to an anti-Flag immunoprecipitation and the presence of FFLuc/RLuc was detected by luciferase assay. Total protein expression was verified by anti-Flag immunoblotting. For comparison, proteins, such as GNGT1, which did not interact with Nkd in LUMIER were also tested, and consistent with the HTP assay, did not score positively in manual retesting (Fig. 7B). The fold change over control values from the manual LUMIER assays were then compared with screen mLIR values for 28 distinct protein pairs (Fig. 7B and 7C). In comparing manual fold change over control values with screen mLIR values, it can be seen that the interactions detected by the PPI screen could be manually repeated. For example, we re-tested hNKD1 interactions with hNKD1, PLEKHA2 and GNGT1, which had diverse mLIR values of 34.09, 2.93, and 0.77, respectively (Fig. 5 and 7). The manual LUMIER repeats yielded corresponding fold change over manual values of 80.14, 6.10 and 1.36, respectively. Since manual LUMIER repeats for Nkd protein partners with lower mLIR values also gave low manual fold over control values, it is likely that the screen had a low frequency of false negatives. The screen was also successful at identifying previously described interactions, such as the Nkd-Dvl interaction (Yan et al., 2001a), as evidenced by a high hNKD1 mLIR value for DVL3 (19.07), which was manually repeated to yield a strong corresponding fold change over control value (76.05). Moreover, the previously unpublished hNKD1-Axin1 interaction, with an mLIR of 7.23, was also recapitulated manually with a high fold change over control value (28.08).
Figure 5: Validation of Nkd binding partners by LUMIER.

HEK293T cells were transiently-transfected with (A) hNKD1-FFLuc or (B) mNkd2-FFLuc and 3xFlag-tagged constructs. Cell lysates were subjected to anti-Flag immunoprecipitation (IP) and associated hNKD1- or mNkd2-FFLuc was detected by measuring luciferase activity. Luciferase activity was normalized to β-galactosidase activity (expressed as average +/- range, n=2). Total hNKD1-FFLuc and mNkd2-FFLuc expression were measured from total cell lysates (Totals) and levels of 3xFlag constructs were confirmed by anti-Flag immunoblotting.
**Figure 6: Validation of Nkd binding partners by LUMIER**

HEK293T cells were transiently-transfected with (A) Axin1-FFLuc, (B) β-catenin-FFLuc, (C) RLuc-SMURF1 or (D) RLuc-SMURF2 and 3xFlag-Nkd. Cell lysates were subjected to anti-Flag immunoprecipitation (IP) and associated FFLuc/RLuc protein was detected by measuring luciferase activity. Luciferase activity was normalized to β-galactosidase activity (expressed as average +/- range, n=2). Total FFLuc/RLuc expression was measured from total cell lysates (Totals) and levels of 3xFlag constructs were confirmed by anti-Flag immunoblotting.
Figure 7: Validation of Nkd binding partners by LUMIER

HEK293T cells were transiently-transfected with (A) hNKD1-FFLuc and 3xFlag-Nkd constructs. Cell lysates were subjected to anti-Flag immunoprecipitation (IP) and associated hNKD1-FFLuc was detected by measuring luciferase activity. Luciferase activity was normalized to β-galactosidase activity (expressed as average +/- range, n=2). Total hNKD1-FFLuc expression was measured from total cell lysates (Totals) and levels of 3xFlag constructs were confirmed by anti-Flag immunoblotting. Comparisons of screen mLIR (median LUMIER Intensity Ratio) versus manual fold change over control values for (B) hNKD1 and (C) mNkd2 are listed. The mLIR data was provided by Dr. Bryan Miller.
Figure 7
Taken together, the process of manually verifying Nkd interactions by LUMIER proved successful, which suggests that PPIs detected by HTP screen were experimentally reliable. Because the mechanism by which Nkd antagonizes canonical Wnt signalling is poorly understood, the data collected from the HTP screens provides the opportunity to gain further comprehension. The Nkd protein partners identified by the PPI screen will be especially valuable towards gaining insight into the role that Nkd may hold in canonical signalling.

3.2 Overexpressed hNKD1 blocks Wnt3a-dependent activation of the Topflash reporter.

Analysis of results shown in the data tables in figures 4B, 4D and 4E suggests that hNKD1 plays an important role in Wnt signalling. For instance, hNKD1 interacts with DVL, a key component of canonical signalling (Clevers, 2006) and modulating hNKD1 levels by cDNA overexpression and siRNA results in observable effects on Topflash reporter activity. Most studies previously focused on the role of Nkd in *Drosophila*, and more recently in Zebrafish, therefore, we sought to study the effects of Nkd1 in canonical Wnt signalling in a mammalian system.

In vertebrates, Nkd1 and Nkd2 are the orthologs of *Drosophila* Nkd (dNkd). Nkd1 and Nkd2 have been previously reported as functionally equivalent (Van Raay et al., 2007), but the normalized functional screen data (Fig. 4E) indicated that they behaved differently. Specifically, normalized cDNA expression data revealed that hNKD1 has a more pronounced effect than mNkd2 on the Topflash reporter, as evidenced by a decrease versus no change/slight increase in reporter activity, respectively. This difference is reflected in the cDNA overexpression P-values (Fig. 4B and 4D) for hNKD1 (3.73) and mNkd2 (1.28). These screen results were confirmed in manual assays (data not shown, but see Fig. 8 and 11 below). To confirm that the differential
effect was not due to species differences, we cloned hNKD2 and transiently-transfected HEK293T with the Topflash reporter in the presence of empty vector (CTL), N-terminally 3xFlag-tagged hNKD1 (3xFlag-hNKD1) or N-terminally 3xFlag-tagged hNKD2 (3xFlag-hNKD2). Cells were then treated for 16 hours with control- or Wnt3a-conditioned media and Topflash reporter activity was measured by luciferase assay. We found that when overexpressed, 3xFlag-hNKD1 blocked Wnt3a-induced Topflash reporter activity, as observed by a decrease in luciferase activity (Fig. 8A). In contrast, overexpressed 3xFlag-hNKD2 had minimal effects on Topflash reporter activity as compared to 3xFlag-hNKD1. Together, this suggests that hNKD1 and hNKD2 may affect canonical Wnt signalling differently, with overexpressed hNKD1 resulting in stronger antagonistic effects.

N-myristoylated Nkd2 has been shown to be involved in trafficking Transforming Growth Factor alpha (TGFα) to the basolateral plasma membrane of polarized epithelial cells (Li et al., 2004). Generally N-myristoylation occurs during protein synthesis. After the initiating methionine is removed, myristate is linked through an amide bond to the N-terminal glycine (Resh, 2004). As such, it is expected that introduction of an N-terminal tag should prevent myristoylation. Although it has not been reported that Nkd1 is myristoylated, as Nkd1 also contains the consensus site for myristoylation (Wharton et al., 2001; Yan et al., 2001a), we next tested the effects of tagging hNKD1 N- or C-terminally. If the putative myristoylation site at the N-terminus of hNKD1 was involved in canonical signalling, then we would expect to see a difference in Topflash reporter activity between the differentially tagged forms. In fact, we found that overexpression of either N- or C-terminally 3xFlag-tagged hNKD1 in HEK293T cells blocked Wnt3a-dependent activation of the Topflash reporter similarly (Fig. 8B).
Figure 8: Overexpressed hNKD1 blocks Wnt3a-dependent activation of the Topflash reporter.

HEK293T cells were transiently-transfected with a Wnt-responsive reporter (Topflash) with empty vector (CTL) or (A) hNKD1-3xFlag or hNKD2-3xFlag, (B) amino- or carboxy-tagged hNKD1, (C) untagged hNKD1 or (D) increasing amounts of 3xFlag-hNKD1 DNA from 0 to 0.28ug per well of a 24-well dish. Topflash activity was measured by luciferase assay. Luciferase activity was normalized to β-galactosidase activity (expressed as average +/- range for n=2). Total protein expression was determined by anti-Flag or anti-hNKD1 immunoblotting.
This suggests that the putative myristoylation site is not required for hNKD1 repressive activity. To confirm that tagging hNKD1 did not affect signalling, we also tested untagged hNKD1 and observed that overexpressed untagged hNKD1 also blocked Topflash reporter activity in HEK293T cells (Fig. 8C). In addition, co-transfecting HEK293T cells with the Topflash reporter and increasing amounts of 3xFlag-hNKD1 suggests that repression by hNKD1 is dose-dependent (Fig. 8D). Together, these results show that overexpression of hNKD1 blocks Wnt3a-dependent activation of the Topflash reporter in mammalian cell culture, which is in agreement with previous vertebrate studies (Wharton et al., 2001; Yan et al., 2001a), and that this overexpression effect is not strictly dependent on the N-terminus. Moreover, we also show that overexpressed Nkd2 does not block the Topflash reporter to the same extent as Nkd1.

3.3 Determination of regions on hNKD1 required for blocking Wnt3a-dependent activation of the Topflash reporter.

Overexpressed hNKD1 blocks Wnt3a-dependent activation of the Topflash reporter, thus we next investigated whether specific regions of Nkd1 were required for this effect. Based on domains previously delineated in *Drosophila* (Rousset et al., 2002), we generated a series of N- and C-terminally truncated 3xFlag-hNKD1 constructs (Fig. 9). To study the effects of these constructs, HEK293T cells were transiently-transfected with the Topflash reporter together with empty vector (CTL), full length 3xFlag-hNKD1 (WT) or the truncated versions of 3xFlag-hNKD1 (Fig. 10A). Following Wnt3a treatment, cells were lysed and lysates were subjected to luciferase assay to determine Topflash reporter activity. Expression of hNKD1 constructs was verified by anti-Flag immunoblotting.
A schematic representation of hNKD1 and mNkd2 deletion constructs is shown. The EF hand (EF), ‘X’, ‘Y’ and histidine-rich (H) domains are marked. Numbering refers to corresponding amino acids in indicated isoforms.
Figure 10: The N-terminal and C-terminal regions of hNKD1 are not sufficient to block Wnt3a-dependent activation of the Topflash reporter to the extent of wild type hNKD1.

HEK293T cells were transiently-transfected with a Wnt-responsive reporter (Topflash) with empty vector, (A) single 3xFlag-hNKD1 constructs or (B) pairs of 3xFlag-hNKD1 constructs. Topflash activity was measured by luciferase assay. Luciferase activity was normalized to β-galactosidase activity (expressed as average +/- range, n=2). Total protein expression was determined by anti-Flag immunoblotting. (C) Schematic of the 3xFlag-hNKD1 construct pairings used in the Topflash reporter assay.
Figure 10
Results revealed that all of the truncations yielded versions of hNKD1 that were compromised in the ability to block the Wnt3a-dependent activation of the Topflash reporter. Specifically, if the reduction in Topflash activity for WT hNKD1 was set to 100%, none of the truncation mutants was able to reduce Wnt3a dependent reporter activity by greater than 53%. This included hNKD1 constructs 119-471 and 1-454, which lack only the N-terminus or short histidine-rich tail respectively, suggesting that both amino- and carboxy-terminal regions of hNKD1 are required for the repressive effect on Topflash reporter activity.

We next examined whether the repressive effect of Nkd1 on the Topflash reporter effect could be rescued by co-expression of two complementary truncations, such that the domains missing from one construct would be compensated by the domains expressed by the second construct. HEK293T cells were transiently-transfected with the Topflash reporter with empty vector or pairs of constructs with either overlapping or non-overlapping domains (Fig. 10B and 10C). We observed that co-expression of hNKD1 truncated constructs was insufficient to restore the full repressive activity of WT hNKD1.

It has been suggested that Nkd specifically binds and thereby inhibits Dsh/Dvl, an activator of canonical signalling (Rousset et al., 2001; Yan et al., 2001a). Further, results from binding assays suggest that the EF-hand and downstream sequence on Nkd interacts with Dsh/Dvl to mediate repressive activity (Rousset et al., 2001; Wharton et al., 2001). Previous work also shows that disruption of the EF-hand through point mutations prevents the antagonistic activity of Nkd1 on Wnt signalling in HEK293 cells (Yan et al., 2001a). Our Topflash reporter analysis has revealed that multiple domains, which include the EF-hand, are required for repressive activity. Specifically, it seems that both N- and C-terminal hNKD1 regions are required to block Topflash
reporter activity. Thus, this work indicates that the Nkd-Dsh/Dvl interaction is not sufficient for Nkd to block Wnt signalling.

### 3.4 Substitution of the histidine-rich tail with that of hNKD1 confers repressive activity to mNkd2 on the Topflash reporter.

Screen data and manual Topflash reporter assays (Fig. 4B, 4D and 8A) suggest that overexpressed Nkd2 is unable to block Wnt signalling to the same extent as Nkd1. Indeed, analysis (Fig. 8A) indicates that overexpression of hNKD2 has modest effects on Topflash reporter activity. To study Nkd2 further, HEK293T cells were transfected with the Topflash reporter and empty vector, 3xFlag-mNkd2 full length or truncated mutants (Fig. 11A). Consistent with the inability of hNKD2 to block Topflash reporter activity, neither wild type nor truncated versions of mNkd2 are able to block Topflash reporter activity as hNKD1 does.

Our analysis (Fig. 10) of hNKD1 truncations reveals that the hNKD1 C-terminal 18 amino acid region is required to block the Topflash reporter. Interestingly, this tail region contains a stretch of histidine residues (Fig. 11 B) that has not been functionally characterized. Since our data suggests that Nkd1, but not Nkd2, blocks canonical signalling, we next examined whether the Nkd1 histidine-rich tail, in the context of Nkd2, could confer the ability to block Topflash reporter activity. We thus generated a chimera that is comprised of amino acids 1-453 from mNkd2 followed by amino acids 465-471 from hNKD1. HEK293T cells were transiently-transfected with the Topflash reporter and empty vector (CTL), 3xFlag-hNKD1, 3xFlag-mNkd2 or 3xFlag-mNkd2/1 chimera (Fig. 11C).
Figure 11: A mNkd2 chimera expressing the poly-His domain from hNKD1 blocks Wnt3a-dependent activation of the Topflash reporter.

HEK293T cells were transiently-transfected with the Topflash reporter with empty vector (CTL) or (A) 3xFlag-mNkd2 deletion constructs or (B) 3xFlag-Nkd constructs. Topflash activity was measured by luciferase assay. Luciferase activity was normalized to β-galactosidase activity (expressed as mean +/- SD, n=3). Total protein expression was determined by anti-Flag immunoblotting. (C) Schematic of the Nkd poly-His domain. Regions of major differences in vertebrate Nkd are highlighted.
We observed that overexpression of the mNkd2/1 chimera was able to block Topflash activity similarly to hNKD1. Therefore, these results indicate that the short histidine-rich region from hNKD1 is able to confer to mNkd2 the ability to block Topflash activity. As overexpressed Nkd1 has a more pronounced effect on canonical Wnt signalling as compared to Nkd2, we focused our continuing efforts towards additional analysis of Nkd1.

3.5 siRNA mediated knockdown of hNKD1 blocks Wnt3a-dependent activation of the Topflash reporter.

Nkd is thought to function as an inducible antagonist in canonical Wnt signalling (Chan et al., 2007; Zeng et al., 2000). A previous RNAi study in Drosophila showed that when nkd double-stranded RNA (dsRNA) was injected into wild-type embryos, the majority of injected embryos developed a moderate or strong nkd phenotype (Rousset et al., 2001). In particular, the embryos developed with greatly reduced denticles (Rousset et al., 2001), a phenotype also observed in embryos exposed to excess Wg (Noordermeer et al., 1992). Indeed, our Topflash assays show that when overexpressed, hNKD1 blocks Wnt3a-dependent Topflash reporter activity (Fig. 8). In the RNAi HTP screen conducted by Dr. Miller, HEK293T cells transiently-transfected with the Topflash reporter and siRNA targeted to hNKD1 (sihNKD1), a decrease in Topflash activity was observed (Fig. 4E). Based on the in vivo studies in Drosophila, this result in mammalian cells was unexpected, so we sought to study siRNA mediated knockdown of hNKD1 further.

For this, we first validated the screen results by transiently-transfecting HEK293T cells with the Topflash reporter with control siRNA (siCTL) or the pool of 4 sihNKD1 duplexes used in the screen, in the absence or presence of hNKD1-3xFlag (Fig. 12A). If Nkd1 acts solely as an antagonist in canonical signalling, then we expect that abrogating hNKD1 expression by siRNAs
will suppress the antagonistic effects and in turn lead to an increase in Topflash reporter activity. However, consistent with screen results, we observed that sihNKD1 pool transfection blocked Topflash reporter activity, suggesting that hNKD1 may be required for canonical signalling. Anti-Flag immunoblotting with lysates from transiently-transfected HEK293T cells confirms that sihNKD1 successfully knocks down exogenously expressed hNKD1-3xFlag. Thus, both siRNA mediated knockdown of hNKD1 and hNKD1 overexpression blocks Topflash reporter activity.

To examine whether hNKD1 knockdown and overexpression results were general effects or were specific for the Wnt pathway, we employed the Fopflash reporter, which is identical to the Topflash reporter except that Fopflash contains 3 LEF1/TCF binding sites harbouring point mutations (Labbe et al., 2007) that render the reporter unresponsive to Wnt3a. HEK293T cells were transiently-transfected with the Fopflash or Topflash reporters in the presence of siCTL, sihNKD1 pool, hNKD1-3xFlag, or empty vector (Fig. 12B). We observed that transient-transfection of siCTL, sihNKD1 pool and hNKD1-3xFlag had negligible effects on Fopflash activity, suggesting that the siRNA effects are specifically associated with Wnt signalling.

We next studied the effects of siRNA mediated knockdown of hNKD1 in RKO human colon carcinoma cells that stably expressed a firefly luciferase-based β-catenin-activated reporter (pBAR) (Major et al., 2007). Because a previous study demonstrated that RKO cells do not express hNKD1 (Li et al., 2005), this was an ideal cell line to test sihNKD1 specificity. RKO cells stably expressing pBAR were transiently-transfected with siCTL, sihNKD1, siβ–catenin and siAxin1 (Fig. 12C). Transient transfection of siβ–catenin and siAxin1 were included as controls. Indeed, we observed that sihNKD1 transient transfection in RKO cells did not affect pBAR activity suggesting that sihNKD1 was specifically targeting hNKD1.
Figure 12: Knocking down hNKD1 expression by pooled siRNA transfection blocks Wnt3a-dependent activation of the Topflash reporter.

(A) HEK293T cells were transiently-transfected with the Topflash reporter with control siRNA (siCTL) or pooled siRNA directed to hNKD1 (sihNKD1) in the presence of empty vector or hNKD1-3xFlag. Topflash activity was measured by luciferase assay. Luciferase activity was normalized to β-galactosidase activity (expressed as average +/- range, n=2). Total protein expression was determined by anti-Flag immunoblotting. (B) HEK293T cells were transiently transfected with the Topflash reporter or its non-responsive mutated variant (Fopflash) with empty vector, hNKD1-3xFlag, control siRNA or pooled siRNA directed to hNKD1. Topflash and Fopflash activity was measured and normalized as above (expressed as average +/- range, n=2). Total protein expression was determined by anti-Flag immunoblotting. (C) RKO cells stably expressing a firefly luciferase-based β-catenin-activated reporter (pBAR) were transiently transfected with the indicated siRNA. The cells also stably expressed Renilla luciferase under the control of a constitutively active thymidine kinase promoter. Firefly luciferase activity was normalized to Renilla luciferase (expressed as mean +/- SEM for 3 independent experiments).
Figure 12
Figure 13: Knocking down hNKD1 expression by single siRNA duplex transfection blocks Wnt3a-dependent activation of the Topflash reporter.

(A) HEK293T cells were transiently-transfected with the Topflash reporter in the presence of control siRNA (siCTL) or siRNA directed to hNKD1 (sihNKD1). Pooled (pl) and single (1-4) duplexes were transfected as indicated. Topflash activity was measured by luciferase assay. Luciferase activity was normalized to β-galactosidase activity and corrected to empty vector transfection (expressed as mean +/- SEM for 2-3 independent experiments). (B) HEK293T cells were transiently transfected with the Topflash reporter or its non-responsive mutated variant (Fopflash) with empty vector, control siRNA or siRNA directed to hNKD1. Pooled (pl) and single (1-4) duplexes were transfected as indicated. Topflash and Fopflash activity was measured and normalized as above (expressed as average +/- range, n=2). (C) HEK293T cells were transiently-transfected with hNKD1 with control siRNA or siRNA directed to hNKD1. Expression of hNKD1 was determined by anti-hNKD1 immunoblotting of total cell lysates. (D) HEK293T cells were transiently-transfected with control siRNA or siRNA directed to hNKD1 and subjected to quantitative real-time PCR analysis of hNKD1 expression. Expression levels were normalized to GAPDH and presented as mean +/- SD.
Figure 13
Our initial signalling assays with sihNKD1 were conducted using a pool of 4 siRNA duplexes designed to target hNKD1 and so, we sought to deconvolve the pool and analyze the effects of individual sihNKD1 duplexes on the Topflash reporter. All four siRNA duplexes were designed to target regions of the hNKD1 open reading frame (ORF). HEK293T cells were transiently-transfected with the Topflash reporter in the presence of pooled or individual sihNKD1 duplexes, siCTL or hNKD1. This analysis revealed that three of the four single sihNKD1 duplexes blocked Topflash reporter activity in a manner comparable to when the duplexes were pooled (Fig. 13A). A Fopflash reporter assay confirmed that the observed effects were specific to the Wnt pathway (Fig. 13B). To determine whether the Topflash reporter effects correlated with hNKD1 knockdown, we next examined whether the individual sihNKD1 duplexes were capable of knocking down overexpressed hNKD1. Lysates from HEK293T cells transiently transfected with hNKD1, siCTL, sihNKD1 pool and sihNKD1 duplexes 1-4, as indicated, were subjected to SDS-PAGE (Fig. 13C). Anti-hNKD1 immunoblotting demonstrated that the sihNKD1 pool and individual duplexes were successful at knocking down exogenous hNKD1 expression levels. To assess the effect of sihNKD1 duplexes on endogenous hNKD1 expression, quantitative RT-PCR was performed using primers for hNKD1 and GAPDH on RNA isolated from HEK293T cells that were transiently-transfected with siCTL or sihNKD1 duplexes 1-4 (Fig. 13D). GAPDH levels were measured for normalization. QPCR data demonstrated that transfecting HEK293T with individual sihNKD1 duplexes led to a decrease in endogenous hNKD1 mRNA levels, when compared to untransfected or siCTL transfected conditions. Of note, the endogenous knockdown efficiency of individual sihNKD1 duplexes observed by QPCR paralleled the efficiency observed in overexpression knockdown analysis. Since three out of four single sihNKD1 duplexes blocked the Topflash reporter and as off-target effects are specific to each individual
siRNAs, our results are consistent with the model that in HEK293T cells, siRNA mediated knockdown of hNKD1 expression blocks Wnt3a-dependent Topflash reporter activity.

3.6 siRNA mediated knockdown of hNKD1 represses the expression of Axin2, a Wnt3a-target gene.

We next sought to determine whether the effects observed with sihNKD1 transfection were assay or cell-type specific. Previous groups, including our own, have shown that Axin2 is a Wnt3a responsive target gene (Jho et al., 2002; Labbe et al., 2007; Lustig et al., 2002; Yan et al., 2001b). Thus, we next measured Axin2 levels in MDA MB 231 cells, a cell line derived from human breast adenocarcinoma, to determine the effects of modulating hNKD1 levels on a downstream endogenous Wnt3a target gene.

Pooled siRNA duplexes present many advantages over using single duplexes including reduced off-target activity and enhanced knockdown efficiency (Straka and Boese, 2008 (online)). Our previous Topflash reporter and QPCR assays (Fig. 13) showed that three out of the four sihNKD1 duplexes blocked reporter activity and knocked down endogenous hNKD1 levels. We thus pooled these duplexes (2, 3, and 4) for subsequent assays. MDA MB 231 cells were transiently-transfected with siCTL or sihNKD1 and treated with control or Wnt3a conditioned medium for either 2 or 4 hours. Following RNA isolation and reverse transcription, QPCR was performed with primers for hNKD1, Axin2, and GAPDH (Fig. 14). Again, GAPDH levels were measured for normalization. We observe that when MDA MB231 cells were transfected with siCTL, Axin2 mRNA levels increased upon Wnt3a treatment. Similarly, we noticed a modest increase in hNKD1 mRNA levels upon Wnt3a treatment, in line with previous studies that show Nkd is induced by Wnt3a (Yan et al., 2001a; Zeng et al., 2000). Consistent with our Topflash
Figure 14: Knocking down hNKD1 expression by siRNA transfection in represses the expression of Axin2, a Wnt3a-target gene.

MDA MB231 cells were transiently-transfected with control siRNA (siCTL) or pooled siRNA directed to hNKD1 (sihNKD1). Cells were then treated with control- or Wnt3a-conditioned media for (A) 2 hours or (B) 4 hours and subjected to quantitative real-time PCR analysis of hNKD1 and Axin2 expression. Expression levels were normalized to GAPDH and presented as mean +/- SD.
reporter assays in HEK293T cells, we observed a decrease in Wnt3a induced Axin2 mRNA levels in cells transfected with sihNKD1 after 2 and 4 hour Wnt3a treatments. Basal Axin2 mRNA levels for conditions treated with control medium remained comparable and analysis of hNKD1 mRNA levels showed that endogenous hNKD1 was successfully knocked down. These results further support our hypothesis that hNKD1 is required for canonical signalling, as evidenced by results with an exogenous reporter and an endogenous target gene in two mammalian cell lines.

### 3.7 Characterizing the Nkd-Nkd interaction

Protein interactions are crucial for proper protein function. Moreover, our Topflash reporter data demonstrates that multiple domains on hNKD1 are required for the repressive effect (Fig. 10), suggesting the possibility that hNKD1 may act through more than one partner. To better understand how hNKD1 can elicit both positive and negative effects on canonical signalling, we next sought to study Nkd1 partner protein interactions.

Data from the PPI screen using hNKD1-FFLuc as bait indicated that hNKD1 may homodimerize, as denoted by a hNKD1 LUMIER mLIR value of 34.09 (Fig. 7B). As this interaction had not been previously described, we sought to characterize the hNKD1 homodimerization. Nkd contains a single EF-hand motif (Fig. 2) and EF-hand motifs are common calcium-binding motifs that often occur in even numbered clusters (Lewit-Bentley and Rety, 2000). Based on the behaviour of other EF-hand containing proteins (Gifford et al., 2007), we hypothesized that Nkd1 dimerization might occur through EF-hand pairing.

Lysates from HEK293T cells co-transfected with hNKD1-FFLuc and a series of 3xFlag-hNKD1 deletion mutants were subjected to LUMIER (Fig. 15). Anti-Flag immunoprecipitation and
subsequent luciferase assay confirmed the hNKD1 homodimerization. We observed that 3xFlag-hNKD1 mutant 1-192, which contains the ‘EFX’ region, could immunoprecipitate full length hNKD1-FFLuc. However, mutant 1-165, which contains the EF-hand but not the adjacent ‘X’ region, was unable to immunoprecipitate hNKD1-FFLuc, as evidenced by a drop in luciferase activity. Together, this data suggests that hNKD1 dimerization may occur via the ‘EFX’ region.

On the other hand, analysis of N-terminally truncated versions of hNKD1 showed that the interaction persisted in the absence of the ‘EFX’ region. The 3xFlag-hNKD1 mutant 248-471, which lacks the N-terminal region and the EF-hand, was able to immunoprecipitate full length hNKD1-FFLuc, suggesting that hNKD1 may homodimerize via a C-terminal region. Thus, either the ‘EFX’ or C-terminal region appears to be sufficient to allow hNKD1 to dimerize.

In addition to hNKD1 homodimerization, the screen data also indicated that Nkd heterodimerizes (Fig. 7B and 7C). To recapitulate this novel interaction, LUMIER was performed on lysates from HEK293T cells that were co-transfected with hNKD1-FFLuc and 3xFlag-mNkd2 deletion constructs (Fig. 16A). Similar to hNKD1 homodimerization results, we observed that 3xFlag-mNkd2 mutant 1-188, which contains the ‘EFX’ region, was able to associate with hNKD1-FFLuc. A summary of interaction mapping for hNKD1 and mNkd2 with indicated interacting partners is shown in Figure 17.

We previously showed that the hNKD1 histidine-rich tail, in the context of mNkd2, could confer the ability to block Topflash reporter activity (Fig. 11B). To examine whether this region had a role in hNKD1 homodimerization, LUMIER was performed using lysates from HEK293T cells co-transfected with 3xFlag-hNKD1 truncation mutants and hNKD1ΔH-FFLuc, which spans amino acids 1-454 followed by a firefly luciferase encoding region (Fig. 16B). We observed that hNKD1ΔH-FFLuc immunoprecipitated with C-terminally truncated 3xFlag-hNKD1 mutants
similarly to full length hNKD1-FFLuc, indicating that hNKD1 lacking the histidine-rich tail can associate with truncated NKD1 mutants retaining the ‘EFX’ region. However, our data also showed that hNKD1ΔH-FFLuc was unable to immunoprecipitate with N-terminally deleted 3xFlag-hNKD1 mutants 166-471 and 248-471, neither of which contain the ‘EFX’ region.

Thus, our LUMIER data shows that if either only the ‘EFX’ or histidine-rich region is deleted from one of the hNKD1 constructs, dimerization persists. However, a construct lacking one of the regions, such as hNKD1ΔH-FFLuc, cannot dimerize with another construct lacking the other region, such as hNKD1 mutant 166-471, which lacks the ‘EFX’ region. Altogether, this suggests that Nkd dimerizes via two distinct domains, namely the ‘EFX’ region and the C-terminal histidine-rich tail.
HEK293T cells were transiently-transfected with hNKD1-FFLuc and 3xFlag-hNKD1 constructs. Cell lysates were subjected to anti-Flag immunoprecipitation (IP) and associated hNKD1-FFLuc was detected by measuring luciferase activity. Luciferase activity was normalized to β-galactosidase activity (expressed as average +/- range, n=2). Total hNKD1-FFLuc expression was measured from total cell lysates (Totals) and levels of 3xFlag-hNKD1 constructs were confirmed by anti-Flag immunoblotting.
**Figure 16: Mapping the Nkd dimerization domain.**

HEK293T cells were transiently-transfected with (A) hNKD1-FFLuc and 3xFlag-mNkd2 constructs or (B) hNKD1ΔH-FFLuc and 3xFlag-hNKD1 constructs. Cell lysates were subjected to anti-Flag immunoprecipitation (IP) and associated hNKD1-FFLuc was detected by measuring luciferase activity. Luciferase activity was normalized to β-galactosidase activity (expressed as average +/- range, n=2). Total FFLuc expression was measured from total cell lysates (Totals) and levels of 3xFlag-Nkd constructs were confirmed by anti-Flag immunoblotting.
Figure 16
Figure 17: Naked domains and interactions.

A schematic representation of hNKD1 and mNkd2 deletion constructs (left). The EF-hand and the poly-His (H) domains are marked, along with amino acid numbering. The interactions between (A) hNKD1 and (B) mNkd2 constructs with full length hNKD1, DVL2 and Axin1, as determined by LUMIER, are summarized based on Figures 15, 16, 18, 19 (right). N.D., not determined; o, no interaction. (C) A schematic representation of hNKD1 and putative regions required to interact with hNKD1, DVL2 and Axin1.
3.8 Characterizing the Dishevelled-Nkd interaction

In canonical signalling, Dsh/Dvl functions to block phosphorylation of β-catenin, which ultimately leads to the activation of Wnt target gene transcription (Logan and Nusse, 2004). Previous groups have reported the Nkd-Dsh/Dvl interaction and it was suggested that Nkd binds to specifically inactivate Dsh/Dvl function in canonical signalling (Rousset et al., 2001; Yan et al., 2001a). Accordingly, when HEK293T cells are transiently transfected with the Topflash reporter in the presence of empty vector, 3xFlag-DVL2 or 3xFlag-hNKD1, we observed that DVL2 overexpression enhanced Topflash reporter activity and yielded a loss of Wnt3a-dependent activation (Fig. 18A). Moreover, a dampened enhancement of Topflash reporter activity was observed when 3xFlag-hNKD1 and 3xFlag-DVL2 were co-expressed.

Previous, binding assays have suggested that Nkd ‘EFX’ region interacts with the ‘PDZ’ and adjacent basic region on Dsh/Dvl (Rousset et al., 2002; Wharton et al., 2001; Yan et al., 2001a). To date, the Dsh/Dvl-Nkd interaction has been largely studied using yeast two-hybrid and GST pulldown assays (Rousset et al., 2002; Wharton et al., 2001; Yan et al., 2001a). Thus, we next mapped the regions on hNKD1 required to interact with DVL2 in a mammalian system by LUMIER using the truncated hNKD1 constructs described above (Fig. 9). Anti-Flag immunoprecipitations were performed on lysates from HEK293T cells that had been co-transfected with DVL2-FFLuc and the indicated 3xFlag-tagged hNKD1 mutants. The immunoprecipitates were then subjected to the luciferase assay to detect associated DVL2-FFLuc (Fig. 18C). The LUMIER data showed that DVL2-FFLuc and WT 3xFlag-hNKD1 co-immunoprecipitated, as evidenced by the strong luciferase activity measured from anti-Flag
**Figure 18: Mapping the DVL2 binding domain to the hNKD1 internal region.**

(A) HEK293T cells were transiently-transfected with the Topflash reporter with empty vector (CTL) or indicated 3xFlag-constructs. Topflash activity was measured by luciferase assay. Luciferase activity was normalized to β-galactosidase activity (expressed as mean +/- SD for n=3). Total protein expression was determined by anti-Flag immunoblotting. (B) A schematic of DVL is shown. The DIX, PDZ, basic and DEP regions are marked. The putative binding region for Nkd is indicated (C) HEK293T cells were transiently-transfected with DVL2-FFLuc and 3xFlag-hNKD1 constructs. Cell lysates were subjected to anti-Flag immunoprecipitation (IP) and associated DVL2-FFLuc was detected by measuring luciferase activity. Luciferase activity was normalized to β-galactosidase activity (expressed as average +/- range, n=2). Total DVL2-FFLuc expression was measured from total cell lysates (Totals) and levels of 3xFlag-hNKD1 constructs were confirmed by anti-Flag immunoblotting.
Figure 18
Figure 19: Mapping the DVL3 binding domain to the hNKD1 internal region.

HEK293T cells were transiently-transfected with DVL3-FFLuc and 3xFlag-hNKD1 constructs. Cell lysates were subjected to anti-Flag immunoprecipitation (IP) and associated DVL3-FFLuc was detected by measuring luciferase activity. Luciferase activity was normalized to β-galactosidase activity (expressed as average +/- range, n=2). Total DVL2-FFLuc expression was measured from total cell lysates (Totals) and levels of 3xFlag-hNKD1 constructs were confirmed by anti-Flag immunoblotting.
immunoprecipitates (IP). Analysis with C-terminally truncated hNKD1 mutants revealed that deletion of the histidine-rich tail did not affect the ability to interact with DVL2, however carboxy-terminal deletions up to amino acids 247 and 192 weakened the interaction. Additional deletions to amino acids 165 and 118 resulted in a marked loss of interaction with DVL2. Mapping with the N-terminally truncated hNKD1 mutants showed that deletion of the region N-terminal of the EF-hand did not affect the hNKD1-DVL2 association. An N-terminal deletion up to amino acid 248, such that the construct lacked the ‘EFX-Y’ region, reduced but did not abolish the interaction with DVL2. This suggests that the ‘EFX-Y’ region of hNKD1 is not absolutely required to interact with DVL2. A summary of the interaction mapping is shown in Figure 17. Similar results were observed for DVL3-FFLuc (Fig. 19).

Taken together, our data suggests that the hNKD1-DVL2 interaction is mediated by multiple points of contact within the hNKD1 ‘X-Y’ region and C-terminal region between amino acids 248 and 471. These results are consistent with a previous study in which mutations in the EF-motif domain in mNkd did not impair the ability of mNkd1 and endogenous Dvl1, 2 or 3 to co-immunoprecipitate in Cos7 cells (Yan et al., 2001a). Moreover, GST-pull down and Y2H assays performed with Drosophila Nkd suggest that a Nkd construct, spanning the ‘EFX-Y’ region and the adjacent downstream 79 amino acids, could interact with Dsh comparably to WT Nkd (Rousset et al., 2002). Of note, our signalling data suggests that 3xFlag-hNKD1 deletion constructs capable of immunoprecipitating DVL2-FFLuc are unable to block the Topflash reporter to the same extent as WT 3xFlag-hNKD1 (Fig. 10A). Altogether, this suggests that while hNKD1 and DVL2 do associate, this interaction alone is not sufficient for hNKD1 to antagonize canonical Wnt signalling.
3.9 Characterizing the Axin-Nkd interaction

Upon analysis of the hNKD1 LUMIER screen data, it was exciting to note a previously unreported interaction of Nkd1 and Nkd2 with Axin1, a pivotal component of the β-catenin destruction complex. Furthermore, Axin1 ranked in the top 15 of hNKD1 interacting partners, when sorted by P-values (Fig. 4B). In canonical Wnt signalling, Axin functions as a scaffold and brings β-catenin in proximity to the protein kinases CKIα and GSK3, which results in β-catenin phosphorylation and subsequent degradation (Clevers, 2006). It is proposed that Wnt signal induces Axin recruitment to LRP5/6 at the plasma membrane, where it may associate with Dishevelled and thus lead to the disruption of the β-catenin destruction complex (Clevers, 2006).

Consistent with previous studies (Kishida et al., 1999a; Kishida et al., 1998; Luo and Lin, 2004), we observed that in HEK293T cells transiently-transfected with the Topflash reporter with empty vector or 3xFlag-Axin1, overexpression of Axin blocks activation of the Wnt-dependent Topflash reporter (Fig. 20A).

We had previously verified the novel interaction detected between Axin and hNKD1 by repeating the LUMIER assay manually (Fig. 6A). To further validate this interaction using a different approach, a co-immunoprecipitation followed by immunoblotting was also performed. To this end, HEK293T cells were transiently-transfected with hNKD1-HA in the absence or presence of 3xFlag-Axin1 and lysates were subjected to anti-Flag immunoprecipitation (Fig. 20B). Subsequent anti-HA immunoblotting confirmed that hNKD1-HA co-immunoprecipitated with 3xFlag-Axin1. We next sought to use LUMIER to determine regions on hNKD1 that were required for this novel interaction. HEK293T cells were co-transfected with Axin1-FFLuc and a series of 3xFlag-hNKD1 truncated constructs (Fig. 20C). We observed that 3xFlag-hNKD1 was unable to co-immunoprecipitate Axin1-FFLuc in the absence of the hNKD1 C-terminal region.
Figure 20: Mapping the Axin1 binding domain to the hNKD1 C-terminal region.

(A) HEK293T cells were transiently-transfected with the Topflash reporter with empty vector (CTL) or 3xFlag-Axin. Topflash activity was measured by luciferase assay. Luciferase activity was normalized to β-galactosidase activity (expressed as mean +/- SD for n=3). Total protein expression was determined by anti-Flag immunoblotting. (B) HEK293T cells were transiently-transfected with hNKD1-HA in the presence or absence of 3xFlag-Axin1. Cell lysates were subjected to anti-Flag immunoprecipitation and associated hNKD1-HA was detected by anti-HA immunoblotting. (C) HEK293T cells were transiently-transfected with Axin1-FFLuc and 3xFlag-hNKD1 constructs. Cell lysates were subjected to anti-Flag immunoprecipitation (IP) and associated Axin1-FFLuc was detected by measuring luciferase activity. Luciferase activity was normalized to β-galactosidase activity (expressed as average +/- range, n=2). Total Axin1-FFLuc expression was measured from total cell lysates (Totals) and levels of 3xFlag-hNKD1 constructs were confirmed by anti-Flag immunoblotting.
Figure 20
Figure 21: Mapping the Axin1 binding domain to the mNkd2 C-terminal region.

HEK293T cells were transiently-transfected with Axin1-FFLuc and 3xFlag-mNkd2 constructs. Cell lysates were subjected to anti-Flag immunoprecipitation (IP) and associated Axin1-FFLuc was detected by measuring luciferase activity. Luciferase activity was normalized to β-galactosidase activity (expressed as average +/- range, n=2). Total Axin1-FFLuc expression was measured from total cell lysates (Totals) and levels of 3xFlag-hNKD1 constructs were confirmed by anti-Flag immunoblotting.
This was evidenced by the lack of luciferase activity following anti-Flag immunoprecipitations with C-terminally deleted 3xFlag-hNKD1 constructs 1-165, 1-192, 1-247 and 1-454. On the other hand, N-terminally truncated 3xFlag-hNKD1 constructs were able to co-immunoprecipitate Axin1-FFLuc, as observed with constructs 119-471, 166-471 and 248-471. When LUMIER was performed with lysates from HEK293T cell co-transfected with Axin1-FFLuc and 3xFlag-mNkd2 truncated constructs, similar results were observed, with the exception of 3xFlag-mNkd2 construct 115-462, with which the interaction was persistently weaker (Fig. 21). Together, our data shows that Axin1 and hNKD1 are novel interacting partners and in particular, Axin1 interacts with the hNKD1 C-terminal region histidine-rich tail. A summary of interaction mapping for hNKD1 and mNkd2 is shown in Figure 17.

3.10 Nkd localization at the plasma membrane

To date, hNKD1 localization nor a function for the hNKD1 N-terminal myristoylation consensus sequence has been thoroughly examined. In light of studies that report Axin1 and Dishevelled, both hNKD1 interacting partners, are recruited to the plasma membrane (Schwarz-Romond et al., 2007; Zeng et al., 2008), we therefore sought to examine the cellular localization of overexpressed hNKD1 by immunofluorescence. Previous studies show that Nkd2, and not Nkd1, recognizes TGFα and targets TGFα-containing vesicles to the lower lateral membrane of polarized MDCK cells (Li et al., 2004; Li et al., 2007). The sorting to the plasma membrane appears to be dependent on myristoylation at the N-terminus. We transfected non-polarized HEK293T cells with hNKD1-3xFlag, incubated cells with mouse anti-Flag or rabbit anti-hNKD1 antibodies and subsequently stained with anti-mouse or anti-rabbit AlexaFluor488-labelled secondary antibodies (Fig. 22). Cells were also stained with DAPI to mark DNA and phalloidin, which binds F-actin to monitor cell shape. Immunofluorescence images show staining of
overexpressed hNKD1-3xFlag in the cytoplasm and at cell edges, which may be indicative of membrane staining. Similar staining was observed for mNkd2-3xFlag. Although, quite preliminary, this data suggests that overexpressed hNKD1 can localize to the cytoplasm and plasma membrane. Taken together with our signalling an interaction data, we speculate that hNKD1 may function at the plasma membrane, where it may interact with DVL2 and Axin1, a novel interacting partner.
Figure 22: hNKD1 cellular localization at the membrane.

HEK293T cells were transiently-transfected with empty vector (pCMV5) or indicated Nkd-3xFlag constructs and fixed with paraformaldehyde. Following permeabilization with Triton X-100, cells were blocked and stained with anti-Flag (A, A’, A”) or anti-hNKD1 (B, B’, B”) antibodies. Cells were then treated with anti-mouse or anti-rabbit AlexaFluor488 for visualization (centre). Cells were also stained with DAPI (left) and phalloidin (right).
Figure 22
4 Discussion

4.1 General discussion

Wnt signalling is critical throughout development and adult life. Aberrant activation of the canonical pathway is known to lead to a variety of human diseases, such as colon carcinomas (Clevers, 2006). To this end, many efforts have been dedicated to examining the mechanisms by which the canonical Wnt pathway is regulated. In particular, many groups have invested research into pathway regulators, such as GSK3β or APC (Clevers, 2006; Logan and Nusse, 2004; Wodarz and Nusse, 1998). The work presented in this thesis aims to examine the role of Nkd, a putative negative regulator in canonical signalling (Wharton et al., 2001; Zeng et al., 2000).

A survey of the literature concerning Nkd in Wnt signalling illustrates that our current understanding of how Nkd proteins behave in canonical signalling is, for the most part, derived from studies in Drosophila. Binding assays show that the dNkd internal region containing the EF-hand motif, termed ‘EFX’, binds to the basic and ‘PDZ’ domains of Dsh, an activator in canonical Wnt signalling (Rousset et al., 2001; Wharton, 2003). In turn, this interaction is thought to inactivate Dsh in canonical signalling (Rousset et al., 2001; Rousset et al., 2002; Yan et al., 2001a), however the mechanism through which this inhibition occurs is unknown.

Through physical and functional studies, we examined vertebrate Nkd in mammalian cells to understand the manner by which Nkd regulates canonical signalling. In line with other studies (Rousset et al., 2001; Wharton et al., 2001; Zeng et al., 2000), our observations suggest that at high levels of expression, Nkd acts as a negative regulator, but excitingly, at low levels, Nkd may also act as a positive regulator. Moreover, we identified and initiated characterization of two novel hNKD1 interacting partners. Taken together, this work suggests that Nkd has a dual role in
regulating Wnt signalling and that the mechanism by which this is effected is more complex than simply binding to and antagonizing Dsh/Dvl.

4.2 Physical and functional high throughput screens involving hNKD1 and mNkd2

Despite the large efforts that have been focussed towards studying canonical/β-catenin Wnt signalling in recent years (Klaus and Birchmeier, 2008), many aspects of this signalling pathway remain poorly understood. To this end, Dr. Bryan Miller from our lab recently conducted a series of HTP screens designed to investigate Wnt signalling. The screens were conducted using a subset of 640 genes enriched for signalling components, including Nkds, and explored interaction partner proteins as well as functional variations in response to gene expression modulation. While many interaction HTP screens in the past have been conducted with Y2H (Stelzl and Wanker, 2006), this study examined PPIs in mammalian cells using LUMIER (Barrios-Rodiles et al., 2005). Two of the eleven baits used in the interaction screen were hNKD1 and mNkd2 and subinteractomes for each protein were generated. Because a limitation of HTP screens is the presence of false positives, it is critical to assess assay performance through manual verification of hits. As such, various interactions involving hNKD1 and mNkd2 were manually verified. The interactions identified by the screen were repeated with remarkable concordance, which illustrates the experimental reliability of the screen. As expected, previously described Nkd interacting partners, such as DVL1, DVL2 and DVL3 (Yan et al., 2001a), were identified. Furthermore, novel interacting partners, such as Axin1, were revealed using this method. Because protein interactions are so crucial to protein function and given the fidelity of the screen results, analysis of the Nkd subinteractomes served as a valuable tool to investigate how Nkds may behave in Wnt signalling. Moreover, the large number of novel interacting
partners for hNKD1 and mNkd2 supports our hypothesis that Nkd is involved in a more complex mechanism than simply binding to Dvl. The subinteractomes also showed that hNKD1 has a greater number of interacting partners than mNkd2, providing evidence that the isoforms may behave differently. While this contrasts to previous genetic-based studies suggesting redundant roles (Van Raay et al., 2007; Zhang et al., 2007), it agrees with another study in mammalian cells that indicate some distinct features (Li et al., 2004; Li et al., 2007).

The functional screens carried out in parallel to the interaction screen also offered insights into Nkd function. For instance, normalized data from the screen suggested that when overexpressed, hNKD1 blocked Wnt signalling and further analysis revealed the unexpected finding that Nkd1 and Nkd2 may have different functions in the Wnt pathway. Individually, the interaction and functional screens were useful, but much stronger conclusions can be drawn from integrated analysis. A statistical Combined Pathway Score (CPS) for each screened gene was thus derived and was used to predict components of the Wnt pathway. Because this value reflected individual performances in all three screens, a high CPS value denoted a higher probability that the given gene was involved in the Wnt pathway, as illustrated by higher CPS obtained for DVL2 (21.31) and the new candidate Nkd interacting partner, Axin1 (12.91). Altogether, the validation work presented in this thesis contributed towards showing the experimental reliability of the HTP screens and the accuracy of the integration method at predicting interaction partners and functional components of the Wnt pathway. This work also provided new avenues to pursue for investigating the role of hNKD1 in Wnt signalling.
4.3 The differential effects of Nkd1 and Nkd2

Both Nkd forms have been assumed to function similarly in Wnt signalling, as suggested by overexpression and morpholino experiments in Zebrafish and partial knockout studies in mice (Van Raay et al., 2007; Zhang et al., 2007). The differential signalling effects we observed with Nkd1 and Nkd2 disagreed with previous in vivo Zebrafish data, which suggests a similar antagonistic role for both Nkds in canonical and non-canonical signalling (Van Raay et al., 2007). Misexpression of mouse Nkd1 in Drosophila has been shown to antagonize Wg function (Wharton et al., 2001), but a similar assay for mouse Nkd2 has yet to be reported. In fact, few studies relating to Nkd2 in Wnt signalling exist. Of note, Nkd2, and not Nkd1, has been shown as required in the delivery and fusion of TGFα containing vesicles to the lower lateral membrane of polarized cells (Li et al., 2004; Li et al., 2007). The work presented in this thesis shows that while the Nkds are related, they may have different functions. In particular, Nkd2 has moderate effects on Wnt signalling, when compared to Nkd1. Our hypothesis is further supported by the PPI screen data, which shows that the isoforms have different interacting partners. It is proposed that as a result of early duplicated genes during early vertebrate evolution, isoforms may have undergone degenerative losses or development of novel functions (Hurley et al., 2005). As a consequence, it is possible that Nkd1 may behave more similarly to dNkd than Nkd2 and that each isoform may have evolved to develop unique functions. This hypothesis may explain why Nkd2, and not Nkd1, is involved in mammalian TGFα trafficking or why Nkd1 is able to block canonical Wnt signalling.

Interestingly, a mNkd2/1 chimera construct, comprised of mNkd2 with the histidine-rich tail substituted from that of Nkd1, was able to block the Topflash reporter to the same extent as hNKD1. The amino acid sequences for hNKD1 and hNKD2 share 43.8% identity, but the
histidine-rich regions share 85% identity (Katoh, 2001), which is unexpected since the substitution of this short region conferred different signalling effects. Upon closer examination, a notable difference between the Nkd histidine-rich region is a tyrosine residue present in Nkd1, but absent from Nkd2 (Fig. 11C). A function has not been characterized for the histidine-rich region, but it would be interesting to study whether this tyrosine residue in Nkd1 is a candidate phosphorylation site and whether phosphorylation is required for Nkd1 to block Wnt signalling.

In all, our overexpression data suggests that Nkd1 yields a more apparent effect on canonical signalling than Nkd2 and since our primary interest was with canonical signalling, we continued our efforts towards examining Nkd1.

4.4 hNKD1 as a positive regulator in canonical Wnt signalling

Normalized data from Dr. Miller’s screen suggested that knockdown of hNKD1 by siRNA blocked Wnt3a-dependent signalling. This result was unexpected since Nkd was presumed to be an inducible antagonist in Wnt signalling (Chan et al., 2007; Zeng et al., 2000) and our data showed that signalling is blocked by hNKD1 overexpression. Consistent with the screen, manual verification also suggested that siRNA-mediated knockdown of hNKD1 blocked Topflash reporter activity. Two other lines of evidence further supported this observation. First, we observed that sihNKD1 also repressed the expression of Axin2, a previously characterized endogenous Wnt3a target gene (Jho et al., 2002; Labbe et al., 2007; Lustig et al., 2002; Yan et al., 2001b) indicating the effect was not specific to an artificial transcriptional reporter. Second, our siRNA experiments were conducted in human embryonic kidney cells (HEK293T) and breast cancer cells (MDA MB 231), two distinct cell lines, providing evidence that the observed effect is not specific to one cell type.
In examining Axin2 mRNA levels in response to sihNKD1, we observed a repression of Wnt signalling after only 2 hours of Wnt3a ligand stimulation, when compared to control. The short time frame required to elicit this effect suggests that sihNKD1 may have direct effects on the Wnt target gene. It will be worthwhile to study shorter and longer time points to decipher whether hNKD1 is implicated in initiating events for Wnt signalling in this system. Examining various other Wnt3a target genes, such as CD44 or cyclin D1 (Vlad et al., 2008), in other mammalian cell lines in response to sihNKD1 will also be necessary to validate this result. Nevertheless, our siRNA-mediated knockdown data suggests that endogenously, hNKD1 is required for canonical Wnt signalling.

While the use of RNAi has provided a powerful technique to manipulate endogenous mRNA expression and analyze loss-of-function phenotypes, this method also presents numerous limitations, including off-target effects (Cullen, 2006; Moffat and Sabatini, 2006). Off-target effects can result from non-specific activation of innate immune responses, saturation of the cell’s RNAi machinery and inadvertent knockdown of a non-target mRNA (Cullen, 2006). To address these concerns, it is necessary to minimize off-target effects and confirm the specificity of an RNAi result.

Because the use of pooled siRNA duplexes has been shown to minimize off-target effects (Straka and Boese, 2008 (online)), we initially employed a pool of 4 unique siRNA duplexes targeting the hNKD1 ORF (Open Reading Frame). We showed that the sihNKD1 pool was successful at knocking down hNKD1 and our signalling assays suggest that the effects observed were specifically associated with Wnt signalling. To confirm the specificity of the pooled sihNKD1, the pool was deconvoluted and we observed that three of the four duplexes behaved similarly in blocking Wnt signalling and knocking down exogenous and endogenous hNKD1 expression.
Excitingly, results using RKO human colon carcinoma cells, a Wnt3a responsive cell line from the Angers lab that does not express hNKD1 (Li et al., 2005; Major et al., 2007), provide evidence to support the specificity of sihNKD1. Specifically, in these cells, canonical Wnt signalling was unaffected by sihNKD1 transfection, as measured by a stably-expressed β-catenin-responsive luciferase reporter. Taken together, our siRNA-mediated knockdown studies suggest that at low endogenous levels, hNKD1 may promote canonical Wnt signalling.

To further evaluate the specificity of sihNKD1 and thereby support a positive role for Nkd in Wnt signalling, it will be important to plan a study whereby the Nkd phenotype can be rescued by introducing sihNKD1-resistant cDNA. Because our data suggests that both overexpression and siRNA mediated knockdown of hNKD1 repress canonical Wnt signalling, this study will require carefully titrating exogenous sihNKD1-resistant Nkd1, such as mNkd1 (data not shown), and monitoring Topflash reporter activity in Wnt-responsive cells subjected to sihNKD1 transfection. If Nkd1 is required at low levels for Wnt signalling, then we expect that as mNkd1 expression is increased, we will observe an increase in Wnt3a-dependent Topflash reporter activity followed by a dose-dependent decrease. This could be achieved by generating stable cell lines, such as those available from Clonetech, in which mNkd1 expression can be dose-dependently-induced by the introduction of doxycycline, a tetracycline derivative.

Previous RNAi-related studies involving reducing Nkd expression have employed double-stranded RNA (dsRNA) in Drosophila (Rousset et al., 2001) and antisense morpholino oligonucleotides (MO) in Zebrafish (Van Raay et al., 2007). Injecting nkd dsRNA into Drosophila wild-type embryos led to a decreased number of denticles (Rousset et al., 2001), a phenotype observed with embryos exposed to excess Wg (Noordermeer et al., 1992). Similarly, a reduction of maternal Zebrafish Nkd1 by MO injections resulted in a modest increase in the arc
of gsc expression at 30% epiboly, which is indicative of an increase in maternal Wnt signalling in the Zebrafish embryo (Van Raay et al., 2007). While both studies yielded related results, neither study presented assays to specifically confirm Nkd expression reduction or knockdown. A rescue experiment, whereby the phenotype of embryos subjected to Nkd knockdown is rescued to the wild type phenotype by overexpressing increasing amounts of Nkd, has also not been reported in either study. Interestingly, in Zebrafish, the injection of Nkd1 MOs and nkd1 RNA both resulted in an expansion of gsc expression at 30% and 50% epiboly, respectively (Van Raay et al., 2007). It is hypothesized that Nkd1 MO injections expanded the positive influence of maternal signalling while overexpression reduced the negative influence of zygotic signalling (Van Raay et al., 2007). Considering the dissimilar results from Nkd expression knockdown studies between previous groups (Rousset et al., 2001; Van Raay et al., 2007) and our group, it could be argued that the opposing outcomes may be due to species, assay and knockdown specificities. For instance, the groups that observed an increase in Wnt signalling in response to Drosophila and Zebrafish Nkd knockdown studied phenotypic readouts, such as denticle number (Rousset et al., 2001) and embryonic dorsal organizer staining (Van Raay et al., 2007), while we observed a decrease in Wnt signalling in response to human NKD1 knockdown by studying direct effects on a Wnt-responsive transcriptional reporter and an endogenous Wnt target gene. Interestingly, two mouse Nkd1 knockout studies to date have reported conflicting phenotypes, which suggests an in vivo function for Nkd1, although both reported that Nkd1 may be dispensable for development (Li et al., 2005; Zhang et al., 2007). In the first study, Nkd1 knockout mice exhibited defective sperm maturation, reduced testis size and a decrease in fertility (Li et al., 2005). In the second study, Nkd1 knockout mice did not display changes in spermatogenesis, but instead exhibited altered cranial bone morphology and reduced fertility (Zhang et al., 2007). Of note, neither study used complete Nkd1 knockout mice and employed
mice expressing internally deleted or truncated mNkd1, resulting in residual protein expression. Interestingly, mice homozygous for truncating mutations in both Nkd1 and Nkd2 were also viable and exhibited a similar phenotype to the single Nkd1 truncation within the same study (Zhang et al., 2007). A complete Nkd1 knockout study is yet to be reported and worthwhile pursuing to study effects on development. The possibility of redundancy between Nkd1 and Nkd2 could also be studied using this method.

### 4.5 hNKD1 homodimerization

With the data collected from the HTP PPI screen, we sought to gain functional insight by examining novel and previously described hNKD1 interacting proteins. Using LUMIER, we verified and mapped hNKD1 interactions with hNKD1, DVL2 and Axin1. We first explored hNKD1 homodimerization, a previously unreported interaction, and mapped the interaction to the EFX domain and histidine-rich tail. Based on the behaviour of other EF-hand motif containing proteins (Gifford et al., 2007), we expected that the interaction would occur through EF-hand motif pairing. However, our data suggests hNKD1 homodimerizes via the EFX domain and the histidine-rich tail. Our data also suggests that Nkd1 may heterodimerize with Nkd2 in a similar fashion. A role has not been elucidated for the histidine-rich tail on Nkd, but multihistidine regions are known to bind divalent metals (Sovago and Osz, 2006). Since EF-hand motifs are also known to bind to divalent metals, such as calcium and magnesium (Lewit-Bentley and Rety, 2000), it would be interesting to study whether hNKD1 homodimerization and activity are dependent on metal association. This could be performed by examining dimerization and signalling activity in the presence of chelating agents, such as EDTA or EGTA, followed by titrating amounts of candidate divalent metals, such as Ca$^{2+}$, Mg$^{2+}$, Ni$^{2+}$, Cu$^{2+}$ or Zn$^{2+}$ (Lewit-Bentley and Rety, 2000; Sovago and Osz, 2006). Interestingly a study in Drosophila suggests
that Zn\(^{2+}\), and not Ca\(^{2+}\), is involved in the interaction between Nkd and Dsh (Rousset et al., 2002). While our data suggests that Nkds dimerize via two distinct domains, the function of this association requires further study. It may be worth generating Nkd1 constructs harbouring point mutations, such that dimerization is abolished, and studying subsequent effects on Wnt signalling.

4.6 hNKD1 interacts with DVL2

Epigenetic studies have placed Nkd downstream from Wg/Wnt, upstream of armadillo/β-catenin and downstream of, or at the level of, Dsh/Dvl (Rousset et al., 2001; Wharton et al., 2001). Nkd and Dsh/Dvl have been shown to interact and this interaction is thought to inactivate Dsh/Dvl, but the mechanism is unclear (Rousset et al., 2001; Rousset et al., 2002; Wharton et al., 2001; Yan et al., 2001a). Our interaction mapping shows that multiple regions, including the ‘X-Y’ region and an uncharacterized region downstream of amino acid 247, on hNKD1 are required for interacting with DVL2. When considered in parallel to our signalling data, we noted that hNKD1 truncation mutants capable of associating with DVL2 were compromised in the ability to repress Wnt signalling. In fact, our functional mapping with hNKD1, using a series of truncated mutants, showed that all of the truncated mutants tested were compromised in the ability to repress signalling. For instance, constructs lacking the N-terminus or short histidine-rich tail, which retained full DVL2 binding, were impaired in the ability to block Topflash reporter activity, as compared to WT hNKD1, suggesting that both amino- and carboxy-terminal regions of hNKD1 are required for repressing Wnt signalling. Moreover, co-expression of these regions using separate constructs was unable to restore an antagonistic effect comparable to that of WT hNKD1, suggesting the regions may require co-linear expression. As such, in contrast to a previously proposed hypothesis (Rousset et al., 2001; Rousset et al., 2002), our data suggests that
the interaction between hNKD1 and DVL2 alone is not sufficient to inhibit canonical Wnt signalling. This is in agreement with another study in which point mutations to the EF-hand motif in vertebrate Nkd1 disturbed the ability to block canonical signalling but did not affect the association with Dvl (Yan et al., 2001a).

One caveat of this work is the possibility that some of the truncation mutants may not yield properly folded hNKD1 forms. In particular, it is possible that in mutant 1-165, the EF-hand motif is not properly folded and requires the adjacent ‘X’ domain, which is present in mutant 1-192 for proper folding. Nonetheless, we suspect that multiple hNKD1 regions, including the EF-hand motif and the N- and C-terminals, are required to block Wnt signalling. To investigate this possibility further, more hNKD1 mutants harbouring specific mutations, such as internal deletions, in these regions should be studied.

Binding assays have shown that Nkd1 interacts with the internal PDZ region in Dvl (Rousset et al., 2001; Yan et al., 2001a). This PDZ region has also been shown to interact with canonical pathway positive regulators, such as CK1ε (Price, 2006; Wharton, 2003), and it has been theorized that Nkd1 may block the access of these proteins to Dvl (Wharton, 2003). Alternatively, and in line with our signalling data, other proteins, associated with Nkd1 on regions separate from those interacting with Dvl, may be recruited to Dvl to positively or negatively regulate downstream canonical signalling. Furthermore, considering Dvl is described as core component of the planar cell polarity pathway (Strutt, 2003), it will be interesting to investigate a role for Nkd in non-canonical signalling. Indeed, it has been suggested that Nkd may act as a switch between canonical and non-canonical signalling by inhibiting canonical Dvl and thereby activating non-canonical Dvl activities (Yan et al., 2001a). However, a recent study
in Zebrafish suggests that Nkd antagonizes both canonical and non-canonical signalling (Van Raay et al., 2007).

Dvl is thought to be activated through phosphorylation in response to Wnt signal (Gao et al., 2002; Price, 2006; Sun et al., 2001; Takada et al., 2005). This phosphorylation can be seen as a mobility shift by SDS-PAGE separation followed by immunoblotting for endogenous Dvl (Bryja et al., 2007; Gonzalez-Sancho et al., 2004; Lee et al., 1999) and our preliminary data in two human cell lines reveals that this endogenous Dvl band shift is unaffected by hNKD1 overexpression (data not shown). This result suggests that hNKD1 may act downstream of the activating phosphorylation or in an unrelated mechanism, a conjecture that requires further study.

4.7 hNKD1 interacts with Axin1

Originally identified as a canonical signalling inhibitor that also regulated embryonic axis formation (Zeng et al., 1997), Axin is now described as a multidomain scaffold protein that interacts with multiple proteins and regulates many signalling pathways (Luo and Lin, 2004). It was thus exciting to note that our HTP PPI screen identified an interaction between Axin1 and hNKD1. We manually verified this novel interaction and using LUMIER, we showed that the C-terminal histidine-rich tail on hNKD1 was required for the interaction with Axin1. To further refine this interaction, constructs harbouring point mutations in the histidine-rich region should be generated. In addition, Axin1 truncation constructs should be generated to map regions that are required for interacting with hNKD1. Whether the interaction with hNKD1 restricts or promotes association with other Axin1 interacting partners, such as Dsh/Dvl, has yet to be examined.
4.8 hNKD1 as a mediator between Axin and Dvl

The interaction between Dsh/Dvl and Axin is thought to promote β-catenin accumulation but the mechanisms by which Dsh/Dvl is activated and Axin is in turn inhibited are poorly understood. It has been shown that *Drosophila* Dsh is required for the Wnt-induced recruitment of Axin to the plasma membrane (Cliffe et al., 2003), but attempts to co-immunoprecipitate Axin and Dsh/Dvl have had varying results. One group observed that the direct biochemical interaction depended on precise buffer conditions (Smalley et al., 1999) while another group reported an inability to get robust and specific co-immunoprecipitation signals (Schwarz-Romond et al., 2007). This suggests that this interaction at the plasma membrane may be highly dynamic and may include mediating components. Taken together with the work presented in this thesis, we propose that hNKD1 may serve as a mediating component between Dvl and Axin to promote canonical Wnt signalling (Fig. 23A). In response to Wnt signal when hNKD1 is present in the cell at low concentrations, we propose that hNKD1 may recruit Axin1 away from the destruction complex and towards DVL2 at the plasma membrane to activate signalling. This is supported by our siRNA assays that suggest hNKD1 promotes Wnt signalling and by our preliminary immunofluorescence data that shows hNKD1 may localize to the plasma membrane in non-polarized cells. To reconcile the antagonistic signalling effects observed in response to overexpressed hNKD1, we speculate that when expressed at higher concentrations, hNKD1 sequesters DVL2 and Axin1 separately and in turn inhibits the Dvl-Axin interaction required to activate canonical signalling (Fig. 23B). This would account for our data which suggests that hNKD1 regions that associate with DVL2 are not sufficient to block signalling. While this model is highly speculative, it presents an intriguing working hypothesis to direct future studies.
Figure 23: Proposed dual role for hNKD1 in canonical Wnt signalling.

(A) When expressed at low levels, hNKD1 aids in coordinating the movement of Axin and DVL to the receptor complex, leading to signalling activation. (B) When overexpressed, hNKD1 sequesters DVL and Axin1 separately, thus inhibiting the interaction required to activate signalling.
The function of hNKD1 dimerization remains to be clarified, but the association may render other binding sites on hNKD1 unavailable and in turn, sequester DVL2 and Axin1 separately. This possibility could be examined through binding affinity assays, in which Axin1-DVL2, Axin1-hNKD1 and DVL2-hNKD1 interactions are studied in response to increasing amounts of hNKD1. It will be worth performing further immunofluorescence studies that include monitoring hNKD1 localization in response to Wnt signal in polarized cells and concurrently examining DVL2 and Axin1 staining. We propose that while the Nkd isoforms may have differential signalling functions, Nkd1 may behave similarly to Nkd2, in the ability to target partner proteins to the plasma membrane (Li et al., 2004; Li et al., 2007).

A previous study proposed that Nkd may act to delicately regulate Dvl protein levels and activities to effect downstream signalling events reliably (Zhang et al., 2007). We and others have data that suggest hNKD1 is a Wnt3a target gene (Yan et al., 2001a; Zeng et al., 2000). These observations support a negative feedback role for hNKD1 in canonical Wnt signalling, given that hNKD1 overexpression results in repressed signalling. In our model, we hypothesize that hNKD1 acts to fine tune Wnt signalling, but in lieu of negatively regulating DVL only, hNKD1 has a dual role involved in positively and negatively regulating signalling through interactions with Axin1 and DVL2. Preliminary data obtained from SW480 cells suggest that hNKD1 functions upstream of the scaffolding protein APC, which is mutated in these cells to result in constitutively active canonical signalling (data not shown). Our data with RKO cells shows that Wnt signalling can persist in the absence of hNKD1, suggesting that hNKD1 is not absolutely required, but is instead a component of a delicate mechanism to enhance canonical signalling. We speculate that hNKD1 may promote signalling in specific contexts. For example, hNKD1 may enhance signalling in certain cells during development or in tumours in which
elevated hNKD1 expression and constitutively active canonical Wnt signalling have been reported (Koch et al., 2005; Yan et al., 2001b). To further support this speculative model, efforts should be put towards immunoprecipitating a trimeric DVL-hNKD1-Axin complex, in addition to the experiments suggested above. Many specific details remain to be clarified but nonetheless, it will be interesting to study the dynamics of this complex in the absence and presence of Wnt signal.

4.9 General conclusions

In summary, the work presented in this thesis explored the role of hNKD1 in canonical Wnt signalling in mammalian cells. Presently, the hNKD1 is believed to act as an inducible antagonist in Wnt signalling, but the mechanism through which hNKD1 behaves is unclear. To this end, we referred to normalized data sets from a series of recently completed HTP screens, which included hNKD1, to gain functional and physical insights. Follow-up studies performed with hNKD1 aided to support the reliability and reproducibility of these screens. Further study focussed on analysing hNKD1 through functional studies and examining interacting partners, two of which were previously unpublished. Excitingly, our siRNA-mediated knockdown assays suggest that hNKD1 is required for canonical signalling. These results contrast previous studies, in which Nkd1 has been described solely as a signalling antagonist (Chan et al., 2007; Rousset et al., 2001; Van Raay et al., 2007; Wharton et al., 2001; Yan et al., 2001a). Instead, we suspect that at low levels, hNKD1 acts to mediate the Dvl-Axin interaction at the plasma membrane and thus promote Wnt signalling. At higher levels, hNKD1 functions as a pathway antagonist, including a role in a negative feedback mechanism. While many experiments remain to be completed, altogether, our studies provide novel evidence that hNKD1 may dynamically regulate canonical Wnt signalling both positively and negatively in mammalian cells. Because elevated hNKD1
expression has been previously described in Wnt-related colon cancers and hepatoblastomas (Koch et al., 2005; Yan et al., 2001b), our work also contributes towards understanding inappropriate Wnt signalling and subsequent effects in human diseases.
References


