The Search for Novel Wnt Pathway Modulators

by

Peter Poliszczuk

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

© Copyright by Peter Poliszczuk 2010
The Search for Novel Wnt Pathway Modulators

Peter Poliszczuk
Master of Science
Department of Biochemistry
University of Toronto
2010

Abstract

Signaling pathways are complex and function to transmit signals from the extracellular environment into the cell. Analysis of results obtained from a high throughput siRNA screen led to the identification of Membrane protein palmitoylated 3 (MPP3) and Leukocyte Tyrosine Kinase (LTK) as novel negative regulators of the Wnt pathway. MPP3 is a MAGUK family protein and domain mapping studies indicated that the Guk domain plays a role in the negative regulation of the pathway. LTK, a receptor tyrosine kinase, has several transcript variants one of which lacks the entire kinase domain (LTKΔKD). While LTKΔKD interacted with the Wnt receptor Frizzled7, the full length LTK did not, suggesting distinct modes of pathway regulation. Analysis of neuronal cells, NIE115 and Neuro2a, demonstrated LTK is expressed and that cells are Wnt3a responsive, thereby providing a neuronal model system appropriate for further studies on the mechanism and biological role of LTK as a negative regulator of the Wnt pathway.
Acknowledgments

First I would like to start things off by thanking my supervisor Dr. Liliana Attisano for providing me with this wonderful opportunity to work and study in her lab. It has been a growing experience in which I was able to develop as both a person and scientist. Dr. Attisano provided wonderful guidance and insight into various aspects of my project. She is a great source of knowledge and helped me strive to do my best. I would like to follow by thanking the many people that have come and gone within the lab that provided me with great guidance and support throughout the years in both the ups and downs. I would also like to thank my family for their full support, guidance, and understanding over the past years. Lastly, all my friends that were there for me, supported me, and accompanied me for those various intellectual conversations over a pint or two, thank you. This has all been a great experience and hopefully the next is the same.

Thank you!
# Table of Contents

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

Table of Contents ........................................................................................................ iv

List of Tables ................................................................................................................ vii

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

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

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

1.1 Wnt Signaling ...................................................................................................... 1

1.1.1 Canonical Wnt Signaling .............................................................................. 1

1.1.2 Non-canonical Wnt Signaling ...................................................................... 3

1.2 Wnt Glycoproteins ............................................................................................ 6

1.3 Frizzled Transmembrane Receptors ................................................................ 7

1.4 LRP5/6 ............................................................................................................... 8

1.5 Other unconventional Wnt receptors ............................................................... 9

1.5.1 RYK ........................................................................................................... 9

1.5.2 ROR .......................................................................................................... 10

1.5.3 PTK7 ......................................................................................................... 11

1.5.4 LTK .......................................................................................................... 12

1.6 MAGUKs .......................................................................................................... 15

1.6.1 MPP3 ....................................................................................................... 17

1.7 Thesis perspective ............................................................................................. 18

2 Methods and Materials ............................................................................................ 19

2.1 Mammalian expression constructs ................................................................. 19

2.2 Production of Wnt3a conditioned medium ..................................................... 20

2.3 Cell culturing .................................................................................................... 20
2.4 Transfection of cells.............................................................................................................. 20
2.5 LUMIER assay......................................................................................................................... 21
2.6 TOPflash Reporter Assay ....................................................................................................... 22
2.7 siRNAs....................................................................................................................................... 22
2.8 Real time quantitative reverse transcription PCR (QPCR)..................................................... 22
2.9 β-catenin stabilization and Dvl upshift assay.......................................................................... 24
3 Results ....................................................................................................................................... 26
  3.1 The use of a high-throughput screening approach to search for new Wnt pathway modulators .............................................................................................................. 26
  3.2 MPP3 acts as a negative regulator of the Wnt pathway.......................................................... 34
  3.3 The Guk domain of MPP3 is important for MPP3 function in the Wnt pathway ............... 39
  3.4 LTK is a Negative regulator of the Wnt pathway...................................................................... 49
  3.5 LTKΔKD but not full length LTK interacts with FZD7............................................................ 57
  3.6 LTKΔKD does not inhibit LRP6 enhanced TOPflash activity.............................................. 67
  3.7 Wnt3a and Wnt5a do not enhance LTKΔKD homodimerization........................................... 70
  3.8 LTK and LTKΔKD do not interact with Vangl1 or Vangl2.................................................... 70
  3.9 LTK does not enhance the Wnt5a induced inhibition of canonical Wnt signaling .......... 73
  3.10 Neuro2a and NIE 115 cell lines are Wnt responsive and express LTK.............................. 77
4 Discussion ................................................................................................................................. 83
  4.1 Application of HTP screening to identify novel signaling pathway modulators .............. 83
  4.2 MPP3 is a negative regulator of canonical Wnt signaling.................................................... 84
  4.3 LTK is a negative regulator of canonical Wnt signaling....................................................... 86
  4.4 LTKΔKD interacts with Frizzled receptors............................................................................. 87
  4.5 The influence of Wnt3a and Wnt5a on LTK dimerization..................................................... 90
  4.6 The role of LTK in Wnt induced neuronal development.................................................... 91
  4.7 Potential models for the regulation of canonical Wnt signaling by LTK............................ 92
4.8 General conclusion........................................................................................................................................... 93
References......................................................................................................................................................... 95
List of Tables

Table 1: siRNAs used in work presented ................................................................. 23
List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>The canonical Wnt signaling pathway.</td>
</tr>
<tr>
<td>2</td>
<td>Tyrosine kinase dendrogram.</td>
</tr>
<tr>
<td>3</td>
<td>High throughput screening detects known signaling pathway regulators.</td>
</tr>
<tr>
<td>4</td>
<td>siRNA directed towards LTK and MPP3 modulate the Wnt but not the TGFβ and BMP pathways.</td>
</tr>
<tr>
<td>5</td>
<td>siRNA directed towards CASK and DLG1 modulate the TGFβ but not the Wnt and BMP pathways.</td>
</tr>
<tr>
<td>6</td>
<td>siRNA directed towards MPP2 and DUSP5 modulated the BMP but not the Wnt and TGFβ pathways.</td>
</tr>
<tr>
<td>7</td>
<td>siRNA directed towards PSKH2 had no effect on the TGFβ, Wnt, and BMP pathways.</td>
</tr>
<tr>
<td>8</td>
<td>Comparison of the primary screen data to the manual verification data.</td>
</tr>
<tr>
<td>9</td>
<td>MPP3 is a negative regulator of the Wnt pathway.</td>
</tr>
<tr>
<td>10</td>
<td>Increasing amounts of MPP3 does not down regulate the Wnt pathway.</td>
</tr>
<tr>
<td>11</td>
<td>A schematic of MPP3 deletion constructs.</td>
</tr>
<tr>
<td>12</td>
<td>Deletion of one Lin2/7 domain and the PDZ domain of MPP3 do not influence the Wnt pathway.</td>
</tr>
<tr>
<td>13</td>
<td>The Guk domain of MPP3 plays a negative regulatory role in the Wnt pathway.</td>
</tr>
<tr>
<td>14</td>
<td>LTK transcript variants and protein coding regions.</td>
</tr>
<tr>
<td>15</td>
<td>LTK is a negative regulator of the Wnt pathway.</td>
</tr>
<tr>
<td>16</td>
<td>siRNA resistant LTK cDNA rescues wildtype TOPflash activity.</td>
</tr>
</tbody>
</table>
Figure 17: LTKΔKD negatively regulates TOPflash activity. .................................................. 59

Figure 18: Full length LTK does not interact with FZD7....................................................... 62

Figure 19: LTKΔKD interacts with FZD7........................................................................... 64

Figure 20: LTKΔKD interacts with other Frizzleds. .............................................................. 66

Figure 21: LTKΔKD does not decrease the LRP6 induced increase in TOPflash activity. ...... 69

Figure 22: Wnt3a and Wnt5a do not enhance LTKΔKD dimerization. .............................. 72

Figure 23: LTK and LTKΔKD do not interact with Vangl1 and Vangl2............................... 74

Figure 24: Full length LTK does not enhance the antagonistic effect of Wnt5a on Wnt3a- induced canonical signaling. ....................................................................................... 76

Figure 25: NIE115 and Neuro2a cells express Ltk and are Wnt responsive........................ 78

Figure 26: Knockdown of Ltk does not influence β-catenin stabilization and Dvl3 phosphorylation in NIE115 and Neuro2a cells. ................................................................. 81

Figure 27: Proposed working models for LTK in the canonical Wnt Pathway.................... 89
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKT</td>
<td>v-akt murine thymoma viral oncogene homolog</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>Arg</td>
<td>Arginine</td>
</tr>
<tr>
<td>Asn</td>
<td>Asparagine</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>CamKII</td>
<td>Calcium/calmodulin kinase</td>
</tr>
<tr>
<td>CASK</td>
<td>Calcium/Calmodulin-dependent serine protein kinase</td>
</tr>
<tr>
<td>CCK-4</td>
<td>Carcinoma kinase-4</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CK1</td>
<td>Casein kinase 1α (CK1)</td>
</tr>
<tr>
<td>CK2</td>
<td>Casein kinase 1</td>
</tr>
<tr>
<td>CPS</td>
<td>Combined pathway score</td>
</tr>
<tr>
<td>CRD</td>
<td>Cysteine-rich domain</td>
</tr>
<tr>
<td>Cthrc1</td>
<td>Collagen triple helix repeat containing 1</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>Dgo</td>
<td>Diego</td>
</tr>
<tr>
<td>DKK</td>
<td>Dickkopf</td>
</tr>
<tr>
<td>DLG1</td>
<td>Discs Large Homology 1</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DUSP5</td>
<td>Dual Specificity Phosphate 5</td>
</tr>
<tr>
<td>DVL</td>
<td>Dishevelled</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>FFluc</td>
<td>Firefly luciferase</td>
</tr>
<tr>
<td>Fmi</td>
<td>Flamingo</td>
</tr>
<tr>
<td>FZD</td>
<td>Frizzled</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>Gly</td>
<td>Glycine</td>
</tr>
<tr>
<td>GPCR</td>
<td>G coupled protein receptor</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase 3β</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>Guk</td>
<td>Gyanylate kinase like</td>
</tr>
<tr>
<td>HEK</td>
<td>Human Embryonic Kidney</td>
</tr>
<tr>
<td>HTP</td>
<td>High-throuphput</td>
</tr>
<tr>
<td>ICD</td>
<td>Intracellular domain</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin like</td>
</tr>
<tr>
<td>IGFR1</td>
<td>Insulin-like growth factor 1 receptor</td>
</tr>
<tr>
<td>IRS-1</td>
<td>Insulin receptor substrate-1</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun-N-terminal kinase</td>
</tr>
<tr>
<td>LA</td>
<td>LDLR type A</td>
</tr>
</tbody>
</table>
LEF1: Lymphoid enhancer binding factor-1

LRP: LDL-receptor related protein

LTK: Leukocyte tyrosine kinase

LUMIER: LUminescence-based

Mammalian IntERactome

MAGUK: Membrane-associated guanylate kinase homologs

MAPK: Mitogen activated protein kinase

MPP2: Membrane protein palmitoylated 2

MPP3: Membrane protein palmitoylated 3

Nkd1: Naked 1

NSCLC: Non-small cell lung cancer

Pak1: p21-activated kinase

PCP: Planar cell polarity

PCR: Polymerase chain reaction

PDE: Phosphodiesterase

PDZ: Post-synaptic density-95 Discs-large-Zonula occludens-1

Phe: Phenylalanine

PI3K: Phosphoinositide 3-kinase

Pk: Prickle

PKC: Protein kinase C

PLC: Phospholipase C

Pro: Proline

PSKH2: Protein Serine Kinase H2

PTK: Protein tyrosine kinase

PTK7: Protein tyrosine kinase-7

Q-PCR: quantitative PCR

ROR: Receptor tyrosine kinase-like orphan receptor

RYK: Related to tyrosine kinases

Ser: Serine

sFRP: Secreted frizzled-related protein

SH3: Src homology 3

siAKT2: AKT2 siRNA

siCASK: CASK siRNA

siCTL: control siRNA

siDLG1: DLG1 siRNA

siDUSP5: DUSP5 siRNA

siLTK: LTK siRNA

siMPP2: MPP2 siRNA

siMPP3: MPP3 siRNA

siPAK1: PAK1 siRNA

siPSKH2: PSKH2 siRNA

siRNA: Small interfering RNA

SS: Signal sequence

Stan: Starry night

Stbm: Starbismus

TCF: T-cell specific factor

Thr: Threonine
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>β-TrCP</td>
<td>β-transducin repeat-containing protein</td>
</tr>
<tr>
<td>trkB</td>
<td>Neutrophic tyrosine kinase receptor type 2</td>
</tr>
<tr>
<td>TSLC1</td>
<td>Tumor suppressor in lung cancer 1</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>TβRII</td>
<td>TGFβ-receptor II</td>
</tr>
<tr>
<td>Ube2m</td>
<td>Ubiquitin-conjugating enzyme E2M</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>Val</td>
<td>Valine</td>
</tr>
<tr>
<td>Vangl</td>
<td>Vang-like</td>
</tr>
<tr>
<td>WIF-1</td>
<td>Wnt inhibitory factor-1</td>
</tr>
<tr>
<td>WLS</td>
<td>Wntless</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
1 Introduction

1.1 Wnt Signaling

1.1.1 Canonical Wnt Signaling

Signaling pathways are a fundamental aspect of how cells communicate with one another and respond to their environment, influencing cell growth, cellular differentiation and apoptosis (Clevers, 2006; Logan and Nusse, 2004). In the simplest form, a signaling pathway functions through the binding of a ligand to its specific receptor which in turn activates the receptor in order to elicit an intracellular response. The Wnt family of secreted glycoproteins are the ligands (Figure 1) which promote cell proliferation, cell polarity, neural differentiation, and cell fate determination during embryonic development and tissue homeostasis (Clevers, 2006; MacDonald et al., 2009). β-catenin is a fundamental player in the canonical Wnt pathway. In the presence of ligand, β-catenin is bound to the destruction complex which is composed of several proteins including a scaffolding protein Axin, adenomatous polyposis coli (APC), casein kinase 1α (CK1), and glycogen synthase kinase 3β (GSK3β). When bound, β-catenin is phosphorylated on Serine (Ser) 45 by CK1α which primes β-catenin for the sequential phosphorylation of Threonine (Thr) 42, Ser 39, and Ser 37 by GSK3β. This phosphorylation of β-catenin promotes the recognition by β-TRCP, an E3 ubiquitin ligase, which leads to the ubiquitination of β-catenin and proteasomal degradation. This continuous degradation prevents cytoplasmic β-catenin from translocating into the nucleus, binding to transcription factors T-cell factor and lymphoid enhancer factor 1 (TCF/LEF-1), and stimulating Wnt responsive genes. In the presence of Wnt glycoproteins, Wnts bind simultaneously to the seven-pass transmembrane Frizzled receptors (Fzd) and the lipoprotein receptor-related protein 5 or 6 (LRP5/6) co-receptor. The assembly of this Wnt-Fzd-LRP6 receptor complex promotes the translocation and binding of
Figure 1: The canonical Wnt signaling pathway.

(A) In the off state, β-catenin is bound by the destruction complex composed of APC, Axin, GSK3β, and CK1α. CK1α phosphorylates β-catenin which primes β-catenin for subsequent phosphorylation by GSK3β. The phosphorylation of β-catenin allows it to be recognized by the E3 ubiquitin ligase β-TRCP which polyubiquitinates β-catenin targeting it for degradation by the proteasome. The degradation of β-catenin prevents it from translocating to the nucleus, binding to TCF/LEF-1 transcription factors, and stimulating the transcription of Wnt dependent genes.

(B) In the on state, Wnt binds to the FZD receptor and LRP5/6 co-receptor forming a heteromeric complex. This receptor complex formation recruits Dvl to FZD which then promotes the dissociation of the destruction complex thus preventing the targeted degradation of β-catenin and allowing for cytoplasmic accumulation. β-catenin is then able to translocate into the nucleus, bind to TCF/LEF-1 transcription factors, and promote the transcription of Wnt dependent genes.
Dishevelled (Dsh) to the Fzd receptor, resulting in the dissociation of the destruction complex by the association of Axin to conserved PPP(S/T)P motifs on LRP5/6. This disruption of the destruction complex stabilizes β-catenin, which then accumulates in the cytoplasm. The increased levels of cytoplasmic β-catenin allows for translocation into the nucleus, binding to TCF/LEF-1 transcription factors, and transcription of Wnt responsive genes (Figure 1) (Clevers, 2006; Logan and Nusse, 2004).

1.1.2 Non-canonical Wnt Signaling

Wnt proteins also activate several β-catenin independent or noncanonical pathways. Insights into the molecular mechanisms of these pathways are currently limited as in vitro methods have not been fully developed. Nonetheless, it is a growing field of interest as researchers try to understand the biological outcomes of noncanonical Wnt signaling. Unlike the β-catenin dependent pathway that functions through β-catenin stabilization, the β-catenin independent pathway functions through three different mechanisms (Klein and Mlodzik, 2005; Veeman et al., 2003; Wang and Malbon, 2003). First, specific Wnt ligands in combination with specific frizzled receptors act through a Wnt/calcium pathway in order to activate calcium/calmodulin kinase II (CamKII) and protein kinase C (PKC) (Kuhl et al., 2000b; Wang and Malbon, 2003). Second, the non-canonical pathway may also act through the activation of phospholipase C (PLC) and phosphodiesterase (PDE) by the recruitment of heterotrimeric GTP-binding proteins (Klein and Mlodzik, 2005). Last, the third mechanism functions through the Wnt/Planar Cell Polarity (PCP) pathway that involves the activation of the Jun-N-terminal kinase (JNK) (Kohn and Moon, 2005). Although the full molecular mechanisms of noncanonical Wnt signaling are not understood, the current consensus is that noncanonical Wnt signaling antagonizes the β-catenin dependent pathway.
1.1.2.1 Calcium/Calmodulin-Dependent Kinase II (CamKII) and Protein Kinase C

It was first observed in Zebrafish that upon injection of embryos with Wnt5a or Wnt11 mRNA, an intracellular flux of Ca\(^{2+}\) occurred (Slusarski et al., 1997b; Westfall et al., 2003). The flux in Ca\(^{2+}\) activates CamKII and PKC, two enzymes that are regulated by intracellular Ca\(^{2+}\) levels (Kuhl et al., 2000a; Sheldahl et al., 1999). From this observation it was concluded that the β-catenin independent pathway uses Ca\(^{2+}\) as a second messenger in order to fully activate the pathway. The Wnt/Ca\(^{2+}\) pathway has been shown to be involved in a variety of cellular processes that include dorso-ventral patterning of the embryo and the regulation of cell migration (Larabell et al., 1997; Weeraratna et al., 2002). Furthermore, the Wnt/Ca\(^{2+}\) pathway has been implicated in the promotion of cell invasiveness and malignant progression of cells in cancer (Kuhl, 2004). Specifically, the Wnt5a/PKC branch of the Wnt/Ca\(^{2+}\) pathway was shown to increase motility in melanoma cells by increasing Snail and vimentin expression and decreasing E-cadherin expression (Dissanayake et al., 2007).

1.1.2.2 Heterotrimeric GTP-Binding Proteins

The Frizzled receptor family of transmembrane proteins resemble conventional G coupled protein receptors (GPCRs) (Slusarski et al., 1997a). Like typical GPCRs, Frizzled receptors contain seven transmembrane spanning hydrophobic segments, the typical predicted N-terminal glycosylation sites, and predicted phosphorylation sites on the C-terminal tail required for cyclic AMP-dependent protein kinase (PKA), protein kinase C (PKC), and casein kinase 2 (CK2) activation (Bhanot et al., 1996; Dale, 1998; Slusarski et al., 1997a; Slusarski et al., 1997b; Wang et al., 1996). Unlike typical GPCRs, the frizzled receptors lack the Asn-Pro-X-X-Tyr motif (where X is any amino acid) at the end of the seventh spanning transmembrane segment, along with the conserved Asp-Arg-Tyr motif present at the second intracellular loop used for G protein
coupling (Angers and Moon, 2009). Thus, Frizzled receptors were not considered true GPCRs but were hypothesized to function through small GTP-binding proteins. To support this hypothesis, it was observed that Fzd2 and Wnt5a are able to enhance the intracellular calcium release in zebrafish embryos, a response that can be induced through G-protein signaling (Slusarski et al., 1997a). Upon treatment of zebrafish embryos with pertussis toxin, a G protein inhibitor, Ca\(^{2+}\) flux induced by Wnt5a and Fzd2 interactions was prevented (Slusarski et al., 1997a). In addition, elongation of Xenopus explants was inhibited by Wnt11 over-expression and upon co-expression of the pertussis toxin elongation was restored (Penzo-Mendez et al., 2003).

1.1.2.3 Wnt/Planar Cell Polarity

The Wnt/PCP pathway was originally identified in the Drosophila and is crucial for tissue polarity of epithelial and mesenchymal cells in multiple organisms (Seifert and Mlodzik, 2007; Vinson and Adler, 1987). The Wnt/PCP pathway provides positional information to cells of tissues in order to generate polarization of structures and information to help cells orient themselves in a specific fashion (Seifert and Mlodzik, 2007). Many genes involved in the Wnt/PCP pathway have been identified and include Fzd, the transmembrane protein Starbismus/Vang-like (Stbm/Vangl), the adaptor protein Prickle (Pk), and the atypical cadherin Flamingo/Starry night (Fmi/Stan) (Seifert and Mlodzik, 2007). Furthermore, mutations in the genes Wnt5b (pipe tail) (Rauch et al., 1997), Wnt11 (silberblick) (Heisenberg et al., 2000), glypican 4 (knypek) (Topczewski et al., 2001), and Vang-like2 (Vangl2;trilobite) (Jessen et al., 2002) disrupt proper gastrulation movements of convergence and extension, an aspect of development controlled by the Wnt/PCP pathway (Dale et al., 2009). In Drosophila, PCP mutants exhibit disruption in the orientation of cells in the eye, wing, and abdomen (Seifert and Mlodzik, 2007). Within the Drosophila eye, PCP mutants show defects in the arrangement of
photoreceptors and the hairs present on the wing exhibit a swirly pattern as opposed to the characteristic proximal-distal orientation. During Drosophila development and cell morphogenesis, it is essential that the PCP components exhibit asymmetric localization. Within the wing, the proteins Fzd, Dsh, and Diego (Dgo) are expressed together on the distal cell membranes where as Pk and Stbm are enriched on the proximal membrane. The proper localized expression of those proteins is essential in establishing proper orientation and development of cells within the Drosophila wing (Amonlirdviman et al., 2005).

1.2 Wnt Glycoproteins

The first ever discovered Wnt gene was formerly known as Int1 and is now known as the mouse Wnt1 gene (Nusse and Varmus, 1982). The Wnt family of glycoproteins are found in most metazoans and play key roles in numerous aspects of development (Logan and Nusse, 2004). The range of Wnt family members is vast as there are 5 members found in the worm, 7 in flies, 15 in zebrafish, and a total of 19 members in mice and humans (Korkut and Budnik, 2009). The family of Wnt proteins can be divided into two classes, the canonical activating Wnts or the noncanonical activating Wnts. At the primary sequence level all Wnts share an invariant pattern of 23 Cys residues, all contain a signal sequence and several potential N-glycosylation sites (Miller, 2002). The glycosylation of Wnt proteins is not fully understood but it is thought to play a role in intracellular trafficking and targeting of Wnts to the proper exocytic route and may also aid in the extracellular spreading of the Wnts once released from the cell (Hausmann et al., 2007). The Wnt glycoproteins contain two conserved amino acids, Cys 77 and Ser 209, which are lipid modified by acetylation with the addition of palmitic acid and palmitoleic acid, respectively (Takada et al., 2006; Willert et al., 2003). It is thought that the acylation of Cys77 aids in the activity of the Wnt protein as mutations of Cys77 to Ala, severely diminished Wnt
signaling (Willert et al., 2003). In contrast, the acylation of Ser209 is required for the correct intracellular trafficking and secretion of Wnt as substitution of Ser209 with Ala, inhibited secretion of Wnt3a, restricting it to the endoplasmic reticulum (Takada et al., 2006). The study of Wnt proteins has been difficult due to their hydrophobic nature but recent investigations have given some insights into the production and secretion of Wnt proteins. Factors that are involved in the secretion and maturation of Wnt proteins include lipoprotein particles, a retromer complex, a multipass transmembrane protein Wntless (WLS) specific for Wnt secretion, and the acyltransferase, Porcupine, which acylates Cys77 and Ser209 (Coudreuse et al., 2006; Panakova et al., 2005; Prasad and Clark, 2006; Takada et al., 2006).

1.3 Frizzled Transmembrane Receptors

The frizzled receptor is one of the key components of both the β-catenin dependent and independent pathways and in humans and mice there are ten different genes that encode for ten different frizzleds (FZD1-10) (Wang et al., 2006). The Frizzled receptors display three basic regions, the N-terminal exofacial region that can participate with or without a co-receptor in the binding of agonist (Wnts) or antagonist (Secreted Frizzled-related protein family [sFRP], Wnt inhibitory factor-1 (WIF-1), Cerberus, and members of Dickkopf [DKK]) (Hsieh, 2004). The Frizzled receptors contain a transmembrane central core that contains seven hydrophobic α-helices, and the three intracellular loops (iLoop1, iLoop2, and iLoop3), and a C-terminal tail that communicates the downstream effects upon ligand binding (Wang et al., 2006). All the frizzleds are similar to each other ranging in amino acid length of 537 to 706 with Hfz-4 being the shortest and Hfz-6 being the longest (Wang et al., 2006). The exofacial N-terminus region of all frizzleds contain a cystein-rich domain (CRD) which is involved in the binding of Wnt ligands and is also the site of N-glycosylation, a characteristic of typical GPCRs (Morris and Malbon, 1999). Also,
the extracellular loops between transmembrane segments II and III and transmembrane segments IV and V are linked by a di-sulfide bridge (Moxham and Malbon, 1985; Wang et al., 2006). On the cytoplasmic side of the frizzled receptor, Frizzleds-1,2,4,5,7,8, and 10 contain the C-terminal PDZ binding motif “Ser/Thr-Xxx-Val” whereas Frizzleds-3,6, and 9 are devoid of such a motif (Wang et al., 2006). The greatest divergence amongst the Frizzled receptors lies in the C-terminal portion of the receptors as the length varies, with Hfz-1 being the shortest (24 amino acids) and Hfz-6 the longest (212 amino acids). Last, all frizzled receptors contain the C-terminal KTxxxW motif which directly binds to the PDZ domain of Dvl, the central player in Fzd-induced signal transduction of both canonical and noncanonical Wnt signaling (Wallingford and Habas, 2005; Wong et al., 2003).

1.4 LRP5/6

The Wnt co-receptors, LDL receptor-related proteins 5 and 6 (LRP5/6) and the Drosophila ortholog, Arrow, are type I single-span transmembrane proteins composed of 1615, 1613, and 1678 amino acids respectively (Brown et al., 1998; Wehrli et al., 2000). The two receptors, LRP5 and LRP6, share 73% and 64% identity in the extracellular and intracellular domains, respectively, with Arrow sharing a 40% total identity to both LRP5 and LRP6 (Brown et al., 1998; Wehrli et al., 2000). The extracellular regions of LRP5/LRP6/Arrow contain three different domains which include the YWTD (Tyr-Trp-Thr-Asp) type β-propeller domain, an epidermal growth factor (EGF) – like domain, and a LDLR type A (LA) domain (Jeon et al., 2001; Springer, 1998). The intracellular region of LRP5, LRP6, and Arrow are composed of 207, 218, and 209 amino acids respectively and are all rich in prolines and serines which make up 15-20% of the amino acid composition (Tamai et al., 2004). The intracellular regions of the receptors share no sequence similarities with other LDLR proteins, lack any recognizable
catalytic motifs, but contain five reiterated PPP(S/T)P motifs that are used for axin binding which is essential for receptor signaling (Tamai et al., 2000; Tamai et al., 2004). It has been demonstrated that the PPP(S/T)P motif is the minimal requirement for the activation of the Wnt pathway as cells transfected with a truncated LDLR receptor, which plays no role in Wnt signaling, fused to one PPP(S/T)P motif was observed to bind Axin and stimulate the Wnt/β-catenin pathway (Tamai et al., 2000; Tamai et al., 2004). Furthermore, Axin preferentially binds to phosphorylated PPP(S/T)P motifs. Upon Wnt binding the PPP(S/T)P motifs are phosphorylated, promoting axin binding, which then directly controls the phosphorylation and degradation of β-catenin (Tamai et al., 2000).

1.5 Other unconventional Wnt receptors

It is firmly established that the receptors required for Wnt signaling include those of the Frizzled family and the LRP5/6 co-receptor (Clevers, 2006). Recently, studies have shown that other non-conventional Wnt co-receptors are also involved in both canonical and non-canonical Wnt signaling pathways. These receptors include the receptor tyrosine kinases RYK, ROR, and PTK7 (Billiard et al., 2005; Lu et al., 2004a; Shnitsar and Borchers, 2008). LTK is closely related to the receptor tyrosine kinases RYK, ROR, and PTK7 according to a kinase dendrogram (Figure 2) and the work presented in this thesis strongly argues that LTK is a new unconventional Wnt receptor.

1.5.1 RYK

Related to tyrosine kinases (RYK) belongs to a subclass of catalytically inactive receptor tyrosine kinases which contains a glycosylated extracellular domain, a transmembrane domain, and an intracellular kinase domain that contains amino acid substitutions that prevent ATP binding thus rendering the receptor catalytically inactive (Hovens et al., 1992; Katso et al.,
The extracellular domain of RYK contains sequence homology to Wnt inhibitory factor (WIF) which was the first indication that RYK may bind to Wnt glycoproteins (Patthy, 2000). Researchers then demonstrated that RYK binds to Wnt1, Wnt3, Wnt3a, and Wnt5a (Keeble et al., 2006; Lu et al., 2004a; Schmitt et al., 2006). The binding of these Wnt proteins to RYK was shown to influence the expression of T cell Factor (TCF), affect medial-lateral retinotectal topographic mapping, induce neurite outgrowth, and was required for axon guidance (Keeble et al., 2006; Lu et al., 2004a; Schmitt et al., 2006). An interesting mechanism of function was observed by Lyu et al in which binding of Wnt to RYK induces intracellular cleavage of the receptor by γ-secretase. The release of the RYK intracellular domain (ICD) allows for the interaction of the ICD with Cdc37 which promotes the stabilization and translocation of the ICD into the nucleus (Lyu et al., 2009). The translocation of the ICD into the nucleus then stimulates transcription that is required for neurogenesis (Lyu et al., 2008).

1.5.2 ROR

The receptor tyrosine kinase-like orphan receptors (RORs) are a family of type I transmembrane receptor tyrosine kinases which have two structurally similar members, ROR1 and ROR2 (Masiakowski and Carroll, 1992). Both receptors are involved in developmental processes which include skeletal and neuronal development, cell movement, and cell polarity (Green et al., 2008). The ROR receptors are composed of an extracellular domain which contains an immunoglobulin domain, a Cystine rich domain, and a Kringle domain, and an intracellular domain which contains a Tyrosine kinase domain and a Proline rich domain which is flanked by two Ser/Thr-rich domains (Masiakowski and Carroll, 1992). Both ROR1 and ROR2 are catalytically active and several splice variants which lack the extracellular or the transmembrane and intracellular domains have been reported (Masiakowski and Carroll, 1992; Oishi et al., 1999; Reddy et al., 1996). The extracellular Cystine-rich domain of the ROR proteins is highly similar
to those of the Wnt-binding FZD receptors also suggesting that Wnts were potential ligands to
these previously ligand orphan receptors (Masiakowski and Carroll, 1992; Roszmusz et al.,
2001; Saldanha et al., 1998; Xu and Nusse, 1998). Nonetheless, evidence demonstrates that,
ROR2 binds to both Wnt1 and Wnt3 which in turn functionally antagonized Wnt1- and Wnt3-
mediated stabilization of cytosolic β-catenin in osteoblastic cells (Billiard et al., 2005). ROR2 is
able to activate the non-canonical Wnt5a/JNK pathway by directly binding to Wnt5a which
induces receptor homodimerization and activation (Liu et al., 2008; Oishi et al., 2003).
Additionally, ROR1 and ROR2 form a heterodimer mediated by Wnt5a, as demonstrated through
immunoprecipitation experiments using whole brain lysates from E16 mouse embryos (Paganoni
et al., 2010). The presence of both ROR1 and ROR2 is required for proper synapse formation in
hippocampal neurons of mice as decreasing receptor expression through siRNA mediated gene
silencing reduced the amount of synapse formation (Paganoni et al., 2010). Furthermore, ROR1
and ROR2 are expressed mainly in the nervous system during various stages of mouse
embryogenesis (E8-E17) (Oishi et al., 1999).

1.5.3  PTK7
Protein tyrosine kinase-7 (PTK7), also known as colon carcinoma kinase-4 (CCK-4) (Jung et al.,
2002; Mossie et al., 1995), contains an extracellular domain with seven immunoglobulin like (Ig)
loops and an intracellular kinase domain that is catalytically inactive due to the lack of the Asp-
Phe-Gly triplet required for catalytic activity (Kroicher et al., 2001; Park et al., 1996; Shnitsar and
Borchers, 2008). PTK7 is a PCP pathway regulator and a novel non-conventional Wnt receptor.
Mutations in the mouse PTK7 gene disrupts neural tube closure and stereociliary bundle
orientation and is required for Xenopus neural convergent extension and neural tube closure (Lu
et al., 2004b). Also, in Xenopus, it was observed PTK7 recruits DVL to the plasma membrane
and is required for FZD7-mediated dsh localization (Shnitsar and Borchers, 2008).
1.5.4 LTK

Given the close relationship Leukocyte Tyrosine Kinase (LTK) shares with RYK, ROR, and PTK7 (Figure 2) and the supporting evidence of their involvement in neuronal development, makes LTK a candidate unconventional Wnt receptor. LTK was first isolated from leukocytes and is related to several tyrosine kinase receptor genes that are part of the insulin receptor family. LTK was first reported to encode a receptor devoid of an extracellular domain (Ben-Neriah and Bauskin, 1988) but further studies revealed the presence of several transcript variants which encode an extracellular domain containing a signal sequence, a single spanning transmembrane domain, and an intracellular kinase domain. Furthermore, one human transcript variant was reported to encode a receptor that lacks the intracellular kinase domain (Toyoshima et al., 1993).

LTK is a ligand orphan receptor which is mainly expressed in pre-B and B lymphocytes, the brain, and in human leukaemias (Ben-Neriah and Bauskin, 1988; Bernards and de la Monte, 1990). The fact that LTK is a ligand orphan receptor, make studies into the functional mechanisms difficult. Such difficulty may be overcome by the generation of a chimeric receptor in which the extracellular portion of a receptor with a known ligand is fused to the transmembrane and intracellular portion of the ligand orphan receptor, allowing for receptor activation. This method was employed in early studies to understand the functional mechanisms of LTK. Upon stimulation, it was observed that Tyrosine 485 and Tyrosine 862 are phosphorylated which promoted the interaction of insulin receptor substrate-1 (IRS-1) and Shc with those residues, respectively. Furthermore, the binding of IRS-1 and Shc promoted the activation of the Ras pathway where binding by IRS-1 alone suppressed apoptosis of hematopoietic cells (Ueno et al., 1996). In addition, phosphorylation of Tyrosine 753 promoted the direct binding of the p85 subunit of Phosphoinositide 3-kinase (PI3K), which in turn activated the PI3K pathway (Ueno et al., 1997). The activation of LTK tyrosine kinase activity
Figure 2: Tyrosine kinase dendrogram.

(A) A kinase dendrogram adapted from Manning *et al.* depicting a portion of the tyrosine kinase tree (Manning *et al.*, 2002). LTK is highlighted in red and boxed. Receptor tyrosine kinases relevant to Wnt signaling are highlighted in red. (B) A schematic of receptor tyrosine kinases mentioned within the work presented.
is sufficient to promote neurite outgrowth by catalyzing reactions in the PI3K and MAPK pathways (Yamada et al., 2008). This suggested that the phosphorylation of Tyr 753 activates the PI3K/AKT pathway which leads to neurite outgrowth and neuronal survival (Yamada et al., 2008).

1.6 MAGUKs

Axin is the scaffolding protein which brings the core regulatory components of the canonical Wnt-pathway together in order to control β-catenin levels in the cytosol (Clevers, 2006). The MAGUKs (membrane-associated guanylate kinase homologs) are a scaffolding protein family that aid in various cell signaling pathways by bringing together components at the plasma membrane and several members have been implicated in Wnt signaling (See below Section 1.6.1). MAGUKs have been show to localize in several types of junctions and are required for junction formation which includes synaptic and neuromuscular junctions along with epithelial and tight junctions (Dimitratos et al., 1999). The MAGUKs are generally composed of several distinctive binding domains which include, from N- to C-terminus, one to three PDZ domains, an SH3 domain, and a region that is homologous to yeast and mammalian cytoplasmic gyanylate kinases termed the Guk domain (Anderson, 1996; Dimitratos et al., 1999). Gyanylate kinases contain GMP and ATP binding sites which convert GMP to GDP by the addition of a phosphate group acquired from ATP (Berger et al., 1989; Klenow and Lichtler, 1957; Stehle and Schulz, 1990). The GuK domain of MAGUKs lack this catalytic activity due to mutations in the GMP and/or ATP binding sites which prevent GMP and/or ATP from binding (Kistner et al., 1995; Willott et al., 1993; Woods and Bryant, 1991). MAGUKs also contain a HOOK domain, a short amino acid stretch located between the SH3 and Guk domains that is required for localization to the plasma membrane of imaginal disc cells and for epithelial growth regulation (Hough et al.,
Some MAGUKs contain a C-terminal CaM kinase and calmodulin-binding site and a Lin-7-binding domain, also known as the Veli-binding domain in mammals, which binds to proteins that interact with transmembrane receptors (Dimitratos et al., 1997). The MAGUK family of scaffolding proteins is further divided into four subfamilies based on domain content and sequence similarities. These families include the Dlg-like MAGUKs that contain three PDZ domains, an SH3 domain, GuK domain, and in some cases a HOOK domain, the ZO-1-like MAGUKs which contain three PDZ domains, a SH3 domain, a GuK domain, and a C-terminal proline rich extension. The ZO-1 like subfamily is composed of ZO-1, ZO-2, and ZO-3 which are localized at tight junctions in mammals and septate junctions of Drosophila. Also, the P55-like MAGUKs were first identified as a palmitoylated protein isolated from erythrocyte membrane cytoskeleton. Members of this subfamily contain one PDZ domain, a SH3 domain, and a GuK domain. Last, the Lin-2-like MAGUKs contain one PDZ domain, a SH3 domain, a GuK domain, and an N-terminal calmodulin-binding domain which is followed by a Lin-7-binding region (Dimitratos et al., 1999). The proteins in the Lin-2-like subfamily are orthologous to C. elegans Lin-2 with the C-terminal portion of the protein being homologous to p55 (Cohen et al., 1998; Dimitratos et al., 1999). The MAGUKs help organize cytoplasmic proteins and membrane-associated proteins into distinct multimolecular complexes in order to increase interaction efficiency (Kornau et al., 1995; Tejedor et al., 1997; Tsunoda et al., 1997). These protein complexes are also localized to special regions of the plasma membrane such as cell junctions and the apicolateral and basolateral surface, where they can be targeted to the appropriate receptors and thereby increasing receptor efficacy (Wilson et al., 1996). This increases the efficiency of signal transduction through localization of specific receptor molecules to regions of the cell surface that are exposed to the respective ligand. In addition, it allows signaling cascades to be spatially restricted, allowing for the use of the same intermediate
components by different signaling cascades to elicit a downstream response (Carraway and Carraway, 1995; Dimitratos et al., 1999).

1.6.1 MPP3

Membrane protein palmitoylated 3 (MPP3) is a 585 amino acid long cytoplasmic protein among the MAGUK family members which has high homology to Dlg3 and thus belongs to the Dlg-like subfamily (Fukuhara et al., 2003; Masuda et al., 2002; Smith et al., 1996). MPP3 is composed of two Lin2/7 domains, a PDZ domain, a SH3 domain, and a GuK domain and has been shown to bind to the Tumor Suppressor In Lung Cancer 1 (TSLC1) protein, which is a glycoprotein that is expressed and predominantly distributed in the lateral membrane of polarized epithelial cells (Masuda et al., 2002). TSLC1 is a tumor suppressor in non-small cell lung cancer (NSCLC) as suppression of TSLC1 expression induces tumorigenicity of A549 cells, a NSCLC cell line (Kuramochi et al., 2001). Wnt signaling has also been implicated in lung cancer formation as evidence shows upregulation of Dvl in NSCLC cells, and both Wnt1 and Wnt2 are over expressed in NSCLC cell lines and primary tissue (He et al., 2004; Mazieres et al., 2005; Uematsu et al., 2003; You et al., 2004). MPP3 may influence tumor suppression through the Wnt pathway by linking TSLC1 with Wnt pathway components in order to suppress tumorigenicity. Additionally, MPP3 has been reported to be recruited to the MPP5 protein scaffold that is located at the retinal outer limiting membrane (Kantardzhieva et al., 2006). MPP5, a MAGUK protein also known as PALS, forms a complex with Par6 which is important in non-canonical Wnt signaling enduced cell polarity (Bachmann et al., 2001; Etienne-Manneville et al., 2005). Given the protein interactions reported between MPP3, TSLC1, and MMP5 and the implication and involvement of these interacting partners in Wnt signaling (Bachmann et al., 2001), make MPP3 a potential modulator of the Wnt signaling pathway.
1.7 Thesis perspective

Signaling pathways play crucial roles in a wide variety of cellular processes such as cell differentiation, proliferation, growth and death (Clevers, 2006; Moustakas and Heldin, 2009; Xiao et al., 2007). For example, the Wnt pathway functions in diverse processes throughout development such as planar cell polarity and axon guidance (Keeble et al., 2006; Simons and Mlodzik, 2008). Most pathways are capable of influencing each other, thus promoting “cross-talk” that makes each pathway even more complex and interesting with a plethora of known and unknown players which co-operate in a variety of contexts. To provide some insights, investigators have used high-throughput (HTP) screening approaches to unravel these intricacies and identify novel modulators of various pathways. With the use of a HTP siRNA screen of the human kinome performed by a previous post doctoral fellow of the lab, Dr. Mary Erclik, I identified MPP3 and LTK as potential negative regulators of the Wnt signaling pathway. Overall, the objective of this thesis was to manually verify hits obtained from the screen, establish MPP3 and LTK as a negative regulator of the canonical Wnt signaling pathway, and gain insight into potential mechanisms of function of LTK in the canonical Wnt signaling pathway.
2 Methods and Materials

2.1 Mammalian expression constructs

Axin-3xFlag and LRP6-Myc were kindly provided by Dr. Bryan Miller. IGFR1-FFluc, Vangl1-3xFlag, and Vangl2-3xFlag constructs were obtained from J. Wrana’s lab, at SLRI. Dr. Monika Podkowa generously provided the TβRII-3xFlag construct. Mr. Peter Ching provided the following constructs: FZD2-FFluc, FZD4-FFluc, FZD5-FFluc, FZD6-FFluc, FZD7-FFluc, FZD8-FFluc, FZD9-FFluc, FZD10-FFluc, FZD7-3xFlag, and FZD7∆C-3xFlag. TOPflash reporter in pGL3 (pOT) was obtained from B. Vogelstein.

To generate MPP3-3xFlag, MPP3 was amplified from MGC clone 64841 (Accession No. BC056865) by PCR with the addition of a consensus start sequence and inserted into a pCMV5c backbone containing a C-terminal 3xFlag tag using the restriction sites MluI and XbaI. MPP3 mutant derivatives were created using MPP3-3xFlag as template and inserted into a pCMV5c backbone containing a C-terminal 3xFlag tag using the restriction sites MluI and XbaI. LTK∆KD-3xFlag and FL LTK-3xFlag were generated by PCR amplification of LTK from MGC clone 33146 (Accession No. BC045607) followed by insertion into a pCMV5c backbone containing a C-terminal 3xFlag tag using restriction sites MluI and XbaI. A consensus start sequence was inserted into both constructs. The LTK∆KD-3xFlag was generated by PCR amplification of the MGC clone resulting in a protein containing amino acids 1-478. The FL LTK-3xFlag construct was generated by PCR amplification and re-construction of three pieces as the MGC clone of LTK contained two unspliced introns. LTK-3xFlag and LTK∆KD-3xFlag were used in the generation of LTK-FFluc and LTK∆KD-FFluc by subcloning LTK and LTK∆KD into a pCMV5c backbone containing a C-terminal FFluc encoding region using MluI and NotI restriction sites.
2.2 Production of Wnt3a conditioned medium

Mouse fibroblast L cells stably expressing mouse Wnt3a were generated (Labbe et al., 2007) by stably transfecting using pPGK-neo/Wnt3a (Shibamoto et al., 1998). Cells were cultured in DMEM supplemented with 0.2% FBS, 0.2% Amphotericin B, and 1% penicillin/streptomycin, cultured at 5% CO\textsubscript{2} at 37°C for 3 days. Medium from control cells and Wnt3a expressing cells was collected and centrifuged to remove cell debris. Wnt3a conditioned medium was tested to ensure ligand activity using the TOPflash reporter assay. Once activity was confirmed, the medium was stored at 4°C.

2.3 Cell culturing

Human Embryonic Kidney (HEK) 293T cells were cultured in DMEM supplemented with 10% FBS. Neuro2a cells were cultured in DMEM supplemented with 10% FBS and split once 80% confluence was reached. NIE115 cells were cultured in DMEM without sodium pyruvate supplemented with 10% inactive FBS. All cells were incubated at 37°C in 5% CO\textsubscript{2}.

2.4 Transfection of cells

HEK293T cells were transfected using the calcium phosphate precipitation method. Cells were plated at a density of 80,000 cells/well in a 24 well plate format and transfected with a total of 0.67 µg of DNA composed of control and expression vectors. The DNA mixture was suspended in 22.5 µl of distilled H\textsubscript{2}O (dH\textsubscript{2}O) to which 2.5 µl of 2.5 M CaCl\textsubscript{2} was added and then vortexed. Subsequently, 25 µl of HEBS buffer (280 mM NaCl, 50 mM HEPES acid, 1.5 mM Na\textsubscript{2}HPO\textsubscript{4}, and 1000 ml dH\textsubscript{2}O pH 7.05) was added with bubbling and samples were then incubated at room temperature for 15 minutes. DNA precipitate was added to cells at a volume of 50 µl per well. Medium was changed approximately 24 hours post-transfection. NIE115 and Neuro2a cells were transfected using 5 µl Lipofectamine 2000 (Invitrogen) per 20 nM of siRNA per well of a
6-well dish or 10 µl Lipofectamine 2000 (Invitrogen) per 1.0 µg DNA per well of a 6-well dish according to the manufacturer’s protocol.

2.5 LUMIER assay

Protein-protein interactions were detected using the LUminescence-based Mammalian IntEＲactome (LUMIER) assay (Barrios-Rodiles et al., 2005; Miller et al., 2009). HEK293T cells seeded at a density of 80,000 cells/well in a 24 well plate format were transfected with 0.14 µg of FFluciferase tagged “bait” protein, 0.14 µg of 3X-Flag tagged “prey” protein, 0.025 µg of β-galactosidase reporter and pCMV5 empty vector to 0.67 µg using the calcium phosphate precipitation method. Medium was changed 24 hours post-transfection and cells were lysed 48 hours post-transfection in 250 µl lysis buffer (50 nM Tris-HCl, 150 nM NaCl, 1 mM EDTA, 0.1% Triton X-100, 10% glycerol and protease inhibitors). Aliquots of cell of lysates (150 µl) were immunoprecipitated using anti-Flag M2 monoclonal antibody (1:1000, Sigma) for one hour followed by incubation with Protein G sepharose 4 Fast Flow (GE Healthcare) for one hour both at 4°C. Immunoprecipitates were subsequently washed three times with wash buffer (5 nM Tris-HCL, 15 nM NaCl, 0.1 nM EDTA, 0.1% Triton X-100, and 10% glycerol) followed by resuspension in 50 µl of wash buffer. In order to quantify associated FFluciferase activity, 25 µl of luciferase substrate was added to 10 µl of immunoprecipitate and the reaction was measured in an EG&G Berthold microplate luminometer. Luciferase readings were normalized to β-galactosidase readings obtained from an aliquote of whole cell lysate. To ensure equivalent prey protein expression, an aliquot of the whole cell lysate was separated by SDS-PAGE and visualized using anti-Flag (1:3000, Sigma) immunoblotting.
2.6 TOPflash Reporter Assay

The TOPflash reporter consists of multiple TCF/LEF-1 binding sites upstream a promoter that drives the expression of a FFLuciferase gene. The activity of the FF luciferase gene can be quantified as expression is dependent on β-catenin levels, thus being Wnt responsive. HEK293T cells were typically transfected with 0.05µg of TOPflash reporter, 0.025 µg of β-galactosidase reporter, the indicated amount of pCMV5/3X-Flag DNA construct, 20 nM of siRNA if indicated, and empty vector pCMV5 to 0.67 µg. Cells were starved 24 hours post-transfection with DMEM supplemented with 0.2% FBS, 0.2% Amphotericin B, and 1% penicillin/streptomycin for a minimum of 2 hours. Cells were then treated with control or Wnt3a-conditioned medium overnight for a maximum of 16 hours. Cells were then lysed with lux lysis buffer (25 mM Tris-phosphate, 2 mM DTT, 2 mM DCTA, 10% glycerol and 1% Triton X-100) and luciferase activity was measured and normalized as previously described (LUMIER assay).

2.7 siRNAs

siRNA oligonucleotide complexes directed towards the designated genes were ordered from Dharmacon (Table 1) as a pool of four duplexes or as four individual duplexes. A control siRNA duplex (siCTL) was designed with a sequence of G GGC AAG ACG AGC GGG AAG dTdT, and was purchased from Dharmacon. siRNAs were used at a concentration of 20 nM for transfection experiments.

2.8 Real time quantitative reverse transcription PCR (QPCR)

HEK293T cells were plated in 6 well dishes and transfected with 20 nM of siRNA using the calcium phosphate DNA precipitation method. Total RNA was collected using the RNeasy QIAGEN RNA isolation kit. Total RNA was quantified on a spectrophotometer (Eppendorf),
Table 1: siRNAs used in work presented.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Genome</th>
<th>siRNA</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKT2</td>
<td>Human</td>
<td>siAKT2</td>
<td>M-003001-01-0005</td>
</tr>
<tr>
<td>CASK</td>
<td>Human</td>
<td>siCASK(1)</td>
<td>D-005311-01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>siCASK(2)</td>
<td>D-005311-02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>siCASK(3)</td>
<td>D-005311-05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>siCASK(4)</td>
<td>D-005311-06</td>
</tr>
<tr>
<td>DLG1</td>
<td>Human</td>
<td>siDLG1(1)</td>
<td>D-009415-01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>siDLG1(2)</td>
<td>D-009415-02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>siDLG1(3)</td>
<td>D-009415-03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>siDLG1(4)</td>
<td>D-009415-04</td>
</tr>
<tr>
<td>DUSP5</td>
<td>Human</td>
<td>siDUSP5(1)</td>
<td>D-003566-01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>siDUSP5(2)</td>
<td>D-003566-02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>siDUSP5(3)</td>
<td>D-003566-03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>siDUSP5(4)</td>
<td>D-003566-05</td>
</tr>
<tr>
<td>LTK</td>
<td>Human</td>
<td>siLTK(1)</td>
<td>D-003152-21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>siLTK(2)</td>
<td>D-003152-06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>siLTK(3)</td>
<td>D-003152-06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>siLTK(4)</td>
<td>D-003152-07</td>
</tr>
<tr>
<td>Ltk</td>
<td>Mouse</td>
<td>siLtk(1)</td>
<td>D-063855-01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>siLtk(2)</td>
<td>D-063855-02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>siLtk(3)</td>
<td>D-063855-03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>siLtk(4)</td>
<td>D-063855-04</td>
</tr>
<tr>
<td>MPP2</td>
<td>Human</td>
<td>siMPP2(1)</td>
<td>D-009729-01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>siMPP2(2)</td>
<td>D-009729-02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>siMPP2(3)</td>
<td>D-009729-03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>siMPP2(4)</td>
<td>D-009729-05</td>
</tr>
<tr>
<td>MPP3</td>
<td>Human</td>
<td>siMPP3(1)</td>
<td>D-010612-01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>siMPP3(2)</td>
<td>D-010612-02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>siMPP3(3)</td>
<td>D-010612-03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>siMPP3(4)</td>
<td>D-010612-05</td>
</tr>
<tr>
<td>Non-targetting</td>
<td>Human/Mouse</td>
<td>siCTL</td>
<td>Custom</td>
</tr>
<tr>
<td>Pak1</td>
<td>Mouse</td>
<td>siPak1(1)</td>
<td>D-048101-01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>siPak1(2)</td>
<td>D-048101-02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>siPak1(3)</td>
<td>D-048101-03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>siPak1(4)</td>
<td>D-048101-04</td>
</tr>
<tr>
<td>PSKH2</td>
<td>Human</td>
<td>siPSKH2</td>
<td>M-005366-00-0005</td>
</tr>
</tbody>
</table>
and 1 µg was DNaseI treated (Fermentas) followed by priming 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). Quantitative PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems) using the ABI Prism 7000 or 7900 sequence detection system (Applied Biosystems) with primers at 100 nM. Primers used were directed towards hLTK (Forward: TAT CTA CCG GGC CAG TTA TTA CCG CA, reverse: TTG GAT GTG AAG ATG CCC TCC AGG AA), hMPP3 (forward: TCA AAG GGC ACT ATG TGG CTG GT, reverse: AGG ACA TCT TTC CTT CCT GCG A) and hGAPDH (forward: ACA TCA AGA AGG TGG TGA AGC AGG, reverse: ACG AAT TTG GCT ACA GCA ACA GGG, (Labbe et al., 2007)). Neuro2a and NIE115 cells were plated in 6 well dishes and transfected with 20 nM of siRNA. Total RNA was collected and treated as described above and quantitative PCR was performed. Primers used were directed towards mLtk (forward: CTC ATT CTG CTG GAG CTG ATG TCT, reverse: TGC AAT AGG TCC TGC ATG GTC AGA), mPak1 (forward: TGA CGA TGC TAC CCC ACC T, reverse: AGT TGG AGT AAC AGG AAG TGG TT) and mGAPDH (forward: GAG ACA GCC GCA TCT TCT TGT, reverse: CAC ACC GAC CTT CAC CAT TTT (Labbe et al., 2007)). Relative quantitation of gene expression levels was calculated by the $\Delta\Delta C_t$ method normalized to GAPDH levels (docs.appliedbiosystems.com/pebiodocs/04303859.pdf).

2.9 β-catenin stabilization and Dvl upshift assay

Neuro2a and NIE115 cells were plated in 6 well dishes at a density of 120,000 cells/well and 100,000 cells/well, respectively. After 24 hours post-transfection, cells were washed with DMEM supplemented with 0.2% FBS. At 48 hours post-transfection, cells were treated with control or Wnt3a-conditioned media for two hours. Cell lysates were collected, separated on an
SDS-PAGE gel, and then visualized using anti β-catenin (1:2000, BD Biosciences) or anti-Dvl3 (1:1000, Santa Cruz) immunoblotting for β-catenin stabilization and Dvl upshift assays respectively. Anti-Actin (1:10000, Sigma-Aldrich) immunoblotting was used as loading control.
3 Results

3.1 The use of a high-throughput screening approach to search for new Wnt pathway modulators

In order to identify novel positive or negative regulators of the Activin, Wnt, and BMP signaling pathways, a previous Post-Doctoral Fellow in the lab, Dr. Mary Erclik, performed a high-throughput (HTP) screen using siRNAs directed towards the human kinome and phosphatome (Figure 3A). The siRNA library consists of over 800 siRNA pools directed towards an individual kinase, kinase-associated protein, or phosphatase. Gene knockdown was performed in HEK293T cells in a 384-well plate format and the influence on the Activin, Wnt, and BMP pathways was measured using pathway specific Luciferase reporter constructs (Figure 3B). The reporters included 3TP (Activin), TOPflash (Wnt), and IBRE (BMP) which upon stimulation by specific ligands, Activin, Wnt3a, and BMP2, respectively, induce activation of a Luciferase reporter (Figure 3C). The reporters contain pathway specific enhancer elements located upstream of a promoter that drives Firefly luciferase (FFlux) transcription upon pathway specific ligand stimulation (Figure 3B). To measure luciferase activity, luminescence was measured after cell lysis and addition of a luciferase substrate. All luciferase readings were normalized to β-galactosidase activity provided by a coexpressed unregulated β-galactosidase reporter to adjust for transfection efficiencies. Once the raw data from the screens was obtained, B-scores were calculated for each individual gene, and the average for triplicate runs of the Activin and BMP screens and quadruplicate runs of the Wnt screen was determined. A B-score is like a Z-score but it also takes into account within-plate systematic effects which may be used to remove row, column or well effects. Unlike the Z-score, the B-score cannot be easily calculated using standard spreadsheet formulas and is based on an iterative algorithm (Birmingham et al., 2009;
Day 1
Cell Seeding in 384 well plate

Day 2
Cell transfection
- siRNA
- β-galactosidase
- TOPflash

Day 3
Ligand treatment
+/− Ligand

Day 4
Assay Development
- Luciferase readings
- β-galactosidase readings

<table>
<thead>
<tr>
<th>Gene</th>
<th>3TP (Activin)</th>
<th>TOPflash (Wnt3a)</th>
<th>IBRE (BMP2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSKH2</td>
<td>10.59</td>
<td>-0.64</td>
<td>-2.06</td>
</tr>
<tr>
<td>AKT2</td>
<td>1.37</td>
<td>-0.57</td>
<td>-1.61</td>
</tr>
<tr>
<td>LTK</td>
<td>2.20</td>
<td>0.42</td>
<td>0.57</td>
</tr>
<tr>
<td>MPP3</td>
<td>0.90</td>
<td>3.84</td>
<td>1.84</td>
</tr>
<tr>
<td>CASK</td>
<td>-0.93</td>
<td>-0.15</td>
<td>-1.20</td>
</tr>
<tr>
<td>DLG1</td>
<td>4.47</td>
<td>3.40</td>
<td>1.82</td>
</tr>
<tr>
<td>MPP2</td>
<td>0.19</td>
<td>0.10</td>
<td>2.14</td>
</tr>
<tr>
<td>DUSP5</td>
<td>-0.01</td>
<td>2.36</td>
<td>-2.26</td>
</tr>
</tbody>
</table>

Figure 3
Figure 3: High throughput screening detects known signaling pathway regulators.

(A) Flow chart of screening procedure. (B) Luciferase reporter constructs used in the primary screens and manual verification experiments. The screen was performed on the TGFβ (3TP), Wnt3a (TOPflash), and BMP2 (IBRE) signaling pathways. (C) Confirmation of luciferase reporter activation to respective ligands. (D) AKT2 is identified as a regulator of the Wnt and TGFβ pathways. Average B-score of triplicate runs (TGFβ and BMP2) or quadruplicate runs (Wnt) from primary screen. Positive B-scores indicate enhanced reporter activity and negative B-scores indicate suppressed reporter activity. (E) Manual verification of the effect of siAKT2 on the TGFβ, Wnt, and BMP pathways. (F) Average B-scores of selected hits from primary screens. TGFβ and BMP screens were performed in triplicate for -/+ ligand treatment, while Wnt screen was performed in quadruplicate for -/+ ligand treatment.
Brideau et al., 2003). Arbitrary cut-offs of B-scores of 3 and -3 were used to define a set of genes that fell above or below these cut-off marks for further investigation (Figure 3D).

In order to ensure that the screening procedure was performing as expected, the effects of siRNAs towards known regulatory components of the investigated signaling pathways were examined. AKT2 has been reported as a negative regulator of the Activin pathway and a positive regulator of the Wnt pathway (Fukumoto et al., 2001; Song et al., 2006). Consistent with this, knockdown of AKT2 yielded an average B-score of 5.11 and -3.12 in the Activin and Wnt runs, respectively (Figure 3D,E). HTP screens using siRNA gene knockdown often yield false-positives and to eliminate these selected hits were manually verified. For this, screen hits were re-tested in a non-HTP manner using the same luciferase signaling assay. For this, HEK293T cells were transiently-transfected with siRNAs, signaling reporter, and β-galactosidase reporter. Cells were then treated overnight with and without appropriate ligand and reporter activity was measured by luciferase assay.

Screen hits displaying a variety of properties were selected from the HTP screen (Figure 3D) and manually verified to ensure the reproducibility of the selected hits. The siRNAs directed towards Leukocyte Tyrosine Kinase (LTK) and Membrane Protein Palmitoylated 3 (MPP3), specifically influenced the Wnt pathway by enhancing TOPflash activity in the primary screen indicating a negative regulatory role in the Wnt pathway (Figure 4A,C). Activin responsive 3TP activity was decreased and increased in cells treated with siRNAs directed towards Calcium/Calmodulin-Dependent Serine Protein Kinase (CASK) and Discs Large Homolog 1 (DLG1) respectively, indicating a positive regulatory role for CASK and a negative regulatory role for DLG1 in the Activin pathway (Figure 5A,C). Furthermore, siRNAs directed towards Membrane Protein Palmitoylated 2 (MPP2) and Dual Specificity Phosphatase 5 (DUSP5) influenced a negative and
Figure 4: siRNA directed towards \textit{LTK} and \textit{MPP3} modulate the Wnt but not the TGFβ and BMP pathways.

(A,C) Average B-scores from primary screens. B-score cut-off marks were arbitrarily set at 3 and -3 and genes falling over the cut off were further investigated. Average B-score of triplicate runs (TGFβ and BMP2) or quadruplicate runs (Wnt) were used. (B,D) Analysis of Luciferase reporter activity by manual assays. HEK293T cells were transiently-transfected with an siRNA pool directed towards \textit{LTK} or \textit{MPP3} and the effect on pathway-specific reporter activation was determined. The mean +/- standard deviation for triplicates of a representative experiment repeated a minimum of three times is shown.
Figure 5: siRNA directed towards CASK and DLG1 modulate the TGFβ but not the Wnt and BMP pathways.

(A,C) Average B-scores from primary screens. B-score cut-off marks were arbitrarily set at 3 and -3 and genes falling over the cut off were further investigated. Average B-score of triplicate runs (TGFβ and BMP2) or quadruplicate runs (Wnt) were used. (B,D) Analysis of Luciferase reporter activity by manual assays. HEK293T cells were transiently-transfected with an siRNA pool directed towards CASK or DLG1 and the effect on pathway-specific reporter activation was determined. The mean +/- standard deviation for triplicates of a representative experiment repeated a minimum of three times is shown.
positive effect on the BMP pathway respectively (Figure 6A,C). The results obtained from the manual assays verified that the screen performed well as siRNA knockdown of LTK and MPP3 increased TOPflash activity (Figure 4B,D), siRNA knockdown of CASK and DLG1 decreased and increased 3TP activity respectively (Figure 5B,D), and siRNA knockdown of MMP2 and DUSP5 increased and decreased IBRE activity respectively (Figure 6B,D).

Protein Serine Kinase H2 (PSKH2) was originally identified as a potential negative regulator of the Activin pathway as siRNA directed towards PSKH2 increased 3TP activity (Figure 7A). However in manual verification tests, siRNA knockdown of PSKH2 had minimal influence on 3TP activity as compared to cells not transfected with siRNA (Figure 7B), indicating PSKH2 was a false-positive obtained from the HTP screen. The reason for this may be due to experimental inconsistency as the B-scores amongst triplicate runs were highly variable.

These results demonstrate that the HTP screening procedure is highly reliable as all the screening results were manually verified with the exception of PSKH2 (Figure 8). This shows that this HTP screen can be used to search for new components of various signaling pathways.

3.2 MPP3 acts as a negative regulator of the Wnt pathway

MPP3 is a scaffolding protein which is part of the Membrane-Associated Guanylate Kinase (MAGUK) family of proteins. MAGUKs are important in the coupling of signals from the extracellular environment to intracellular signaling pathways and the cytoskeleton (Anderson, 1996). Results from gene knockdown from the primary screen showed that siMPP3 increased TOPflash activity suggesting that MPP3 plays a negative regulatory role in the Wnt pathway. The siRNAs used in the primary screen and in the subsequent manual verification were comprised of a pool of four individual siRNAs that target four different regions of the gene. To
Figure 6: siRNA directed towards MPP2 and DUSP5 modulated the BMP but not the Wnt and TGFβ pathways.

(A,C) Average B-scores from primary screens. B-score cut-off marks were arbitrarily set at 3 and -3 and genes falling over the cut off were further investigated. Average B-score of triplicate runs (TGFβ and BMP2) or quadruplicate runs (Wnt) were used. (B,D) Analysis of Luciferase reporter activity by manual assays. HEK293T cells were transiently-transfected with an siRNA pool directed towards MPP2 or DUSP5 and the effect on pathway-specific reporter activation was determined. The mean +/- standard deviation for triplicates of a representative experiment repeated a minimum of three times is shown.
Figure 7: siRNA directed towards PSKH2 had no effect on the TGFβ, Wnt, and BMP pathways.

(A) Average B-scores from primary screens. B-score cut-off marks were arbitrarily set at 3 and -3 and genes falling over the cut off were further investigated. Average B-score of triplicate runs (TGFβ and BMP2) or quadruplicate runs (Wnt) were used. (B) Analysis of Luciferase reporter activity by manual assays. HEK293T cells were transiently-transfected with an siRNA pool directed towards PSKH2 and the effect on pathway-specific reporter activation was determined. The mean +/- standard deviation for triplicates of a representative experiment repeated a minimum of three times is shown.
Figure 8: Comparison of the primary screen data to the manual verification data.

A summary of the manual verification of hits obtained from the screen are shown. “-“ indicates the screening results were not manually reproduced and “+” indicates the screening results were manually reproduced. This shows that the screening results are reliable. * Note that the B-scores for triplicate runs of PSKH2 were highly variable.
detect the potential existence of off-target effects, the pool of siRNAs was deconvolved and the effect on TOPflash activity of each individual siRNA was investigated in HEK293T cells using the luciferase reporter assay. This analysis revealed that three of the four siRNAs, siMPP3#2, siMPP3#3 and siMPP3#4, increased TOPflash activity with siMPP3#2 and #4 yielded the most significant increase in TOPflash activation (Figure 9A). To ensure that the individual siRNAs target the gene of interest, Q-PCR analysis was performed to determine the extent of endogenous gene knockdown. HEK293T cells were transiently-transfected with each individual and the pool of siRNAs directed towards MPP3 for 48 hours. Total RNA was collected from cell lysates and reverse transcription was performed on DNaseI treated total mRNA to produce cDNA. Primers that are intron spanning and produce a fragment between 100-200 base pairs (bp) in size were designed and Q-PCR was performed. All individual siRNAs and the pool of siRNAs directed towards MPP3 decreased endogenous MPP3 at levels ranging from 50% to 80% as compared to cells transfected with a non-targeting control siRNA (Figure 9B). The siRNAs siMPP3#1 and siMPP3#3 yielded the lowest knockdown efficiency and consistent with this, these siRNAs comparatively had the least influence on TOPflash activity (Figure 9). In general, it is expected that at least two independent siRNAs targeting the same gene yielding the same biological effect is indicative of a true hit. Thus, these results are consistent with a negative regulatory role for MPP3 in the Wnt pathway.

3.3 The Guk domain of MPP3 is important for MPP3 function in the Wnt pathway

We next examined the effect of overexpression of MPP3 on TOPflash activity. For this, a 3xFlag-tagged MPP3 construct was generated and the effect of expressing increasing amounts of MPP3 on TOPflash activity was determined. HEK293T cells were transiently-transfected with
Figure 9: MPP3 is a negative regulator of the Wnt pathway.

(A) Knockdown of MPP3 increases TOPflash activity. HEK293T cells were transiently-transfected with 20 nM control, each of the 4 individual MPP3-targetting siRNA or pool of MPP3-targetting siRNA, together with TOPflash, and a β-galactosidase reporter using the CaPO4 method. Cells were treated overnight with control or Wnt3a-conditioned media and TOPflash activity was measured by luciferase assay normalized to β-galactosidase activity. The mean +/- standard deviation for triplicates of a representative experiment repeated a minimum of three times is shown. (B) MPP3-targetting siRNA decreases endogenous MPP3 levels. HEK293T cells were transiently-transfected with 20 nM of control siRNA, each of the 4 individual MPP3-targetting siRNA, or pool of MPP3-targetting siRNA for 48 hours and MPP3 levels were analyzed by Quantitative PCR (Q-PCR). The mean +/- standard deviation for triplicates of a representative experiment repeated a minimum of three times is shown.
increasing amounts of MPP3 cDNA, cells were treated overnight with control or Wnt3a-conditioned media and then luciferase activity was measured using the luciferase assay. No difference in TOPflash activity was observed (Figure 10B) suggesting that there is sufficient expression of endogenous MPP3 to elicit a negative regulatory effect.

MPP3 is a scaffolding protein that contains several protein-protein interaction domains. In order from N-terminal to C-terminal these domains include two Lin2/7 domains, a PDZ domain, a SH3 domain and a Guk domain (Figure 10A). The Lin2/7 domains bind to Lin-2 and Lin-7 proteins which are scaffolding proteins capable of forming complexes to aid in protein-protein interactions (Harris et al., 2002). PDZ domains bind to PDZ binding motifs which are generally found on the extreme carboxy terminal end of proteins however, internal PDZ binding motifs have been reported (Anderson, 1996). SH3 domains bind to polyproline motifs and the MAGUK Guk domains are homologous to that of the Saccharomyces cerevisiae guanylate kinase (Guk1) enzyme that catalyzes the conversion of GMP to GDP but MAGUK Guks lack the binding ability for GMP and/or ATP or lack the catalytic activity to transfer the phosphate group from ATP to GMP. To determine whether these domains play a role in Wnt signaling, dominant negative versions of 3xFlag-tagged MPP3 were generated that harbor deletions of the second Lin2/7 domain (MPP3ΔC-Lin2/7), PDZ domain (MPP3ΔPDZ), or the Guk domain(MPP3ΔGuk) (Figure 11A). Expression of deletion constructs in transiently-transfected HEK293T cells was examined by anti-Flag immunoblotting and confirm equal expression levels as compared to wild type (WT) MPP3 (Figure 11B). The effect of over expression of these deletion constructs on TOPflash activity was then investigated by transiently-transfecting HEK293T cells with increasing amounts of individual deletion constructs and the TOPflash reporter. It was observed that overexpression of either MPP3ΔC-Lin2/7 or MPP3ΔPDZ had no effect on TOPflash activity (Figure 12A,C), suggesting that the two domains do not play an essential role in modulating Wnt
Figure 10
Figure 10: Increasing amounts of MPP3 does not down regulate the Wnt pathway.

(A) A schematic of MPP3 with the Lin2/7, PDZ, SH3, and Guk domains indicated in their respective amino acid position. (B) Over expression of MPP3 does not affect TOPflash activity. HEK293T cells were transiently-transfected with increasing amounts of 3X-Flag tagged MPP3 cDNA construct, TOPflash, and a β-galactosidase reporter using the CaPO₄ method. Cells were treated overnight with and without Wnt3a and TOPflash activity was measured by luciferase assay normalized to β-galactosidase activity. The mean +/- standard deviation for triplicates of a representative experiment repeated a minimum of three times is shown. (C) Increasing amounts of exogenous MPP3 was detected using immunoblotting. HEK293T cells were transiently-transfected with increasing amounts of 3X-Flag tagged MPP3 cDNA construct using the CaPO₄ method and lysates were used to demonstrate increased expression of exogenous protein with increasing amounts of cDNA tranfected.
Figure 11
Figure 11: A schematic of MPP3 deletion constructs.

(A) MPP3 and deletion constructs with the Lin2/7, PDZ, SH3, and Guk domains are depicted with domains indicated in their respective amino acid position. (B) MPP3 deletion constructs are expressed at equal levels as wild type. HEK293T cells were transiently-transfected with 3X-Flag tagged wild type MPP3 or MPP3 deletion constructs ΔC-Lin2/7, ΔPDZ, and ΔGuk using the CaPO₄ method. Exogenous protein expression was analyzed by anti-Flag immunoblotting.
Figure 12
**Figure 12:** Deletion of one Lin2/7 domain and the PDZ domain of MPP3 do not influence the Wnt pathway.

(A,C) Over expression of MPP3ΔPDZ (A) and MPP3ΔC-Lin2/7 (B) do not affect TOPflash activity. HEK293T cells were transiently-transfected with increasing amounts of a 3X-Flag tagged MPP3ΔPDZ or MPP3ΔC-Lin2/7 cDNA construct along with TOPflash, and a β-galactosidase reporter using the CaPO₄ method. Cells were treated overnight with and without Wnt3a and TOPflash activity was measured by luciferase assay normalized to β-galactosidase activity. The mean +/- standard deviation for triplicates of a representative experiment repeated a minimum of three times is shown.  (B,D) Increasing amounts of exogenous ΔPDZ (C) and ΔC-Lin2/7 (D) were detected using immunoblotting. HEK293T cells were transiently-transfected with increasing amounts of 3X-Flag tagged ΔPDZ or ΔC-Lin2/7 cDNA construct using the CaPO₄ method and lysates were used to demonstrate increased expression of exogenous protein with increasing amounts of cDNA transfected.
signaling. In contrast, overexpression of MPP3ΔGuk decreased TOPflash activity as compared to cells transfected with increasing amounts of WT MPP3 (Figure 13A), suggesting that MPP3ΔGuk functions in a dominant negative manner to inhibit Wnt signaling. The Guk domain binds to Guk-binding proteins that include guanylate kinase-associated protein, guanylate kinase-associated kinesin, and in addition they may bind to their SH3 domain in an intramolecular fashion (Funke et al., 2005). Knowing that the Guk domain facilitates in interaction between proteins it is possible that the Guk domain is involved in an indirect negative regulatory role in Wnt signaling by mediating the interaction between negative modulators of the Wnt pathway.

3.4 LTK is a Negative regulator of the Wnt pathway

LTK is part of the ros/insulin family of tyrosine kinases which contains an N-terminal signal sequence, a single spanning transmembrane domain, and an intracellular protein tyrosine kinase domain (Figure 14A) (Ben-Neriah and Bauskin, 1988). LTK is a ligand orphan receptor tyrosine kinase (RTK) which is expressed mainly in the pre B-cells and the brain (Ben-Neriah and Bauskin, 1988; Bernards and de la Monte, 1990). Studies show LTK to be involved in neurite outgrowth, with various splice variants being reported (Figure 14B) (Yamada et al., 2008). The results of gene knockdown obtained from the primary screen along with preliminary manual analysis (Figure 4A,B) identified LTK as a negative regulator of the Wnt pathway. The siRNAs used in the primary screen and in the manual verification were comprised of a pool of four individual siRNAs that target four different regions of the gene (Figure 15A). To test for potential off-target effects, the pool of siRNAs were deconvolved and the effect on TOPflash activity of each individual siRNA was investigated in HEK293T cells using the Wnt-responsive luciferase TOPflash reporter assay. This analysis revealed that three of the four siRNAs,
Figure 13: The Guk domain of MPP3 plays a negative regulatory role in the Wnt pathway.

(A) Over expression of ΔGuk increases TOPflash activity. HEK293T cells were transiently-transfected with increasing amounts of a 3X-Flag tagged ΔGuk cDNA construct along with TOPflash, and a β-galactosidase reporter using the CaPO₄ method. Cells were treated overnight with and without Wnt3a and TOPflash activity was measured by luciferase assay normalized to β-galactosidase activity. The mean +/- standard deviation for triplicates of a representative experiment repeated a minimum of three times is shown. (B) Increasing amounts of exogenous ΔGuk was detected using immunoblotting. HEK293T cells were transiently-transfected with increasing amounts of 3X-Flag tagged ΔGuk cDNA construct using the CaPO₄ method and lysates were used to demonstrate increased expression of exogenous protein with increasing amounts of cDNA tranfected.
Figure 14: LTK transcript variants and protein coding regions.

(A) LTK is located on chromosome 15 with four different splice variants being reported (Toyoshima et al., 1993). The longest form of LTK reported is Variant 1. Variant 2 and Variant 3 lack one and two introns, respectively, compared to Variant 1 (Red blocks). The fourth transcript variant contains an inframe stop codon that produces a truncated version of Variant 1 that lacks the kinase domain. Experiments performed on LTK were using transcript Variant 1 (LTK) and Variant 4 (LTK∆KD). (B) LTK transcript variants 1, 2 and 3 encode a N-terminal signal sequence (SS), a single pass transmembrane domain (TM), and a protein tyrosine kinase (PTK) domain. The short form of LTK (LTK∆KD) encodes for a SS and the TM domain, followed by the truncation of the PTK domain.
**Figure 15: LTK is a negative regulator of the Wnt pathway.**

(A) Targeted regions of siRNAs directed towards LTK. (B) Knockdown of LTK increases TOPflash activity. HEK293T cells were transiently-transfected with 20 nM control siRNA, each of the 4 individual LTK-targeting siRNA, or a pool of LTK-targeting siRNA, TOPflash, and a β-galactosidase reporter. Cells were treated overnight with control or Wnt3a-conditioned media and TOPflash activity was measured by luciferase assay normalized to β-galactosidase activity. (C) LTK-targeting siRNA decreases endogenous LTK levels. HEK293T cells were transiently-transfected with 20 nM of control siRNA, each of the 4 individual LTK-targeting siRNA, or pool of LTK-targeting siRNA for 48 hours and LTK levels were analyzed by Q-PCR. (D) Overexpression of LTK decreases TOPflash activity. HEK293T cells were transiently-transfected with increasing amounts of LTK, TOPflash, and β-galactosidase reporter. Cells were treated overnight with and without Wnt3a and TOPflash activity was measured by luciferase assay normalized to β-galactosidase activity. LTK expression was detected in aliquots of cell lysate by anti-Flag immunoblotting. For all plots (B-D), the mean +/- standard deviation for triplicates of a representative experiment repeated a minimum of three times is shown.
siLTK(2), siLTK(3) and siLTK(4), increased TOPflash activity with siLTK(4) providing the most significant increase (Figure 15B). To confirm that the individual siRNAs target the gene of interest, Q-PCR analysis was performed to determine endogenous gene knockdown. HEK293T cells were transiently-transfected with each individual siRNA as well as the pool of siRNAs directed towards LTK for 48 hours. Total RNA was collected from cell lysates and reverse transcription was performed on DNaseI treated total mRNA to produce cDNA. Primers that are intron spanning and produce a fragment between 100-200 base pairs (bp) in size were designed and Q-PCR was performed. All individual and the pool of siRNAs directed towards LTK decreased the levels of endogenous LTK ranging between a 70% to 85% decrease as compared to cells transfected with siCtl, a non-targeting control siRNA (Figure 15C). The siRNAs, siLTK(2) and siLTK(4) yielded the greatest knockdown efficiency and had the greatest increase in TOPflash activity (Figure 15B,C).

To further strengthen the evidence for a negative regulatory role of LTK in the Wnt pathway, the effect of overexpressing LTK on TOPflash activity was examined. A 3xFlag-tagged LTK construct was generated and HEK293T cells were transiently-transfected with increasing amounts of LTK cDNA. Cells were incubated overnight with control or Wnt3a-conditioned media and luciferase activity in cell lysates was then measured. With increasing amounts of LTK, a decrease in TOPflash activity was observed (Figure 15D) indicating that LTK inhibits the Wnt pathway. Next, a rescue experiment was performed using an siLTK resistant LTK construct. For this, nucleotides in the target site of siLTK(4) were mutated such that the protein coding sequence would not be altered but the siLTK(4) nucleotide recognition sequence was changed (Figure 16A). HEK293T cells were co-transfected with siLTK(4) and the siLTK(4) resistant LTK construct, treated overnight with and without Wnt3a, and TOPflash activity was determined. This analysis revealed that the siLTK(4) resistant LTK construct rescued TOPflash
Figure 16
Figure 16: siRNA resistant LTK cDNA rescues wildtype TOPflash activity.

(A) Schematic of the region on LTK cDNA targeted by siLTK(4). Nucleotides highlighted in red indicate silent mutations inserted to create siLTK(4) resistant LTK cDNA construct. (B) LTK rescues siLTK(4)-mediated activation of TOPflash. HEK293T cells were transiently co-transfected with full length or siLTK(4) resistant LTK cDNA construct, 20 nM of pooled siLTK or siLTK(4), TOPflash, and β-galactosidase reporter. Cells were treated overnight with control or Wnt3a-conditioned media and TOPflash activity was measured by luciferase assay normalized to β-galactosidase activity. The mean +/- standard deviation for triplicates of a representative experiment repeated a minimum of three times is shown. (Bottom panel) LTK expression was detected in aliquots of cell lysate by anti-Flag immunoblotting.
activity observed in cells transfected with siLTK(4) alone to the level of cells transfected with non-targeting siCTL (Figure 16B). Altogether, these results clearly show that LTK functions as a negative regulator of Wnt signaling as gene knockdown increases and protein overexpression decreases TOPflash activity (Figure 15).

One splice variant of LTK (Variant 4) encodes for a short form of LTK that lacks the intracellular kinase domain (LTK\(\Delta\)KD) due to an inframe stop codon (Figure 14). Next, I examined whether overexpression of LTK\(\Delta\)KD altered TOPflash activity. For this, a 3xFlag-tagged LTK\(\Delta\)KD construct was generated and transiently-transfected into HEK293T cells in increasing amounts. Cells were treated overnight with control or Wnt3a-conditioned media and luciferase activity in cell lysates was then measured. While LTK\(\Delta\)KD inhibited TOPflash, this effect was not as robust as that obtained with full length LTK, as greater amounts of LTK\(\Delta\)KD were required to decrease TOPflash activity (Figure 17A). The target of siLTK(4) is in the 3’UTR of LTK\(\Delta\)KD, thus a rescue experiment was also performed using this kinase-deleted construct (Figure 17B). In HEK293T cells co-transfected with LTK\(\Delta\)KD and siLTK(4), LTK\(\Delta\)KD decreased the siLTK(4) induced increase in TOPflash activity (Figure 17C). This further strengthens the evidence for a negative role of LTK in the Wnt pathway suggesting that the kinase domain can enhance the inhibitory effect on Wnt signaling.

3.5 LTK\(\Delta\)KD but not full length LTK interacts with FZD7
RYK and ROR are two receptor tyrosine kinases that are related to LTK (Figure 2) and have been implicated in Wnt signaling as Wnt co-receptors (Kim et al., 2008; Li et al., 2008). RYK is known to interact with both FZD7 and FZD8 (Keeble et al., 2006; Kim et al., 2008) and ROR2 has been shown to cooperate with FZD2 (Li et al., 2008). These observations provided evidence into possible interactions between LTK and FZD7. In order to test for a potential interaction
Figure 17

(A) TOPflash

(B) LTKΔKD

(C) TOPflash

Figure 17
Figure 17: LTKΔKD negatively regulates TOPflash activity.

(A) Over-expression of LTKΔKD decreased TOPflash activity. HEK293T cells were transiently-transfected with increasing amounts of LTKΔKD, TOPflash, and β-galactosidase reporter. Cells were treated overnight with and without Wnt3a and TOPflash activity was measured by luciferase assay normalized to β-galactosidase activity. The mean +/- standard deviation for triplicates of a representative experiment repeated a minimum of three times is shown. (B) Schematic of the region on LTKΔKD cDNA targeted by siLTK(4). (C) LTKΔKD rescues wildtype TOPflash activity. HEK293T cells were transiently co-transfected with cDNA construct of LTKΔKD comprised only of coding sequence and an siRNA targeting the 3’UTR of LTKΔKD (siLTK(4)), TOPflash, and β-galactosidase reporter. Cells were treated overnight with control or Wnt3a-conditioned media and TOPflash activity was measured by luciferase assay normalized to β-galactosidase activity. The mean +/- standard deviation for triplicates of a representative experiment repeated a minimum of three times is shown.
between LTK and FZD7, the LUMIER assay was performed in which HEK293T cells were co-transfected with 3xFlag-LTK or 3xFlag-TβRII as a negative control and FZD7-FFluc. Cell lysates were subject to anti-Flag immunoprecipitation and then followed by luciferase assay to detect interactions. If an interaction between the two proteins occurs, the Ffluc tagged protein will be isolated through the immunoprecipitation steps and detected using the luciferase assay.

LTK did not interact with FZD7 as the luciferase readings were similar to that observed in the negative control, TβRII (Figure 18A). TβRII and FZD7 are two receptor proteins that do not interact, therefore the low level of luciferase activity observed is most likely due to aggregate formation. Similar results were obtained when the tags were reversed with LTK being FFluc-tagged and FZD7 being Flag-tagged (Figure 18B).

Next, the interaction between LTKΔKD and FZD7 receptor was examined using LUMIER. Interestingly a strong interaction was observed between LTKΔKD and FZD7 (Figure 19A,B).

To determine if LTKΔKD interaction with FZD7 requires the tail of FZD7, HEK293T cells were co-transfected with LTKΔKD-FFluc and FZD7-3xFlag that lacks the C-terminal tail (FZD7ΔC). The interaction between LTKΔKD and FZD7ΔC was maintained (Figure 19A,B) suggesting that LTKΔKD interacts with FZD7 via the transmembrane domain or the extracellular portion of the receptor.

RYK is capable of interacting with more than one FZD receptor (Keeble et al., 2006; Kim et al., 2008), thus LTKΔKD was screened for potential interactions between other receptors of the FZD family using the LUMIER assay. It was observed that LTKΔKD interacted with all the FZD receptors at various strengths with interactions between FZD7 and FZD4 being the strongest (Figure 20).
Figure 18
Figure 18: Full length LTK does not interact with FZD7.

(A,B) Full length LTK does not interact with FZD7. (A) HEK293T cells were transiently-transfected with FFLuc-tagged FZD7 or FFLuc vector and 3xFlag-tagged LTK or 3xFlag-tagged TβRII as negative control. (B) HEK293T cells were transiently-transfected with 3xFlag-tagged FZD7 or 3xFlag-tagged FZD7 containing cytoplasmic tail deletion (FZD7ΔC) and FFLuc-tagged LTK or FFLuc-tagged IGFR1 as negative control. (A,B) Anti-flag immunoprecipitation was performed followed by luciferase assay to detect interactions. The mean +/- standard deviation for triplicates of a representative experiment repeated a minimum of three times is shown. Expression of Flag-tagged baits were detected using anti-Flag immunoblotting.
Figure 19
Figure 19: LTK∆KD interacts with FZD7.

(A) HEK293T cells were transiently-transfected with FFluc-tagged FZD7 or FFluc vector and 3xFlag-tagged LTK∆KD or 3xFlag-tagged TβRII as negative control. Anti-Flag immunoprecipitation was performed followed by luciferase assay to detect interactions. The mean +/- standard deviation for triplicates of a representative experiment repeated a minimum of three times is shown. Expression of Flag-tagged bait was detected using anti-Flag immunoprecipitation. LTK∆KD and TβRII expression was detected using anti-Flag immunoblotting. (B) The cytoplasmic tail of FZD7 does not facilitate the interaction between LTK∆KD and FZD7. HEK293T cells were transiently-transfected with FFluc-tagged LTK∆KD and 3xFlag-tagged FZD7 or 3xFlag-tagged FZD7∆C. Anti-Flag immunoprecipitation was performed followed by luciferase assay to detect interactions. The mean +/- standard deviation for triplicates of a representative experiment repeated a minimum of three times is shown. FZD7 and FZD7∆C expression was detected using anti-Flag immunoblotting.
Figure 20: LTK\(\Delta\)KD interacts with other Frizzleds.

LTK\(\Delta\)KD interacts with FZD2, FZD4, FZD5, FZD6, FZD8, FZD9, and FZD10. HEK293T cells were transiently-transfected with FF\(\text{Fluc}\)-tagged FZD2, FZD4, FZD5, FZD6, FZD8, FZD9, or FZD10 and 3xFlag-tagged LTK\(\Delta\)KD. Anti-Flag immunoprecipitation was performed followed by luciferase assay to detect interactions. The mean +/- standard deviation for triplicates of a representative experiment repeated a minimum of two times is shown. LTK\(\Delta\)KD expression was detected using anti-Flag immunoblotting.
The observation that LTK does not interact with FZD7 whereas LTK\(\Delta\)KD does, suggests that the two forms of the receptor are able to inhibit the Wnt pathway via two different mechanisms. Full length LTK may function through the binding of a ligand which subsequently activates the receptor, stimulating events that in turn inhibit the Wnt pathway. On the other hand, LTK\(\Delta\)KD may interact with FZD7, which inhibits the activation of the Wnt pathway by preventing the LRP6/FZD7 complex formation.

3.6 LTK\(\Delta\)KD does not inhibit LRP6 enhanced TOPflash activity

Wnt3a-mediated heterodimerization of LRP6 and FZD7 promotes the activation of the Wnt pathway, an effect that also occurs upon overexpression of the LRP6 receptor alone. As observed in Figure 23, transient transfection of LRP6 dramatically increases basal and Wnt3a stimulated TOPflash activity in HEK293T cells. If LTK\(\Delta\)KD inhibits Wnt signaling by binding to LRP6, thus preventing LRP6/FZD7 complex formation, a decrease in TOPflash activity would be observed in cells overexpressing LRP6 and LTK\(\Delta\)KD. To test this, HEK293T cells were co-transfected with decreasing amounts of Myc-LRP6 and a fixed amount of 3xFlag-LTK\(\Delta\)KD. Cells were incubated overnight with control or Wnt3a-conditioned media and luciferase activity in cell lysates was then measured. The coexpression of LTK\(\Delta\)KD with LRP6 appeared to reduce the increased TOPflash activity induced by LRP6, suggesting that LTK\(\Delta\)KD was preventing the LRP6/FZD7 interaction (Figure 21). However anti-Flag and anti-Myc immunoblotting of exogenous proteins, revealed a reduction in LRP6 expression when co-transfected with LTK\(\Delta\)KD (Figure 21). Therefore, the reduction in TOPflash activity observed is due to the decreased expression of LRP6 and not due to an effect of LTK\(\Delta\)KD expression.
Figure 21
Figure 21: LTKΔKD does not decrease the LRP6 induced increase in TOPflash activity.

HEK293T cells were transiently-transfected with decreasing amounts of Myc-tagged LRP6 and constant amount of 3xFlag-tagged LTKΔKD, TOPflash, and β-galactosidase reporter. Cells were treated with control or Wnt3a-conditioned media overnight and TOPflash activity was measured by luciferase assay normalized to β-galactosidase activity. The mean +/- standard deviation for triplicates of a single experiment is shown. Expression of LRP6 and LTKΔKD was detected using anti-Myc and anti-Flag immunoblotting, respectively.
3.7 Wnt3a and Wnt5a do not enhance LTKΔKD homodimerization

Wnt3a and Wnt5a have been shown to bind to ROR2 with Wnt5a inducing homodimerization and activation of ROR2 (Liu et al., 2008). To determine if Wnt3a or Wnt5a increased LTK dimerization, HEK293T cells were co-transfected with 3xFlag-LTKΔKD and FFluc-LTKΔKD and treated with control, Wnt3a-conditioned, or Wnt5a-conditioned media and LUMIER was performed. It was observed that LTK was able to homodimerize in the absence of Wnt addition and that neither Wnt3a nor Wnt5a treatment enhanced the homodimerization of LTKΔKD (Figure 22). This suggests that neither Wnt3a nor Wnt5a increase LTKΔKD homodimerization. However, given that the receptor was transfected, ligand induced effects may be masked by the spontaneous interactions observed by overexpression.

3.8 LTK and LTKΔKD do not interact with Vangl1 or Vangl2

Vangl2 is a membrane protein which contains four transmembrane domains and a large intracellular domain containing a PDZ-domain-binding motif at the carboxy terminus (Kibar et al., 2001; Torban et al., 2004). Ror2 and Vangl2 have been shown to be genetically linked through collagen triple helix repeat containing 1 (Cthrc1) and play a role in the Wnt PCP pathway by stabilizing the Wnt-Receptor complex (Yamamoto et al., 2008). In addition, the Ryk receptor promotes convergent extension movements in Xenopus lavis in which the non-canonical Wnt pathway is known to play an important role (Kim et al., 2008; Myers et al., 2002). The close relationship between LTK and the receptors RYK and ROR (Manning et al., 2002) raised the possibility that LTK might inhibit canonical Wnt signaling through the activation of the non-canonical pathway, as the non-canonical Wnt pathway antagonizes the canonical Wnt pathway. Thus, the potential interaction between LTK or LTKΔKD with Vangl1 or Vangl2 was investigated using LUMIER. HEK293T cells were co-transfected with Vangl1-3xFlag or
Figure 22
Figure 22: Wnt3a and Wnt5a do not enhance LTK∆KD dimerization.

HEK293T cells were transiently-transfected with FFluc-tagged LTK and 3xflag-tagged LTK. Cells were treated overnight with and without Wnt3a or Wnt5a and cell lysates were subjected to anti-Flag immunoprecipitation followed by luciferase assay to detect interaction. The mean +/- standard deviation for triplicates of a representative experiment repeated a minimum of three times is shown. Expression of Flag-tagged LTK∆KD was detected using anti-Flag immunoblotting.
Vangl2-3xFlag and LTK-FFluc or LTKΔKD-FFluc, lysates were subjected to anti-Flag immunoprecipitation followed by a luciferase assay to detect interactions. Insulin-like growth factor type 1 receptor (IGFR1), which a receptor tyrosine kinase that is also closely related to LTK but not involved in Wnt signaling, was used as a negative control. LTK and LTKΔKD did not interacted with Vangl1 and Vangl2 as the luciferase readings were similar to that observed in the negative control, IGFR1 (Figure 23). Although it appeared that LTKΔKD interacted with both Vangl1 and Vangl2, the total expression of LTKΔKD-FFluc was significantly higher than the expression of both LTK-FFluc and IGFR1-FFluc. Therefore if Immunoprecipitated (IP) over Totals were plotted the luciferase readings would be more representative to each other (Data not shown). Although LTK did not interact with the non-canonical pathway component, Vangl1/2, it is still possible that LTK inhibits canonical Wnt signaling through the non-canonical PCP pathway via other PCP proteins such as Prickle or Starry night.

3.9 LTK does not enhance the Wnt5a induced inhibition of canonical Wnt signaling

Wnt5a activates the non-canonical pathway and inhibits the activation of the Wnt3a induced activation of the canonical pathway. Therefore, I next investigated whether LTK enhances Wnt5a mediated inhibition of Wnt3a induced TOPflash activity. HEK293T cells were transiently-transfected with various amounts of 3xFlag-LTK. Cells were incubated overnight with media containing three parts control and one part Wnt3a-conditioned media or a ratio of three parts Wnt5a-conditioned to one part Wnt3a-conditioned media, and luciferase activity in cell lysates was then measured. A decrease in TOPflash activity was observed in cells that were treated with the Wnt5a/Wnt3a media as compared to cells treated with only Wnt3a media. The decrease in TOPflash activity was further enhanced when cells were transiently-transfected with increasing amounts of LTK and treated with the Wnt5a/Wnt3a media (Figure 24A). This
Figure 23: LTK and LTKΔKD do not interact with Vangl1 and Vangl2.

HEK293T cells were transiently-transfected with 3xFlag-tagged Vangl1 or 3xFlag-tagged Vangl2 and FFluc-tagged LTK, FFluc-tagged LTKΔKD, or FFluc-tagged IGFR1 as negative control. Anti-Flag immunoprecipitation was performed followed by luciferase assay to detect interactions. The mean +/- standard deviation for triplicates of a representative experiment repeated two times is shown. Vangl1 and Vangl2 expression was detected using anti-Flag immunoblotting.
Figure 24
Figure 24: Full length LTK does not enhance the antagonistic effect of Wnt5a on Wnt3a-induced canonical signaling.

(A) HEK293T cells were transiently-transfected with 0.05µg, 0.1µg, and 0.4µg of 3xFlag-tagged LTK, TOPflash and β-galactosidase reporter. Cells were treated overnight with and without Wnt3a and Wnt5a at a ratio of 1:0 or 1:3 (Wnt3a:Wnt5a) and TOPflash activity was measured by luciferase assay normalized to β-galactosidase activity. The mean +/- standard deviation for triplicates of a representative experiment repeated two times is shown. Expression of LTK was detected using anti-Flag immunoblotting. (B) Fold difference in TOPflash activation of the various treatments in A.
suggests that LTK increases the Wnt5a induced inhibition of TOPflash activity but when the Wnt5a mediated fold inhibition is calculated this was not the case. The fold inhibition is equal in all of the different treatments (Figure 24B) indicating the effect observed in Figure 24A is actually due to an additive effect of Wnt5a and LTK inhibition and not a synergistic effect between Wnt5a and LTK. This indicates that LTK inhibits the canonical Wnt pathway through a different mechanism and not through Wnt5a mediated inhibition.

3.10 Neuro2a and NIE 115 cell lines are Wnt responsive and express LTK

Wnt signaling has been implicated in a wide variety of aspects of neuronal development. Examples include the regulation of posterior-directed growth of corticospinal tract (Liu et al., 2005), regulation of neuronal differentiation during cortical neurogenesis (Lyu et al., 2008), and signaling during axon guidance (Bovolenta et al., 2006). Given that RYK is involved in all these cases, there is a close relationship between RYK and LTK (Manning et al., 2002), and that LTK is highly expressed in the brain (Bernards and de la Monte, 1990) suggest the possibility that LTK might also have a role in neuronal development. Thus, I next focused on identifying a relevant system in which to study the effects and functions of LTK in a neuronal system. In order to do this, I first examined whether the mouse neuronal cell lines, Neuro2a and NIE115, express Ltk by performing Q-PCR analysis on total RNA. Both cells express higher amounts of Ltk as compared to HEK293T cells (Figure 25A). Ltk expression was normalized to the housekeeping gene GAPDH which may be expressed at varying levels across cell lines. However, the levels of GAPDH displayed minimal variation among the three cell lines, suggesting that the Neuro2a and NIE115 cells express higher amounts of Ltk compared to HEK293T cells. Next, I determined if these two cell lines are Wnt3a responsive by performing the β-catenin stabilization and Dvl upshift assays. Upon Wnt3a treatment Dvl is phosphorylated resulting in a shift in the
Figure 25: NIE115 and Neuro2a cells express \( Ltk \) and are Wnt responsive.

(A) The neuronal NIE115 and Neuro2a cell lines express \( Ltk \). Total RNA was isolated from HEK293T, NIE115, and Neuro2a cells and \( Ltk \) expression levels were analyzed using Q-PCR.

(B) NIE115 and Neuro2a cells are Wnt responsive. Wnt3a induces β-catenin is stabilization and DVL3 phosphorylation in NIE115 and Neuro2a cells. NIE115 and Neuro2a cells were treated with and without Wnt3a for 2, 3, 4, and 5 hours, cells were lysed, and β-catenin Dvl3 was detected using anti-β-catenin and anti-Dvl immunoblotting, respectively.
protein’s mobility during SDS-PAGE and β-catenin is stabilized within the cytoplasm. Neuro2a and NIE115 cells were treated with control or Wnt3a-conditioned media for two, three, four, and five hours and cell lysates were analyzed by anti-Dvl3 and anti-β-catenin immunoblotting. Both Neuro2a and NIE115 cells are Wnt responsive as Dvl was phosphorylated as indicated by the observed band mobility shift and β-catenin levels increased upon Wnt3a treatment (Figure 25B). These results show Neuro2a and NIE115 cells express LTK and have a Wnt response allowing for studies into the role LTK may play in Wnt signaling using neuronal model systems. Additionally, Dvl2 upshift was not observed in Neuro2a and NIE115 cells upon Wnt3a treatment and increased levels of β-catenin and Dvl2/3 upshift were not observed upon Wnt5a treatment in both cell lines (Data not shown), suggesting that this is mainly a canonical Wnt response.

I next examined whether knocking down Ltk would affect either Dvl upshift or β-catenin stabilization. Cells were transiently-transfected with siRNA directed towards mouse Ltk for 48 hours, treated with control or Wnt3a-conditioned media for 2 hours, and cell lysates were analyzed with anti-Dvl3 and anti-β-catenin antibodies via immunoblotting. Knockdown of Ltk did not have an effect on either Dvl3 phosphorylation or β-catenin stabilization (Figure 26C). In parallel total RNA was isolated and Q-PCR analysis was performed to determine the knockdown efficiency of Ltk. An efficiency of 65% and 75% in the Neuro2a and NIE115 cells respectively was achieved (Figure 26A,B). Therefore, the lack of effect on Dvl3 phosphorylation and β-catenin stabilization may be due to inefficient knockdown of Ltk as a higher knockdown efficiency might be required to observe potential effects on Dvl3 phosphorylation and β-catenin stabilization. The effect of over-expression of LTK∆KD on Dvl phosphorylation and β-catenin stabilization was also tested. Interestingly, overexpression of LTK∆KD decreased the amount of total Dvl and β-catenin detected by immunoblotting while the actin loading control was unaltered
Figure 26

A) Q-PCR Neuro2a Cells

B) Q-PCR NIE115 Cells

C) Western Blot Analysis

- β-catenin
- Actin
- P-Dvl3
- Dvl3

Neuro2a

NIE115

Wnt3a

Ltk mRNA levels

Pak1 mRNA levels
Figure 26: Knockdown of Ltk does not influence β-catenin stabilization and Dvl3 phosphorylation in NIE115 and Neuro2a cells.

(A) Ltk-targeting siRNAs decrease endogenous Ltk expression in Neuro2a and NIE115 cells. Neuro2a and NIE115 cells were transiently-transfected with 20 nM control or a pool of Ltk-targeting siRNA, treated overnight with control or Wnt3a-conditioned media 24 hours post transfection, and Ltk levels were analyzed by Q-PCR at 48 hours post transfection. Pak1 knockdown and expression was used as positive control. (B) Knockdown of Ltk does not influence β-catenin stabilization and Dvl3 phosphorylation. NIE115 and Neuro2a cells were transiently-transfected with a pool of siRNAs directed towards Ltk. Cells were treated with and without Wnt3a for 2 hours and β-catenin stabilization and Dvl upshift was detected using anti-β-catenin and anti-Dvl immunoblotting respectively.
under all treatments (Figure 26C). This suggests that LTK may influence β-catenin and Dvl expression or protein stability.

Altogether, the data presented confirms LTK as a negative regulator of the canonical Wnt signaling pathway. The interaction observed between LTKΔKD and FZD7 points to a potential mechanism for the inhibition of the canonical Wnt pathway. On the other hand, insight into how full length LTK regulates the Wnt pathway remains to be determined. The use of both neuronal cell lines will be very useful in providing insights into the biological function of LTK and the role in Wnt signaling.
4 Discussion

4.1 Application of HTP screening to identify novel signaling pathway modulators

Signaling pathways are very complex containing an abundance of known and unknown proteins that function to mediate signals. In order to discover novel regulators and gain insight into already known signaling pathways, investigators have developed various HTP screening methods. Such screens include protein interaction screens, chemical screens and functional screens that investigate the effects of overexpression of a protein or siRNA knockdown on a selected signaling pathway (Miller et al., 2009; Moffat and Sabatini, 2006; Stelzl and Wanker, 2006). In our lab, a Post Doctoral fellow, Dr. Mary Erclik, performed a HTP siRNA screen on a variety of signaling pathways, with the goal of identifying new pathway modulators that influence more than one signaling pathway. In this thesis, I focused on using the results from the screens to identify novel regulators of the Wnt pathway. It is widely accepted that HTP screens are prone to various experimental and/or theoretical limitations that result in false positives and false negatives (Cusick et al., 2005). Therefore, I manually verified selected hits from the screen to confirm them. The manual verification steps that I performed showed that seven of the eight hits chosen for analysis yielded results that were consistent with those obtained from the primary screens. Although a larger number of hits would need to be tested to more accurately determine performance, at least for the selected hits, the screen data was reliable.

Recently, Miller et al. demonstrated that the incorporation of data sets from multiple screens helps to eliminate false-positives and enhance screening performance using an analytical method termed combined pathway score (CPS) (Miller et al., 2009). In this case, results obtained from a protein-protein interaction screen as well as cDNA overexpression and siRNA screens were integrated to narrow down true hits and decrease the chances of false positives and false
negatives. Among the many hits observed, this method helped to identify the new Wnt pathway modulator Ube2m and provide insight into the function of Nkd1 in the Wnt pathway (Miller et al., 2009). In future work, if multiple functional screens were performed and integrated with the data from the screens conducted by Dr. Mary Erclik using the CPS, the number of false positives would be reduced. This would also allow selection of hits with greater confidence, increasing the chances of identifying new pathway modulators. Nonetheless, the screens performed identified MPP3 and LTK as potential negative regulators of the Wnt pathway. In this thesis, follow up experiments were directed towards these two hits, with the majority of the focus directed towards LTK.

4.2 MPP3 is a negative regulator of canonical Wnt signaling

The Wnt signaling pathway is involved in a variety of developmental and cellular processes which influence differentiation, growth, and proliferation (Clevers, 2006). Deregulation of the pathway at any point which disrupts one of these processes may be disastrous to an organisms' well being as the onset of a variety of cancers may develop. MPP3 is a MAGUK scaffolding protein composed of multiple protein-protein interaction domains (Fukuhara et al., 2003; Smith et al., 1996). Scaffolding proteins help to organize proteins involved in signaling pathways by bringing them into close proximity to each other such as the case of Axin in the Wnt signaling pathway (Clevers, 2006) and the Ste5 scaffolding protein in the MAPK pathway in *Saccharomyces cerevisiae* (Choi et al., 1994; Errede and Ge, 1996). Deregulating the role of Axin by preventing the formation of the destruction complex allows cytosolic β-catenin to accumulate and in turn activate the Wnt pathway. The screens performed in our lab identified MPP3 as a potential negative regulator of the Wnt pathway. Although the mechanisms of action of MPP3 was not determined, as a member of the MAGUK family it is tempting to speculate that
MPP3 may influence the Wnt pathway by bringing negative regulators of the Wnt pathway in close proximity to the plasma membrane, which could affect the pathway at the receptor level.

Analysis of the effect of abrogating expression of MPP3 by siRNA revealed that Wnt transcriptional activity was increased, therefore, it was expected that overexpression of MPP3 would decrease activity, but this was not the case (Figure 10B). One explanation for this could be that the expression of endogenous MPP3 may be sufficient to elicit a negative effect on Wnt signaling. A similar observation was made when the colocalization of MPP3 with tumor suppressor in lung cancer 1 (TSLC1) was examined by Fukuhara et al. (Fukuhara et al., 2003). Over-expression of MPP3 did not increase TSLC1 colocalization to the plasma membrane, as levels of TSLC1 at the membrane were similar when compared to colocalization of TSLC1 with endogenous MPP3 (Fukuhara et al., 2003). MPP3 is composed of various domains (Figure 10A), therefore, as an alternative I generated versions of MPP3 lacking the various binding domains in anticipation that these would act as dominant negatives to block Wnt signaling.

Indeed, the deletion of the Guk binding domain of MPP3 increased TOPflash activity (Figure 13A) while MPP3 lacking the PDZ domain or the second Lin2/7 domain (C-Lin2/7) had no effect on activity (Figure 12 A,C). This suggests the Guk domain is involved in the negative regulatory role of MPP3 on Wnt signaling. The affect of MPP3 lacking the SH3 domain on TOPflash activity was not investigated and remains to be determined. A reason for why no effect on TOPflash activity was observed when only one Lin2/7 domain was deleted may be due to the fact that the two Lin2/7 binding domains may play redundant roles. Lin2/7 domains are able to associate with each other and facilitate the formation of a higher order scaffolding structure (Harris et al., 2002; Roh et al., 2002) Thus, the deletion of one Lin2/7 domain could be compensated by the remaining Lin2/7 domain. If this were true, then deletion of only the first Lin2/7 domain (N-Lin2/7) would yield the same results observed when only the C-Lin2/7
domain was deleted. Therefore, in future work the deletion of both Lin2/7 domains, and the affect on TOPflash activity, should also be investigated.

MPP3 contains high homology to DLG3 and belongs to the DLG-like subfamily of the MAGUK proteins (Fukuhara et al., 2003; Smith et al., 1996). A variety of DLG proteins have been demonstrated to be involved in the regulation of Wnt signaling. For example, DLG1 binds to APC, which modulates the antiproliferative effects of APC and DLG3 has been reported to regulate Wnt signaling through DLG3-mediated β-catenin degradation (Brennan et al., 2010; Hanada et al., 2000; Ishidate et al., 2000). Also, PSD-95, another MAGUK protein interacts with a range of Frizzled receptors, which may serve the purpose of recruiting molecules of the Wnt pathway into the vicinity of the receptor, in turn helping to regulate the Wnt pathway (Hering and Sheng, 2002). These studies indicate that MAGUK proteins may have a general role in the regulation of the Wnt pathway and that continued studies on MPP3 would be warranted.

4.3 LTK is a negative regulator of canonical Wnt signaling

Wnt signaling is primarily mediated by two main receptors, the FZD family of receptors and the LRP5/6 receptors which form heterodimers upon Wnt binding, inducing dissociation of the destruction complex and β-catenin stabilization which leads to the activation of Wnt target genes (Clevers, 2006). More recently, a variety of unconventional Wnt receptors have been reported which include the receptor tyrosine kinases, RYK and ROR (van Amerongen et al., 2008). These two receptors influence the non-canonical Wnt pathway (Lu et al., 2004a; Paganoni and Ferreira, 2005), play a role in a variety of neuronal related aspects of development (Billiard et al., 2005; Lu et al., 2004a), and are closely related to LTK according to a kinase dendrogram (Figure 2A) (Manning et al., 2002). The results presented in this thesis show that siRNA mediated
knockdown of *LTK* increased Wnt transcriptional activity (Figure 4 A,B), while overexpression of LTK decreased Wnt transcriptional activity (Figure 15D). Altogether, this work demonstrated that LTK is a negative regulator of the Wnt pathway.

### 4.4 LTKΔKD interacts with Frizzled receptors

*LTK* is subject to alternative splicing that includes a full length version and a variant that encodes for a receptor devoid of the kinase domain referred to as LTKΔKD herein (Figure 14B). In the case of LTK that lacks the kinase domain, overexpression decreased TOPflash activity but not as strongly as the full length receptor (Figure 17A). Therefore, LTKΔKD must elicit a negative effect on Wnt signaling through a mechanism that does not involve activation of kinase activity. RYK was reported to interact with FZD7 and FZD8 (Keeble et al., 2006; Kim et al., 2008) while ROR2 interacted with FZD2 (Li et al., 2008). These observations suggested the possibility that LTK might also interact with FZD receptors and indeed LUMIER experiments confirmed this hypothesis (Figure 19, 20). This provides a possible model for how LTKΔKD might regulate the Wnt pathway in which the interaction between LTKΔKD/FZD7 might prevent FZD7-LRP5/6 interaction, which would in turn prevent activation of the Wnt pathway (Figure 27B). Such mechanisms in which splice variants of receptors that lack the cytoplasmic tyrosine kinase domain or are subject to posttranslational modifications in which the kinase domain is cleaved have been previously reported for trkB protein kinase (Klein et al., 1990; Middlemas et al., 1991), Fibroblast Growth Factor receptor (Hou et al., 1991), and Hepatocyte Growth Factor receptor (c-met) (Prat et al., 1991). Interestingly, receptors which lack the intracellular kinase domain have also been shown to interact with membrane-bound cytoplasmic kinases and through
Figure 27
Figure 27: Proposed working models for LTK in the canonical Wnt Pathway.

(A) The full length splice variant of LTK may inhibit the Wnt pathway in one of many ways. LTK may prevent the binding of Wnt ligands to FZD receptors or prevent the binding of DVL to the FZD receptor upon Wnt binding. Alternatively, full length LTK may activate a component of the non-canonical Wnt pathway which may mediate downregulation of the Canonical Wnt pathway. Full length LTK may also inhibit Wnt signaling through the PI3K pathway as it has been shown to bind to components of the PI3K pathway.

(B,C) The short form of LTK may inhibit the Wnt pathway in one of two ways. It may bind to FZD which in turn prevents receptor complex formation between FZD and LRP5/6 or prevents the interaction between FZD and DVL. Another potential mechanism may be the binding of short form of LTK to a lipid anchored signaling protein that may inhibit canonical Wnt signaling by directly acting on the pathway or by indirectly inhibiting the canonical Wnt pathway through the activation and action of the non-canonical Wnt pathway or PI3K pathway.
this association elicit an intracellular response as in the case of the CD4, and CD8 receptors binding to Lck kinase (Veillette et al., 1989) and Interleukin 2 receptors which bind the Lck kinase (Hatakeyama et al., 1991). These observations provide other possible models for how LTK∆KD may regulate the Wnt pathway. For example, LTK∆KD may also bind to membrane-associated kinases which could activate non-canonical signals, which in turn antagonize canonical Wnt signaling (Figure 27C). To examine this possibility, it would be necessary to be determine if LTK∆KD has other interacting partners other then the Frizzled receptors. If so, I would test if preventing the interaction would influence LTK function. Furthermore, LTK∆KD interacted with other members of the Frizzled family (Figure 20). This may provide a link to the non-canonical pathway as certain Frizzled receptors have been linked to non-canonical Wnt signaling. The interaction between non-canonical Frizzleds could stimulate the non-canonical pathway, inturn antagonizing canonical Wnt signaling. In future, it would be interesting to determine the in vivo expression pattern of the splice variants and whether the negative effect of LTK∆KD may be biologically relevant.

4.5 The influence of Wnt3a and Wnt5a on LTK dimerization

In contrast, unlike LTK∆KD, FL LTK did not associate with FZD7 and therefore must regulate the Wnt pathway through a different mechanism. This could occur through the activation of the intracellular kinase domain of LTK which would stimulate downstream events. A common mechanism of receptor activation involves ligand binding and receptor homo- or heterodimerization, therefore, in this thesis, I tested whether Wnt3a or Wnt5a could induce LTK receptor dimerization. In the case of LTK∆KD, without ligand treatment dimerization was observed and there was no increase when cells were treated with either Wnt3a or Wnt5a-conditioned media. This may be due to the fact that the receptors were overexpressed, however,
even when up to 100 fold decreased amounts of receptor was transfected, neither ligand enhanced the consistent levels of dimerization (Figure 21). A potential reason as to why enhanced dimerization was not observed upon Wnt3a or Wnt5a treatment is that these studies were done using LTKΔKD and it may be that the kinase domain facilitates receptor dimerization. Therefore, in future studies it would be worthwhile to repeat these experiments using FL LTK. Alternatively, the binding of Wnt3a or Wnt5a may not be required for receptor dimerization, rather it may only be required for receptor activation. This type of phenomenon was demonstrated by Paganoni et al. as heterodimerization between Ror1 and Ror2 and homodimerization of Ror2 was observed, and co-expression of Wnt5a ligand with receptors did not increased complex formation. On the other hand, the coexpression of Wnt5a and receptors was necessary for receptor activation (Paganoni et al., 2010). Thus, it was suggested that Ror receptor dimers form but in order to activate the complexes, binding of Wnt5a is required. This may also be the case with LTK. This further iterates the need to perform the dimerization experiments using FL LTK. In addition, downstream targets of LTK would need to be identified in order to determine receptor activation.

4.6 The role of LTK in Wnt induced neuronal development
Wnt signaling is starting to emerge as a pathway which regulates various aspects of neuronal development such as Wnt5a mediated axon guidance (Keeble et al., 2006), the requirement of Wnt3a for neurite outgrowth (Lu et al., 2004a), and the influence of Wnt5a on synapse formation in hippocampal cells (Paganoni et al., 2010). Interestingly, all these emerging roles of Wnt signaling in neuronal development involve the receptor tyrosine kinases, RYK or ROR. This strongly suggests that LTK may play a role in Wnt mediated neuronal development as LTK is also highly expressed in the brain (Ben-Neriah and Bauskin, 1988; Bernards and de la Monte,
Thus, it is important to identify a biologically relevant system to study the potential role of LTK in neuronal development. Here, I identified two neuronal cell lines which express \textit{LTK} and are Wnt responsive (Figure 24). The NIE115 and Neuro2a cell lines will be extremely useful in helping to identify if LTK plays a role in neuronal development and in providing insights into a biological role for LTK. For example, it could be investigated if LTK influences neuronal differentiation or is required for Wnt3a induced neurite outgrowth.

### 4.7 Potential models for the regulation of canonical Wnt signaling by LTK

There are a wide range of potential mechanisms to explain LTK’s ability to negatively regulate Wnt signaling which were not investigated in the work presented. The ligand for LTK is not known which makes responses elicited by receptor activation difficult to study. It is possible to overcome this hurdle by creating a chimeric receptor where the extracellular portion of a receptor with a known ligand is linked to the transmembrane and intracellular portion of the LTK receptor. The intracellular portion of LTK could then be stimulated by the addition of ligand recognized by the extracellular portion of the chimeric receptor. This method was used by Ueno \textit{et al.} to show that LTK interacts with components of the PI3K pathway. The interaction of LTK with the signaling molecules IRS1, Shc, and p85 of the PI3K pathway provides a possible link to the negative regulatory role of LTK on the Wnt pathway. One response of the PI3K pathway is the activation of AKT/PKB, which is able to phosphorylate GSK3β, subsequently inhibiting the activity of GSK3β. Inhibiting GSK3β activity allows for β-catenin to accumulate within the cytoplasm which would stimulate the activation of the Wnt pathway (Grille et al., 2003; Weston and Davis, 2001). Binding of the PI3K pathway components to LTK may potentially sequester the proteins involved in the activation of the PI3K pathway. This would prevent the activation of AKT subsequently preventing the inhibition of GSK3β allowing for downregulation of the Wnt
pathway. Alternatively, the binding of these proteins to LTK may stimulate other unknown mechanisms which regulate the Wnt pathway providing more avenues of investigation to LTKs role in Wnt signaling.

Another potential mechanism in which LTK may function is through the cleavage of an intracellular portion of the receptor. The receptor RYK functions through this mechanism in which ligand binding induces cleavage of the intracellular portion of the receptor by $\gamma$-secretase (Lyu et al., 2008). The cleaved portion of RYK translocates into the nucleus acting as a transcription factor that stimulates transcripton of genes involved in neurite outgrowth (Lyu et al., 2008). This is not the first instance in which a receptor acts through an intracellular cleavage mechanism as ALK, a receptor tyrosine kinase that is highly related to LTK, is also cleaved upon ligand binding (Mourali et al., 2006). Caspase-3 recognizes the amino acid sequence DEVD and cleaves proteins that contain this motif. ALK contains a similar amino acid sequence to that recognized by caspase-3, where the valine is substituted with leucine creating a DELD sequence which is still recognized by caspase-3 allowing for cleavage of ALK at this position (Mourali et al., 2006). Interestingly, LTK also contains a single DELD sequence present at a similar position as in ALK, giving reason to suspect that LTK may function through an intracellular cleavage mechanism as that demonstrated by RYK.

4.8 General conclusion

In summary, HTP screening has proven to be a useful tool in identifying new Wnt signaling pathway components. MPP3 and LTK were identified as hits in a screen performed by Dr. Mary Erclik in the lab and manual verification data presented in this thesis confirmed that both MPP3 and LTK function as negative regulators of the Wnt pathway. The follow up domain mapping experiments conducted on MPP3 showed the Guk domain to be involved in the
negative regulatory role of MPP3 on Wnt signaling. More extensive follow up on LTK provided evidence in support of the idea that LTK is a new unconventional Wnt receptor. The fact that various splice variants of LTK have been reported and the work, herein, showing that these variants may function through different mechanism to elicit a negative effect on Wnt signaling, make it an intriguing area of research to pursue. Furthermore, receptor tyrosine kinases related to LTK involved in Wnt signaling are linked to neuronal development and the data demonstrate that LTK is highly expressed in neuronal cells, strongly suggest that LTK plays a role in Wnt influenced neuronal development.


