CHARACTERIZATION OF PROTEIN TYROSINE KINASE 7 (PTK7) IN DEVELOPMENT AND DISEASE

by

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Abstract

Protein Tyrosine Kinase 7 (Ptk7) is a transmembrane pseudokinase implicated in Wnt signal transduction during embryonic development and in cancer. Ptk7 is a known regulator of mammalian neural tube closure and Xenopus convergence & extension (C&E) movements⁴; however, conflicting reports have indicated both positive and negative roles for Ptk7 in both non-canonical Wnt/PCP and canonical Wnt/β-catenin signaling pathways²⁴. To clarify the function of Ptk7 in vertebrate embryonic patterning and morphogenesis, I generated maternal-zygotic (MZ) ptk7 mutant zebrafish and found that early loss of zebrafish Ptk7 leads to defects in axial C&E, neural tube morphogenesis, and planar cell polarity (PCP). MZptk7 mutant embryos establish normal Wnt-dependent dorsoventral patterning at the onset of gastrulation; however, post-gastrulation MZptk7 mutant embryos display significant up-regulation of β-catenin target gene expression. MZptk7 mutants show expansion of the mesodermal compartment within the tail bud, suggesting an important role for Ptk7 in regulating Wnt/β-catenin-dependent cell fate specification. Furthermore, a plasma membrane-tethered Ptk7 extracellular fragment is sufficient to rescue both PCP morphogenesis and Wnt/β-catenin patterning defects in MZptk7 mutant embryos, indicating that the extracellular domain of Ptk7 acts as an important regulator of both non-canonical Wnt/PCP and canonical Wnt/β-catenin signaling in multiple developmental contexts.

Zygotic ptk7 mutant zebrafish display no obvious embryological defects and develop to adulthood; however, as juveniles these fish develop severe spinal curvatures analogous to human idiopathic scoliosis (IS). Scoliosis is a complex genetic disorder, characterized by three-dimensional rotations of the spine associated with underlying vertebral malformations.
(congenital scoliosis, CS) or with no identifiable underlying abnormalities (idiopathic scoliosis, IS). Vertebral abnormalities are not associated with spinal curvature in zygotic *ptk7* mutant zebrafish; however, MZ*ptk7* mutants display vertebral malformations analogous to those found in humans with congenital scoliosis (CS). These defects are linked to abnormalities in embryonic segmentation, suggesting an important role for maternal *ptk7* in axial patterning. Therefore, MZ*ptk7* and zygotic *ptk7* mutant zebrafish represent novel models of CS and IS (respectively) and suggest that both types of deformity share genetic mechanisms that relate to altered Wnt signal transduction.
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List of Abbreviations

AB, Apical-basal
AIS, Adolescent Idiopathic Scoliosis
AP, anteroposterior
APC, Adenomatous Polyposis Coli
ATF2, Activating Transcription Factor 2
ATP, Adenosine Triphosphate
BMP, Bone Morphogenic Protein
C&E, Convergence and Extension
Ca^{2+}, Calcium
CCK4, Colon Carcinoma Kinase 4
Cdc42, Cell division control protein 42
Celsr, Flamingo-related Cadherin EGF LAG Seven-Pass G-Type Receptor
CHD7, Chromodomain Helicase DNA-binding protein 7
CK1, Casein Kinase 1
CNS, Central Nervous System
COS-1, CV-1 (simian) in Origin, carrying SV40 genetic material
CRD, Cysteine-Rich Domain
CS, Congenital Scoliosis
CVM, Congenital Vertebral Malformation
DEP, Dishevelled, Egl-10 and Pleckstrin domain
Dgo, Diego
DIX, present in Dishevelled and Axin
Dkk1, Dickkopf 1
dpf, days post fertilization
Dsh, Dishevelled
DV, Dorsoventral
ECM, Extracellular Matrix
EGFR, Epidermal Growth Factor Receptor
ER, Estrogen Receptor
FBN, Facial Branchiomotor Neuron
FBN-1, Fibrillin-1
FGF, Fibroblast Growth Factor
FGFR1, Fibroblast Growth Factor Receptor 1
Fmi, Flamingo
Fz, Frizzled
GFP, Green Fluorescent Protein
GH, Growth Hormone
GPR126, G Protein-coupled Receptor 126
GSK3, Glycogen Synthase Kinase 3
GTP, Guanosine Triphosphate
GWAS, Genome Wide Association Study
HCT116, Human Colorectal Carcinoma Transformed 116
HEK293T, Human Embryonic Kidney 293, SV40 T-antigen containing
hpf, hours post fertilization
IG, Immunoglobulin
IGF-1, Insulin-like Growth Factor-1
Int-1, Integration Site-1
IS, Idiopathic Scoliosis
IVD, Intervertebral Disc
JNK, c-Jun N-terminal Kinase
LBX1, Ladybird homeobox 1
LRP5/6, Low-density Lipoprotein Receptor 5 and/or 6
MBT, Mid-Blastula Transition
MicroCT, Micro Computed Tomography
MMP, Matrix Metalloproteinase
MMTV, Mouse Mammary Tumour Virus
MO, Morpholino Oligonucleotide
MRI, Magnetic Resonance Imaging
MT1-MMP, Membrane Type 1 Matrix Metalloproteinase
MTNR1B, Melatonin Receptor 1B
MZ, Maternal-zygotic
Nedd4-1, Neural precursor cell expressed developmentally downregulated 4 – 1
NKD, Naked Cuticle
NTD, Neural Tube Disorder
NTF3, Neurotrophin Factor 3
OTK, Off-track
PAR1, Partitioning defective 1 homolog
PCP, Planar Cell Polarity
PDZ, Post synaptic density protein (PSD95), Disc-large (Dlg), Zona occludens 1 (ZO1) domain
Pk, Prickle
PK, Pseudokinase
PKC, Protein Kinase C
PSM, Presomitic Mesoderm
PTK7, Protein tyrosine kinase 7
RA, Retinoic Acid
Rab, Ras-related proteins in brain
Rac, Ras-related C3 botulinum toxin
RACK1, Receptor of Activated Protein Kinase C 1
RhoA, Ras homolog gene family, member A
Ror, RAR-related Orphan Receptor
(q)RT-PCR, (quantitative) Reverse Transcriptase – Polymerase Chain Reaction
SCD, Spondylocostal Dysostosis
STD, Spondylothoracic Dysostosis
T-ALL, T-cell Acute Lymphoblastic Leukemia
TCF/LEF1, T-Cell Factor/Lymphoid Enhancer-binding Factor 1
TGFβ, Transforming Growth Factor β
tm, Transmembrane
Vang, Van Gogh
Vangl, Van Gogh-like
VEGF, Vascular Endothelial Growth Factor
VEGFR, Vascular Endothelial Growth Factor Receptor
Waif1, Wnt-activated inhibitory factor 1
Wg, Wingless
WISH, Whole-mount in situ hybridization
Wnt, Wingless-related integration site
WT, Wild-type
Xdd1, Xenopus Dishevelled Deletion 1 (DshΔPDZ mutant)
YFP, Yellow Flourescent Protein
ZF, Zinc-finger
ZFN, Zinc-finger Nuclease
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Chapter 1

1. INTRODUCTION

1.1 The Wnt Signaling Pathway

Wnt was first identified in 1982 through screens for genes activated by insertions of Mouse Mammary Tumour Virus (MMTV) and named Int1, corresponding to the specific integration site. Drosophila int1 was expressed in a striped pattern along the body axis and was found to encode the Drosophila Wingless (Wg). Int1 was later named ‘Wnt1’ (Wingless-related integration site 1).

A developmental role for Wnt was discovered when Int1 was implicated in embryonic “organizer” activity in Xenopus laevis. Int1 knock-out mice produced an embryonic lethal phenotype with defects in mid-brain and cerebellar development. 19 Wnt-related genes have been identified in mammals and are expressed at different times and in different tissues. Many have unique roles during development; however, several Wnt orthologs are conserved throughout the entire animal kingdom and have an evolutionarily conserved role in setting up the primary body axis.

In general, the Wnt pathway has been linked to a wide range of biological processes. For example, Wnt pathway components regulate the cellular architecture required for cell adhesion and polarity. The canonical Wnt/β-catenin-dependent branch of the pathway controls gene expression involved in cell division and tissue fate decisions. Non-canonical Wnt pathways influence cellular behavior like polarity and oriented cell divisions. Finally, Wnt has been implicated in asymmetric stem-cell division. The importance of Wnt signaling is highlighted by instances where misregulation leads to developmental abnormalities, disease or cancer.

1.1.1 The canonical Wnt/β-catenin signaling pathway

Wnt proteins are approximately 40 kDa in size and contain several conserved cysteine residues. These glycoproteins are lipid-modified with palmitoleic acid and due to their relative insolubility, Wnts are thought to act as short-range signaling molecules rather than classical long-range morphogens. Heparan sulfate proteoglycans (HSPGs) are cell-surface
extracellular matrix molecules that associate with Wnts and assist in extracellular Wnt gradient formation\textsuperscript{20}.

On target cells, Wnt molecules bind to complexes of Frizzled (Fz) and Low-density lipoprotein receptor 5 or 6 (LRP5/6) (Figure 1.1.1). Fzs are seven-pass transmembrane proteins that consist of a N-terminal cysteine-rich domain (CRD) that interacts with lipids on the Wnt molecule\textsuperscript{21}. Fz forms a large protein family (there are 10 mammalian Fzs) and individual family members are often functionally redundant with each other\textsuperscript{22-24}. Fzs can bind multiple Wnts, but show specific affinities for certain Wnt molecules. This introduces a level of selectivity on signal activation in a receiving cell\textsuperscript{25}. Fz interacts with the single pass membrane protein LRP5/6 in vertebrates (Arrow in Drosophila) to mediate canonical Wnt signaling\textsuperscript{26-28}. Two non-overlapping domains of LRP5/6 may interact with separate Wnt ligands, once again adding an additional level of signal specificity\textsuperscript{29}.

In the absence of Wnt ligands, intracellular β-catenin (Armadillo in Drosophila) is targeted for proteosomal degradation through phosphorylation by casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3)\textsuperscript{30-33}. GSK3 is brought into contact with β-catenin by the scaffolding protein Axin\textsuperscript{34} (Figure 1.1.1). The tumor suppressor adenomatous polyposis coli (APC) also binds β-catenin and recruits it to the Axin-GSK complex through Axin-APC interactions\textsuperscript{35} (Figure 1.1.1). Axin forms large multimers through its DIX (present in Dishevelled and Axin) domain, and creates cytoplasmic “destruction complexes” that keep cytoplasmic levels of β-catenin low\textsuperscript{36}.

Upon Wnt binding, the intracellular portion of LRP5/6 is phosphorylated by CK1 and GSK3, which leads to Axin recruitment away from the Axin-GSK3-APC complex\textsuperscript{37-40}. Dissociation of Axin leads to cytoplasmic accumulation and nuclear translocation of β-catenin. In the nucleus, β-catenin binds TCF/LEF-1 transcription factors\textsuperscript{41,42} and activates tissue specific target gene transcription (Figure 1.1.1). Axin2 is a major conserved β-catenin target, thus highlighting the importance of negative feedback in Wnt pathway regulation\textsuperscript{43}.

The cytoplasmic tail of Fz also recruits Dishevelled (Dsh) to the plasma membrane (through the PDZ domain of Dsh). Dsh oligomerization occurs at the plasma membrane (mediated by the Dsh DIX domain) and leads to formation of large “signalsomes”, which include Fz, LRP5/6, GSK3, Dsh and Axin. Dsh recruits β-arrestin 2, which mediates endocytosis of Fz-LRP5/6-Wnt complexes\textsuperscript{44}, an essential component of signal activation\textsuperscript{45,46}.
Figure 1.1.1. Overview of the vertebrate Wnt signaling pathway. Wnt signals can be transduced through canonical Wnt/β-catenin, non-canonical Wnt/Planar cell polarity (PCP) or non-canonical Wnt/calcium (Ca^{2+}) pathways. Canonical Wnt/β-catenin signaling is mediated by Frizzled (Fz) receptors and low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6) co-receptors. Phosphorylation of β-catenin is mediated by Axin-GSK3-APC complexes and targets the protein for degradation. Wnt binding activates Dishevelled (Dsh), which leads to inhibition of Axin-APC-GSK3 activity. Stabilized β-catenin translocates to the nucleus where it binds TCF/LEF transcription factors and activates target gene transcription. Non-canonical Wnt/PCP signaling involves Wnt-Fz recruitment of Dsh and downstream activation of Rho and Rho-associated kinase (ROCK), thus inducing cytoskeletal rearrangements. Non-canonical Wnt signaling can also activate c-Jun N-terminal kinase (JNK). Together with Fos, phosphorylated c-Jun forms the AP-1 transcription factor complex. Non-canonical Wnt/Ca^{2+} signaling activates phospholipase C (PLC) through Wnt-Fz resulting in the release of intracellular Ca^{2+} from the endoplasmic reticulum (ER). Ca^{2+} release activates protein kinase C (PKC) and calcium-calmodulin-dependent kinase II (CAMKII).
Control of Wnt/β-catenin signaling occurs at multiple levels. Examples of Wnt inhibitors include secreted Frizzled related proteins (sFRPs) that bind and sequester Wnts away from receiving cells\textsuperscript{47} and Dickkopf1 (Dkk1), which is a secreted Wnt antagonist that binds LRP5/6 and prevents Fz-LRP5/6-Wnt complex formation\textsuperscript{48,49}.

### 1.1.2 Wnt/β-catenin signaling and development

Many Wnt signaling components were identified as \textit{Drosophila} segment polarity genes\textsuperscript{50}, which revealed an important role for Wnt/β-catenin signaling in patterning the \textit{Drosophila} embryo. Subsequent genetic screens and epistasis experiments generated an outline of the core Wnt/β-catenin signaling pathway.

\textit{Wingless} (\textit{wg}, a \textit{Drosophila} Wnt homolog) is essential for maintenance of \textit{engrailed} (\textit{en}) gene expression and specification of naked cell fate on the ventral epidermis: embryos that are defective for \textit{wg} signaling are completely covered in denticles on the ventral cuticle\textsuperscript{51}. \textit{Wg} gradients also define cell fates across the wing imaginal disc\textsuperscript{52} and are required for specification of wing marginal cell fate\textsuperscript{53}. In the eye imaginal disc, \textit{wg} signaling functions to define the border between the retina and surrounding head capsule by promoting head fate\textsuperscript{54,55}, while \textit{wg} mutations also cause mis-patterning within the CNS (RP2 motoneurons are not specified), indicating an important role for this pathway in neurogenesis\textsuperscript{56}. Other late stage analyses have revealed important roles for \textit{wg} in organogenesis, including heart development\textsuperscript{57}.

In vertebrates, Wnt/β-catenin signaling plays a conserved role in axial patterning and was initially found to regulate Spemann-Mangold organizer formation in \textit{Xenopus}\textsuperscript{58}. The Spemann-Mangold organizer is a conserved developmental structure that defines spatial pattern formation within the early vertebrate embryo and is required for head mesoderm, notochord and pharyngeal endodermal tissue specification\textsuperscript{59}. Upon fertilization in \textit{Xenopus}, the cortical cytoplasm at the vegetal pole of the embryo shifts in a microtubule dependent manner to the equatorial plane\textsuperscript{60}. This vegetal cortical cytoplasm contains factors that activate Wnt/β-catenin signaling and cytoplasmic accumulation of maternal β-catenin, which marks the site of the future organizer\textsuperscript{61}. \textit{Xenopus wnt11} has been shown to translocate dorsally during cortical rotation and is required upstream of β-catenin for organizer formation\textsuperscript{62}.

Coordination between β-catenin and TGF-β signaling at the Spemann-Mangold organizer induces organizer-specific gene expression (\textit{goosecoid} [\textit{gsc}]) and secretion of ventral BMP, Wnt and Nodal antagonist like Noggin and Chordin\textsuperscript{63}. Transplantation of the organizer or ectopic
expression of β-catenin is sufficient to induce a secondary embryonic axis\textsuperscript{58,59}, whereas inhibition of cortical rotation, removal of the organizer, or knock-down of β-catenin blocks the formation of all dorsal structures\textsuperscript{64,65}.

When zygotic transcription begins at the mid-blastula transition (MBT), Wnt/β-catenin signaling no longer induces dorsal organizer tissue fate, but rather plays an opposing role antagonizing the head organizer to specify ventral/posterior tissue fates. Overexpression of Wnt ligands or β-catenin post-MBT suppresses anterior development\textsuperscript{66,67}, indicating that inhibition of Wnt/β-catenin activity is required to maintain dorsal or anterior fate. Wnt/β-catenin inhibition through overexpression of an antagonist or knock-down using morpholino antisense oligonucleotides (MOs) results in a shortened trunk and neural anteriorization\textsuperscript{68,69}.

Unlike Xenopus, loss of \textit{wnt11} in zebrafish does not lead to dorsoventral (DV) patterning defects\textsuperscript{70} and no other Wnt ligand has been implicated in dorsal specification. However, in zebrafish, maternal β-catenin does accumulate in nuclei of dorsal marginal cells and marks the future dorsal domain (Figure 1.1.2). Overexpression of β-catenin leads to axis duplication\textsuperscript{71}, inhibition leads to embryonic ventralization\textsuperscript{71,72}, and conserved gene targets like \textit{dharma}/\textit{bozozok} (ref. \textsuperscript{73}), \textit{chordin} (ref. \textsuperscript{74,75}) and \textit{goosecoid} (ref. \textsuperscript{76}) function in axis formation through inhibition of ventral BMP and Wnt ligand expression (Figure 1.1.2). During zebrafish gastrulation, Wnt/β-catenin signaling plays a conserved role in posterior fate specification (Figure 1.1.2): \textit{wnt8} is expressed in the ventrolateral domain and required for ventral/posterior tissue fates in zebrafish\textsuperscript{77,78}. Indeed, overexpression of \textit{wnt8} leads to neural posteriorization\textsuperscript{77}.

In the zebrafish embryonic tailbud, Wnt/β-catenin signaling is required for specification and maintenance of posterior tissue in a mesenchymal state through activation of transcriptional targets like \textit{tbx6} (ref. \textsuperscript{79-81}). As the body elongates, Wnt/β-catenin signaling specifies paraxial mesodermal fate from a bi-potential neural/mesodermal progenitor cell pool located within the tail bud\textsuperscript{82,83}, indicating an important role for Wnt/β-catenin signaling in stem-cell dynamics during development.

In addition to dorsoventral patterning, forward genetic screens in zebrafish have identified requirement for Wnt/β-catenin signaling components in later stages of development. For example, Wnt pathway components play a role in liver development (\textit{wnt2bb}), neurogenesis (\textit{tcf3}), anterior brain patterning (\textit{axin2})\textsuperscript{84} and cardiac valve formation (\textit{apc})\textsuperscript{85}.
Figure 1.1.2. Wnt/β-catenin signaling and axial patterning in zebrafish. (a) Maternal β-catenin localizes to the nucleus of cells in the future dorsal organizer (orange in b) prior to the onset of zygotic transcription. (b) Organizer-specific expression of goosecoid (gsc) and chordin (chd) inhibit the ventralizing activity of Wnt and BMP signaling that is activated just prior the onset of gastrulation. Lateral and animal pole views of zebrafish embryos are represented with dorsal (D) and ventral (V) sides are indicated. Dorsoventral (DV) patterning is translated into anterior (A) and posterior (P) tissue fate specification. (c) Dorsal view of somite stage embryo. Posterior Wnt signaling is inhibited by anterior Wnt inhibitors like Dickkopf 1 (Dkk1) and Wnt-activated inhibitory factor 1 (Waif1).
In the mouse epiblast (the embryo proper), Wnt/β-catenin signaling is enriched in the primitive streak at E6.0\textsuperscript{86,87} and embryos lacking Wnts or β-catenin fail to gastrulate\textsuperscript{88,89}. Constitutive activation of β-catenin induces premature transformation of epiblast into mesoderm\textsuperscript{90}, suggesting that in mice, Wnt/β-catenin acts as a mesoderm inducer. However, Wnt/β-catenin signaling also plays a conserved in axis specification: Axin-null embryos and a hypomorphic allele of \textit{Apc} complete gastrulation, but display partial axis duplication\textsuperscript{91,92}.

Inhibition of anterior Wnt/β-catenin signaling is also important for correct patterning of anterior structures in the mouse. Ectopic Wnt/β-catenin activation results in loss of head and/or reduced expression of neural markers\textsuperscript{91,93}, while mice mutant for \textit{Wnt3a} or \textit{LRP6} display posterior truncations\textsuperscript{27,94}, revealing a conserved role for Wnt/β-catenin in posterior tissue fate specification.

Wnt/β-catenin signaling is dynamic surrounding the tail bud, oscillating in phase with formation of somite pairs from the presomitic mesoderm (PSM)\textsuperscript{95}. Interestingly, Wnt/β-catenin signaling regulates Notch signaling in the PSM, which play an important role during somitogenesis\textsuperscript{95}. Mice homozygous for a hypomorphic allele of \textit{Wnt3a} (\textit{vestigial tail (vt)}) display a loss of somite pattern\textsuperscript{96}, revealing an important role for Wnt/β-catenin signaling in axial segmentation.

### 1.1.3 Wnt/β-catenin signaling and cancer

Downstream components of the Wnt/β-catenin signaling pathway are often mutated in human cancer. An early connection between Wnt signaling and human cancer came from positional cloning of inherited human colon cancer genes. Adenomatous polyposis coli (APC) was mapped to mutations in the \textit{APC} gene and is an inherited form of cancer that leads to multiple colon polyps at an early age\textsuperscript{97}. \textit{Apc} mutant mice (\textit{Min}, Multiple intestinal neoplasia) inherit cancer in an autosomal dominant manner\textsuperscript{97}, while in zebrafish, \textit{apc} haploinsufficiency is sufficient to predispose an animal to intestinal, hepatic, and pancreatic tumors\textsuperscript{98}. Mutations in \textit{Axin} and within phosphorylation sites of β-catenin have also been identified in colorectal and other forms of human cancer\textsuperscript{99-102}.

Some Wnt ligands, Fz receptors and other positive regulators of the Wnt/β-catenin pathway are upregulated in multiple forms of human cancer\textsuperscript{103,104}; also, Wnt/β-catenin inhibitors are often downregulated\textsuperscript{103,104}. Therefore, deregulation of Wnt/β-catenin pathway signaling is associated with tumorigenesis, making Wnt/β-catenin pathway components attractive therapeutic targets for cancer therapy.
1.1.4 The non-canonical Wnt/planar cell polarity (PCP) signaling pathway

Planar cell polarity (PCP) refers to polarity across the field of a tissue and is required for diverse cellular processes including epithelial tissue organization, directed cell migration and oriented cell divisions.

Core components of the PCP signaling pathway were identified in *Drosophila*, with PCP mutants displaying disrupted cuticular and/or wing cell hair orientation, as well as misorientation of ommatidial units of the eye\(^{105-107}\). Mutant alleles of *frizzled (fz)*, *disheveled (dsh)*, *prickle (pk)*, *flamingo/starry night (fmi/stan)*, *vang/strabismus (vang/stbm)* and *diego (dgo)* (ref. \(^{106,108-112}\)) were found to disrupt planar polarity. Through epistasis experiments, these components were grouped together and placed into a pathway.

Protein complex formation and asymmetric membrane localization are a hallmark of *Drosophila* PCP (Figure 1.1.4). Fz, Dsh and Dgo form a complex that promotes PCP signaling, while the four-pass transmembrane protein Vang forms a complex with intracellular Pk\(^{113}\) and competes with Dgo to locally antagonize Dsh activity\(^{114}\) (Figure 1.1.4). Asymmetric localization of these signaling complexes results in polarized signal activation. In the fly wing for example, Fz-Dsh complexes localize to the distal side of cells, while Vang-Pk complexes localize to the proximal side\(^{115-117}\) (Figure 1.1.4). The seven-pass membrane cadherin, Fmi/Stan binds to both Fz and Vang and transports them to the plasma membrane\(^{109,118}\). Asymmetric localization of PCP complexes is thought to occur through an initial bias in Fz-Dsh activity combined with directed intracellular trafficking and mutual complex antagonism\(^{119,120}\).

How the initial asymmetry is established remains controversial, and the role for a Wg/Wnt ligand upstream of PCP signaling has been difficult to establish in *Drosophila*. Wing clones simultaneously mutant for *wg, Dwnt2, Dwnt4, Dwnt6, Dwnt10* display wild-type polarity\(^{118}\), suggesting that Wnts are not locally required. Also, cultured wing fragments still polarize\(^{118,121}\), suggesting that no long range signal is required as a directional cue. However, clonal overexpression of both *wg* and *Dwnt4* reorients neighboring wild-type cells towards a clone\(^{122,123}\), suggesting that Wnt ligands can function as an instructive cue for PCP. Generally, mutant alleles for Wg pathway components have been difficult to interpret since the effect on polarity has been hard to separate from the established role in canonical Wg signaling.
Figure 1.1.4. Asymmetric PCP signaling complex localization in *Drosophila* wing epithelia. PCP occurs orthogonal to apical-basal (AB) polarity and is established in *Drosophila* epithelia by the asymmetric distribution of core PCP factors. In wing hair cells, actin-rich hairs emanate from the distal end of the apical surface. Frizzled (Fz), Dishevelled (Dsh) and Diego (Dgo) complexes accumulate at the distal side of the hair cells where they activate actin enrichment necessary for hair growth. Van Gogh (Vang) and Prickle (Pk) complexes accumulate at the proximal side and inhibit Dsh-mediated cytoskeletal rearrangements. Flamingo (Fmi) is localized symmetrically and forms homodimers that are required to localize PCP receptor complexes and transmit polarity cues to adjacent cells.
It was recently reported that loss of a single \(wg\) allele on a hypomorphic \(wg\) and loss-of-function \(dwnt4\) background caused robust PCP defects\(^{123}\). Tissue polarity appears to be oriented towards the Wg/Wnt source, with Wg/Wnt emanating from the wing margin\(^{124}\) and from poles in the eye\(^{125}\), suggesting that Wg/Wnt creates a localized gradient of Fz-PCP activity that is transferred and reinforced across a tissue.

The polarity cue is transferred through intercellular interactions. Fz is required for Vang-Pk localization to the cell surface of adjacent cells, and the ability of Pk to block Dsh cell-autonomously reinforces polarity within these neighboring cells\(^{126}\). In this manner, polarity is translated across a tissue and loss of polarity in PCP mutant clones can affect polarity of surrounding wild-type tissue in a characteristic manner\(^{127}\). \(Vang\) mutant clones reorient proximal cells away from the clone\(^{110}\), whereas \(fz\) mutant clones reorient distal cells towards the clone\(^{105}\). This effect, referred to as domineering non-autonomy highlights the importance of intercellular communication for establishing polarity across a tissue.

Ultimately, localized Fz-Dsh activity leads to polarized activation of the cytoskeleton, forming actin-rich cellular structures at a characteristic location. Downstream effector proteins \(inturned\ (in), fuzzy\ (fy),\) and \(fritz\ (frtz)\) (ref.\(^{128,129}\)) localize to the distal side of the wing cell in response to Fz-Dsh activity\(^{128,130}\), and regionally transduce upstream signals to cytoskeletal effector proteins. A hypomorphic mutation in the RhoA p21 GTPase was found to disrupt polarity in the \(Drosophila\) eye and wing, and could attenuate activated PCP signaling in these tissues, identifying a key modifier of the actin cytoskeleton downstream of PCP signaling\(^{131}\). Disruption of \(Rac1, Rac2, Cdc42\) and \(Mtl\) alleles in combination suggested that multiple Rho GTPases play overlapping roles during planar polarity generation in \(Drosophila\)\(^{132}\). Epistasis experiments also suggested that Rho GTPases signal to the nucleus through JNK/SAPK-like kinases\(^{131}\).

### 1.1.5 PCP signaling in vertebrates

The core PCP signaling components are conserved in vertebrates; however, the existence of ten Fz (Fz 1-10), two Vang (Van Gogh-like 1 and 2 (Vangl1 and Vangl2)) and three Dsh (Dsh1, Dsh2 and Dsh3) homologs have made deciphering the vertebrate pathway challenging. Planar polarity directs hair follicle position and orientation in mammals. For example, mice with \(Fz6\) mutations display misorientation of hairs on their skin\(^{133}\). Additionally, PCP signaling is required for precise organization and orientation of kinocilia and stereocilia of the sensory epithelium within the inner ear. Several PCP mutant mouse strains display misorientation of hair
cells within cochlea of the inner ear\textsuperscript{134,135}. Defects in eyelid closure occur in multiple PCP mutant mice, suggesting an important role for PCP signaling in development of eyelid epithelia\textsuperscript{134,136}.

Research in vertebrate models has identified additional roles for PCP signaling in directed tissue migration and cell intercalation. Evidence also supports vertebrate specific roles for PCP signaling in formation and orientation of cilia\textsuperscript{137-139}, oriented cell divisions\textsuperscript{140,141}, neuronal migration\textsuperscript{142} and axonal pathfinding\textsuperscript{143}.

Importantly, PCP was found to mediate convergence and extension (C&E) movements in \textit{Xenopus} and zebrafish\textsuperscript{70,144-147}. C&E occurs in vertebrates during gastrulation as a result of directed tissue movements that function to narrow and elongate the vertebrate axis (Figure 1.1.5). In \textit{Xenopus}, axial cells elongate and orient themselves along the mediolateral axis with bipolar lamellipodia shortly after involution\textsuperscript{148}. Cells intercalate between their anterior and posterior neighbors, which contributes to axis elongation\textsuperscript{148}. In zebrafish, convergence begins during mid-gastrulation with the directed migration of lateral mesoderm tissue towards the dorsal margin\textsuperscript{149} (Figure 1.1.5). As these cells near the dorsal axis they elongate along their mediolateral axes and undergo coordinated collective migration dorsally. Cell intercalations near the midline contribute to axis elongation\textsuperscript{150} (Figure 1.1.5). In mice, mesodermal C&E occurs by mediolateral cell elongation, bipolar protrusions and cell intercalations between anterior and posterior neighbors\textsuperscript{151}. Disruption of C&E results in reduced axis elongation. Mutants and/or morphants for PCP signaling components display shorter and broader axes\textsuperscript{70,142,145,146,151,152}, and this phenotype is now considered indicative of impaired PCP signaling.

C&E movements also narrow the neural plate\textsuperscript{153} and directed cell intercalations following cell division are an important aspect of neural tube morphogenesis in zebrafish\textsuperscript{154}. Maternal-zygotic (MZ) \textit{vangl2} mutant zebrafish display dysmorphic neural tubes with an accumulation of neural epithelium at the embryonic midline\textsuperscript{154}. In mammals, C&E of the neural plate elongates this structure, which subsequently folds in on itself to form the neural tube. Mouse mutants for \textit{Vangl2, Dvl1} and \textit{Dvl2, Celsr1}, or \textit{Fz3} and \textit{Fz6} display craniorachischisis, a severe open neural tube phenotype that extends the entire length of the body\textsuperscript{134-136,155}. In humans, mutations in \textit{VANGL1, VANGL2, FZ6, PRICKLE1} and \textit{CELSR1} have been identified in cases of
Figure 1.1.5. Convergence and extension (C&E) movements occur during gastrulation and functions to narrow and elongate the vertebrate axis. (a) Individual cell behaviors depend on the tissue and region of the embryo and include mediolateral intercalations, radial intercalations and directed cell migrations. (b-d) Tissue movements during zebrafish gastrulation. Black arrows indicate the direction of involution at the onset of gastrulation (b, 6 hpf). The dorsal organizer (DO) and germ ring (GR) are indicated. C&E movements begin during mid-gastrulation (c, 8 hpf) and involve directed migration of lateral mesendoderm (green) towards the embryonic midline (orange). Cell intercalation at the midline contributes to axial extension (red arrows), while directed migration of anterior tissues like the prechordal plate (PCP) contribute to axial extension without contributing to axial convergence. At the end of gastrulation (d, 10hpf) axial (orange) and paraxial (green) tissue is narrowed and elongated along the mediolateral and anteroposterior axes, respectively. All views are dorsal.
craniorachischisis and in spina bifida, a more mild neural tube disorder (NTD) that occurs only at the cadual extent of the neural tube\textsuperscript{156-161}.

Upon internalization, gastrulating midline mesendoderm cells migrate towards the animal pole and contribute to axis elongation without contributing to dorsal convergence\textsuperscript{149} (Figure 1.1.5). PCP signaling also regulates this aspect of axial morphogenesis, independent of its role in mediolateral cell intercalation. In zebrafish, a singular eye field within the neural plate is split during morphogenesis as a consequence of midline hypoblast tissue extension\textsuperscript{162}. Disruption of anterior elongation results in medial instead of bilateral induction of optic stalks, and incomplete separation of the eyes, or cyclopia\textsuperscript{163}. Thus, cyclopia is another characteristic phenotype of disrupted PCP signaling in zebrafish\textsuperscript{70,163}.

PCP also controls collective migration of individual cells. PCP signaling drives cellular protrusions at the leading edge of migrating neural crest cells in \textit{Xenopus}\textsuperscript{164,165}, although the role for PCP in neural crest migration in mice is less clear. \textit{Vangl2 (Lp)} mutant mice display only minor defects in the distribution of neural crest cells, which may be secondary to overall morphological defects\textsuperscript{166}. This suggests that a requirement for PCP signaling in certain tissues may be species specific. However, a conserved role for PCP exists for proper migration of facial branchiomotor neurons (FBNs) that innervate muscles of the head\textsuperscript{142,167}. \textit{Vangl2} loss-of-function in zebrafish and mouse prevents caudal migration of FBNs from the more anterior rhombomere 4 into rhombomere 6\textsuperscript{167}.

A role for PCP signaling in organogenesis has also been reported. For example, PCP genes are required for normal lung branching morphogenesis\textsuperscript{168}. Mutant mice display smaller and misshapen lungs with fewer branches. Also, multiple cardiovascular defects are evident in \textit{Vangl2 (Lp)} mutant mice, including shared aorta and pulmonary outflow from the right ventricle as well as ventricular septal defects\textsuperscript{166}. The heart in \textit{Lp} mutants fails to loop; however, this is thought to be secondary to defects caused by incomplete axial rotation and reduced cervical flexion\textsuperscript{166}.

Asymmetric localization of PCP components is a conserved aspect of PCP signaling in vertebrates. Epidermal cells on the mouse skin localize Vangl2 and Fz6 asymmetrically along the anteroposterior axis to mediate hair follicle orientation\textsuperscript{169}. Dynamic cellular processes involved in directed cell movements have made asymmetries in protein localization more difficult to track; however, both symmetrical and asymmetrical Dsh localization along the
plasma membrane have been observed in tissue undergoing C&E movements in *Xenopus*\textsuperscript{170,171}. In zebrafish, *Drosophila* Pk tagged with GFP (GFP-Pk) localized to the anterior plasma membrane in mesodermal and neuroectodermal cells\textsuperscript{154,172}, while *Xenopus* Dsh (GFP-Dsh) was enriched at the posterior cell membrane in zebrafish mesodermal cells undergoing C&E movements\textsuperscript{172}. This pattern of asymmetry would suggest anterior-posterior (AP) planar polarization across the field of intercalating tissues. Importantly, GFP-Pk localization is lost in *MZvangl2* mutants\textsuperscript{154}, demonstrating its utility as an *in vivo* live marker of polarized tissue during embryogenesis in zebrafish.

In addition to asymmetrical protein localization, domineering non-autonomy also occurs during vertebrate PCP signaling. In the mouse epidermis, wild-type hair explants fail to polarize their follicles when placed next to mutant tissue\textsuperscript{169}, and wild-type cells transplanted into mutant *vangl2* embryos fail to undergo mediolateral elongation and C&E movements in zebrafish\textsuperscript{142}. In the neural tube of *MZvangl2* zebrafish embryos, transplanted wild-type cells fail to intercalate properly within the neural tube\textsuperscript{154} and localization is disrupted in wild-type cells in *MZvangl2*\textsuperscript{154}, revealing an important role for intercellular transmission of polarity in vertebrates to communicate the signal across the field of a tissue.

One major difference between *Drosophila* and vertebrate PCP signaling is a clear requirement for Wnt ligands in vertebrate signal transmission. Wnt5a mutant mice display characteristic hair cell misorientation within the cochlea\textsuperscript{173}, while loss of *wnt4*, *wnt5* or *wnt11* results in C&E defects in *Xenopus* and zebrafish\textsuperscript{70,144,152,174}. Interestingly, global overexpression of Wnt11 can rescue the *wnt11* mutant phenotype in zebrafish\textsuperscript{70}, suggesting that Wnts play a permissive role during gastrulation; however, *Wnt11* expressed medial to the somites is sufficient to orient myocyte elongation during chick embryogenesis\textsuperscript{175}, revealing an instructive role for Wnt ligands in certain tissues.

Multiple cellular processes have been observed to occur downstream of PCP signaling in vertebrates. Activation of Rho and Rac GTPases was detected in regions undergoing C&E movements, and activation of Rho was blocked using dominant negative Dsh\textsuperscript{176,177}. RhoA can rescue *wnt5* and *wnt11* mutant zebrafish\textsuperscript{178}, and Rho kinase as well as the formin Diaphanous (mDia) function downstream of PCP signaling in zebrafish to mediate C&E\textsuperscript{178,179}.

Cell contacts are also important for directed tissue migration and cell intercalation. PCP signaling promotes Rho-independent cell-cell contact persistence through localized
accumulation of Wnt11, Fz7 and Fmi in the zebrafish animal cap\textsuperscript{180}, while independent experiments suggest that PCP signaling promotes tissue cohesion through Rab5 GTPase-mediated endocytosis of E-cadherin\textsuperscript{181}, an important regulator of cell adhesion and tissue morphogenesis during gastrulation\textsuperscript{182}. Recently, PCP was shown to control septin-mediated localization of cortical actomysin along the mediolateral axis of converging cells\textsuperscript{183}. This effect was associated with intercellular tension during collective tissue movements involved in intercalation and body axis C&E\textsuperscript{183}.

Embryonic tissue migration occurs within the context of extracellular matrix (ECM) and disruption of the ECM has been shown to disrupt tissue morphogenesis\textsuperscript{184}. Interestingly, PCP signaling has been shown to directly influence ECM assembly\textsuperscript{185,186}, which corresponds with the onset of C&E movements\textsuperscript{187}. Vangl2 and prickle1a are required for ECM matrix assembly via inhibition of matrix metalloproteinase (MMP14) proteolytic activity, and experiments suggest that PCP signaling normally inhibits ECM matrix assembly through controlled cadherin mediated cell adhesion\textsuperscript{188,189}. These data suggest that cell-cell and cell-ECM interactions are additional mechanisms through which PCP signaling controls tissue migration and morphogenesis during development.

1.1.6 Wnt pathway specificity

Wnt ligands, Fz receptors and Dsh are shared between Wnt/β-catenin and Wnt/PCP signaling pathways. Because of obvious differences in signal outcome, tremendous effort has gone into determining how a cell controls activation of one pathway versus the other. Traditionally, Wnt ligands have been classified as either canonical or non-canonical based on whether their expression can induce a secondary axis (canonical Wnt1 and Wnt3a) or not (non-canonical Wnt4, Wnt5a, Wnt11). Also, disruption of non-canonical Wnt signaling \textit{in vivo} induces PCP-dependent C&E defects with none of the obvious patterning defects associated with loss of canonical Wnt/β-catenin activation\textsuperscript{70,152,173}. \textit{In vitro} evidence suggests, however, that receptor context rather than the specific Wnt ligand determines signal outcome. Purified non-canonical Wnt5a was shown to inhibit canonical Wnt/β-catenin activity through an alternative Wnt receptor, Ror2\textsuperscript{190}. Interestingly, this same study showed that Wnt5a could activate Wnt/β-catenin signaling in the presence of Fz4 and LRP5\textsuperscript{190}, indicating differential pathway activation depending on the presence or absence of certain receptor molecules. In \textit{Xenopus}, Wnt5a can induce a secondary axis if expressed with the appropriate Fz receptor\textsuperscript{191},
and Wnt11 has been shown to activate the β-catenin pathway to establish the Spemann-Mangold organizer during early embryonic stages.\textsuperscript{62}

To date, a number of Wnt receptors, co-receptors and regulatory proteins have been identified and many of these have been shown to promote one type of Wnt signaling while simultaneously inhibiting the other. Thus, the theory of a functional switch between Wnt/PCP and Wnt/β-catenin pathways exists in the field. LRP5/6 is an essential co-factor involved in Wnt/β-catenin activation; however, in vertebrates LRP6 has also been shown to affect C&E movements through inhibition of PCP signaling.\textsuperscript{192} Ror2 is a transmembrane receptor tyrosine kinase that binds Wnts through its cysteine-rich domain (CRD) and can inhibit canonical Wnt/β-catenin signaling in tissue culture;\textsuperscript{190} however, Ror2 knock-down in \textit{Xenopus} results in C&E defects and \textit{Ror1; Ror2} double-knockout mice have shorter body axes and open neural tubes\textsuperscript{193,194}, suggesting roles in PCP-dependent tissue morphogenesis. Dkk1, inhibits canonical Wnt/β-catenin signaling through interactions with LRP6, but also promotes Wnt/PCP signaling through interaction with the cell surface proteoglycan Glypican 4/Knypek\textsuperscript{195}, an important regulator of vertebrate PCP signaling. Finally, the transmembrane protein Waif1 can attenuate Wnt/β-catenin signaling while promoting Wnt/PCP signaling through active internalization of LRP6\textsuperscript{196}. These examples highlight the fact that at the level receptor expression, alternative Wnt pathways can either be activated or inhibited.

Dsh lies downstream of both canonical Wnt/β-catenin and non-canonical Wnt/PCP receptor complexes. Therefore, differential recruitment and activation of Dsh is an important mechanism through which cellular context can determine signal outcome. Distinct domains of Dsh are used for Wnt/β-catenin versus Wnt/PCP signaling: the DIX domain is required for Wnt/β-catenin signal activation and acts in isolation as a dominant negative to inhibit β-catenin target gene transcription downstream of Wnt-Fz-LRP\textsuperscript{197}. The DEP domain functions in Wnt/PCP signaling through interactions with Rac\textsuperscript{176}, and it can inhibit Wnt/PCP-dependent morphogenesis when expressed alone\textsuperscript{197}. The PDZ domain of Dsh is thought to act as the functional switch between Wnt pathways: interactions with Van Gogh, Prickle, Diego and PAR1 support Wnt/PCP signaling, while CK1, GSK3 and Naked cuticle (NKD) interactions support Wnt/β-catenin signaling\textsuperscript{198}. Expression of a Dsh PDZ domain deletion mutant (Xdd1) disrupts Wnt/PCP signaling. Indeed, Xdd1 is a commonly used functional inhibitor of PCP signaling \textit{in vivo}\textsuperscript{197,199}. Localization of Dsh to the plasma membrane is important for PCP signaling\textsuperscript{170,197,200}, whereas apical versus basolateral Fz/Dsh localization has been shown to activate Wnt/PCP and Wnt/β-
catenin signaling, respectively\textsuperscript{201}. The pool of Dsh is thought to be limiting; therefore, activation of one pathway sequesters Dsh making it unavailable to activate the other. This provides a mechanism through which ligand-receptor complexes can activate one type of Wnt signaling while simultaneously inhibiting the other.

1.2 Protein tyrosine kinase 7 (Ptk7)

Protein tyrosine kinase 7 (Ptk7) is a single-pass transmembrane protein that has been shown to regulate PCP-mediated morphogenetic movements and Wnt/β-catenin signal transduction. Ptk7 is an atypical receptor tyrosine kinase that contains extracellular immunoglobulin (Ig)-like domains and an intracellular catalytically inactive kinase domain\textsuperscript{202,203}.

1.2.1 Ptk7 and human cancer

\textit{PTK7} was initially cloned from normal melanocytes using degenerate primer pairs corresponding to conserved subdomains of protein tyrosine kinases\textsuperscript{204}. Full-length cDNA was also identified in metastatic colon cancer and the gene was named colon carcinoma kinase 4 (\textit{CCK4}) because of its expression in that tissue\textsuperscript{205}. \textit{CCK4} was not expressed in the normal adult colon; however, high levels of gene expression in mouse fetal colon, suggesting that the gene plays a developmental role\textsuperscript{205}. Subsequently, \textit{PTK7} upregulation was seen in gastric, esophageal and lung cancer\textsuperscript{206-209}, as well as in acute myeloid leukemia blast cells\textsuperscript{210}. Conversely, expression is decreased in metastatic melanoma\textsuperscript{211} and deletion of chromosome 6p, the region where \textit{PTK7} is located, were identified in a number of different cancers\textsuperscript{212,213}. \textit{PTK7} is not expressed in pituitary tumors\textsuperscript{214}. Such differential expression means that the role for \textit{PTK7} in cancer progression is not well understood; however, evidence points towards an association between \textit{PTK7} expression and tissue metastasis in certain contexts\textsuperscript{205,215-217}. Regardless, \textit{PTK7} is of current interest as a potential cancer biomarker and as a drug target. \textit{PTK7} fragments are secreted from non-advanced colon cancer cell lines making it a potential marker for early detection of colorectal cancer\textsuperscript{218}. Also, expression in T cell acute lymphoblastic leukemia (T-ALL) makes \textit{PTK7} a potential agent for detection and immunophenotyping\textsuperscript{219}. Finally, \textit{PTK7} has been show to function as an excellent target for aptamers to efficiently deliver molecules and for cell capture\textsuperscript{220-224}, making \textit{PTK7} an intriguing therapeutic target.
1.2.2 Ptk7 during development

*Ptk7* is evolutionarily conserved and has been shown to play multiple roles throughout development. Orthologs have been identified in Hydra (*Lemon*), *Drosophila* (Off-track (*Otk*)), human, mouse, *Xenopus*, and chick (kinase-like gene (*KLG*))\(^{202,204,205,225-227}\).

The *Drosophila* homologue of *Ptk7*, Off-track (*Otk*), was identified in a screen looking for homologs of the *trk* family of mammalian neurotrophin receptors\(^{228}\). *Otk* contains 6 extracellular Ig domains and an extracellular leucine zipper motif, which may mediate receptor dimerization\(^{229}\). The intracellular domain of *Otk* contains sequences highly reminiscent of the catalytic domains of protein tyrosine kinases; however, the catalytic domain lacks an DFG catalytic motif (replaced by TPA) shared by most kinases\(^{230}\). Interestingly, *in vitro* kinase assays indicate that *Otk* is rapidly auto-phosphorylated, suggesting that *Otk* does have protein kinase activity\(^{228}\).

*Otk* expression was detected in neural tissue throughout development and along the germinal band in stripes corresponding to posterior parasegments 0-14\(^{228}\). Induced expression of *otk* in S2 cells resulted in homophilic cell aggregations with *Otk* localizing to sites of cell-cell contact\(^{228}\), suggesting that *Otk* may mediate cell adhesion.

Initial investigations revealed a role for *Drosophila otk* in Plexin/Semaphorin signaling and axon guidance *in vivo*. Indeed, *Otk* can co-immunoprecipitate with *Drosophila* and mammalian Plexins and *otk* mutants generated through P-element excision displayed defects in neuronal branching and morphology within the CNS and at muscle targets\(^{227}\). *Otk* loss-of-function interacted genetically with *PlexA* and *Sema1a* mutant alleles and suppressed the effects of *Sema1a* overexpression\(^{227}\).

Subsequent investigations with the same mutant as well as with *otk\(^{\text{RNAi}}\)* revealed Wnt-dependent tissue patterning and morphogenesis defects. Loss of *otk* led to loss of denticles in the ventral epidermis, while overexpression of *Otk* led to ectopic denticles\(^2\), implicating *Otk* as an inhibitor of Wg/β-catenin signaling. In the fly wing, overexpression of *Otk* led to PCP defects in which wing hairs were improperly oriented; however, loss of *otk* had no effect on wing tissue polarity\(^2\).

Vertebrate Ptk7 is an atypical receptor tyrosine kinase with seven extracellular Ig-like domains, a single transmembrane domain and a catalytically inactive pseudokinase domain. Ten putative N-glycosylation sites (NXS/T) are present within the extracellular domain of mouse and human
Ptk7\textsuperscript{202,205}, and Western blot analysis suggests that Ptk7 is heavily glycosylated\textsuperscript{1,202}. The transmembrane domain of Ptk7 is highly conserved and contains a GxxxG motif, which has been shown to drive transmembrane protein-protein interactions\textsuperscript{231}. However, the transmembrane domain of Ptk7 does not self associated \textit{in vitro}\textsuperscript{232}. The intracellular domains of all vertebrate Ptk7 homologs contain structural features conserved in kinases, but several key conserved motifs are differentially altered depending on the species. In human and mouse Ptk7 for example, the GXGXXG motif and the HRDL motif (required for ATP binding\textsuperscript{230}) are changed to GXSXXG and HKDL, respectively\textsuperscript{202,205}. In all vertebrate Ptk7 orthologs, the catalytic DFG motif required for terminal phosphate cleavage is modified\textsuperscript{202,205,225}. Consistent with this, protein activation by tyrosine phosphorylation was not observed following PTK7 overexpression in COS-1 or HEK293 cells, or in \textit{in vitro} kinase assays\textsuperscript{202,205}. This suggests that Ptk7 is a “defective” or “dead” pseudokinase\textsuperscript{233}; however, the high degree of evolutionary conservation within the kinase domain suggests that it remains functionally important. Finally, Ptk7 contains a conserved PDZ-binding motif at its C-terminus, suggesting possible intracellular interactions with PDZ domain-containing proteins.

In the mouse, \textit{Ptk7} expression is detected in the primitive streak at E7.0\textsuperscript{202}. Later on, mouse \textit{Ptk7} is enriched in the tail bud, limbs, somites, gut and cranio-facial structures\textsuperscript{1,202,234}. Immunostaining has detected membrane-localized Ptk7 during somite stages in the neuroepithelium and lateral mesenchyme\textsuperscript{234}. Very low levels of \textit{Ptk7} transcript were detected in most tissues of the adult mouse; however, significant expression was detected in the lung and unpregnant uterus\textsuperscript{202}. In humans, \textit{PTK7} is highly expressed in the liver, lung, pancreas, kidney, and placenta; at lower levels in the brain and heart; and is barely detectable in the skeletal muscle\textsuperscript{203}.

Evidence from vertebrate models has strongly implicated \textit{Ptk7} as a regulator of Wnt signaling in multiple tissues. Research in this area started with the identification of \textit{Ptk7} gene trap mutant mice that died perinatally and displayed craniorachischisis, a neural tube disorder (NTD) indicative of disrupted PCP signaling\textsuperscript{1}. These mice displayed compressed body axes, suggesting defects in C&E movements, as well as misorientation of hair cells within the inner ear\textsuperscript{1}. In a separate study, the same \textit{Ptk7} mutant allele was observed to cause severe defects in facial branchiomotor neuron (FBN) migration, phenotypically similar to \textit{Vangl2} (loop-tail, Lp) mutant mice\textsuperscript{167}. Mice trans-heterozygous for \textit{Ptk7} and \textit{Vangl2} (Lp) mutant alleles displayed spina bifida\textsuperscript{1}, which suggested that \textit{Ptk7} genetically interacts with the PCP signaling pathway.
A second, ENU-induced mouse mutant (chuzhoi, chz) confirmed the requirement for Ptk7 in C&E and inner ear tissue cell polarity\textsuperscript{234}. Characterization of chz mutant mice identified additional roles for Ptk during organogenesis\textsuperscript{234}; chz mutants had small and misshapen lungs with abnormal branching as well as heart defects in which the aorta and pulmonary trunk exited together from the right ventricle. Septal defects and abnormal heart looping were also observed. Chz mutant mice did not display significant defects in neural crest cell migration, consistent with a lack of neural crest defects in other PCP mutant mouse models\textsuperscript{234}.

*Xenopus PTK7* is expressed in the neuroectoderm from late gastrulation and throughout neurulation\textsuperscript{1}. Knock-down of *PTK7* in *Xenopus* by antisense morpholino oligonucleotides (MO) resulted in defects in neural C&E and failure of neural tube closure in a majority of embryos\textsuperscript{1}, consistent with a role for Ptk7 in PCP-mediated morphogenesis. Interestingly, overexpression of *PTK7* lacking the intracellular domain led to similar phenotypes\textsuperscript{1}, which was suggested to indicate dominant-negative activity.

*PTK7* is expressed in *Xenopus* neural crest cells and gene knock-down in this tissue inhibited migration without affecting specification\textsuperscript{235}, indicating that Ptk7 (and/or PCP) may have evolved different roles with respect to polarized neural crest cell migration. Expression of a truncated PTK7 protein lacking the intracellular domain similarly inhibited neural crest cell migration, again suggesting dominant-negative activity for this mutant construct\textsuperscript{235}. An Activating Transcription Factor 2 (ATF2)-based luciferase reporter assay, used to assess PCP-based JNK activation\textsuperscript{236}, revealed that PTK7 activates JNK in unpolarized *Xenopus* animal cap cells\textsuperscript{2,235}, suggesting that PTK7 promotes PCP signaling. However, the precise role for Ptk7 in *Xenopus* PCP signal transduction remains controversial after a recent study found that PTK7 inhibits JNK reporter activity in whole embryos during neurula stages and inhibits non-canonical Wnt-induced PCP phenotypes\textsuperscript{4}.

Conflicting evidence from *Xenopus* also suggests differential roles for *PTK7* in Wnt/\(\beta\)-catenin signal transduction. Morpholino (MO)-mediated knock-down of *PTK7* in *Xenopus* inhibited Spemann-Mangold organizer formation\textsuperscript{3}, while overexpression of *PTK7* was not sufficient to induce ectopic organizer or \(\beta\)-catenin reporter activity\textsuperscript{3}, suggesting that *PTK7* plays a permissive role in organizer formation. Independent experiments found that PTK7 knock-down inhibited posterior neural tissue fate specification, indicating an activating role for Ptk7 in Wnt/\(\beta\)-catenin signal transduction, albeit at later stages of embryonic development\textsuperscript{4}.
Conversely, independent experiments found that MO knockdown of *Xenopus* *PTK7* in animal pole explants enhanced Wnt/β-catenin reporter activation through ectopic *wnt8* expression\(^2\). Furthermore, *PTK7* overexpression inhibited the ability of *wnt8* expression to induce a secondary axis\(^2\), suggesting that *PTK7* inhibits Wnt/β-catenin signaling. The extracellular domain of *PTK7* was required for this activity\(^2\).

Experiments in human cells were equally conflicting. siRNA-mediated knock-down of endogenous *PTK7* in HCT116 colon cancer cells inhibited Wnt3a-induced TOP-FLASH/luciferase reporter activity\(^3\), while in HEK293T cells, reporter activity was inhibited by *PTK7* overexpression\(^2\). *PTK7* knock-down in HEK293T cells inhibited LRP6-mediated TOP-FLASH/luciferase reporter activation\(^4\), suggesting that *PTK7*’s influence on Wnt/β-catenin signaling pathway is context specific. Ultimately, the role of Ptk7 in Wnt/β-catenin signaling remains to be determined.

### 1.2.3 Ptk7 and Wnt signal transduction

*PTK7* recruits Dsh to the plasma membrane in *Xenopus* animal cap explants and is required for Dsh hyperphosphorylation\(^235\). *PTK7* binds Dsh; however, this interaction requires Fz7\(^235\), suggesting that *PTK7* forms a complex with Fz7 and Dsh to mediate PCP signal transduction. These interactions require the intracellular domain of *PTK7* and the PDZ domain of Dsh\(^235\). In a separate study, RACK1 (receptor of activated protein kinase C 1) was identified in *Xenopus* as a binding partner of *PTK7* and is required for *PTK7*-mediated Dsh translocation through recruitment of protein kinase C δ1 (PKCδ1)\(^237\). Interestingly, PKCδ1 is a molecule previously shown to interact with Dsh and regulate PCP-mediated morphogenesis\(^171\). Notably, these interactions do not require Fz7\(^237\), suggesting a parallel mechanism through which *PTK7* affects PCP signal transduction. *PTK7* expression in *Xenopus* also affects JNK reporter activity\(^2,4\), suggesting that *PTK7* regulates PCP signaling in part through the JNK pathway.

*PTK7* was subsequently shown to interact with Fz7 independent of Dsh\(^2\). In this study, the extracellular domain of *PTK7* was found to co-immunoprecipitate in complex with Fz7 and Wnt3a or Wnt8, but not Wnt5a or Wnt11\(^2\). Independent analysis has shown that *PTK7* also interacts with the canonical Wnt co-receptor LRP6\(^4\), suggesting that *PTK7* selectively interacts with canonical Wnt/β-catenin signaling complexes in certain contexts.

Mouse *Ptk7* is not required for membrane recruitment of Dsh in planar polarized mesoderm or within the inner ear\(^151,238\), nor does JNK mutation interact with *Ptk7*\(^238\). These mechanistic
differences further suggest that the pathway may have evolved differently in mice and *Xenopus* and that the mechanisms involved in Ptk7-mediated signal transduction may be species specific.

*PTK7* likely affects planar polarity by acting on the cytoskeleton. Hair cell orientation within the cochlea is disrupted in *Ptk7* mutants and, in this tissue, *Ptk7* is required for junctional accumulation of contractile myosin heavy chain protein (MIIB). Myosin-dependent contractile force was required in this study for inner hair cell orientation, providing a potential link between *Ptk7* and tissue polarity within the inner ear. C&E movements have been shown to require similar myosin dynamics in *Xenopus*; however, the relationship between *Ptk7* and myosin in this context has not been explored.

Using yeast-two hybrid and co-immunoprecipitation techniques, the intracellular domain of PTK7 was found to associate with β-catenin. Interestingly, this interaction was inhibited by Wnt3a. In MDCK cells, overexpression of Dsh competes for PTK7 binding with β-catenin, suggesting that by binding to different intracellular proteins, PTK7 may further influence cellular context and pathway specific activity.

Interestingly, PTK7 is proteolytically cleaved at multiple sites in human cancer cell lines and cleavage is positively associated with cell invasiveness. Membrane associated, type 1 transmembrane matrix metalloproteinase (MT1-MMP) cleaves PTK7 within the seventh Ig-like domain, generating a soluble ectodomain fragment (65kDa) and a membrane-tethered fragment that includes the pseudokinase domain (50kDa). Full-length PTK7 inhibits invasiveness, while cleavage of the extracellular domain reverses this effect. Further evidence suggests that MT1-MMP cleavage is coupled to cleavage by ADAM proteinases within the C-terminus of the extracellular domain, adjacent to the plasma membrane, in highly invasive fibrosarcoma (HT1090) cells and in colon cancer (SW480) cells. Both extracellular domain cleavages appear to be required for subsequent intracellular γ-secretase-mediated cleavage of PTK7 and this intracellular cleavage product can translocate to the nucleus to enhance proliferation, migration and anchorage dependent colony formation. Therefore, four PTK7 cleavage products made by different enzymes differentially affect cellular behavior and may explain why PTK7 expression alone is not always associated with tumorigenesis. Consequently, PTK7-positive tumors may be differentially invasive based on the profile of PTK7 cleavage products. Such fragments could provide useful diagnostic markers in the future.
1.2.4 Alternative Ptk7 pathways

It is important to note that Ptk7 plays a role in other, non-Wnt-dependent pathways, during vertebrate embryogenesis. In *Xenopus*, PTK7 and PlexinA1 physically interact, with knockdown of both genes independently leading to similar defects in neural crest cell migration\(^{235,240}\). *PlexinA1* and *PTK7* also interact to cause neural crest migration defects when co-expressed\(^{240}\), suggesting a shared pathway requirement for both genes. In chick, PlexinA1 has been shown to form a complex with Ptk7 in the ventricular segment during cardiac development and this interaction inhibits the pro-migratory effect of *Sema6D* expression on cardiac tissue\(^{241}\).

*PTK7* is expressed in vascular endothelial cells in response to vascular endothelial growth factor (VEGF) and plays a role in promoting vascular endothelial cell migration\(^{242,243}\). PTK7 interacts with the VEGF receptor (VEGFR) Flt-1 in a complex mediated by VEGF-A ligand. Knockdown of *PTK7* results in reduced VEGF-induced phosphorylation of Flt-1 and downstream targets of the VEGF signaling pathway\(^{243}\), suggesting an important role for *PTK7* in VEGF signal transduction. Interestingly, knock-down of *Ptk7* in the mouse cornea results in a significant decrease in local angiogenesis\(^{243}\), implicating *Ptk7* as an important angiogenic factor *in vivo*.

1.3 Scoliosis

Scoliosis is defined as lateral curvature of the spine of 10° or greater\(^{5,6}\) and can occur as a consequence of underlying vertebral malformations (congenital scoliosis, CS); neurological disease (*e.g.* Cerebral palsy, paralysis); muscular abnormalities (*e.g.* Duchenne muscular dystrophy); neurofibromatosis; connective tissue abnormalities (*e.g.* Marfan’s syndrome); and/or skeletal dysplasia\(^{5,244}\). However, the majority of structural curves are idiopathic (idiopathic scoliosis, IS) with no known underlying cause\(^{5,244}\).

1.3.1 Congenital Scoliosis (CS)

Congential scoliosis refers to spinal curvature caused by the presence of underlying congenital vertebral malformations (CVMs) and affects an estimated frequency of 0.13-0.5/1,000 live births\(^{245}\).

The types of CVMs observed in humans include hemivertebrae (half of a vertebrae), additional vertebrae, vertebral bar (vertebral fusions), butterfly vertebrae and wedge-shaped vertebrae. Mild cases of CS include isolated CVMs; however, severe forms including spondylocostal
dysostosis (SCD) and spondylothoracic dysostosis (STD) are characterized by multiple CVMs that occur throughout the spine. CVMs may occur in isolation; but are also observed in association with multiple developmental syndromes that include other abnormalities including cardiac, renal or spinal cord malformations\textsuperscript{245}.

### 1.3.2 Somitogenesis and vertebral patterning

Vertebral bodies are derived from somites and several forms of recessive monogenic CS are associated with genes that regulate somitogenesis\textsuperscript{245,246}.

Somites bud off from the anterior presomitic mesoderm (PSM) in a process that involves a mesenchmal to epithelial transition (MET) in response to a periodic wave of gene expression known as the “segmentation clock”\textsuperscript{247-249} (Figure 1.3.2). The “segmentation clock” corresponds to the periodic activation of the Notch signaling pathway within each individual cell of the PSM\textsuperscript{250}. Expression of Notch target genes including \textit{lunatic fringe} (\textit{Lfng}), the \textit{hairy/enhancer of split} family of transcription factors (\textit{Hes/Her/Hairy}) as well as Notch ligands \textit{Delta-like 1 and 3} (\textit{Dll1, Dll3}) (\textit{deltaC (dlc)} in zebrafish), begins in the posterior PSM and travels in an anterior direction on either side of the embryonic midline\textsuperscript{251}. Evidence from zebrafish suggests that oscillations in \textit{deltaC} drive periodic activation of Notch and upregulation of \textit{her1} and \textit{her7} gene expression\textsuperscript{252}. \textit{Hes/Her/Hairy} transcription factors feed back to inhibit their own expression\textsuperscript{252,253} while \textit{Lfng} encodes a glycosyltransferase that inhibits Notch activation\textsuperscript{254}. Thus, Notch signaling oscillates as somitogenesis proceeds along the rostral caudal axis. Disruption of Notch signaling results in a loss of organized somite boundaries and a functional loss of somite polarity\textsuperscript{246,251}.

Wnt pathway target genes have also been shown to oscillate in the PSM out-of-phase and upstream of the Notch pathway: where Notch target gene expression in the mouse PSM is also dependent on \textit{Wnt3a}\textsuperscript{95}. Notch pathway genes including \textit{Dll1, Dll3} and \textit{Notch1} have been shown to be transcriptionally up-regulated by Wnt\textsuperscript{255,256}, providing a functional link between the Wnt and Notch pathways. Inhibitors of the Wnt signaling pathway including \textit{Axin2, Dkk1}, and \textit{Naked 1 (Nkd1)} oscillate out of phase with Notch\textsuperscript{95,257,258}, and are therefore likely to modify the cyclic activation and inhibition of Wnt signaling. \textit{Nkd1} has been identified as a Notch target within the PSM\textsuperscript{258}, indicating that a complex feed-forward/feedback mechnism control somite patterning.
Figure 1.3.2. Overview of axial segmentation during zebrafish embryogenesis. Dorsal view of the embryonic tail bud with anterior up. An anteroposterior (AP) morphogen gradient is formed along the embryonic axis between anterior retinoic acid (RA) and posterior Wnt and FGF molecules. Cells exposed to the AP gradient at a certain threshold (determination front) start the somite differentiation process driven by Notch signaling. The presomitic mesoderm (PSM) and the determination front moves posteriorly with axis elongation. Wnt and FGF signaling influence a posterior pool of progenitors and maintain cells within the PSM in an undifferentiated state. Positive and negative feedback mechanisms drive oscillating expression of Wnt and Notch target genes that regulate the periodic formation of somite pairs at the anterior border of the PSM. Differentiated somite pairs display anterior (A) and posterior (P) polarity and go on to form dermomyotome and sclerotome-derived tissue types including skin, muscle, vertebrae and vertebral-associated structures.
In addition to oscillating gene expression within individual cells of the PSM, an AP morphogen gradient consisting of anterior retinoic acid (RA) and posterior FGF and Wnt gene expression define an AP signal threshold that determines the location of somite boundaries at the anterior extent of the PSM\(^95,259\) (Figure 1.3.2). High levels of FGF and Wnt in the posterior PSM maintain tissue in an undifferentiated state and increasing posterior FGF or Wnt activity results in anterior expansion of the PSM as well as the formation of smaller somites\(^{259-261}\). This function is mediated by transcription factors like *Brachyury/T* and *Tbx6*\(^{262-264}\), which specify and maintain PSM identity through regulation of mesodermal specific genes like *Mesogenin1*\(^{265}\).

Somite formation is coupled to axis elongation so that as the body elongates, caudal FGF and Wnt expressing cells move with the tail bud, and as a result shift the PSM posteriorly\(^95,266\). The simultaneous control of PSM fate and segmentation clock gene expression define Wnt as an link between AP morphogen gradients and the segmentation clock, which implicates Wnt signaling as a important regulator of somite tissue patterning during early embryogenesis.

Cells at the anteriormost extent of the PSM begin to express the transcription factor *mesp2*\(^{267}\). *Mesp2* feeds-back and inhibits Notch signaling\(^{268}\). It also activates transcriptional inhibitors of the *rippy* gene family, which inhibit *tbx6*. Down-regulation of *tbx6* feeds back to decrease *mesp2* expression, which completes the maturation of the newly formed somite. Cell-cell adhesion increases at the anterior end of the PSM\(^{269}\), and each somite appears when a group of cells completes the transition from mesenchyme to a three-dimensional epithelial rosette, known as a somitomere.

Almost immediately after somite formation, the dorsal portion of each somite begins to differentiate into muscle and dermis (dermomyotome), while the ventral portion receives signals from the notochord and differentiates into sclerotome. Sclerotome cells migrate to surround the notochord and differentiate into chondrocytes and connective tissue that contribute to the vertebral bodies, ribs, intervertebral discs and tendons. Each sclerotome displays anterior and posterior (AP) polarity: the anterior half of one sclerotome fuses with the posterior half of the next anterior sclerotome to form one vertebral segment. This causes a phase shift in the vertebrae relative to the muscle, which allows muscle to span adjacent vertebrae and exert force on the spinal column.

Unsurprisingly, many CS related genes are involved in somitogenesis. Mutations in Notch genes *DLL3, MESP2, LFNG,* and *HES7* have been identified in patients with SCD\(^{270-274}\), while
mutations in two PSMs regulatory factors Brachyury/T\textsuperscript{270} and Tbx6\textsuperscript{275} have been identified in individuals with CVMs.

1.3.3 Identification of CS susceptibility genes

Despite an obvious connection between CS and somitogenesis defects, the etiologies of most human CVMs remain unknown and may not be associated with defects in somite patterning.

Abnormalities in neural tube morphogenesis are likely to affect development of surrounding axial tissue, including vertebrae. Interestingly, autopsy of fetusus with neural tube disorders (NTDs) display CVMs\textsuperscript{276} and a family survey of 337 patients with multiple CVMs revealed an increased risk (5-10\%) of NTDs in sibling pairs, suggesting a genetic link between NTDs and CS\textsuperscript{277}.

Complex syndromes associated with CVMs have uncovered other interesting genetic associations not related to somite patterning. Klippel-Feil syndrome, characterized by a short neck and fused cervical vertebrae is linked to mutations in the BMP ligand GDF6 in both familial and sporadic cases\textsuperscript{278}. CHARGE syndrome (Coloboma of the eye and CNS abnormalities; Heart defects; Atresia of the choanae; Retardation of growth; Genital and/or urinary defects; Ear abnormalities or deafness), which often presents with CVMs, is associated with mutations in the gene coding for DNA-binding protein CHD7\textsuperscript{279}.

The notochord acts as a structural base for vertebral development; therefore, it’s not surprising that defects in notochord development lead to vertebral abnormalities in animal models. In the mouse and chicken, loss of the embryonic notochord results in a failure of sclerotome differentiation and loss of vertebrae\textsuperscript{280}. In zebrafish, localized ablation of the notochord also eliminates vertebral body formation\textsuperscript{281}, indicating a conserved requirement for the notochord.

Evidence suggests that the notochord is involved in metameric patterning of the vertebral column. In fused somites (fss) mutant zebrafish, somites lose AP polarity and display defects in boundary formation\textsuperscript{282}. Interestingly, the vertebral bodies (or centra) in fss mutants maintain a normal metameric pattern even though the somites are malformed\textsuperscript{281,282}. Only neural and hemal arches (sclerotome derived) are disorganized\textsuperscript{281,282}, suggesting that notochord is sufficient to instruct segmentation of the vertebral centra in zebrafish even when somite patterning is abnormal. In mouse paraxis mutants, epithelial somites fail to form; however, segmented vertebrae and ribs still develop in the anterior column\textsuperscript{283}, suggesting that, similar to fish, normal
somitogenesis is not required for vertebral segmentation in mice. Late stage dissection of the notochord in amphibian and chick embryos, results in an unsegmented mass of vertebral cartilage\textsuperscript{284,285}, highlighting the conserved requirement for this structure in vertebral segmentation.

Mutation of zebrafish collagen 8a1a (\textit{leviathan, lev/col8a1a}) does not affect segmentation of vertebral centra but results in regionalized bends of the embryonic notochord. This phenotype transitions into vertebral fusions in the adult zebrafish analogous to human cases of CS\textsuperscript{286}. Bending of the notochord is thought to bring together groups of normally spaced osteoblasts and promote ossification of multiple notochord segments together into scoliosis\textsuperscript{286}. This suggests that morphological defects in the shape of axial tissues like the notochord may be sufficient to affect CVMs in humans and should be considered in future analyses.

Multiple environmental factors have been associated with CVMs\textsuperscript{245}. \textit{Hes7}\textsuperscript{+/-};\textit{Mesp2}\textsuperscript{+/-} mice exposed to hypoxia during gestation develop CVMs at a higher rate than control littermates and this hypoxia was shown to affect FGF signaling during somitogenesis\textsuperscript{287}, revealing an interesting link between environmental manipulation and genetic factors controlling somitogenesis. \textit{Axin}\textsuperscript{Fa} heterozygous mice express a hypomorphic allele of \textit{Axin} and display variably kinked tails as a result of posterior CVMs. Interestingly, this phenotype can be partially rescued through maternal methyl supplementation\textsuperscript{278}, suggesting epigenetic modifications like DNA methylation may influence CS severity. Loss of environmental copper affects lysyl oxidases that affect embryonic notochord morphology and CVMs in zebrafish\textsuperscript{286,288,289}, revealing another environmental influence that could affect vertebral segmentation during early stages of human development.

1.3.4 Idiopathic Scoliosis (IS)

Idiopathic scoliosis (IS) is spinal curvature with no underlying abnormality and is quite common, with an incidence in the general population of approximately 3\%\textsuperscript{6,290}. Sub-classification of the disease is based on the age of onset and is categorized as infantile (birth to 3 years old), juvenile (3-11 years) or adolescent idiopathic scoliosis (AIS; 11 years and older). This classification system is not based on any biological marker and it’s believed that later onset, progressive IS develops continuously from juvenile stages through to adolescence. Regardless, late onset scoliosis (AIS) is the most common form of pediatric skeletal disease in the world and affects approximately 2\% of all school age children. Many eventually require
clinical intervention and/or surgical correction. Interestingly, the incidence and severity of AIS is greater in girls than in boys, with a female to male ratio of approximately 2:1 for curves of 10° in magnitude, and 10:1 for curves ≥30°245,291.

For reasons not well understood, some cases of scoliosis are stable or regress, while others rapidly progress leading to severe deformities. Severe scoliosis can impair cardiac and/or pulmonary function292; therefore, early detection and treatment is important. Typical treatment options include initial observation and if a curve progresses, bracing or spinal fusion surgery244.

1.3.5 The etiopathogenesis of idiopathic scoliosis

A number of potential factors have been associated with IS. All cases are not entirely alike and therefore, the disease is likely composed of numerous subgroups, each with unique causes and modifying factors. Certain factors may contribute to the onset of disease or to disease susceptibility while others may only affect curve progression and severity293. Ultimately, IS occurs as a consequence of rotational force upon the spine, which has been attributed to genetic factors, neuromuscular disorders, nervous system pathology, hormonal dysfunction, skeletal growth defects, biomechanical and/or environmental factors.

1.3.5.1 Genetic Factors

An increased incidence of scoliosis occurs in families294-296 and a strong concordence between monozygotic twins297-299 indicates genetic contribution towards IS. Examples of autosomal dominant295, dominant major-gene effect295, X-linked300 and multifactorial inheritance patterns296 have been reported; however, it is generally assumed that IS is a complex trait with multiple contributing factors. Genetic correlations with curve severity have been reported, suggesting that in addition to curve predisposition, genetic factors also contribute to disease progression298. However, phenotypic variability and inconsistent diagnostic criteria have made specific gene candidates difficult to identify.

Genome-wide linkage analyses suggest gene linkage to multiple chromosomes (reviewed in 291,301), while large scale genome-wide association studies (GWAS) have identified unique gene loci associated with AIS in divergent human populations302-304. Candidate gene studies have implicated multiple loci involved in varied biological processes including connective tissue and bone metabolism, melatonin signaling and/or growth control. Many of these associations could not be replicated with larger sample sizes or divergent populations (reviewed in 291); however some interesting candidates are discussed in the relevant sections below.
1.3.5.2 Neuromuscular

Scoliosis is a common deformity in many types of neuromuscular disease and can either be secondary to central or peripheral motor neuron pathology (e.g. cerebral palsy, poliomyelitis) or to myopathy (e.g. Duchenne myopathy or other muscular dystrophy) (reviewed in 305,306).

Neuromuscular scoliosis often presents in a broad thoracolumbar pattern extending down towards the pelvis as a result of trunk muscle weakness, hypertonia or disharmonious control of trunk muscles surrounding the spinal axis. Onset and curve pattern are quite different than typical IS-type curves; however, a few neuromuscular deformities misleadingly resemble IS, leading some to speculate that many cases of IS represent unidentified forms of neuromuscular disease.

Mutations in ROBO3 are linked to horizontal gaze palsy with progressive scoliosis (HGPPS) disease, which is a rare disorder marked by severe scoliosis. Additionally, a GWAS study of 419 AIS families identified associations between AIS and single nucleotide polymorphisms (SNPs) at the Cell Adhesion L1-like (CHL1), Down Syndrome Cell Adhesion Molecule (DSCAM) and Contactin Associated Protein-like 2 (CNTNAP2) gene loci. These genes all participate in axon pathfinding, indicating an important relationship between neuronal connectivity and AIS susceptibility.

It’s also important to consider that an initial asymmetry in spinal alignment could be exacerbated by axial muscle compensatory mechanisms. Therefore, it is probable that neuromuscular imbalances may also contribute to curve severity in many cases.

1.3.5.3 Nervous System

A variety of abnormalities within the CNS have been reported in cases of AIS (reviewed in 301). The most recent analyses have used magnetic resonance imaging (MRI) technology in AIS girls to observe increased cerebellar volumes, cortical thinning, as well as asymmetries in the alignment of vestibular system components. These differences were expected to affect motor control and balance, which may affect IS; however, it has also been suggested these changes are compensatory, given the imbalances associated with the disease. AIS patients also show abnormalities in balance, gait, and somatosensory function, which are thought to negatively impact the growing spine. However, the contribution of these factors towards IS onset are not well understood.
A recent GWAS and follow-up candidate gene study identified a susceptibility locus for AIS in the region containing the ladybird homeobox 1 (LBX1) gene. LBX1 is expressed in the dorsal spinal cord and skeletal muscles. As loss-of-function LBX1 mice fail to differentiate somatosensory neurons, it was proposed that AIS may primarily be associated with somatosensory defects.

Proprioception, or the sense of the relative position of the body parts, has been implicated in IS. Mice mutant for the zinc-finger nuclease Egr3 specifically lack sensory muscle spindles, which are required for proprioception. These mice have gait ataxia and develop scoliosis, demonstrating an important role for postural reflex pathways in spinal alignment. Neurotrophin 3 (NTF3) is expressed in muscle spindles and supports the growth of both spindle and proprioceptive neurons. A promoter polymorphism in the NTF3 gene was associated with curve severity in a Chinese population, suggesting that NTF3 and postural reflex contribute to IS progression in humans.

1.3.5.4 Connective tissue

Marfan’s syndrome, a disease of connective tissue that affects multiple organs, is commonly associated with scoliosis. Dominant mutations in the fibrillin-1 (FBN1) gene have been identified, and it is thought that defects in connective tissue result in increased flexibility of the spine and susceptibility to spinal curvature.

The occurrence of scoliosis in Marfan’s syndrome has led many to speculate that defects in connective tissue metabolism contribute to IS pathogenesis; however, FBN1 was not significantly associated with IS through linkage analysis, nor were other structural proteins of the extracellular matrix, including elastin, collagen I A1 and A2 (COL1A1, COL1A2) and collagen II A1 (COL2A1). Despite this, one study reported a marked decrease in elastic fiber density and organization within the intervertebral ligaments of IS patients, suggesting that elastic fibers may contribute to IS pathogenesis in some patients. A promoter polymorphism in the TIMP2 (tissue inhibitor of metalloproteinase 2) gene was associated with thoracic curve severity in Chinese girls, suggesting that matrix turnover by matrix metalloproteinases may contribute to disease progression.
1.3.5.5 Hormonal

Chicken and rodent models develop scoliosis when melatonin deficient\textsuperscript{323-325} and the popularity of these models have largely contributed to the hypothesis that melatonin plays an important role in the pathogenesis of human IS.

Melatonin is produced from serotonin in the pineal gland and is released in response to darkness. Melatonin signaling has been implicated in circadian rhythm, seasonal affective disorder, sexual maturation, aging, cardiovascular function, cancer progression, and bone metabolism. Melatonin inhibits calmodulin through protein kinase C (PKC)-mediated phosphorylation\textsuperscript{326} and, interestingly, calmodulin antagonists (tamoxifen and trifluoperazine) reduce the rate and magnitude of scoliosis in chicken and mice\textsuperscript{327,328}. In tissue culture, melatonin promotes osteoblast differentiation and bone formation as well as proliferation of chondrocytes\textsuperscript{329,330}, suggesting a possible mechanistic relationship between melatonin and IS pathology.

However, independent results found that physiologically therapeutic doses of melatonin could not rescue scoliosis in chickens\textsuperscript{331} and in many instances, pinealectomized animals do not develop scoliosis, even when plasma levels are at or close to zero\textsuperscript{331,332}. Also, pinealectomy of rhesus monkeys does not lead to scoliosis\textsuperscript{333}, suggesting a differential requirement for melatonin in primates.

The data regarding human melatonin and IS is equally mixed. Melatonin levels in scoliotic patients are not consistently correlated with pathology and melatonin supplementation therapy does not show a significant benefit (reviewed in\textsuperscript{334}). Also, children who have undergone pineal resection for tumor removal do not readily develop scoliosis\textsuperscript{335}. One study linked a melatonin receptor 1B (MTNR1B) gene promoter SNP with AIS incidence in Chinese girls\textsuperscript{336}; however, the same association could not be replicated in other populations\textsuperscript{337,338}. Interestingly, AIS patients display disruptions in G-protein-mediated melatonin signal transduction\textsuperscript{339-341}, and based on their melatonin response profile, can be classified into distinct subgroups\textsuperscript{341}. This kind of profiling may allow for predictive analysis of at risk individuals\textsuperscript{341}; however, the relationship between melatonin signaling and scoliosis remains to be fully understood.

Other hormonal influences have been considered with respect to IS pathology. Girls with AIS display increased circulating levels of growth hormone (GH)\textsuperscript{342,343} and polymorphisms in the estrogen receptor (ER) gene are associated with IS onset and severity\textsuperscript{344-346}. Recently, polymorphisms in the G protein-coupled estrogen receptor 1 (GPER1) gene were linked to IS
severity\textsuperscript{347}, further suggesting that estrogen signaling may contribute to exacerbation of the phenotype. Similarly, candidate gene studies have associated polymorphism of the insulin-like growth factor-1 (IGF-1) gene with curve severity in girls\textsuperscript{348}. Both ER and IGF-1 affect bone growth and metabolism and could be linked to scoliosis progression through both mechanisms.

1.3.5.6 Calmodulin
Calmodulin is a calcium-binding protein that regulates muscle contraction through interaction with actin and myosin, as well as through control of calcium fluxes from the sarcoplasmic reticulum of muscle fibers. Calmodulin levels were found to be significantly higher in platelets of patients with progressive IS as compared to patients with stable scoliosis\textsuperscript{349}, suggesting a relationship between calmodulin and curve progression in humans. Interestingly, asymmetric distribution of calmodulin in paraspinal muscles was found in patients with AIS\textsuperscript{350,351}; however, whether calmodulin is the primary cause of this phenotype or merely representative of compensatory muscle contractions has not been addressed.

Calmodulin1 (CALM1) gene polymorphisms were associated with AIS in 100 Chinese patients\textsuperscript{346,352}. Interestingly, different curve types were linked to different gene polymorphisms: double curves and lumbar curves were associated with a promoter site polymorphism (rs12885713) that inhibits CALM1 expression\textsuperscript{353}, and thoracic curves with a rs5871 polymorphism\textsuperscript{346,352}. Recently, three more intronic polymorphisms in the CALM1 gene have been linked to AIS susceptibility in another Chinese cohort\textsuperscript{354}, further supporting a role for calmodulin metabolism in disease progression. In addition to muscle, CALM1 expression has been detected in cultured chondrocytes and cartilage\textsuperscript{353}, suggesting that multiple tissues may be affected by disruption of Ca\textsuperscript{2+}/calmodulin signaling.

1.3.5.7 Bone metabolism
Abnormalities in bone metabolism are associated with multiple types of spinal curvatures, including AIS. Patients with osteoporosis often develop scoliosis, and spinal curvatures are thought to progress as a consequence of bone loss\textsuperscript{355}. In an early study, osteopenia was associated with curvature in one third of female AIS patients\textsuperscript{356}, while subsequent analysis has found low bone mineral density to be a good predictor of curve severity\textsuperscript{357,358}. Using micro computed tomography (microCT), bone volumes and trabecular bone thickness were found to be reduced in patients with IS and this was correlated with a reduction in osteoblasts and osteocytes from bone biopsies\textsuperscript{359}. 
GWAS identified a SNP (rs6570507) at the G Protein-Coupled Receptor 126 (GPR126) locus to be associated with AIS\textsuperscript{302}. GPR126 is expressed in cartilage, and MO-mediated knock-down in zebrafish led to delayed bone ossification\textsuperscript{302}, suggesting that abnormal bone development plays a significant role in AIS pathogenesis.

Children with AIS display differential growth patterns as compared to age matched controls, implicating growth as a contributing factor in curve progression\textsuperscript{301}. In one longitudinal study, AIS girls displayed lower body weight, greater body height, longer arm span and lower body mass index than controls\textsuperscript{360}. These girls were shorter and had a smaller arm span at age 12, but caught up and overtook the control group by 14-16 years\textsuperscript{360}. Therefore, curve severity was positively correlated with the speed of skeletal growth\textsuperscript{360}.

This accelerated growth may also be disproportionate. Magnetic resonance imaging (MRI) found that girls with AIS had longer vertebrae in the anterior thoracic region relative to controls, coupled with larger intervertebral spaces in the posterior thoracic region, indicating faster anterior growth and slower posterior growth of the spinal column\textsuperscript{361}. Lengthening of the spinal column in AIS does not necessarily occur concomitantly with lengthening of the spinal cord\textsuperscript{362}, contributing to a theory that uncoupled neuro-osseous growth contributes to IS\textsuperscript{363}. This theory suggests that anatomically, a shorter posterior column or a shorter spinal cord acts as a tether that may not support rapid elongation of the anterior column\textsuperscript{364}. Tethering creates abnormal forces on the spinal column that can lead to spinal curvature.

Secondary to curve onset, gross pathological changes in the vertebrae have been documented in cases of IS\textsuperscript{365-367}. These changes include vertebral shape distortions, thickening of the bone, and intervertebral disc migrations\textsuperscript{365}. In human IS, vertebral “wedging” surrounding the apex of the curve is defined by a decreased length on the concave side of the vertebrae with no significant length change on the convex side. This phenomenon has been explained by the Heuter-Volkmann principle whereby vertebral growth is inhibited by mechanical compression and/or stimulated by reduced compression\textsuperscript{368}. Therefore, asymmetrical bone metabolism is most often secondary to the curve. It has been shown that intervertebral disc wedging largely contributes to early curve progression, whereas vertebral wedging occurs after the curve acceleration phase\textsuperscript{301}.
1.3.5.8 Biomechanics

The shape of the human spine is thought to be a major determining factor with respect to IS pathogenesis. Spinal loading across the cranio-caudal axis is required to induce spinal curvature in many animal models\textsuperscript{369,370} and is discussed further below.

The shape of a child’s spine changes from thoracic kyphosis (dorsally directed sagital curve, Figure 1.3.5) at age 8 and reaches minimal kyphosis at approximaly age 12\textsuperscript{371,372}. Interestingly, this time frame correlates with maximal vertebral growth in girls and the typical age of AIS onset\textsuperscript{373}. Male vertebrae are much thicker across the transverse plane during this period as compared to females, and boys experience maximal vertebral growth five years later than girls\textsuperscript{373}. As a result, females tend to have more slender vertebral column during adolescence, which may partially explain the extreme sexual dimorphism observed in AIS.

Finally, the predominant pattern of spinal curvature in AIS is a right-sided thoracic curve whereby the mid/lower thoracic vertebrae are rotated to the right accompanied by a compensatory left-sided thoracic/lumbar curve\textsuperscript{374}. In the normal spine, a mild natural rotation to the right in the mid/lower thoracic vertebrae exists, suggesting that the pattern of IS proceeds along a pre-determined pattern\textsuperscript{375}. This curve pattern is reversed in patients with \textit{situ inversus}, leading some to speculate that left-right patterning mechanisms predispose individuals to certain types of spinal curvature.

1.3.6 Animal models of idiopathic scoliosis

A major complication in IS research is the fact that IS-like deformities in traditional animal models do not develop spontaneously. To induce IS-like curves in most animals requires surgical or systemic interventions that often preclude relevance to the human disease (reviewed in \textsuperscript{369,370}). The first reported case of experimental scoliosis was in 1888. In this case, nerve dissection was used to induce thoracolumbar scoliosis in rabbits\textsuperscript{376}. Since then, chickens have become the most widely used animal model; however, rabbits, rats and mice are also commonly used\textsuperscript{369,370}. Rather extreme interventions are required to induce scoliosis in almost all models: rib resections; anatomical tethering; surgical dissection of nerves, muscles and/or ligaments; bracing; and the injection of toxins have all been used\textsuperscript{369,370}. This is in contrast to human IS that appears to develop without obvious external manipulation required.

Heritable forms of “naturally occurring” scoliosis have been described in rabbits\textsuperscript{377} and chicken\textsuperscript{378-380}; however, limited characterization of these animals suggest limitations in their
Figure 1.3.5. The human spine. Lateral (sagittal) and frontal (coronal) views of the adult spine. Four natural curves support the spine: cervical lordosis, thoracic kyphosis, lumbar lordosis and sacral kyphosis. The spine is straight when viewed from the front (coronal plane); however, scoliosis develops as a consequence of rotational force(s) (arrows) that can occur along the entire length of the spine.
applicability as models. Mouse models display various types of spinal curves: for example, the (ky) mutant displays a progressive form of neuromuscular kyphoscoliosis with spinal curvature secondary to degenerative muscular myopathy\textsuperscript{381}, making (ky) a better model of neuromuscular-type scoliosis. Importantly, the majority of mouse models with spinal curvature display underlying congenital malformations, which lessen their relevance to human IS\textsuperscript{370}.

From animal studies, it is now generally accepted that bipedalism with an upright posture predisposes humans to three-dimensional spinal curvatures. Bipedal chickens develop scoliosis when made melatonin deficient through pinealectomy\textsuperscript{323,382}, making this one of the most commonly used models of IS-like disease progression. Melatonin deficiency in rodent models also leads to spinal curvature, but only when the animals are forced to walk upright by removal of forelimbs and/or the tail\textsuperscript{383,384}. These experiments highlight the importance of upright biomechanics and explain why traditional laboratory animals do not readily develop scoliosis without external manipulation.

The human spine is straight in the coronal plane (viewed from the front, Figure 1.3.5). At birth there is a mild kyphosis (dorsally directed sagital curve) from the head to the base of the spine; however with walking, four natural curves develop. Cervical lordosis (ventrally directed, which supports the head), thoracic kyphosis, lumbar lordosis and sacral kyphosis support the spine\textsuperscript{385} (Figure 1.3.5); however, this orientation directs a large amount of sheer force posteriorly onto the lower spinal column, making it susceptible to rotational forces. Rabbits normally display cervicothoracic kyphosis, but have a tendency to develop lordoscoliosis if tethered into the reverse, lordosis conformation\textsuperscript{386,387}. Similarly, rats display a tendency to develop spinal lordosis when made bipedal\textsuperscript{383,388} and this conformation is an essential precursor to pathological scoliosis that develops upon further manipulations like pinealectomy\textsuperscript{383}.

Other anatomical differences also preclude relevance of certain animal models. Bipedalism in the chicken, makes this animal the most representative of human spinal loading; however, chickens only have eight thoracic vertebrae; lumbar vertebrae that are entirely fused; and two intervertebral discs. This morphology limits spinal mobility and restricts the types of curvatures that can develop. As a result, spinal curvature in chicken is conformationally quite different than in most cases of human IS.
1.3.6.1 Fish as a model of scoliosis

Instead of bipedalism, it has been suggested that IS-type scoliosis depends on spinal loading applied along the cranio-caudal axis and interaction with unknown factors that cause spinal curvature. This comes from the observation that spinal curvature is naturally very common among teleosts (reviewed in ). Fish do not experience gravity, but instead swim against a dense medium (water) with sheer force exerted along the spinal axis from head to tail. Lordosis, kyphosis and rotational scoliosis have been documented in over 20 species of fish, including salmon, halibut, guppy and medaka (reviewed in ), making teleosts an interesting candidate model for the study of human IS.

Pinealectomy in salmon induces spinal curvature, suggesting conserved pathophysiology with respect to melatonin signaling; however, most other cases of reported spinal curvature in fish are anecdotal and their relevance to human IS pathogenesis remains unknown. Curvature is also commonly observed in older laboratory fish with an undescribed pathology. Interestingly, this phenotype may imitate the late stage thoracic kyphoses seen in geriatric patients, further highlighting similarities between humans and fish with respect to spinal loading and biomechanics.

Most importantly, instances of heritable spinal curvature have been described in fish, suggesting that important gene candidates may be uncovered using fish as a model.  medaka and curveback guppy display recessive lordosis and posterior kyphosis with no underlying vertebral abnormalities associated in either case. In curveback, the onset of curvature begins at a variable age and can either stabilize or progress until sexual maturity. Also, a female bias for curve severity in curveback implies that the sexual biases seen in humans can translate to fish models. The curves are primarily within the sagittal plane, making curveback a likelier model of Scheuermann kyphosis; however, coronal (mediolateral) deviation and axial rotation does occur in some fish. In curveback, a major recessive quantitative trait locus (QTL) containing over 100 genes controlling phenotypic susceptibility has been identified; however, no gene candidates have been tested so far, making the etiology of the phenotype difficult to elucidate.
Both *wavy* medaka and *curveback* guppy display variability in curve severity; therefore, understanding phenotypic modification in fish may uncover important genetic, environmental and/or epigenetic factors that influence IS severity.

1.3.6.2 Zebrafish as a model of idiopathic scoliosis

The zebrafish is a well-established system for genetic studies and has several advantages with respect to their use for skeletal patterning and morphological studies. This includes easy accessibility for direct observation, as well as simple and sensitive tools to visualize skeletal structures in live fish\(^397\).

In mammals and chick, the axial skeleton develops from a ventral mesenchymal somite population called the sclerotome that moves to surround the embryonic notochord and differentiates into bone and cartilage. In zebrafish, the sclerotome comprises only a very small portion of the somite and each segmental unit is built around a single vertebral centrum that develops by direct mineralization of the embryonic notochord\(^398,399\). Segmented centra provide a base for the development of vertebral bodies carried out by sclerotome-derived osteoblasts. Neural arches and spines extend dorsally and surround the spinal cord, while hemal arches extend ventrally and act as attachment sites for ribs in the anterior body region.

The vertebral column is regionalized along the AP axis with the human spine consisting of 33 vertebrae: seven cervical, 12 rib-associated thoracic, five lumbar, five fused sacral and five coccygeal vertebrae. Zebrafish have 33 vertebrae: the first four are called Weberian vertebrae and are a unique adaptation of many teleosts that function to transmit vibrations from the swim bladder to the inner ear\(^400\). Zebrafish have 10 trunk vertebrae that are associated with ribs, and 14 caudal (tail) vertebrae that have ventral hemal arches and dorsal spines. The most posterior caudal fin vertebrae are modified to support the caudal fin and form earlier than the trunk and caudal vertebrae through a cartilaginous precursor\(^399,401\).

The vertebral centra of trunk and tail are formed sequentially from anterior to posterior with calcification starting at the boundary of each segment and expanding in both AP directions\(^397\). Vertebral bodies consist mostly of acellular calcified bone that surround a notochordal canal located at the center of each vertebrae\(^402\). Like mammals, the vertebral bodies are concave ventrally and dorsally; however, zebrafish vertebrae are also concave at both the anterior and posterior ends. This differs with respect to mammals that have centra with flat ends. This is
believed to more effectively distribute compressive forces. Intervertebral discs (IVDs) are derived from the notochord and positioned between adjacent vertebrae to support mobility.

1.3.6.3 Identification of scoliosis susceptibility genes in zebrafish
Through linkage association, CHD7, a chromodomain helicase DNA-binding protein, was found to be associated with IS. MO-mediated knock-down of zebrafish chd7 led to abnormalities in vertebral segmentation and mineralization, suggesting a role for chd7 in axial patterning and bone formation. Similarly, MO-mediated knock-down of zebrafish gpr126, a gene associated with AIS in multiple human populations, led to delayed vertebral ossification at 14 dpf. These experiments reveal how zebrafish may prove useful for testing of candidate IS susceptibility genes; however, vertebral abnormalities suggest that these two zebrafish models more closely represent CS-like deformity. Nonetheless, novel loss-of-function zebrafish models may lead to the identification of new IS susceptibility genes relevant to human populations.

1.3.7 The relationship between IS and CS
CS and IS have traditionally been thought of as separable diseases with different pathogenic mechanisms contributing to each curve type. However, family members of individuals with CS display an increased susceptibility for IS, suggesting possible commonalities between both diseases. Indeed, this evidence suggests shared genetic pathways are involved in the pathogenesis of each disease subgroup. To date, however, the genetics are not well understood and specific susceptibility loci have not been identified.

CHD7 reveals one interesting genetic candidate. CHD7 mutations have previously been identified in multiple patients with CHARGE syndrome, a developmental disorder that commonly presents with CVMs; however, the pathological relationship between CHD7, CS and IS has not been explored.

The relationship between CS and IS-type deformities is an intriguing aspect of spinal development and may contribute to the understanding of scoliosis pathogenesis in the future.

Here, I characterize the role for Ptk7 throughout zebrafish development. I describe a loss-of-function mutant model for ptk7 and its use to determine requirements for Ptk7 during embryogenesis, larval as well as juvenile stages of development. I found that early loss of ptk7 in maternal-zygotic (MZ) mutant embryos led to patterning and morphogenesis defects consistent with a role for ptk7 in Wnt signaling. MZptk7 mutant zebrafish also displayed
vertebral abnormalities associated with segmentation defects similar to those found in human patients with CS. Zygotic ptk7 zebrafish did not display any obvious embryological abnormalities; however, ptk7 mutants developed a severe form of late onset spinal curvature that was analogous to human IS. I characterize MZptk7 and ptk7 mutant zebrafish as models of CS and IS, respectively, and suggest a shared genetic basis involved during scoliosis pathogenesis.
Chapter 2

2. INVESTIGATING THE ROLE OF PROTEIN TYROSINE KINASE 7 (PTK7) IN DEVELOPMENTAL WNT SIGNALING

Some of the data presented in this chapter were published in:

2.1 Abstract

Using zebrafish, I have characterized the function of Protein Tyrosine Kinase 7 (Ptk7), a transmembrane pseudokinase implicated in Wnt signal transduction during embryonic development and in cancer. Ptk7 is a known regulator of mammalian neural tube closure and *Xenopus* convergence & extension (C&E) movements\(^1\). However, conflicting reports have indicated both positive and negative roles for Ptk7 in regulating canonical Wnt/β-catenin signaling\(^2\)\(^-\)\(^4\). To clarify the function of Ptk7 in vertebrate embryonic patterning and morphogenesis, I generated maternal-zygotic (MZ) *ptk7* mutant zebrafish using a Zinc-finger nuclease (ZFN) gene targeting approach. Early loss of zebrafish Ptk7 leads to defects in axial C&E, neural tube morphogenesis, and planar cell polarity (PCP). Contrary to published reports, MZ*ptk7* mutant embryos establish normal Wnt-dependent dorsoventral patterning at gastrulation. However, at late gastrula stages and during somitogenesis, I observed significant up-regulation of β-catenin target gene expression and demonstrate a clear role for Ptk7 in attenuating canonical Wnt/β-catenin signaling *in vivo*. MZ*ptk7* mutants display expanded mesodermal cell fate within the tail bud, suggesting an important role for Ptk7 in regulating Wnt/β-catenin-dependent tissue fate specification. Furthermore, I demonstrate that a plasma membrane-tethered Ptk7 extracellular fragment is sufficient to rescue both PCP morphogenesis and Wnt/β-catenin patterning defects in MZ*ptk7* mutant embryos. These results indicate that the extracellular domain of Ptk7 acts as an important regulator of both non-canonical Wnt/PCP and canonical Wnt/β-catenin signaling in multiple developmental contexts.
2.2 Brief Introduction and Rationale

Wnt signaling controls a diverse range of developmental processes from tissue specification to axial morphogenesis\textsuperscript{407,408}. Tight control over pathway activation is important and misregulation has been implicated in a variety developmental abnormalities including CNS, neural tube and cardiac defects\textsuperscript{153,409-411}. Wnt signaling is also required for stem cell maintenance and adult tissue homeostasis, with perturbations frequently implicated in cancer formation\textsuperscript{412,413}.

The Wnt signaling pathway consists of a large set of signaling regulators that are highly conserved among metazoans and activate signaling through stabilization of the transcriptional activator β-catenin. During development, Wnt/β-catenin signaling is essential in early patterning, and more recently has been implicated in the continuous specification of mesoderm from a bi-potential neural/mesodermal precursor pool present in the tail bud during zebrafish tail out growth\textsuperscript{82}. A major lineage of tail bud stem-cell derived mesodermal cells are somites, which were absent posteriorly following pathway inhibition\textsuperscript{82}, revealing an important role for Wnt signaling in formation of somite – a cell population that later differentiates to form skeletal muscle, bone and dermis\textsuperscript{414}. Similar neural/mesodermal fate decisions made within the tail bud have been shown to also take place in Xenopus and mice\textsuperscript{415,416}, suggesting possible conserved canonical Wnt-dependent fate specification within posterior stem cell pools post-gastrulation.

Multiple β-catenin-independent signaling pathways can be activated upon Wnt binding Fz receptors\textsuperscript{417,418}. The best characterized of these is the planar cell polarity (PCP) pathway. PCP coordinates the uniform orientation of cell structure and cell movement within the plane of a tissue\textsuperscript{419}. In vertebrates, this is required to drive polarized cell movements that narrow and elongate the body axis during gastrulation and neurulation – a process termed convergent and extension (C&E)\textsuperscript{153,420,421}. Mutation of vertebrate PCP genes block these cell movements, resulting in broader and shorter tissues, and neural tube closure defects\textsuperscript{421,422}. A core “cassette” of proteins establish PCP across animal species. Many factors are shared with the Wnt/β-catenin signaling pathway, including Frizzled (Fz) and Dishevelled (Dsh).

Differential activation of alternative Wnt pathways is context dependent – in some cases, Wnt5a can activate Wnt/β-catenin signaling\textsuperscript{423} and the cellular complement of Fz co-receptors dictates pathway choice\textsuperscript{424}. For example, the presence of the Wnt co-receptor LRP5/6 activates canonical Wnt/β-catenin responses to affect dorsal axis and neural crest specification\textsuperscript{28}, whereas
the Ror transmembrane kinases can specifically bind Wnt5a and inhibit Wnt/β-catenin signaling\textsuperscript{190}. Ror2 can also form a Wnt-induced complex with the PCP regulator Van Gogh-like 2 (Vangl2) to affect tissue polarity\textsuperscript{425}.

In an attempt to identify novel regulators of Wnt pathway specificity, our collaborators performed affinity mass spectrometry and identified protein tyrosine kinase 7 (Ptk7) as a potential interactor of Fz7 (Stephane Angers, unpublished data). Ptk7 is an atypical receptor tyrosine kinase that is upregulated in metastatic colon cancer, acute myeloid leukemia (AML) and multiple other cancer types\textsuperscript{205-207,210,211,426}. In cell culture studies, PTK7 overexpression promotes the survival and migration of leukemic cell lines and primary AML blasts\textsuperscript{427}, while interfering with PTK7 activity blocks the invasive behavior of epithelial carcinoma cells\textsuperscript{215}.

Full length Ptk7 contains an extracellular domain with seven Immunoglobulin (Ig)-like loops, a transmembrane domain, and a catalytically inactive kinase domain. Orthologs of Ptk7 have been identified in human, mouse, Xenopus, chicken (kinase-like gene [KLG]), Drosophila (Off-track, Otk) and Hydra (Lemon), and have been implicated in various morphogenetic processes in each system\textsuperscript{202,204,205,225-227}. Ptk7 is an important regulator of PCP-mediated tissue morphogenesis in multiple contexts\textsuperscript{1-3,234,235}; however, the relationship between Ptk7 and the PCP signaling pathway (activating versus inhibitory) and the functional domains required for signal transduction are not well understood.

Recent evidence also suggests that Ptk7 regulates canonical Wnt/β-catenin signaling. Morpholino (MO)-mediated knock-down of PTK7 during early Xenopus development suppresses Wnt/β-catenin-dependent Spemann organizer formation\textsuperscript{3}, suggesting that PTK7 potentiates canonical Wnt signaling. However, MO knock-down of PTK7 activity in Xenopus animal cap explants enhances the ability of exogenous Wnt8 to activate β-catenin signaling\textsuperscript{2}, suggesting that PTK7 inhibits the Wnt/β-catenin pathway.

Therefore, in order to clarify the function of PTK7 in vertebrate embryonic patterning and morphogenesis, I cloned zebrafish \textit{ptk7} and generated a loss-of-function allele. Through analysis of maternal-zygotic (MZ) \textit{ptk7} mutant embryos, I demonstrate a requirement for Ptk7 in PCP-dependent morphogenesis, and establish a clear role for Ptk7 in attenuating canonical Wnt/β-catenin signaling \textit{in vivo}. Finally, I demonstrate that the highly conserved Ptk7 pseudokinase domain is not required for Ptk7 activity in axial morphogenesis and mesodermal cell fate.
determination. Rather, a plasma membrane-tethered Ptk7 extracellular domain fragment is sufficient to rescue these Wnt-dependent activities in MZptk7 mutant embryos.
2.3 Results

2.3.1 Zebrafish *ptk7* sequence is well conserved

To begin characterizing Ptk7, I cloned the zebrafish homolog from an embryonic cDNA library using primers designed from predicted open reading frame sequence (Ensembl Zv9, zgc:112211). Based on the sequence I obtained (submitted to GenBank as KF006327), I predicted that zebrafish Ptk7 protein contains 1061 amino acids and consists of an extracellular domain with seven immunoglobulin-like loops, a single transmembrane domain and an intracellular domain with kinase homology (Figure 2.3.1.1). Zebrafish Ptk7 contains a number of conserved N-X-S/T motifs in the extracellular domain that may reflect conserved sites for N-linked glycosylation\(^\text{202,225}\) (Figure 2.3.1.1). The intracellular domain of Ptk7 contains a conserved Mg\(^{++}/ATP\)-binding (GXGXXG) motif; however, the DFG triplet believed to be essential for kinase catalytic activity is modified to SLG in zebrafish (Figure 2.3.1.1). Therefore, like all other orthologs, zebrafish Ptk7 would be expected to be a pseudokinase with no catalytic potential. Finally, the C-terminus of zebrafish Ptk7 contains a conserved PDZ-binding motif (S/T–X–Φ, where Φ is any hydrophobic residue; Figure 2.3.1.1), suggesting possible interactions with PDZ domain-containing proteins.

To analyze sequence conservation, I compared zebrafish Ptk7 to other known vertebrate orthologs and found that Ptk7 is highly conserved with chicken (KLG)\(^\text{225}\), *Xenopus*\(^1\), human and mouse proteins\(^\text{202,205}\) (Figure 2.3.1.2). The transmembrane domain is almost completely conserved across species and a high degree of conservation also exists within the intracellular pseudokinase domain (Figure 2.3.1.2).

Since starting this work, two *ptk7* paralogs have since been annotated in the zebrafish genome (Zv9). I cloned what is now annotated as *ptk7a*, which is located on chromosome 22. *Ptk7b* is located within a telomeric region on chromosome 13 and encodes only a partial membrane bound extracellular fragment (176 amino acids), homologous to a small region of vertebrate Ptk7. For this reason, all future analysis was focused *ptk7a* and is referred to in this text as *zebrafish ptk7*. 
Figure 2.3.1.1 Ptk7 protein sequence. Predicted amino acid sequence of zebrafish Ptk7 protein aligned with chicken, *Xenopus*, mouse and human orthologs. Conserved cysteine residues predicted to form disulfide bridges and mediate immunoglobulin (IG)-loop formation are indicated by red asterisks. Conserved sites for N-linked glycosylation are indicated by green boxes. The predicted transmembrane domain is indicated by a single line. The ‘GXGXXG’ motif and modified ‘DFG’ triplet are indicated by red boxes. The PDZ-binding domain is indicated by a purple box.
Figure 2.3.1.2 Ptk7 domain conservation. Domain structure of zebrafish Ptk7 and its homology to chicken (KLG), *Xenopus*, mouse and human orthologues. Numbers indicate percentage amino-acid identity within the immunoglobulin (IG), transmembrane (TM) and intracellular pseudokinase (PK) domains.
2.3.2 Zebrafish *ptk7* is expressed throughout early embryogenesis and localizes to the plasma membrane

Using whole-mount RNA *in situ* hybridization (WISH) and an anti-sense *ptk7* mRNA probe, I detected *ptk7* expression at the two-cell stage (Figure 2.3.2a). Expression at this early stage precedes the initiation of zygotic transcription at the mid-blastula transition (MBT, 3hpf) and indicates strong maternal gene contribution. WISH using a *ptk7* sense control probe resulted in no staining (Figure 2.3.2b).

*Ptk7* is ubiquitously expressed during blastula stages and throughout early gastrulation (Figure 2.3.2c-f). At late-gastrula stages, *ptk7* expression is pronounced along the midline and in paraxial tissue, and is especially enriched within the tail bud (Figure 2.3.2g,h). Post-gastrulation, *ptk7* is expressed in the head and somites, with an especially high level of expression evident within the tail bud (Figure 2.3.2i,j). At 24hpf, *ptk7* is expressed in the head and within the tail bud (Figure 2.3.2k). The expression pattern is similar to that observed in *Xenopus* and mouse[^1], which encouraged me to further examine Ptk7 function using zebrafish as a model system.

Since subcellular localization of PCP signaling complexes is an important aspect of signal transduction, I decided to look at the subcellular localization of exogenous zebrafish Ptk7. I fused GFP to the C-terminal of zebrafish Ptk7 and overexpressed Ptk7-GFP in zebrafish embryos. Using confocal microscopy, I imaged the neural anlage and notochord of post-gastrula stage embryos and found that Ptk7-GFP localized fairly uniformly along the plasma membrane of cells at this stage (Figure 2.3.2l,m), indicating that exogenous Ptk7 does not asymmetrically localize in polarized tissue of the developing embryo.

[^1]: Previous study.
Figure 2.3.2 Ptk7 embryonic expression and subcellular localization. (a-k) WISH of ptk7 expression throughout the first 24 hours of development. (a) Maternal ptk7 expression is indicated at the two-cell stage (0.75 hpf). (b) Ptk7 sense control probe reveals no non-specific WISH staining. (c) Lateral view of blastula stage (sphere stage, 4 hpf) embryo. (d) Lateral and (e) animal pole views of early gastrula stage (shield stage, 6 hpf) embryo. (f) Lateral view of mid-gastrula stage embryo (75% epiboly, 8 hpf). (g) Lateral and (h) dorsal views of late gastrula stage (bud stage, 10 hpf) embryo. Asterisk and arrowhead highlight tail bud and midline expression, respectively. (i) Lateral and (j) dorsal views of somite stage (15 hpf) embryo. Asterisk and arrowhead highlight tail bud and somite expression, respectively. (k) Lateral view of 24 hpf stage embryo. Asterisk highlights tail expression. (l-m) Dorsal confocal images of the (l) neural keel, (m) notochord and adjacent somites of 8-10 somite stage (15 hpf) embryos injected at the one-cell stage with ptk7-GFP mRNA. Anterior (Ant) is indicated.
2.3.3 Ptk7 knock-down by translation blocking MO leads to morphogenesis defects

To initially assess the requirement for zebrafish ptk7, I knocked-down gene expression using anti-sense morpholino oligonucleotides (MO). I first tested the effect of a translation blocking MO (ptk7ATG MO) that was predicted to target the first 24 nucleotides of the open reading frame of ptk7 mRNA and prevent translation of both maternal and zygotic gene products (Figure 2.3.3.1a). I titrated MO levels through injections of 1-6ng and chose 2ng for subsequent analysis, which was an amount of MO that resulted in an embryonic phenotype with minimal lethality. I verified the efficiency of MO-mediated knockdown by expressing Ptk7-GFP through mRNA injections with or without ptk7ATG MO. I visualized GFP expression during gastrula stages; however, in the presence of ptk7ATG MO, I could not detect GFP fluorescence (Figure 2.3.3.1b-e). This indicated that ptk7ATG MO inhibited translation of exogenous ptk7 mRNAs and suggested that ptk7ATG MO could effectively target ptk7 transcript in vivo.

Ptik7ATG MO injections produced a striking and reproducible phenotype (Figure 2.3.3.1f-n). MO-injected embryos were shorter along the antero-posterior (AP) axis and broader along the mediolateral axis compared to uninjected wild-type controls. I verified that this phenotype was specific to ptk7 knockdown by rescuing the shorter body axis through injection of ptk7 mRNA that could not be recognized by ptk7ATG MO due to sense modifications within the first 24 nucleotides (Figure 2.3.3.1a,j). To further visualize the body axis defect, I used WISH to stain hindbrain (krox20) and paraxial mesoderm (myoD) tissue. In ptk7ATG MO-injected embryos the body axis was significantly shorter and broader that wild-type controls (Figure 2.3.3.1o,p), suggesting a role for ptk7 in PCP-mediated C&E movements during zebrafish gastrulation.

To verify the effect of ptk7 knock-down, I tested a second MO that was predicted to target the exon2-exon3 splice acceptor site of unspliced ptk7 pre-mRNA (ptk7SPL MO, Figure 2.3.3.2a). Splice blocking MOs bind and inhibit pre-mRNA processing through inhibition of the splicesome components, thereby inhibiting translation of wild-type gene product. I titrated MO levels through injection of 1-15ng MO; however, I failed to observe the same body axis defects that I had observed in ptk7ATG MO-injected embryos. At 24 hpf, ptk7SPL MO-injected embryos did not display a shorter body axis (Figure 2.3.3.2c,d), suggesting that ptk7SPL MO did not affect PCP-mediated C&E. At MO levels higher that 15ng, I observed a significant amount of non-specific toxicity and embryonic lethality.
To assess the efficiency of ptk7SPL MO, I used RT-PCR to amplify exon2-exon3 spliced and unspliced ptk7 mRNA following injection of 10ng MO/embryo. I found that an increased proportion of ptk7 transcript was unspliced in ptk7SPL MO-injected compared to uninjected controls at 24 hpf (Figure 2.3.3.2b). Spliced ptk7 transcript was barely detectable at 24 hpf, suggesting that ptk7SPL MO efficiently targeted ptk7 pre-mRNA and inhibited translation of full length Ptk7.

Maternal transcripts are deposited already spliced and therefore, not targeted by splice blocking MOs. One possible explanation for the differential phenotypes between ptk7ATG and ptk7SPL morphants would be that maternal ptk7 gene product rescues an early developmental phenotype in ptk7SPL MO-injected embryos. Nevertheless, conflicting results using these two different ptk7 MOs impelled me to pursue genetic loss-of-function analyses to verify the functional requirement for ptk7 during zebrafish embryogenesis.
Figure 2.3.3.1 *Ptk7ATG MO* targets *ptk7* mRNA and leads to defects in axial morphogenesis. (a) Coding sequence of zebrafish *ptk7* illustrating the *ptk7ATG MO* target sequence and nucleotide sense modifications made to prevent exogenous *ptk7* mRNA recognition (*ptk7* untargetted). (b,d) Bright-field and (c,e) fluorescent images of late gastrula stage (b,c) wild-type (WT) and (d,e) *ptk7ATG MO* injected embryos, injected with *ptk7-GFP* mRNA. (ef,h) Lateral and (g,i) dorsal views of 8-10 somite stage (f,g) wild-type (WT) and (h,i) *ptk7ATG MO*-injected embryos. Brackets indicate the mediolateral expanse of the body axis. (j) Lateral view of 8-10 somite stage embryo co-injected with *ptk7ATG MO* and untargeted *ptk7* mRNA. (k,l) Lateral and (m,n) dorsal views of 24 hpf (k,m) wild-type (WT) and (l,n) *ptk7ATG MO*-injected embryos. (o,p) Flat mounts of 10-12 somite stage (o) wild-type (WT) and (p) *ptk7ATG MO*-injected embryos stained for *krox20* (hindbrain, hb) and *myoD* (somite, s) gene expression.
Figure 2.3.2 Ptk7SPL MO targets ptk7 pre-mRNA but does not affect embryonic morphogenesis. (a) Ptk7 pre-mRNA and ptk7SPL MO target site. Arrows indicate primers used in RT-PCR analysis. (b) RT-PCR of total RNA from 24 hpf wild-type (WT) and ptk7SPL MO-injected (MO) embryos. Unspliced ptk7 transcript runs at approximately 2kb and spliced ptk7 transcript runs at approximately 800bp. A 1kb ladder was used. (c,d) Lateral views of 24 hpf (c) wild-type (WT) and (d) ptk7SPL MO-injected embryos. Ptk7SPLMO-injected embryos did not display any obvious morphological abnormalities.
2.3.4 Zinc-finger nucleases target zebrafish ptk7

MO-mediated knock-down suggested a possible role for ptk7 in PCP-mediated morphogenesis; however, variable phenotypes between multiple MOs led me to question the validity of many results. MOs are also known to cause toxicity, off-target effects and the non-specific activation of alternative genetic pathways\(^{428,429}\). Also, the effects of MO are transient, precluding the analysis of late developmental or adult phenotypes. For all of these reasons I decided to generate a genetic loss-of-function ptk7 zebrafish model.

Within the zebrafish community, zinc-finger nucleases (ZFNs) had been used to generate targeted gene knock-outs\(^{430,431}\). This technology uses tandem zinc-finger repeats that are engineered to recognize specific sites within the genome and target FokI endonuclease, which cleaves non-specifically to generate double-stranded DNA breaks (Figure 2.3.4a). Following ZFN-mediated DNA damage, non-homologous repair mechanisms have been shown to introduce a range of heritable mutations including insertions, deletions and frameshift mutations that ultimately affect gene function\(^{430,431}\) (Figure 2.3.4a).

Based on cloned ptk7 sequence, ZiFiT software (http://zifit.patners.org/ZiFiT/) was used to identify two potential target sites: one in the predicted transmembrane domain (nucleotide 2089-2112) and the other in the intracellular pseudokinase domain (nucleotide 2557-2580) (Figure 2.3.4b). Mizue Naito then used the Oligomerized Pool Engineering (OPEN) system\(^{432,433}\) method to engineer site specific Zinc-finger repeats for both target sites. A bacterial one-hybrid assay was used with a ptk7 sequence specific reporter vector to select engineered ZF arrays\(^{431}\), and the selected ZF cassettes were inserted into FokI-containing expression vectors.

ZFN mRNAs for each target site were injected into wild-type embryos at the one-cell stage at levels that induced moderate toxicity in <10% of embryos. I raised multiple founders (F0s) and outcrossed these F0s to wild-type zebrafish to generate F1 populations. I screened embryonic F1 genomic DNA for potential mutations at the ptk7 intracellular pseudokinase (PK) target site using RT-PCR (Table 2.3.4). The ZFN target site surrounded an SfaN1 restriction endonuclease cut site (Figure 2.3.4d); therefore, efficient cleavage and mutation of the locus eliminated SfaN1 restriction digest (Figure 2.3.4c). Two F1 lines exhibited loss of restriction digest at a frequency of 12.5% and 10.4%, suggesting ZFN-induced mutation at the PK site (Table 2.3.4). A similar
screening approach was used to identify targeted mutations at the transmembrane (TM) target site; however, no mutations were identified (Table 2.3.4).

To determine the nature of the potential mutations, I sequenced genomic DNA surrounding the PK target site. The first mutant allele \((ptk7 \text{ B})\) was a 6 base pair deletion that was predicted to result in deletion of amino acids 856C and 857R (Figure 2.3.4). The second mutant allele \((ptk7^{hsc9})\) was a 10 base pair deletion that was predicted to result in a frameshift mutation and multiple premature stop codons immediately downstream (Figure 2.3.4).

I identified adult heterozygous offspring for the 6 bp mutant allele and in-crossed these fish to generate homozygous mutants. Zygotic mutant zebrafish displayed no obvious phenotypic defects as embryos or adults, nor did maternal-zygotic (MZ) homozygous mutants. Given the strong \(ptk7\text{ATG}\) morphant phenotype that I had observed, this result suggested that a two amino acid deletion at the PK target site might not have a functional consequence.

I raised heterozygous zebrafish carrying the 10 base pair deletion allele \((ptk7^{hsc9/+})\) and in-crossed these zebrafish to generate \(ptk7^{hsc9}\) homozygous mutants. I found that \(ptk7^{hsc9}\) homozygous mutant embryos displayed no obvious defects (Figure 2.3.4e) and developed normally until approximately 4 weeks post fertilization. At this time point, mutant juveniles developed axial curvatures (100\%, \(n=34\), Figure 2.3.4f,g), which suggested that \(ptk7^{hsc9}\) affects spinal morphogenesis during later stages of development (further addressed in Chapter 3). The presence of significant wild-type maternal \(ptk7\) transcript (from \(ptk7^{hsc9/+}\) females) in homozygous mutant embryos led me to consider the possibility that maternal gene product might rescue early developmental defects.
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Table 2.3.4. Results of F1 screening to identify ZFN-induced mutations at *ptk7* pseudokinase (PK) and transmembrane (TM) domain target sites.
Figure 2.3.4 Zinc-finger nucleases target ptk7. (a) Overview of approach for zinc-finger nuclease (ZFN) mutagenesis in zebrafish. Tandem Zinc-finger (ZF) repeats (green circles) are designed to target 9 bp of genomic sequence on either side of target cleavage site. ZFs are fused to FokI endonucleases (FokI) that dimerize to induce double stranded DNA breaks within a non-sequence specific 6 bp “spacer region”. Double-stranded DNA breaks induce nonsense mediated decay mechanisms, which lead to targeted gene mutations including sequence deletions and insertions. (b) Schematic of zebrafish Ptk7 and the two ZFN target sites. Arrowheads indicate the ZFN target sites within the transmembrane domain (TM) and pseudokinase domain (PK), respectively. (c) RT-PCR-based characterization of ZFN-induced mutations at the pseudokinase domain target site. SfaN1 PCR genotyping assay on individual embryos derived from ptk7<sup>hsc9/+</sup> in-cross to show expected DNA products from wild-type (WT), ptk7 heterozygous (+/-) and ptk7 mutant (-/-) embryos. (d) Genetic lesions identified in embryos derived from individual crosses between the indicated founder and wild-type zebrafish. In wild-type ptk7 sequence, ZFN recognition sequences are boxed and the SfaN1 restriction site used for RT-PCR-based genotyping is indicated. Black colons indicate the positions of deletions in the mutant sequences. The predicted amino acid sequences surrounding the target site are indicated for both alleles. (e) Lateral view of 24 hpf ptk7<sup>hsc9</sup> homozygous mutant embryo generated from routine ptk7<sup>hsc9</sup> heterozygous in-cross. (f) Ptk<sup>hsc9</sup> heterozygous (ptk7+/+) and (g) ptk7<sup>hsc9</sup> mutant juvenile zebrafish at 2 months post fertilization. Axial curvatures were observed in 100% of ptk7<sup>hsc9</sup> mutant zebrafish at this time point.
2.3.5 MZptk7 zebrafish demonstrate loss of ptk7 function

Zygotic $ptk^7\text{hsc}9$ mutant zebrafish were viable and fertile; therefore, to generate maternal-zygotic $ptk^7\text{hsc}9$ (MZptk $ptk^7\text{hsc}9$) mutant embryos I incrossed homozygous $ptk^7\text{hsc}9$ mutant adult zebrafish. Because the $ptk^7\text{hsc}9$ mutant allele introduces premature termination codons into exon 16 of 20, I investigated whether $ptk7$ mutant transcripts were targeted by nonsense-mediated decay (NMD). Using WISH, I did not detect transcript in one-cell stage MZptk $ptk^7\text{hsc}9$ embryos (100%, n=15, Figure 2.3.5.1a,b), suggesting a strong loss of maternal contribution. During somite stages, I did detect small amounts of $ptk7\text{hsc}9$ in the tail bud; however, at 24 hpf, I did not observe any expression (Figure 2.3.5.1c-e). I used qRT-PCR at early and late gastrula stages, as well as during somitogenesis to confirm that $ptk7\text{hsc}9$ transcript is strongly down-regulated in MZptk $ptk^7\text{hsc}9$ mutant embryos relative to wild-type controls (Figure 2.3.5.1f-h). These data suggested that $ptk7\text{hsc}9$ transcript was subject to NMD, and therefore, represents a strong loss-of-function allele. All of my future analysis of the $ptk7$ mutant phenotype was done using this loss-of-function $ptk7\text{hsc}9$ mutant allele (referred to as $ptk7$).

MZptk7 mutant zebrafish were shorter and wider than wild-type embryos (100%, n=100, Figure 2.3.5.2a-f). Interestingly, loss of both maternal and zygotic $ptk7$ was not embryonic lethal, and MZptk7 mutant embryos survived to larval stages. However, the majority of MZptk $ptk^7\text{hsc}9$ mutant zebrafish died before 3 weeks post fertilization. I recovered only a small number of MZptk7 mutant adults (9%, n=90) that display axial curvatures phenotypically similar to $ptk7\text{hsc}9$ mutant zebrafish (Figure 2.3.5.2g,h).
Figure 2.3.5.1 *Ptk7*\textsuperscript{hsc9} transcript is targeted for non-sense mediated decay (NMD). (a-e) *ptk7* expression in (a,c) wild-type and (b,d,e) MZ*ptk7*\textsuperscript{hsc9} (MZ*ptk7*) mutant embryos, as visualized by WISH at (a,b) one-cell, (c,d) 10-12 somite and (e) 24 hours stages. (f-h) Quantitative RT-PCR (qRT-PCR) reveals a strong reduction in *ptk7*\textsuperscript{hsc9} transcripts in MZ*ptk7*\textsuperscript{hsc9} (MZ*ptk7*) mutants relative to wild-type (WT) at (f) early gastrula (shield, ***P<0.001), (g) late gastrula (bud, ***P<0.001) and (h) 10-12 somite stages (*P=0.0358). Error bars represent the s.e. for the expression level fold change.
Figure 2.3.5.2 MZptk7 zebrafish display developmental abnormalities. (a,c) Lateral and (b,d) dorsal views of 24 hpf (a,b) wild-type and (c,d) MZptk7^{hsc9} (MZptk7) mutant embryos. Brackets indicate mediolateral expanse of anterior trunk. (e,f) Lateral views of 3 dpf (e) wild-type and (f) MZptk7^{hsc9} (MZptk7) mutant embryos. (g,h) Lateral views of (g) wild-type (WT) and (h) MZptk7^{hsc9} (MZptk7) mutant zebrafish. Surviving MZptk7 mutants display spinal curvature phenotype.
2.3.6 MZptk7 mutant embryos have PCP signaling defects

MZptk7 mutant embryos display broader axes than wild-type controls (Figure 2.3.5.2), indicative of defects in PCP-mediated C&E movements and consistent with the observations I made following ptk7ATG MO-mediated gene knock-down. Using WISH to stain hindbrain (krox20) and paraxial mesoderm (myoD), I confirmed that MZptk7 are shorter and wider than wild-type controls (Figure 2.3.6a,b). I measured these tissue dimensions and found a significant compression along the MZptk7 rostral-caudal axis, with concomitant expansion along the mediolateral axis (Figure 2.3.6c,d).

At late gastrula stages, I used WISH to stain the neural plate (dlx3) and notochord (ntl), and found that these tissues are expanded mediolaterally in MZptk7 embryos, indicating a defect in lateral tissue convergence. Also, hgg1 staining revealed that the prechordal plate does not extend to its normal anterior position in MZptk7 mutant embryos (100%, n=16, Figure 2.3.6e-h), indicative of a defect in axial extension.

Using confocal microscopy, I analyzed the morphology of the developing neural tube and found that MZptk7 demonstrate neural tube defects similar to those observed in other PCP mutants. In MZptk7 mutant embryos at 24 hpf, neuroepithelial cells accumulated at the midline (Figure 2.3.6i,j), suggesting a defect in PCP-mediated intercalations following cell divisions at the embryonic midline.

Cells engaged in C&E movements become elongated across the mediolateral axis. To analyze gastrulating cellular morphology and orientation in MZptk7 and wild-type embryos, I measured ectodermal cell length:width ratios (LWR) as well as orientation. I found that in the dorsal ectoderm of wild-type gastrulae, cells elongated (LWR=2.0±0.46, n=74; Figure 2.3.6k) with a strong mediolateral bias in their orientation with respect to the midline (72% within ±15° with respect to the mediolateral axis; Figure 2.3.6l,m). I found that MZptk7 mutant cells were significantly less elongated (LWR=1.2±0.46, n=94, P<0.001) and exhibited less mediolateral bias in their orientation, consistent with a loss of PCP (Figure 2.3.6k-m).

To investigate how loss of Ptk7 affects PCP, I used confocal microscopy to visualize the subcellular localization of GFP-Pk. In wild-type neuroepithelial cells, I confirmed that GFP-Pk localized to discrete puncta at the anterior plasma membrane (47/61 cells, n=4 embryos; Figure 2.3.6n,p). GFP-Pk puncta were largely lost from the anterior plasma membrane in
MZptk7 mutants (puncta present in 2/136 cells, n=6 embryos; Figure 2.3.6o,p), suggesting a requirement for Ptk7 in regulating PCP.
Figure 2.3.6 MZptk7 mutants display PCP signaling defects. (a,b) Flat mounts of 10-12 somite stage (a) wild-type (WT) and (b) MZptk7<sup>esc</sup> (MZptk7) mutant embryos stained for krox20 (hindbrain) and myoD (somite) gene expression. (c) Quantification of the anterior-posterior extent of the myoD expression domain in wild-type (WT) versus MZptk7 mutant embryos (***P<0.001, n=8 for each group). (d) Quantification of the mediolateral extent of krox20 expression in WT versus MZptk7 mutant embryos (***P<0.001, n=8 for each group). MZptk7 mutant embryos display clear defects in C&E. (e-f) Anterior and (g,h) dorsal views of late gastrula (bud, 10hpf) stage (e.g) wild-type (WT) and (f,h) MZptk7 mutant embryos stained for hgg1 (prechordal plate, pcp), dlx3 (prospective neural plate, arrowheads) and ntl (prospective notochord, n). MZptk7 mutants demonstrate defects in the convergence of both neuroectoderm and axial mesoderm tissues, as well as defects in the axial extension of the prechordal plate relative to neuroectoderm. (i,j) Dorsal confocal images of the neural tube and adjacent somites of 24 hpf (i) wild-type (WT) and (j) MZptk7 embryos injected with membrane-localized monomeric RFP (membraneRFP)<sup>434</sup>. MZptk7 mutant embryos display an accumulation of neural progenitors (asterisk in j) at the centre of the neural primordium. Anterior is left. (k) MembraneRFP-labeled cells in the dorsal ectoderm of WT and MZptk7 embryos during late gastrulation (9 hpf, 90% epiboly). Dorsal view, midline to the right and anterior to the top. The length-to-width ratio (LWR) of cells are as indicated for WT (n=74) and MZptk7 (n=94). (l) Rose diagrams for cell orientation relative to the embryonic midline at 90% epiboly in WT and MZptk7 embryos. (m) Graph showing percentage of mediolaterally aligned cells for which the mediolateral axis is oriented ±15° with respect to the embryonic mediolateral axis. (n,o) Dorsal confocal images the neural keel and adjacent somites of 8-10 somite stage (n) wild-type (WT) and (o) MZptk7 mutant embryos scatter-labeled with GFP-Prickle (GFP-Pk) and membraneRFP. Subcellular localization of the PCP marker GFP-Pk is disrupted in MZptk7. Anterior is up. Confocal imaging was carried out at the level of the first to the fifth somite pairs. (p) Quantification of the localization of GFP-Pk puncta in WT (n=4 embryos) versus MZptk7 (n=6 embryos).
2.3.7 *Ptk7* functions cell-autonomously and non-cell-autonomously

To determine if *ptk7* was required for PCP within a single cell, I labeled *ptk7*ATG MO-injected cells using rhodamine dextran and transplanted morphant cells into unlabeled wild-type host embryos prior to the onset of gastrulation (Figure 2.3.7a). Wild-type and *ptk7*ATG morphant cells were visualized at a similar location at the embryonic margin prior to the onset of gastrulation (Figure 2.3.7b); however, I found that wild-type cells migrated towards the dorsal margin faster than *ptk7*ATG morphant cells at late gastrula stages (Figure 2.3.7c). This indicated that *ptk7*ATG MO affected convergence movements of individual cells within a wild-type environment and suggested that Ptk7 functions cell-autonomously to regulate PCP signaling.

Intercellular communication is also necessary to communicate planar polarity across the field of a tissue. Loss of certain core PCP signaling components affect polarity in neighboring wild-type cells, an effect referred to as domineering non-autonomy. To test whether knock-down of *ptk7* can affect PCP in neighbouring wild-type cells, I transplanted rhodamine dextran-labeled wild-type cells into unlabeled *ptk7*ATG MO-injected embryos (Figure 2.3.7a). I found that both wild-type and *ptk7*ATG morphant cells moved at a reduced speed towards the dorsal margin in *ptk7*ATG MO-injected hosts (Figure 2.3.7d). This indicated that loss of *ptk7* in the host embryo affected convergence movements of wild-type cells and suggested that Ptk7 acts non-cell-autonomously to affect PCP in neighboring tissue.
Figure 2.3.7 *Ptk7ATG* MO affects PCP both cell-autonomously and non-cell-autonomously. (a) Transplant strategy used to test cell versus host requirement for *ptk7*. *Ptk7ATG* MO was co-injected with rhodamine red and wild-type embryos were injected with rhodamine green to label *ptk7* morphant and wild-type cells red and green, respectively. Labeled cells were transplanted into either wild-type or *ptk7ATG* MO-injected hosts. (b) Lateral view of early gastrula stage host embryo (shield stage, 6 hpf) containing wild-type (green) and *ptk7ATG* MO-injected (red) cells at the lateral margin. Dorsal is right. (c) Lateral view of late gastrula stage (bud, 10 hpf) wild-type (WT) embryo containing wild-type (green) and *ptk7ATG* MO-injected (red) cells. Dorsal is right. (d) Lateral view of late gastrula stage (bud, 10 hpf) *ptk7ATG* MO-injected embryo containing wild-type (green) and *ptk7ATG* MO-injected (red) cells. Dorsal is right.
2.3.8 Membrane-tethered extracellular Ptk7 is required for PCP signal transduction

In contrast to MZptk7, zygotic ptk7 loss-of-function did not affect embryonic morphogenesis, indicating that maternal ptk7 is sufficient for development until juvenile stages. I found that injection of ptk7 mRNA into one-cell-staged embryos rescued MZptk7 body axis elongation (Figure 2.3.8h), indicating that early ptk7 expression is required for PCP-mediated morphogenesis.

To determine which domains of Ptk7 protein are required for activity in vivo, I generated plasmids expressing multiple deletion and substitution mutants (Figure 2.3.8a). I made an intracellular domain deletion construct (ptk7ΔICD) to test the requirement for the intracellular domain. I substituted the transmembrane domain of ptk7 with that of the zebrafish epidermal growth factor receptor (Egfr) (ptk7egfrTM) to test the requirement for the transmembrane domain. Finally, I made an extracellular domain deletion construct (ptk7ΔECD) and a construct that expressed only the extracellular domain (ptk7ECD) to test the activity of the extracellular domain.

I fused each of these mutant constructs to a C-terminal GFP and verified that each was expressed in vivo following mRNA injection at the one-cell stage (Figure 2.3.8b-e). Ptk7ΔICD-GFP, Ptk7EgfrTM-GFP and Ptk7ΔECD-GFP localized to the plasma membrane throughout development, whereas Ptk7ECD-GFP was visible in extracellular spaces such as the lumen of the neural tube and between the neural tube and adjacent somites at 24 hpf, indicating that it was secreted (Figure 2.3.8b-e).

To analyze the requirement for each conserved domain of Ptk7, I tested the differential ability of these deletion and substitution constructs to rescue the MZptk7 extension defect (Figure 2.3.8f-k). I titrated levels of full-length Ptk7, injected equimolar amounts of each mutant construct and quantified rescue by measuring tail length at 24hpf (Figure 2.3.8l). I found that ptk7ΔICD (lacking the entire intracellular domain) expression could rescue MZptk7 axial extension to the same extent as full-length ptk7 expression (Figure 2.3.8i,l). Similarly, ptk7egfrTM expression (substituting the transmembrane domain for that of Egfr) could rescue MZptk7 mutants (Figure 2.3.8j,l). However, deletion of the Ptk7 extracellular domain (ptk7ΔECD) abrogated its ability to rescue MZptk7 axial extension (Figure 2.3.8k,l), indicating a requirement for the extracellular
domain in regulating PCP signal transduction. Expression of the secreted Ptk7 extracellular fragment (ptk7ECD) also failed to rescue MZptk7 axial extension (Figure 2.3.8l), suggesting that the extracellular domain alone is not sufficient for PCP. These data indicated that plasma membrane-tethered extracellular domain of Ptk7 (Ptk7ΔICD) is both necessary and sufficient to regulate PCP-mediated morphogenesis during zebrafish development.
Figure 2.3.8 The Ptk7 extracellular domain is required for PCP-mediated morphogenesis. (a) Schematic of Ptk7 isoforms generated for structure-function analysis. (b-d) Confocal images of the animal pole of shield stage embryos injected with (b) ptk7ΔICD-GFP mRNA, (c) ptk7egrTM-GFP mRNA and (d) ptk7ΔECD-GFP mRNA. (e) Dorsal confocal images of the neural tube and adjacent somites of 24hpf staged embryo injected with ptk7ECD-GFP mRNA. Arrowheads indicate accumulations of Ptk7ECD-GFP within the lumen of the neural tube and between the neural tube and adjacent somites. Anterior is right. (f-l) Lateral views of (f) wild-type (WT), (g) MZptk7\textsuperscript{hsc9} (MZptk7) mutant and MZptk7 mutant embryos injected with (h) ptk7 (300pg), (i) ptk7ΔICD (200pg), (j) ptk7egrTM (300pg) and (k) ptk7ΔECD (150pg) mRNA. (l) Quantification of tail length measured from the base of the yolk extension to the tail tip of wild-type (WT), MZptk7, MZptk7+ptk7 mRNA, MZptk7+ptk7ΔICD mRNA, MZptk7+ptk7egrTM mRNA, MZptk7+ptk7ΔECD mRNA and MZptk7+ptk7ECD mRNA-injected embryos. Ptk7ΔECD and Ptk7ECD cannot rescue body axis extension in MZptk7. Each point represents a single embryo and horizontal lines represent the mean of each group (n=10 for each group). ***P<0.001, n.s. not significant.
2.3.9 *Ptk7* genetically interacts with the PCP signaling pathway

To determine whether *ptk7* functions in the core PCP signaling pathway or in some parallel pathway to affect planar polarity, I looked for a genetic interaction between *ptk7* and two other core PCP genes, *glypican 4* and *van gogh-like 2 (vangl2)*. I found that injection of *ptk7*SPL MO into wild-type embryos did not lead to any significant shortening of the embryonic axis (Figure 2.3.9); however, *ptk7*SPL MO-injection into *glypican 4* heterozygous embryos led to a noticeable shortening of the embryonic axis (n=10/12) compared to controls (Figure 2.3.9). Similarly, *ptk7*SPL MO-injection into *vangl2* heterozygous embryos also led to shortening of the embryonic axis (n=15/21) (Figure 2.3.9). These data indicate that *ptk7* knock-down genetically interacts with core PCP signaling pathway components and further implicates Ptk7 in PCP signal transduction.
Figure 2.3.9 Ptk7 genetically interacts with the PCP signaling pathway. Flat mounts of 10-12 somite stage (a) wild-type, (b) ptk7SPL MO-injected, (c) glypican 4 heterozygous (glypican 4/+) (d) glypican 4/+ + ptk7SPL MO-injected, (e) van gogh-like 2 heterozygous (vangl2/+) and (f) vangl2/+ + ptk7SPL MO-injected embryos stained for krox20 (hindbrain) and myoD (somite) gene expression.
2.3.10 Ptk7 overexpression affects PCP-mediated morphogenesis

To test the effect of ptk7 overexpression, I injected ptk7 mRNA into one-cell-staged embryos. Overexpression of full-length ptk7 led to defects in axial C&E and dorsal curvatures of the posterior tail (Figure 2.3.10a,b,k), which phenocopied overexpression phenotypes of other PCP components138. Using WISH, I stained the hind-brain (krox20) and paraxial mesoderm (myoD) of somite stage embryos and found that overexpression of ptk7 lead to a significant shortening of the embryonic axis compared to wild-type control embryos (Figure 2.3.10c,d).

Interestingly, overexpression of ptk7 led to partial or complete cyclopia in the majority of injected embryos (68%, n=100, Figure 2.3.10e-g). Cyclopia is caused by incomplete separation of the optic stalk due to a failure in PCP-mediated anterior extension of midline hypoblast tissue70,163. To more closely analyze the effect of ptk7 overexpression on anterior extension, I performed WISH to mark the anteriorly located prechordal plate (hgg1) and the anterior edges of the neural plate (dlx3) during late gastrulation. I found that the prechordal plate was more posteriorly located relative to the neural plate in embryos over-expressing ptk7 compared to wild-type controls (Figure 2.3.10h-j). In some embryos the prechordal plate was laterally displaced (n=3/10, Figure 2.3.10j), which is a phenotype observed following manipulation of other known PCP factors in zebrafish435. The extension defect was further evidenced by a reduction in the distance between the prechordal plate and notochord (Figure 2.3.10i). This result indicated that ptk7 overexpression affected PCP-mediated axial extension and further suggested a role for ptk7 in PCP-mediated morphogenesis.

I overexpressed each of my ptk7 mutant constructs (Figure 2.3.8a) at equimolar levels and found that ptk7ΔICD and ptk7egfrTM overexpression led to a shortening of the body axis and cyclopia to the same extent as full-length ptk7 (Figure 2.3.10k,l). Conversely, overexpression of ptk7ΔECD did not lead to any morphological defects (Figure 2.3.10l), suggesting that the extracellular domain of Ptk7 was required for PCP activity. Interestingly, ptk7ECD overexpression did not affect embryogenesis (Figure 2.3.10l), suggesting differential activity of secreted versus membrane-bound extracellular Ptk7.
Figure 2.3.10 *Ptk7* overexpression affects PCP-mediated morphogenesis. (a,b) Lateral views of 8-10 somite stage (a) wild-type (WT) and (b) *ptk7* mRNA (400pg) injected embryos. (c,d) Flat mounts of 10-12 somite stage (c) wild-type (WT) and (d) *ptk7* mRNA (400pg) injected embryos stained for *krox20* (hindbrain, hb) and *myoD* (somite, s) gene expression. (e-j) Anterior ventral views of 48 hpf (e) wild-type (WT) and (f,j) *ptk7* mRNA (400pg) injected embryos. *Ptk7* overexpression leads to partial (f) or complete (g) cyclopia. (h-j) Dorsal views of late gastrula stage (h) wild-type (WT) and (i,j) *ptk7* mRNA (400pg) injected embryos stained for *hgg1* (prechordal plate, pcp), *dlx3* (prospective neural plate, np) and *ntl* (prospective notochord, n). Arrowheads indicate the anterior edge of the neural plate. Bi-directional arrows indicate the anteroposterior distance between the prechordal plate and the notochord. (k) Lateral views of embryos at 48 hpf. Class of phenotypes induced by *ptk7* overexpression: wild-type/class 1; class 2, axial extension defects as well as dorsal curvatures of the posterior tail; class 3, mild to severe dorsalization. (l) Distribution of phenotypes in embryos injected with *ptk7* (400pg), *ptk7ΔICD* (300pg), *ptk7egfrTM* (400pg), *ptk7ΔECD* (300pg) or *ptk7ECD* (300pg) mRNA.
2.3.11 Ptk7 overexpression promotes PCP signaling

Both knock-down and overexpression of ptk7 led to similar defects in axial extension, consistent with embryonic phenotypes following activation or inhibition of PCP signaling in other contexts. To determine whether ptk7 plays a positive or negative role in PCP signaling, I examined the effect of ptk7 overexpression on exogenous non-canonical Wnt activity. I expressed zebrafish wnt5b mRNA and found that high-levels of wnt5b overexpression interfered with embryonic C&E movements (Figure 2.3.11a). Injection of low wnt5b or ptk7 mRNA levels did not disrupt C&E visualized by WISH of hindbrain (krox20) and paraxial mesoderm (myoD) markers (Figure 2.3.11c,d). Interestingly, co-expression of low wnt5b and ptk7 resulted in strong C&E defects evidenced by a significant shortening of the embryonic axis compared to controls (***P<0.001, Figure 2.3.11c,d).

Similarly, I titrated wnt11 overexpression and found that high levels of wnt11 mRNA caused a shortening of the body axis and cyclopia (Figure 2.3.11b). Low levels of wnt11 overexpression did not affect embryogenesis; however, when co-expressed with low levels of ptk7 mRNA, I observed an increase in both the incidence and severity of cyclopia (Figure 2.3.11e,f). WISH revealed that co-expression of low levels of ptk7 and wnt11 led to reduced anterior migration of the prechordal plate (hgg1) relative to the anterior neural plate (dlx3) (Figure 2.3.11g), consistent with a defect in the axial extension that preceeds cyclopia. Together these data suggested that Ptk7 regulates C&E movements by potentiating non-canonical Wnt/PCP signal activity.
Figure 2.3.11 Ptk7 overexpression promotes PCP signaling. (a) wild-type (WT) and wnt5b mRNA (200pg) injected embryos at 48 hpf. (b) wild-type (WT) and wnt11 mRNA (400pg) injected embryos at 48 hpf. Overexpression of wnt5b or wnt11 leads to PCP-mediated morphogenesis defects. (c) Flat mounts of 10-12 somite stage wild-type (WT) embryos and embryos injected with ptk7 (200pg), wnt5b (50pg) or ptk7 (200pg) + wnt5b (50pg) mRNA stained for krox20 (hindbrain) and myoD (somite) gene expression. (d) Quantification of the anterior-posterior extent of the myoD expression domain in WT, ptk7 (200pg), wnt5b (50pg) or ptk7 (200pg) + wnt5b (50pg) mRNA-injected embryos (**P<0.001). n.s., not significant. (e) Anterior ventral views of embryos at 48 hpf. Cyclopia phenotypes were viewed as class I (no cyclopia), class II (partial cyclopia) and class III (full cyclopia). (f) Quantification of cyclopia phenotypes in WT, ptk7 (200pg), wnt11 (50pg) or ptk7 (200pg) + wnt11 (50pg) mRNA-injected embryos. (g) Dorsal views of late gastrula stage wild-type (WT) embryos and embryos injected with ptk7 (200pg) + wnt11 (50pg) mRNA stained for hgg1 (prechordal plate, pcp), dlx3 (prospective neural plate, np) and ntl (prospective notochord, n). Arrowheads indicate the anterior edge of the neural plate.
2.3.12 Ptk7 overexpression affects dorsoventral patterning

In addition to defects in PCP-mediated morphogenesis, I found that overexpression of ptk7 led to embryonic dorsalization/anteriorization (Figure 2.3.10k, class 3), as evidenced by a reduction in posterior tissue. This phenotype is not normally associated with PCP signaling mutants.

Canonical Wnt/β-catenin signaling strongly influences dorsoventral (DV) patterning, first by promoting dorsal organizer formation prior to the onset of zygotic transcription and then by opposing its maintenance during late-blastula stages\(^{438}\). Using quantitative RT-PCR (qRT-PCR), I looked at the expression levels of bozozok and chordin, direct gene targets of β-catenin during organizer formation (4hpf). I found no significant difference in gene expression relative to wild-type controls following ptk7 mRNA injection (Figure 2.3.12a), suggesting that ptk7 overexpression does not affect Wnt/β-catenin-mediated establishment of the dorsal organizer.

However, at the onset of gastrulation I found that ptk7 mRNA-injected embryos displayed lateral expansion of dorsal chordin expression as well as reduced expression of the ventral Wnt-target gene vox (ref. \(^{439}\)) (Figure 2.3.12b-e), which suggested inhibition of ventral Wnt signaling and consequential expansion of the dorsal domain. During late gastrulation, I found that ptk7 overexpression led to a reduction in the expression of the Wnt target gene, axin2 (Figure 2.3.12f-g), which is required for posterior fate specification. This suggested that ptk7 overexpression attenuates the ventralizing activity of Wnt/β-catenin signaling during early embryogenesis to influence dorsoventral patterning and posterior fate specification.

To test the domains of Ptk7 required for Wnt/β-catenin pathway inhibition, I overexpressed ptk7ΔICD and ptk7egfrTM and found that both led to the same level of dorsalization as full-length ptk7 overexpression (Figure 2.3.10k,l; class 3). To further analyze the domain requirement(s) for Wnt/β-catenin pathway inhibition, I used qRT-PCR and found that ptk7, ptk7ΔICD and ptk7egfrTM overexpression led to a small but significant reduction in relative axin2 mRNA levels compared to wild-type at the onset of gastrulation (Figure 2.3.12h).

Ptk7ΔECD overexpression did not significantly affect axin2 expression (Figure 2.3.12h), suggesting that the extracellular domain of Ptk7 is required for Wnt/β-catenin signal inhibition. Overexpression of the extracellular domain alone (Ptk7ECD) was not sufficient to inhibit Wnt/β-catenin target gene activation (Figure 2.3.12h), suggesting that the extracellular domain must be anchored to the membrane in order to affect both patterning and morphogenesis.
Figure 2.3.12 Ptk7 overexpression affects embryonic DV patterning. (a) qRT-PCR to detect relative chordin ($P=0.87$) and bozozok ($P=0.95$) expression levels at 4 hpf in wild-type (WT) embryos and embryos injected with ptk7 (400pg) mRNA. (b-e) WISH of early gastrula stage (shield stage, 6 hpf) (b,d) wild-type (WT) and (c,e) ptk7 mRNA (400pg) injected embryos stained for (b,c) chordin (chd) and (d,e) vox expression. Arrowheads indicate ventral expansion of the chd domain in ptk7 injected embryos. (f,g) Lateral views of late gastrula stage (bud, 10 hpf) (f) wild-type (WT) and (g) ptk7 mRNA (400pg) injected embryos stained by WISH for axin2 expression. (h) qRT-PCR to detect relative axin2 expression levels at shield stage in uninjected wild-type control embryos and ptk7 (400pg), ptk7ΔICD (300pg), ptk7egrTM (400pg), ptk7ΔECD (300pg) or ptk7ECD (300pg) mRNA injected embryos. Error bars represent the standard error of the fold change. The graph is representative of two independent experiments with three technical replicates each. *$P<0.05$. 

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2.3.13 Ptk7 inhibits exogenous Wnt/β-catenin signal activity

To further validate a role for ptk7 in canonical Wnt/β-catenin signal transduction, I examined the effects of ptk7 overexpression on exogenous wnt8 expression. In zebrafish embryos, ectopic expression of wnt8 produces phenotypes characteristic of Wnt/β-catenin activation; namely, defects in dorsoventral patterning and posteriorization of the neural ectoderm\(^78\) (Figure 2.3.13b). I found that overexpression of ptk7 could fully rescue the loss of forebrain and eye-field associated with wnt8 overexpression (Figure 2.3.13c), suggesting that Ptk7 can inhibit exogenous Wnt/β-catenin activity.

To test the ability of each of the Ptk7 deletion/substitution constructs (Figure 2.3.8a) to rescue wnt8 overexpression phenotypes, I co-expressed both ptk7ΔICD and ptk7egfrTM and found that both rescued the wnt8 eyeless phenotype to the same extent as full-length ptk7 expression (Figure 2.3.13d). This confirmed that the Ptk7 intracellular and transmembrane domains were not required for Ptk7-mediated inhibition of Wnt/β-catenin activity. Conversely, overexpression of ptk7ΔECD and ptk7ECD were unable to rescue the wnt8 eyeless phenotype (Figure 2.3.13d), suggesting that the extracellular domain is required yet insufficient for Ptk7-mediated Wnt/β-catenin inhibition.
Figure 2.3.13 The extracellular domain of Ptk7 is required for exogenous Wnt/β-catenin signal inhibition. (a-c) Lateral anterior views of 48 hpf (a) wild-type (WT), (b) wnt8 (10pg) and (c) wnt8 (10pg) + ptk7 (200pg) mRNA injected embryos. Overexpression of wnt8 disrupts CNS pattern, as demonstrated by loss of eyes and reduced forebrain. (d) Quantification of phenotypes observed upon injection of wnt8 (10pg) mRNA, and upon co-injection of wnt8 with full-length ptk7 (300pg), ptk7ΔICD (200pg), ptk7egrTM (300pg), ptk7ΔECD (150pg) or ptk7ECD (200pg) mRNA. Embryos were scored as being ‘eyeless’ if the eye was <25% the size of uninjected controls.
2.3.14 *Ptk7* inhibits Wnt3a-induced β-catenin signaling in HEK293T cells

To assess the conservation of Ptk7-mediated Wnt/β-catenin inhibition, I analyzed the effect of Ptk7 overexpression in a human cell line (HEK293T) with Dr. Avais Daulat (Angers lab, Department of Pharmacy at the University of Toronto). I expressed full-length *ptk7-GFP* through plasmid transfections and verified expression as well as plasma membrane localization using confocal microscopy (Figure 2.3.14.1a).

Recombinant Wnt3a activated β-catenin-dependent TCF/LEF luciferase (TOPFlash) reporter activity in HEK293T cells (Figure 2.3.14.1b), consistent with Wnt/β-catenin activation in this assay. I found that expression of *ptk7* abrogated Wnt3a-stimulated TOPFlash reporter activity (Figure 2.3.14.1b), suggesting that Ptk7 can inhibit Wnt/β-catenin signaling in a human cell line, similar to zebrafish embryos. *Ptk7ΔICD* and *ptk7egfrTM* expression had the same inhibitory effect as full length *ptk7*; however, *ptk7ΔECD* expression failed to inhibit TOPFlash reporter activity (Figure 2.3.14.1b), indicating that the extracellular domain is required to inhibit Wnt/β-catenin signaling in HEK293T cells. These results were consistent with the observations I made using zebrafish embryos and suggests a conserved role for Ptk7 in Wnt/β-catenin pathway inhibition. Interestingly, *ptk7ECD* expression was sufficient to inhibit TOPFlash reporter activity in HEK293T cells (Figure 2.3.14.1b), suggesting differential activity in human tissue culture versus zebrafish embryos.

To test the effect of endogenous PTK7 knockdown on Wnt/β-catenin signal transduction in HEK293T cells, I transfected siPTK7 (ref. 3) and verified that PTK7 was effectively knocked down through Western blot analysis (Figure 2.3.14.2a). Again, recombinant Wnt3a activated TOPFlash reporter activity, consistent with activation of Wnt/β-catenin activity in this assay. However, I found that TOPFlash reporter activity increased following PTK7 knockdown (Figure 2.3.14.2b), suggesting that loss of endogenous PTK7 enhanced Wnt/β-catenin activity *in vitro*. Together these data suggested a conserved role for PTK7 as a functional inhibitor of Wnt/β-catenin pathway activity in human cells.
Figure 2.3.14.1 Ptk7 inhibits Wnt3a-induced luciferase activity in HEK293T cells. (a) Confocal image of scattered Ptk7-GFP expression in confluent HEK293T cells. (b) To activate Wnt/β-catenin signaling Wnt3a-conditioned medium (Wnt3a CM) was used. Cells were transfected with empty vector, ptk7, ptk7ΔICD, ptk7egfrTM, ptk7ΔECD or ptk7ECD expression plasmids. The graph represents TOPFlash luciferase activity in three independent experiments. The luciferase activity of Wnt3a-treated empty vector (CS2+)-transfected cells was set to 1. Error bars represent s.e.m. ***$P<0.001$. n.s., not significant.
Figure 2.3.14.2 siPTK7 enhances Wnt3a-induced luciferase activity in HEK293T cells. (a) Western blot analysis of human PTK7 in HEK293T cells transfected with either control siRNA (siCTRL) or siPTK7 (ref. 3). Actin was used as a loading control. (b) Wnt3a conditioned media was used to activate Wnt/β-catenin signaling in HEK293T cells. Cells were transfected with control siRNA (siCONTROL) or siPTK7. The graph is representative of two independent experiments with three technical replicates each. The luciferase activity of control media-treated cells was set to 1. Error bars represent s.e.m. *P<0.05.
2.3.15 Ptk7 is not required for anterior CNS or dorsoventral patterning

Overexpression of Ptk7 led to dorsoventral (DV) patterning defects and suggested that Ptk7 can inhibit zygotic Wnt/β-catenin target gene transcription during zebrafish embryogenesis. siRNA-mediated knock-down of PTK7 in HEK293T cells also suggested that Ptk7 is a functional inhibitor of Wnt/β-catenin signal transduction. However, MZptk7 mutant embryos at 24 hpf do not display obvious DV patterning defects associated with activated Wnt/β-catenin signaling like CNS patterning abnormalities including loss of forebrain and/or eyefields. I confirmed that CNS patterning is normal in MZptk7 mutant embryos through use of WISH to mark anterior and posterior domains of the anterior CNS (Figure 2.3.15a,b).

I used qRT-PCR to quantify β-catenin-dependent dorsal organizer gene expression in wild-type and MZptk7 mutant embryos. Prior to onset of gastrulation at 4 hpf, I observed a small but significant upregulation of the β-catenin-dependent dorsal expression of bozozok (boz) and chordin (chd) in MZptk7 mutants (Figure 2.3.15c). This suggested an inhibitory role for ptk7 in dorsal organizer formation. Using WISH, I detected a slight expansion of the boz expression domain at the site of the future dorsal organizer at 5 hpf (100%, n=20; Figure 2.3.15d) and confirmed boz upregulation at this stage in MZptk7 mutants using qRT-PCR (Figure 2.3.15d).

However, by the onset of gastrulation (6 hpf), I could no longer detect significant differences in the dorsal expression of chd or goosecoid (gsc) (Figure 2.3.15f,g), nor did I observe differences in ventral axin2 or vox expression using WISH and qRT-PCR in MZptk7 relative to wild-type controls (Figure 2.3.15f,g). These data suggested that ptk7 might play a role in defining the dorsal organizer prior to the onset of gastrulation; however, genetic loss of ptk7 did not have a significant effect on subsequent maintenance of DV domains during early stages of zebrafish gastrulation.
Figure 2.3.15 MZptk7 mutant embryos do not display CNS defects or defects in DV patterning. (a,b) WISH of 24 hpf (a) wild-type (WT) and (b) MZptk7<sup>hsc9</sup> (MZptk7) embryos stained for emx1, pax2.1 and krox20 to mark the forebrain, midbrain-hindbrain boundary and posterior rhombomeres, respectively. (c) Chordin (chd) and bozozok (boz) expression is slightly increased in MZptk7 embryos immediately following MBT (onset of zygotig transcription and dorsal organizer formation), as assayed by qRT-PCR of embryos at 4 hpf (**) \( P<0.01 \). (d) Dorsal view of boz expression in wild-type (WT) and MZptk7 embryos prior to the onset of gastrulation (5 hpf). (e) qRT-PCR of boz expression in MZptk7 relative to wild-type (WT) prior to the onset of gastrulation (5 hpf, \( **P=0.0078 \)). (f) WISH for dorsal organizer genes chordin (chd) and goosecoid (gsc), and ventral genes axin2 and vox during gastrulation (shield, 6 hpf). Embryos are viewed from the animal pole with dorsal to the right (for chd, axin2 and vox). Gsc expression is shown as a dorsal view. (g) qRT-PCR assays of chd (\( P=0.4675 \)), gsc (\( P=0.9515 \), axin2 (\( P=0.07 \)) and vox (\( P=0.3974 \)) expression in wild-type (WT) versus MZptk7 embryos during gastrulation. No significant differences were observed. Error bars represent the s.e. for the expression level fold change. All graphs are representative of two independent experiments with three replicates each.
2.3.16 MZptk7 mutants display Wnt/β-catenin-dependent tail bud patterning defects

Following gastrulation, Wnt/β-catenin signaling is required for posterior vertebrate axis development\textsuperscript{81,82,442,443}. Ptk7 is enriched in the tailbud; therefore, I decided to investigate a role for Ptk7 in posterior canonical Wnt/β-catenin activity post-gastrulation. Using WISH, I observed upregulated levels of \textit{axin2} expression within the presomatic mesoderm (PSM) of somite stage embryos (100\%, \textit{n}=17; Figure 2.3.16.1a,b). Using qRT-PCR, I found that Wnt-target genes \textit{axin2}, \textit{lef1}, and \textit{cycinD1} were upregulated relative to wild-type controls during late gastrula (bud stage) and somite stages (Figure 2.3.16.1c,d). These data indicated that Wnt/β-catenin signaling was enhanced in MZptk7 mutant embryos post-gastrulation and supports my previous observations that \textit{ptk7} inhibits Wnt/β-catenin activity.

Wnt/β-catenin signaling in the posterior tailbud specifies paraxial mesodermal cell fate from a bi-potential neural/mesodermal stem cell pool\textsuperscript{82}. Additionally, high levels of Wnt in the posterior pre-somatic mesoderm (PSM) maintains tail bud tissue in an undifferentiated state through the activation of transcription factors like \textit{Tbx6} and \textit{Mesogenin1}\textsuperscript{264,265}. Using WISH, I found that the posterior Wnt/β-catenin-dependent \textit{tbx6} expression domain was anteriorly expanded relative to total body length in MZptk7 mutants compared to wild-type controls (Figure 2.3.16.2a-e). I found that \textit{mesoderm posterior protein b (mespb)} expression, which marks the anterior border of the presomatic mesoderm (PSM), was similarly anteriorly shifted in MZptk7 mutants (Figure 2.3.16.2f-h). These data indicated an increase in posterior mesodermal tissue in MZptk7 mutant embryos and suggest a role for \textit{ptk7} in Wnt/β-catenin-dependent cell fate decisions at the tailbud.

In the embryonic tail bud, Wnt/β-catenin signaling drives continuous specification of mesoderm from a bi-potential neural/mesodermal precursor pool present in the tail bud during zebrafish tail outgrowth\textsuperscript{82}. To test if neural fate specification in the MZptk7 mutant tail bud was compromised as a result of expanded mesoderm, I stained neural precursors with \textit{sox2} and found that although the \textit{sox2} expression domain was more diffuse in the MZptk7 mutant tail bud, the number of \textit{sox2} positive cells appeared similar to controls (Figure 2.3.16.2i-l). Therefore, even though complete loss of \textit{ptk7} led to an expansion of mesodermal tissue fate in the tail bud, it did not appear to cause an obvious defect in neural fate specification within this tissue.
Figure 2.3.16.1 MZptk7 embryos display increased Wnt/β-catenin signaling activity post-gastrulation. (a,b) WISH of axin2 expression in 10-12 somite stage (a) wild-type (WT) and MZptk7<sup>hsc9</sup> (MZ) embryos. (c) Late gastrula stage expression of axin2 (**P=0.0026), lef1 (**) and cyclinD1 (**P=0.0028) in MZptk7 relative to wild-type (WT). (d) 10-12 somite stage expression of axin2 (**P=0.0026), lef1 (**P=0.0035) and cyclinD1 (**P=0.0028) in MZptk7 relative to wild-type (WT). Each graph is representative of two independent experiments with three replicates each. Error bars represent the s.e. for the expression level fold change.
Figure 2.3.16.2 MZptk7 embryos display tail bud patterning defects. (a,c) Lateral and (b,d) tail bud views of WISH of tbx6 expression in 8-10 somite stage (a,b) wild-type (WT) and (c,d) MZptk7
mutant embryos. (e) Quantification of the size of the tbx6 expression domain (bracket in a) relative to the total body length (dashed line in a) in wild-type (WT) and MZptk7
mutant embryos. The tbx6-positive presomitic mesoderm domain is significantly expanded in MZptk7 embryos (**P<0.001). Error bars represent s.d. (f,g) Lateral views of WISH for mespb expression (marking the anterior extent of the pre-somatic mesoderm) in (f) wild-type (WT) and (g) MZptk7 mutant embryos. (h) Quantification of the distance between the most posterior part of the embryonic tail and the posterior extent of mespb expression (bracket in f) relative to total body length (dashed line in f) in wild-type (WT) and MZptk7. Mespb expression is anteriorly shifted in MZptk7 mutant embryos (**P=0.0016). Error bars represent s.d. (i,k) Lateral and (j,l) tail bud views of WISH of sox2 expression in 8-10 somite stage (i,j) wild-type (WT) and (k,l) MZptk7 embryos.
2.3.17 PCP signaling is not required for tail bud patterning in zebrafish

Since MZптk7 mutant embryos display a strong C&E defect, I reasoned that the tail patterning defects could be secondary to abnormal tail morphogenesis. To test whether morphogenesis affects posterior tissue patterning, I looked at Wnt/β-catenin signaling activity and posterior tissue patterning in a specific Wnt/PCP pathway mutant, vangl2.

Wnt/β-catenin target gene transcription was not upregulated post-gastrulation in vangl2 mutant embryos relative to vangl2 heterozygous controls (Figure 2.3.17a), indicating that loss of PCP was not sufficient to promote Wnt/β-catenin activity. Furthermore, vangl2 mutant embryos did not display anterior expansion of the tbx6 expression domain at the tail bud (Figure 2.3.17b-d), nor was there any change in posterior sox2 expression (Figure 2.3.17e-h). These data indicated that tail bud patterning is not affected by PCP-dependent morphogenesis and suggests that the expansion of mesodermal tissue fate in MZптk7 embryos is specific to a defect in Wnt/β-catenin patterning.
Abnormal PCP signaling does not disrupt Wnt/β-catenin signaling or posterior tissue patterning. (a) qRT-PCR to analyze expression of Wnt/β-catenin target genes in vangl2 mutants relative to wild-type (WT). No difference in expression levels of axin2 (P=0.6314), lef1 (P=0.3469), or cyclinD1 (P=0.8825) was observed. Error bars represent s.e. of the expression level fold change. The graph is representative of two independent experiments with three replicates each. (b,c) Lateral views of WISH tbx6 expression in (b) wild-type (WT) or vangl2+/− and (c) vangl2 mutant embryos. (d) Quantification of the size of the tbx6 expression domain relative to the total body length in wild-type (WT) or vangl2+/− and vangl2 mutant embryos. The tbx6-positive presomitic mesoderm is not significantly expanded in vangl2 mutants. (e,g) Lateral and (f,h) tail bud views of WISH of sox2 expression in 8-10 somite stage (e,f) wild-type (WT) or vangl2+/− and (g,h) vangl2 mutant embryos.
2.3.18 Membrane-tethered Ptk7 is required for Wnt-dependent mesodermal specification in the embryonic tailbud

The extracellular domain of Ptk7 is required to inhibit exogenous Wnt/β-catenin signaling. Consistent with Ptk7 acting as a functional inhibitor of Wnt/β-catenin signaling, I found that full-length ptk7 expression could rescue tbx6 domain expansion in MZptk7 mutant embryos (Figure 2.3.18a-f). I also tested the ability of my deletion and substitution constructs (Figure 2.3.8a) to rescue the MZptk7 tail patterning defect through injections of equimolar amounts of each mutant construct followed by measurement of the tbx6 expression domain relative to total body axis length. I found that both ptk7ΔICD and ptk7egfrTM expression rescued the tail patterning defect to the same extent as full-length ptk7 expression (Figure 2.3.18g). Consistent with previous observations, expression of the Ptk7 extracellular domain deletion (ptk7ΔECD) or the secreted Ptk7 extracellular fragment (Ptk7ECD) failed to rescue the MZptk7 tail patterning defect (Figure 2.3.18g), indicating a requirement for membrane bound Ptk7 in Wnt/β-catenin-dependent tissue patterning. These data support my earlier observations and indicate that the plasma membrane-tethered extracellular domain of Ptk7 (Ptk7ΔICD) is sufficient to inhibit Wnt/β-catenin signaling in vivo.
Figure 2.3.18 The Ptk7 extracellular domain is required for Wnt/β-catenin tail bud patterning. (a,c,e) Lateral and (b,d,f) tail bud views of WISH of tbx6 expression in 8-10 somite stage (a,b) wild-type (WT), (c,d) MZptk7ΔICD (MZptk7) mutant and (e,f) MZptk7 mutant embryos injected with ptk7 (300pg) mRNA. (g) Quantification of the tbx6 expression domain relative to total body axis length in wild-type (WT), MZptk7, MZptk7+ptk7 (300pg) mRNA, MZptk7+ptk7ΔICD (200pg) mRNA, MZptk7+ptk7egfrTM (300pg) mRNA, MZptk7+ptk7ECD (150pg) mRNA, and MZptk7+ptk7ECD (200pg) mRNA-injected embryos. Tbx6 is expanded in MZptk7 and this can be rescued by full-length Ptk7 and Ptk7ΔICD but not by Ptk7ΔECD. ***P<0.001. n.s. not significant. Error bars represent s.d.
2.4 Discussion

The Wnt pathway consists of a complex network of signaling molecules that integrate extracellular information through a variety of cell surface proteins. The presence or absence of a surface component can dictate the context of a cellular response, favoring activation of canonical Wnt/β-catenin, non-canonical Wnt/PCP signaling, or other alternative pathways. Recent studies implicate Ptk7 as a novel Wnt co-receptor that can influence signal specificity during vertebrate embryogenesis. However, conflicting reports have indicated both positive and negative roles for Ptk7 in regulating Wnt/β-catenin activity.

In this chapter, I cloned and characterized the zebrafish ptk7 ortholog. Zebrafish ptk7 is strongly expressed at the one-cell-stage; it is expressed ubiquitously throughout gastrulation; and strong ptk7 expression is maintained in the tail bud throughout segmentation stages. Using zinc-finger nucleases (ZFNs), I generated a ptk7 mutant allele (hsc9) that was subject to nonsense mediated decay, indicating a strong loss-of-function allele.

2.4.1 Ptk7 and non-canonical Wnt/PCP signaling

Zygotic ptk7<sup>hsc9</sup> mutant embryos develop normally, with no appreciable defects in embryonic patterning or morphogenesis. However, maternal zygotic ptk7<sup>hsc9</sup> mutant embryos display axial C&E defects, abnormal neural tube morphogenesis, and loss of PCP. These results provide further evidence that Ptk7 is an essential regulator of vertebrate PCP. More importantly, I demonstrated that Ptk7 enhances the activity of exogenous Wnt5 and Wnt11 ligands, indicating that Ptk7 regulates PCP by potentiating non-canonical Wnt signal transduction.

Although the molecular mechanisms by which Ptk7 promotes PCP remain ill defined, overexpression studies in Xenopus have implicated a key role for the highly conserved Ptk7 pseudokinase domain: it interacts with receptor of activated protein kinase C 1 (RACK1, a known effector of Dsh required for Xenopus neural tube closure<sup>171,237</sup>), and it is required for Ptk7-mediated translocation (and subsequent hyperphosphorylation) of Dsh at the plasma membrane<sup>235</sup>. I used an in vivo rescue-based strategy to perform structure-function analyses of Ptk7. Strikingly, I found that full-length Ptk7 and Ptk7ΔICD constructs have equivalent abilities to rescue MZptk7 C&E defects, whereas deletion of the Ptk7 extracellular domain eliminated function. Therefore, the kinase domain is
dispensable for Ptk7 activity in PCP-mediated C&E. Rather, my data indicate that a plasma membrane-tethered Ptk7 extracellular domain (Ptk7ΔICD) is sufficient to promote normal PCP, and highlight the importance of extracellular protein-protein interactions for Ptk7 function.

This work complements recent studies of mammalian Ptk7, which demonstrate that Ptk7 is not required for Dsh2 membrane localization in planar polarized cells of the mouse mesoderm or the auditory epithelium. Therefore, functions for the Ptk7 kinase domain in Dsh localization and PCP might have evolved differently in Xenopus. Alternatively, Ptk7 might have context-dependent PCP functions. The specific role of Ptk7 intracellular domains in oriented cell division, radial intercalation, cilia orientation, and facial branchiotor neuron migration remain to be determined.

2.4.2 Ptk7 and canonical Wnt/β-catenin activity

I demonstrated that overexpression of zebrafish Ptk7 disrupts dorsoventral patterning, inhibits Wnt/β-catenin-dependent gene expression in early gastrula-stage embryos and inhibits the effects of wnt8 overexpression. Furthermore, loss of Ptk7 activity in MZptk7 mutant embryos results in increased Wnt/β-catenin target gene transcription at early segmentation stages. Ptk7 is strongly expressed in the tailbud, where Wnt/β-catenin signals specify paraxial mesoderm cell fate from bi-potential neural/mesodermal progenitor cell pools. In MZptk7 embryos, I observed an expanded domain of presomitic mesoderm differentiation that is independent of PCP-mediated morphogenesis defects. These data therefore indicate that Ptk7 attenuates Wnt/β-catenin signaling during vertebrate embryogenesis, with essential roles in regulating Wnt/β-catenin-dependent stem cell fate decisions in the developing tailbud.

Wnt/β-catenin patterning defects have not been reported in three independent analyses of Ptk7 mutant mice. However, these studies focused predominantly on PCP-related phenotypes. Of note, Paudyal et al. (2010) did report a shortened spinal cord and body axis in Ptk7/chuzoi mutant mouse embryos, which would be consistent with misregulated Wnt/β-catenin signaling during posterior paraxial mesoderm differentiation and tailbud outgrowth.

In Xenopus, MO-based analysis has yielded conflicting results. Peradziryi et al. (2011) demonstrated that knockdown of Ptk7 in animal cap explants stimulated β-catenin target gene
transcription in response to exogenous Wnt signals. This study supports our findings that Ptk7 inhibits Wnt/β-catenin signaling in vivo; however, endogenous Wnt/β-catenin patterning defects were not reported for Xenopus PTK7 MO-injected embryos. In a second study, MO knockdown of PTK7 activity caused defects in Spemann organizer formation, suggesting that Ptk7 potentiates canonical Wnt signaling. Although this finding is difficult to reconcile with my analysis of MZptk7 mutant embryos, PCP factors such as Vangl2 have been implicated in the asymmetric distribution of maternal transcripts within Xenopus oocytes, which is required for dorsoventral pattern formation. As Ptk7 is a well-established regulator of PCP, defects observed in the latter study may reflect a similar requirement for maternal Ptk7 in Xenopus oocyte polarity.

Of interest, despite an early expansion of Wnt/β-catenin-dependent dorsal organizer gene expression in MZptk7 mutant embryos, I did not observe later dorsoventral or anterior CNS patterning defects. As Wnt/β-catenin signals also restrict dorsal organizer activity in later-staged blastula, it is possible that potentiation of ventralizing Wnt/β-catenin signals in MZptk7 mutants might compensate for early expansion of the dorsal organizer. Alternatively, there may be functional redundancy between Ptk7 and other Wnt/β-catenin inhibitors. For example, the transmembrane protein Waif1 has recently been shown to attenuate Wnt/β-catenin signaling while promoting PCP. Zebrafish Wnt-activated inhibitory factor 1 (waif1) is broadly expressed during gastrulation but becomes restricted to the neuroectoderm during segmentation and these domains overlap with ptk7 expression.

I also demonstrated that the Ptk7 pseudokinase domain is dispensable for Wnt/β-catenin signal inhibition, as assayed by rescue of MZptk7 tailbud patterning defects. Rather, a plasma membrane-tethered Ptk7 extracellular domain is sufficient for this activity. This result contrasts work by Puppo et al. (2011), which suggested that physical interactions between the pseudokinase domain and intracellular β-catenin might provide a mechanistic link between Ptk7 and canonical Wnt signal transduction pathways. However, Ptk7 can form a complex with Fz (ref. 235), and the extracellular domain of Ptk7 can selectively bind canonical ligands Wnt3a and Wnt8 (ref. 2). This suggests that Ptk7 may function as a canonical Wnt co-receptor to affect pathway specificity in vivo. My results highlight the importance of the Ptk7 extracellular domain in both Wnt/β-catenin and non-canonical Wnt/PCP signal transduction pathways during vertebrate embryonic development.
2.4.3 Ptk7, cancer, congenital malformations and disease

Wnt/β-catenin signaling plays an oncogenic role in the development of colon cancer, hepatocellular carcinoma and multiple other tumor types\textsuperscript{448}. By contrast, non-canonical Wnt pathways antagonise Wnt/β-catenin signaling, and are thought to drive morphogenetic cell behaviours associated with tumor invasion and metastasis – pathogenic events that account for ~90\% of cancer deaths\textsuperscript{449}. My data demonstrate that Ptk7 not only promotes PCP, but also inhibits Wnt/β-catenin signaling during vertebrate development. Therefore, these results could have significant implications for Ptk7 misregulation observed in colon carcinoma, acute myeloid leukemia (AML) and multiple other cancer types\textsuperscript{205-207,210,211,215,426,427}. For example, during cancer progression Ptk7 could initiate a switch from β-catenin-mediated tumour formation and cell proliferation towards PCP-directed changes in cell adhesion, polarity and motility that are associated with metastasis.

Zygotic \textit{ptk7} mutant embryos display no obvious defects in embryonic patterning or morphogenesis. Rather, maternal Ptk7 is sufficient to rescue early development. This maternal rescue will now permit investigation into the role for Ptk7 (and consequently, misregulated Wnt/β-catenin and PCP signaling) in juvenile growth, patterning and adult physiology. For example, the late onset of axial deformity observed in zygotic \textit{ptk7} mutants suggests that they occur independently of early tailbud patterning defects. Thus, zygotic \textit{ptk7} mutant zebrafish may prove to be a useful model for determining the consequences of abnormal Wnt signal transduction in adult/late-onset disease.
Chapter 3

3. CHARACTERIZATION OF CONGENITAL AND LATE ONSET SPINAL CURVATURE IN PTK7 MUTANT ZEBRAFISH MODELS

The data in this chapter have been submitted and are currently under review as:

3.1 Abstract

Scoliosis is a complex genetic disorder, characterized by three-dimensional rotations of the spine\(^5\). Curvatures caused by underlying vertebral malformations (congenital scoliosis, CS) are apparent at birth\(^5,6,246\), while scoliosis with no obvious underlying abnormality (idiopathic scoliosis, IS) commonly develops during adolescence\(^5,7\). Here, I describe maternal zygotic (MZ) and zygotic \(ptk7\) mutant models that recapitulate CS and IS, respectively. I found that MZ\(ptk7\) mutant zebrafish displayed vertebral abnormalities at larval stages analogous to those found in human CS patients. Zygotic \(ptk7\) mutant zebrafish do not display embryological abnormalities; however, these mutants develop severe late-onset three-dimensional spinal curvatures analogous to human idiopathic scoliosis (IS). I characterized MZ\(ptk7\) and \(ptk7\) mutant zebrafish as models of CS and IS, respectively, and suggest that CS and IS share common genetic mechanisms related to Wnt signal transduction.
3.2 Brief Introduction and Rationale

Scoliosis refers to curvature of the spine and can occur as a consequence of underlying vertebral malformations; underlying congenital or juvenile disease; or with no identifiable primary cause. The majority of human scoliosis is idiopathic (idiopathic scoliosis, IS) and affects approximately 3% of the population\textsuperscript{6,290}. The majority of IS presents during adolescence (adolescent idiopathic scoliosis, AIS) and for poorly understood reasons, curves either regress, stabilize or progress up to sexual maturity. Interestingly, the incidence and severity of AIS is greater in girls than it is in boys, suggesting sex specific mechanisms involved during pathogenesis.

IS is known to have a genetic component; however, it is assumed to be a complex trait in most cases, with multiple contributing factors. Linkage analysis suggests multiple contributing loci\textsuperscript{291}, while genome wide association studies (GWAS) have identified genetic candidates involved in axon guidance (ROBO3, DSCAM)\textsuperscript{303}, proprioception (LBX)\textsuperscript{313} and/or bone metabolism (GPR126)\textsuperscript{302}. Ultimately, IS is caused by rotational force upon the spine and diverse hypotheses regarding the primary cause, including neuromuscular, CNS, bone, connective tissue, hormonal and/or biomechanical abnormalities, have been proposed\textsuperscript{245,301}.

One major challenge regarding IS has been the lack of good animal models. Rodents, the prevalent laboratory animal, are quadrupeds and the structure of their spine, centre of gravity and the biomechanics of spinal loading are quite different from humans. As a result, induction of IS-like deformities in these animals requires drastic surgical or systemic interventions that preclude relevance to human disease\textsuperscript{369,370}. In contrast, teleosts experience spinal loads that are similar to humans (cranial-to-caudal, generated as a function of swimming forward through dense media/water coupled with caudal tail-propulsion), and are naturally susceptible to late-onset spinal curvatures\textsuperscript{389}. Recent observations of heritable scoliosis-like phenotypes in fish have raised the intriguing possibility that teleosts can effectively model human IS\textsuperscript{389,390}.

Scoliosis accompanied by vertebral malformations is referred to as congenital scoliosis (CS) and affects 0.13-0.5/1,000 live human births\textsuperscript{6,245}. Vertebral malformations including hemivertebrae, wedge shaped vertebrae or vertebral bar (vertebral fusions) occur in isolation, with other tissue malformations, or as a part of an underlying syndrome or chromosomal abnormality\textsuperscript{6}.
In mammals and chicken, vertebral bodies are derived from the somites and many cases of familial CS involve genes that regulate somitogenesis\textsuperscript{245,246}. Somites bud off from the presomitic mesoderm (PSM) as a result of cyclic activation/repression of Wnt and Notch signaling pathways. Anteroposterior (AP) gradients of Wnt, FGF and retinoic acid define the anterior extent of the PSM and the point at which a somite border is defined. Therefore, Wnt signaling is considered an important mediator between PSM fate decisions and the oscillating expression network that is necessary to effectively segment the developing vertebrate axis. Despite an obvious correlation between somitogenesis and vertebral malformations, the basis for the majority of vertebral malformations is unknown. Importantly, IS and CS have traditionally been though of as separable diseases; however, instances of both deformities running together in families suggest commonalities between the two\textsuperscript{406,450}.

Here, I report my characterization of \textit{ptk7} and \textit{MZptk7} mutant zebrafish as functional models of IS and CS, respectively. Zygotic \textit{ptk7} mutant zebrafish displayed a fully penetrant form of late-onset spinal curvature that was variable in both time of onset and severity. This spinal defect was not associated with vertebral malformations and, therefore, structurally analogous to human IS. Conversely, complete loss-of-function \textit{MZptk7} mutant larvae displayed CS-like vertebral malformations that may be attributed to defects in embryonic segmentation. Manipulations of both PCP and Wnt/\textbeta-cat signaling phenocopied these segmentation defects, suggesting that deregulated Wnt signaling contributes to the vertebral malformations in \textit{MZptk7} mutant zebrafish. Therefore, my data suggest a genetic link between CS and IS and highlight the usefulness of zebrafish as a genetic model to study different types of spinal deformities.
3.3 Results

3.3.1 Zygotic ptk7 mutant zebrafish display late-onset spinal curvatures

Homozygous ptk7 mutant embryos displayed no obvious defects in embryonic patterning or morphogenesis, because maternal Ptk7 was sufficient to rescue early stages of development. However, during late larval and early juvenile stages (8-10mm length, approximately 4 weeks post fertilization) all ptk7 mutant zebrafish developed three-dimensional axial curvatures of variable magnitude. To more closely visualize the spines of ptk7 heterozygous and ptk7 mutant adults, I stained skeletons using alizarin red and confirmed that ptk7 mutant zebrafish displayed deviation of the spine in both the sagittal (dorsal-ventral) and coronal (left-right) plane (Figure 3.3.1a,b). I grouped live fish based on the magnitude of axial curvature at sexual maturity into four classes (type 1-4) representative of progressively more severe curves (Figure 3.3.1c-h). Mild-moderate curves (type 1 and 2) occurred only in the sagittal plane and were kyphosis-like (Figure 3.3.1d,e), while more severe curves (type 3 and 4) occurred in both the sagittal and coronal planes and represented true scoliosis (Figure 3.3.1f,g). Curve onset was variable among individuals and did not progress after sexual maturity (3 months post fertilization). Interestingly, almost all of the females I recovered displayed a progressed or extreme curve phenotype (93% type 3 and 4, n=58), whereas males tended to display more moderate curves (42% type 2, n=92) (Figure 3.3.1h). This recapitulated human adolescent idiopathic scoliosis (AIS), where girls tend to display curves of greater severity. Late-onset spinal curvature, phenotypic variability and sexual dimorphism are all hallmarks of human AIS; therefore, ptk7 mutant zebrafish may represent a novel animal model of human AIS.
Figure 3.3.1 *Ptk7* mutant zebrafish display spinal deformities. (a,b) Lateral views of trunk and tail skeleton stained with alizarin red for (a) *ptk7/+* and (b) *ptk7* mutant male zebrafish at approximately 6 months of age. (c-g) Adult *ptk7/+* and zygotic *ptk7* mutant zebrafish representative of each curve severity class: (c) unaffected *ptk7* heterozygotic zebrafish (type 0); (d) mild curvature (type 1); (e) moderate curvature (type 2) primarily isolated to the tail; (f) progressed curvature (type 3) with trunk, tail and mild mediolateral rotations; (g) severe curvature (type 4) with extreme trunk, tail and mediolateral rotations. Scale bars represent 1 mm. (h) Graph depicting phenotypic and gender distributions for type 1-4 classification based on curve severity at sexual maturity (approximately three months of age). Quantification is represented as a percentage of the total for male (n=92) and female (n=58) zebrafish.
3.3.2 *PtK7* mutant zebrafish do not display vertebral malformations

To further define the nature of the axial curvature phenotype in adult *ptk7* mutant zebrafish, I decided to directly visualize vertebral formation in *ptk7* mutant and control animals. I used calcein (a vital, fluorescent calcium-binding chromophore\(^{397}\)) to label the developing skeleton during larval stages. I did not observe any differences in vertebral patterning or formation between *ptk7* mutant and control larvae up to 7.8 mm standard length (approximately 20 dpf) \((n=73, \text{Figure 3.3.2a-f}).\) Mineralized vertebrae formed sequentially from anterior to posterior starting at about 4 mm (approximately 6 dpf) (Figure 3.3.2a,b) and were fully formed, with dorsal and ventral spines, by 7.8 mm standard length (Figure 3.3.2e,f).

To analyze adult vertebrae at high resolution, *ptk7* mutant and control zebrafish were imaged using high-resolution micron-scale computed tomography (microCT) at The Centre for Phenogenomics (Toronto, Ontario). Using this microCT data, I generated three-dimensional skeletal reconstructions of twelve *ptk7* mutant adults and three *ptk7* heterozygous siblings (Figure 3.3.2g-k). These images provided high-resolution evidence of the spinal deformities typical in *ptk7* mutant zebrafish and confirmed that overall, vertebral malformations are not associated with spinal curvature in *ptk7* mutants (Figure 3.3.2l,m). I observed isolated vertebral fusions in three *ptk7* mutant zebrafish that were localized to the caudal tail (Figure 3.3.2n); however, this occurred in a minority of fish and was not associated with anterior curvature in these isolated individuals.

Asymmetric or “wedge-shaped” vertebral distortions are a general aspect of structural curves in human AIS that occur secondary to abnormal force distribution on vertebral bodies\(^{451,452}\). To analyze individual vertebral morphologies in *ptk7* mutant zebrafish, I used microCT reconstructions to measure the dorsal, ventral, left and right length dimensions for each vertebral body and quantified dorsal:ventral and left:right aspect ratios. Ratios greater or lesser than one were representative of vertebral shape distortions and corresponded to the shape of the spinal curvature in the fish (Figure 3.3.2o,p). The affected vertebrae were found in the vicinity of the curve apex and occurred along the entire length of the trunk and tail (Figure 3.3.2o,p), which revealed no strong positional bias in the localization of spinal curvature in *ptk7* mutant zebrafish.
Figure 3.3.2 Ptk7 mutants do not display vertebral malformations but do display vertebral shape distortions that correspond to curve shape. (a-f) Live calcein staining of (a,b) 4 mm (c,d) 4.5 mm and (e,f) 6.5 mm (a,c,e) ptk7 heterozygous (ptk7/*+) and (b,d,f) zygotic ptk7 mutant larvae. All scale bars equal 0.5mm. (g-k) Lateral and (g’-k’) dorsal views of three-dimesional microCT renderings of adult (g) ptk7 heterozygous (ptk7/*+) as well as (h) type 1, (i) type 2, (j) type 3, and (k) type 4 ptk7 mutant zebrafish. (l-n) Lateral views of caudal vertebrae from three-dimensional microCT renderings of adult (l) ptk7 heterozygous (ptk7/*+) and (m,n) ptk7 mutant zebrafish. Asterisk highlights one isolated vertebral fusion found in ptk7 mutant zebrafish. Ptk7 mutant vertebrae display a wedge-shaped morphology at the apex of curves. (o,p) Graphs depicting dorsal:ventral and left:right length ratios of trunk and tail vertebrae in (o) ptk7 heterozygous (ptk7/*+, n=3) and (p) ptk7 mutant zebrafish (n=12). Horizontal dotted lines demarcate arbitrary ±0.1, determined by the maximum deviation from 1 in ptk7/*+ controls and used to illustrate the deviation of ptk7 vertebrae from normal. Deviation from 1±0.1 is evident at multiple vertebrae in three dimensions along the entire length on the spine. Type 1-4 ptk7 mutants are grouped by point color, and each individual fish is represented by a unique point shape and color. The graph’s horizontal axis spans anterior (A) to posterior (P), from the first abdominal vertebrae to the last caudal vertebrae. The Weberian and caudal tail vertebrae were excluded from the analysis.
3.3.3 Human PTK7 and scoliosis

To investigate whether PTK7 contributes to human IS, Dr. Carol Wise (Sarah M. and Charles E. Seay Center for Musculoskeletal Research, Texas Scottish Rite Hospital for Children) sequenced the PTK7 coding exons of 96 human IS samples and identified 4 distinct missense mutations (Table 3.3.3). Two SNPs (rs34021075 and rs34865794) were genotyped in a follow-up cohort of 736 non-Hispanic white (NHW) AIS cases and 740 NHW controls, but neither SNP was significantly associated with IS (p=0.086 and p=0.313). A novel PTK7 sequence variant in exon 11 was identified in one patient, predicting a proline to alanine substitution (PTK7P545A) within an evolutionarily conserved residue of the sixth extracellular Ig loop. This rare variant was interesting because my previous analysis found that the extracellular domain of zebrafish Ptk7 is essential for Ptk7-mediated Wnt signal transduction.

I cloned PTK7P545A using human PTK7 (MGC clone 30347652) as a template and inserted both wild-type PTK7 and PTK7P545A into zebrafish pCS2+ expression vectors. I made mRNA and injected equal amounts into one-cell stage embryos. In western blots of whole embryo lysates at early gastrula stages, I observed that PTK7P545A accumulated at greater levels than wild-type PTK7 and ran higher on an SDS-PAGE gel (n=3, Figure 3.3.3a), suggesting increased stability and differential modification of PTK7P545A protein. I quantified PTK7 protein normalized to actin during gastrulation and confirmed a significant increase in PTK7P545A protein relative to wild-type control (P=0.0036, Figure 3.3.3b). Using confocal microscopy, I found that plasma membrane localization of YFP-tagged PTK7P545A was increased compared to wild-type PTK7 (normalized to co-injected membrane-localized mRFP, Figure 3.3.3c-h), suggesting that the P545A variation affects trafficking and/or turn-over of PTK7 protein at the plasma membrane.

To determine the functional consequence of the PTK7P545A mutation in vivo, I compared the activity of both wild-type and mutant alleles following mRNA injection into zebrafish embryos. Injection of 100pg of PTK7 mRNA into one-cell staged MZptk7 embryos significantly rescued the PCP-mediated axial extension defects of mutant embryos; however, injection of equal amounts of PTK7P545A mRNA had no effect (Figure 3.3.3i-k). I also tested the ability of PTK7P545A to induce Ptk7 overexpression defects like cyclopia in wild-type embryos (Figure 2.3.10). Injection of PTK7P545A mRNA did not induce PCP defects like cyclopia to the same extent as PTK7 in wild-type embryos (Figure 3.3.3l-n), indicating that the P545A variant
disrupts non-canonical Wnt/PCP signaling activity. Finally, I found that $PTK7^{P545A}$ expression was unable to rescue the forebrain and eye-field defects associated with $wnt8$ overexpression (Figure 3.3.3o-r). This indicated that the P545A variant also disrupted the inhibitory activity of PTK7 on canonical Wnt/β-catenin signaling. Together, these data suggest that PTK7 may contribute to IS pathogenesis through Wnt signal transduction.
Table 3.3 PTK7 variants in IS samples. PTK7 was sequenced in 96 IS case samples (Carol Wise). Results are shown as allele frequencies alongside public results. NHLBI-ESP = National Heart Lung Blood Institute Exome Sequencing Project; EA = European American; AA = African American.

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<td>A/G</td>
<td>missense</td>
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<td>1038</td>
<td>3113</td>
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<td>310/4406</td>
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The P545A variant affects PTK7 protein stability, trafficking and Wnt signaling activity. (a) Western blot analysis of human PTK7 in total cell lysates made from gastrula stage (shield, 6 hpf) wild-type (WT) embryos that were injected with 250pg of either wild-type \textit{PTK7} or \textit{PTK7}^{P545A} mRNA. Actin is used as a loading control. Anti-human PTK7 recognizes PTK7^{P545A} and fails to cross react with zebrafish Ptk7 (uninjected control). (b) Quantification of relative PTK7^{P545A} protein compared to wild-type PTK7 (n=3, **p=0.0036). Protein levels were normalized to actin and wild-type PTK7 was set to 1. (c-h) Confocal images of the animal cap of embryos at 60% epiboly co-injected at the one cell stage with membrane-localized monomeric RFP (membrane RFP)\textsuperscript{434} and either YFP-tagged \textit{PTK7} (250pg) or \textit{PTK7}^{P545A} (250pg) mRNA. Co-expressed (c) membrane RFP and (d-e) YFP-PTK7 visualized at (d) low (720V) and (e) high (920V) intensity. Co-expressed (f) membraneRFP and (g) YFP-PTK7^{P545A} at low (720V) and (g) high (920V) intensity. At high (920V) intensity the YFP-PTK7^{P545A} was above saturation. YFP-PTK7^{P545A} is enriched at the plasma membrane compared to YFP-PTK7. Lateral views of 24 hpf (i) MZ\textit{ptk7} and (j) MZ\textit{ptk7} embryos injected with \textit{PTK7} (100pg) mRNA. Numerical values represent average membrane YFP intensity normalized to membrane RFP fluorescent intensity (n=30 cells for each sample). (k) Quantification of tail-length (mm) at 24hpf measured from the base of the yolk extension to the tail tip in MZ\textit{ptk7} mutant embryos or MZ\textit{ptk7} mutant embryos injected with either \textit{PTK7} (100pg) or \textit{PTK7}^{P545A} (100pg) mRNA. PTK7 can rescue the tail extension defect in MZ\textit{ptk7} (**p<0.0001, Student’s t-test). 100pg of \textit{PTK7}^{P545A} mRNA has no effect on axial extension, suggesting reduced activity of PTK7^{P545A} in the PCP signaling pathway. (l,m) Anterior ventral views of 48 hpf (l) wild-type (WT) and (m) \textit{PTK7} (300pg) mRNA-injected embryos. \textit{PTK7} overexpression induces cyclopia. (n) Quantification of cyclopia phenotype in wild-type (WT) embryos and wild-type (WT) embryos injected with \textit{PTK7} (300pg) or \textit{PTK7}^{P545A} (300pg) mRNA. (o-q) Lateral views of 48 hpf (o) wild-type (WT) embryos and embryos injected with (p) \textit{wnt8} (10pg) or (o) \textit{wnt8} (10pg) plus \textit{PTK7} (250pg) mRNA. Overexpression of \textit{wnt8} disrupts CNS pattern, as demonstrated by loss of eyes and reduced forebrain. Co-injection of \textit{PTK7} (250pg) mRNA can rescue \textit{wnt8}-induced phenotypes. (r) Quantification of phenotypes observed with injection of \textit{wnt8} (10pg) mRNA, and with co-injection of \textit{wnt8} with \textit{PTK7} (250pg) or \textit{PTK7}^{P545A} (250pg) mRNA. Embryos were scored as being ‘eyeless’ only if the eye was completely absent. \textit{PTK7}^{P545A} is less efficient than \textit{PTK7} at rescuing \textit{wnt8}-induced phenotypes, indicating reduced activity of \textit{PTK7}^{P545A} in Wnt/β-catenin inhibition.
3.3.4 MZptk7 mutants display vertebral patterning defects

To better understand the role for ptk7 in spinal morphogenesis, I examined maternal-zygotic ptk7 (MZptk7) mutant zebrafish, which represent an early and complete loss-of-function ptk7 mutant model. Unexpectedly, MZptk7 larvae displayed a very different phenotype than that described for zygotic ptk7 mutants.

Using calcein staining, I observed severe skeletal abnormalities in the majority of MZptk7 mutant larvae (Figure 3.3.4a-i). MZptk7 mutants displayed an initial delay in the onset of vertebral mineralization (100%, n=8; Figure 3.3.4a,b), while at approximately 14 dpf, MZptk7 larvae displayed severe vertebral malformations that included missing or smaller vertebral segments, hemivertebrae and/or vertebral fusions (Figure 3.3.4a-f). These malformations were similar to those seen in human CS patients and led me to consider MZptk7 a good representation of CS-like deformities.

Around 2 weeks post fertilization, the majority of MZptk7 mutants died (n=81/90). The few escapers that survived displayed some isolated vertebral malformations that were very mild compared to the representative population. At 20 dpf, two surviving MZptk7 mutant larvae displayed isolated vertebral malformations visualized as shorter or misshapen vertebral bodies immediately adjacent to vertebrae of normal morphology (2/9; Figure 3.3.4h,i). The majority of MZptk7 mutant larvae recovered at this stage did not display vertebral abnormalities; however; all adult MZptk7 mutant zebrafish displayed axial curvatures phenotypically similar to zygotic ptk7 mutant adults (9/9, Figure 2.3.5.2h).

To better understand the role for ptk7 in vertebral patterning, I generated chimeric embryos through transplantation of MZptk7 mutant cells into the ventral domain (future tail bud region) of wild-type embryos (Figure 3.3.4j). I screened transplanted host embryos at 24 hpf and raised chimeric zebrafish with significant MZptk7 cell contribution within the embryonic tail (Figure 3.3.4k). Interestingly, at larval stages I visualized multiple or isolated vertebral malformations in a number of chimeric larvae (n=8/30, Figure 3.3.4l), suggesting that broad tissue loss of ptk7 in the tail is sufficient to disrupt vertebral patterning at later stages of development. Together, these data suggest that early loss of ptk7 function can lead to CS-like vertebral malformations.
Figure 3.3.4 MZptk7 mutant zebrafish display vertebral malformations. Lateral views of 6 dpf (a) wild-type (WT) and (b) MZptk7<sup>lacZ</sup> (MZptk7) mutant larvae fluorescently labeled with calcein (a calcium-binding fluorophore) to visualize to developing skeleton. The yolk typically auto-fluoresces. (c-f) Calcein staining of 12 dpf (d) wild-type (WT) and (d-f) MZptk7 larvae. Insets highlight representative vertebral malformations present in MZptk7 mutants. Asterisks and arrowheads highlight missing and hemivertebrae, respectively. Bracket highlights vertebral fusions. Hashtags highlight smaller vertebral segments. (g-i) Calcein staining of 20 dpf (g) wild-type (WT) and (h,i) MZptk7 mutant larvae. Isolated vertebral malformations are evident in surviving MZptk7 mutants (i, hashtag). (j) Transplant strategy used to generate MZptk7/wild-type chimeric larvae. MZptk7 mutant cells were labeled with rhodamine-red and transplanted at 4 hpf into the tail bud-fated region (V, ventral) of wild-type (WT) hosts at 6 hpf, as previously described<sup>82</sup>. D indicates dorsal. (k) Lateral view of 24 hpf chimeric embryo, where MZptk7 mutant cells (fluorescent rhodamine-red) are present in the tail of an otherwise wild-type (WT) host embryo. (l) Lateral view of caudal vertebrae of chimeric larvae at 21 dpf. MZptk7 cell contribution to the tail leads to vertebral malformations in otherwise wild-type larvae. Horizontal bracket highlights the affected region where multiple vertebral malformations including smaller segments and mispaterned vertebral spines (asterisks) are visible.
3.3.5 MZptk7 mutant embryos display defects in axial segmentation

Patterning of the zebrafish spine is derived from the segmented organization of the notochord and the periodic organization of the somites from the presomitic mesoderm (PSM) in response to an internal “segmentation clock”249,281. In zebrafish, the vertebral centra initially form through direct mineralization of the notochord, while structures associated with the vertebrae like the hemal and neural arches are derived from the sclerotome (somite derived)280,281. Therefore, patterning abnormalities of the vertebral centra in MZptk7 (Figure 3.3.4) are indicative of defects in notochord segmentation, whereas mispattering of the hemal and neural arches (Figure 3.3.4l) suggests a more broad segmentation defect that includes abnormalities in somite patterning.

To determine if somitogenesis is disrupted in MZptk7 mutant embryos, I performed WISH of her7 and dlc, direct reporters of the Notch segmentation mechanism within the PSM252. During early somitogenesis (10-12 somite stage), her7 and dlc expression was expanded along the AP axis, consistent with my earlier observation that the PSM is expanded in MZptk7 mutant embryos (Figure 3.3.5a,b). Expression was also mediolaterally expanded, consistent with previously characterized defects in C&E movements (Figure 3.3.5a,b). Cyclic gene expression appeared normal in MZptk7 mutants compared to wild-type with periodic “waves” of her7 and dlc gene expression spreading along the length of the PSM (n=25, n=12; Figure 3.3.5a,b). Furthermore, I used qRT-PCR and found that her7 and dlc gene expression levels were not significantly affected in MZptk7 mutants relative to wild-type (Figure 3.3.5d). I also found that rippy1, a marker of posterior somite identity, was expressed in a segmental pattern in MZptk7 mutant embryos (Figure 3.3.5c), suggesting that the somites were effectively polarized along the AP axis.

At later somite stages (18-20 somite stage), I observed morphological abnormalities in the expression domains of her7 and dlc as well as mild asymmetries; however, cyclic gene expression was still maintained and both her7 and dlc were expressed at levels comparable to wild-type controls (Figure 3.3.5e,f). Therefore, MZptk7 mutants differ from mutants/morphants of classic regulators of somite patterning, in which the cyclic and/or symmetric pattern of gene expression is grossly disrupted453-456, however, obvious abnormalities in the morphology of the PSM may ultimately affect both somite and subsequent vertebral patterning.
To directly assess somite patterning in MZptk7, I used WISH and markers of somite boundaries and detected severe defects in the regular spacing and separation of the somites in MZptk7 mutant embryos during somitogenesis (100%, n=12; Figure 3.3.5g,h) and at 40 hpf (100%, n=17; Figure 3.3.5i-k). These results suggested that defects in somite segmentation may contribute to the vertebral malformations in MZptk7 mutants.
Figure 3.3.5 MZptk7 mutant embryos display segmentation defects. (a-c) WISH of somite patterning gene expression at the 10-12 somite stage. All images are posterior views with the embryonic tail bud pointing towards the bottom of the field of view. Multiple phases of (a) her7 and (b) dlc expression are represented for both wild-type (WT) and MZptk7 (MZ) mutant embryos. (c) ripply1 expression in wild-type (WT) and MZptk7 (MZ) mutant embryos. The expression domains of all three markers are medio-laterally expanded in MZptk7 mutants consistent with C&E defects. (d) qRT-PCR of her7 (P=0.426) and dlc (P=0.436) expression levels in 10-12 somite stage MZptk7 mutant embryos relative to wild-type (WT). Graph represents average fold change across three independent experiments with three technical replicates each. Error bars represent s.d. (e,f) WISH of (e) her7 and (f) dlc expression at the 18-20 somite stage. Multiple phases of gene expression are represented for both wild-type (WT) and MZptk7 (MZ) mutant embryos. Morphological differences and mild asymmetric gene expression (white asterix) are evident in MZptk7. (g,h) Flat-mounts of 10-12 somite stage (g) wild-type (WT) and (h) MZptk7 embryos stained for krox20 (hind-brain) and myoD (somite) expression to visualize axial segmentation. MZptk7 display defects in the regular spacing and morphology of the somites at this stage. Vertical bracket highlights abnormally spaced segments. Asterisk highlights fused segments. (i-k) Lateral view of WISH for Xirp2a expression to mark somite boundaries in (i) wild-type (WT) and (j,k) MZptk7 mutant embryos at 40hpf. Incomplete somite boundary formation is evident in MZptk7 mutant embryos as well as abnormal spacing and morphology of somites. The horizontal brackets highlight somite fusions and the asterisks highlight examples of incomplete somite border formation. Scale bars equal 100µm.
3.3.6 Manipulation of PCP signaling leads to defects in vertebral segmentation

*Ptk7* regulates both noncanonical Wnt/PCP and canonical Wnt/β-catenin signal transduction and MZ*ptk7* mutant embryos display defects in PCP-mediated morphogenesis as well as activated Wnt/β-catenin signaling within the embryonic tail bud. Therefore, I assessed whether deregulated Wnt signaling could contribute to vertebral malformations in zebrafish.

To analyze the effect of misregulated Wnt/PCP signaling, I examined segmentation in a mutant for a specific PCP factor, *vangl2*, as well as in embryos injected at the one-cell stage with a dominant-negative form of Dsh that disrupts PCP signaling (Xdd1)\(^9\). Using WISH to mark somite boundaries at 40hpf, I found that disruption of PCP signaling resulted in defects in the regular spacing and separation of the somites, similar to what I observed in MZ*p tk7* mutant embryos (*vangl2* \(n=8/8\) and Xdd1 \(n=15/18\); Figure 3.3.6a-d). This result revealed a previously undescribed role for PCP signaling during embryonic segmentation.

To analyze gene patterning during somitogenesis in *vangl2* mutant embryos, I performed WISH for *her7*, *dlc*, and *ripply1* gene expression. Like MZ*p tk7* mutant embryos, *her7* and *dlc* were expressed at normal levels within the PSM in *vangl2* mutants \((n=10\) and \(n=13\), respectively; Figure 3.3.6e-h) and *ripply1* is expressed in a defined segmental pattern (Figure 3.3.6i,j), indicating normal AP somite patterning in a specific loss-of-function PCP mutant.

*Vangl2* mutant or *Xdd1* mRNA-injected zebrafish are embryonic lethal. Therefore, in order to analyze the effect of disrupted PCP signaling on vertebral patterning during larval stages, I transplanted MZ*vangl2* or *Xdd1* mRNA-injected cells into the tail buds of wild-type hosts. At larval stages, I found that both MZ*vangl2* mutant and *Xdd1* mRNA-injected/wild-type chimeras \((n=8/12\) and \(9/16\), respectively) displayed vertebral malformations including smaller vertebral segments, missing and/or hemivertebrae, analogous to what I observed in MZ*p tk7* mutant and MZ*p tk7*/wild-type chimeric larvae (Figure 3.3.6k-n). These results indicate that disrupted PCP signaling can induce vertebral segmentation defects, which suggests that PCP signaling plays an important role in vertebral patterning during development.
Figure 3.3.6 Abnormal non-canonical Wnt/PCP signaling leads to defects in vertebral segmentation. (a-d) Lateral view of WISH for Xirp2a expression to mark somite boundaries\textsuperscript{457} in (a,b) vangl2 mutant embryos and (c,d) embryos injected with Xdd1 (150pg) mRNA. Asterisks highlight examples of incomplete somite boundary formation. Scale bars equal 100µm. Disruption of non-canonical Wnt/PCP signaling results in somite fusions as well as abnormalities is the spacing and morphology of somites similar to MZptk7. (e-j) WISH of somite patterning gene expression at the 10-12 somite stage. All images are posterior views with the embryonic tail bud pointing towards the bottom of the field of view. (e,g,i) vangl2 heterozygous (vangl2/+) and (f,h,j) vangl2 mutant embryos stained for (e,f) her7, (g,h) dlc, and (i,j) ripply1 expression. Ripply1 expression in the posterior somites is segmented, indicating that anterior-posterior somite patterning is established in vangl2 mutants. The expression domains of all three markers are mediolaterally expanded in vangl2 mutants consistent with C&E defects. (k-n) Lateral views of live calcein stained mutant/wild-type (WT) chimeric larvae that were pre-screened for (k,l) MZvangl2 mutant cells or (m,n) cells injected with Xdd1 (150pg) mRNA in the embryonic tail. Donor cells were transplanted into the ventral margin (future tail bud) of WT host embryos as previously described\textsuperscript{82}. White brackets highlight the affected regions.
3.3.7 Activation of Wnt/β-catenin signaling affects vertebral segmentation

MZptk7 mutant embryos displayed activation of Wnt/β-catenin signaling in the embryonic tail bud. Therefore, to test the effect of activated Wnt/β-catenin signaling on vertebral segmentation, I treated embryos for short intervals during somitogenesis with two GSK3β antagonists, lithium chloride (LiCl)\(^{458}\) and 6-bromoidirubin-3’-oxime (BIO)\(^{459}\). Using WISH to mark somite boundaries at 40hpf, I found that LiCl and BIO treated embryos displayed fused somites as well as abnormal somite shape similar to MZptk7 (Figure 3.3.7a,b). I also used a heat-shock inducible wnt8 transgenic zebrafish line (Tg(hs:Wnt8GFP)) to activate Wnt/β-catenin signaling during somitogenesis. At 48 hpf, I observed similar somite segmentation defects in heat-shocked embryos, including somite fusions and somite shape distortions (Figure 3.3.7c,d), confirming that activation of Wnt/β-catenin signaling affects somite patterning.

Induced wnt8 expression during somitogenesis led to embryonic AP patterning defects, which precluded larval-stage analysis; however, careful titrations of drug allowed me treat embryos during somitogenesis and grow drug treated fish until larval stages. I observed multiple vertebral malformations in drug treated larvae including smaller segments and vertebral fusions (Figure 3.3.7e,f), indicating that activated Wnt signaling affects vertebral patterning. These vertebral malformations were analogous to what I observed in MZptk7 mutants, suggesting that both Wnt/PCP and Wnt/β-catenin signaling affects segmentation and contributes to vertebral malformations in MZptk7 mutant larvae.
Figure 3.3.7 Activated Wnt/β-catenin signaling leads to defects in vertebral segmentation. (a,b) Lateral view of WISH at 40 hpf for Xirp2a expression to mark somite boarders of embryos treated during somitogenesis with canonical Wnt/β-catenin agonists (a) LiCl (0.1M) or (b) BIO (10µM). Brackets highlight segmental fusions. Scale bars equal 100µm. (c,d) Lateral views of 48 hpf Tg(hs:Wnt8GFP) embryo heat-shocked at the 10-12 somite stage. Brackets highlight segmentation defects including fusions and somite shape distortions (somites appear rounded). (e,f) Lateral views of live calcein staining in wild-type larvae treated during somitogenesis with (e) LiCl (10µM) or (f) BIO (1µM). Activation of canonical Wnt/β-catenin signaling led to vertebral abnormalities phenotypically similar to MZptk7 loss-of-function including smaller vertebral segments (white asterisk) and/or vertebral fusions (white bracket).
3.3.8 *Ptk7* mutant embryos do not display defects in somite patterning

*MZptk7* mutant larvae display defects in axial segmentation that likely underlie the vertebral abnormalities observed during later larval stages. Conversely, *zygotic ptk7* mutant zebrafish display a late onset spinal curvature phenotype that is not associated with vertebral malformations. To determine if subtle defects in somitogenesis underlie the *ptk7* phenotype, I performed WISH of somite patterning genes *her7, dlc* and *ripply1*. I did not observe any consistent differences in gene expression patterns or PSM morphology in *ptk7* mutant embryos compared to *ptk7/+* controls (Figure 3.3.8a-c), indicating that, overall, somitogenesis occurs normally in *ptk7* mutant embryos. To assess somite patterning and morphology in *ptk7* mutant embryos, I used WISH to mark somite boundaries and observed no defect in regular spacing and/or morphology of somites in *ptk7* mutants compared to heterozygous controls (Figure 3.3.8d-f).

Maternal Ptk7 rescue of axial segmentation in *ptk7* mutant zebrafish suggests that the late onset spinal curvatures in zygotic loss-of-function mutants are the result of a different biological mechanism. Thus, *MZptk7* and *zygotic ptk7* mutants model two different types of spinal deformity depending on the timing of loss of *ptk7* gene function (CS and IS, respectively), and point to a genetic link between the two human diseases even though the biological basis may differ with respect to the etiopathogenesis.
Figure 3.3.8 *Ptk7* mutants do not display segmentation defects. (a-c) WISH of somite patterning gene expression at the 18-20 somite stage. All images are posterior views with the embryonic tail bud pointing towards the bottom of the field of view. Multiple phases of (a) *her7* and (b) *dlc* expression are represented for both *ptk7* heterozygous (*ptk7/*+) and *ptk7* mutant embryos. (c) *ripply1* expression at 12-14 somite stage in *ptk7* heterozygous (*ptk7/*+) and *ptk7* mutant embryos. (d,e) Flat-mounts of 12-14 somite stage (e) *ptk7* heterozygous (*ptk7/+*) and (f) *ptk7* mutant embryos stained for *myoD* (somite) expression to visualize segmentation during somitogenesis. *Ptk7* mutants do not display defects in the regular spacing and morphology of the somites at this stage. (f,g) Lateral views of WISH for *Xirp2a* expression to mark somite boundaries in (f) *ptk7* heterozygous (*ptk7/+*) and (g) *ptk7* mutant embryos at 40hpf. No obvious defects in somite patterning or morphology are evident in *ptk7* mutant embryos.
3.4 Discussion

As a result of unexpected phenotypes, I characterized spinal deformities in both zygotic and maternal-zygotic (MZ) loss-of-function *ptk7* mutant zebrafish. In zebrafish, maternal gene products supplied to the egg have been shown to drive early patterning and morphogenesis\(^{460}\), making maternal contribution an important consideration when studying embryonic gene function. In the previous chapter, I characterized the role for maternal *ptk7* in early embryonic development and described how zygotic *ptk7* mutant embryos were phenotypically normal due to maternal rescue of the embryonic defects. Since zygotic *ptk7* mutants were viable, I was able to study larval, juvenile and adult mutant phenotypes and uncover an important role for Ptk7 during later stage spinal morphogenesis. Interestingly, I observed three isolated vertebral fusions in zygotic *ptk7* mutant zebrafish (3/12; Figure 3.3.2n) that were localized to caudal tail vertebrae. This likely reflects an eventual loss of maternal gene product during later stage patterning of posterior axial segments and further illustrates the differential roles for maternal versus zygotic *ptk7* during axial segmentation and spinal morphogenesis (Figure 3.4.1).

Zygotic *ptk7* mutant zebrafish lack obvious embryological defects, but develop late onset three-dimensional spinal curvatures that recapitulate human IS. Like IS patients, *ptk7* mutants do not display underlying vertebral malformations or any other identifiable primary defect, but do exhibit spinal curves of variable magnitude that have a female bias in their severity. Characteristic asymmetrical vertebral shape distortions occur along the spine and correspond to curve shape; however, unlike other teleost IS models\(^{395}\), *ptk7* mutants display no strong bias in the shape or position of these deformities, making *ptk7* mutants a more faithful representation of all the curve types found in IS populations. Therefore, in this project, I have characterized a genetically defined animal model of IS, linking Ptk7 to curve susceptibility in zebrafish and highlighting the potential for *ptk7* mutants as a candidate genetic model for future studies of IS pathogenesis.

Phenotypic variability and genetic heterogeneity have made it difficult to identify causative genes for late onset IS in human populations. Few genetic animal models that effectively model IS have been identified, which has made IS pathogenesis difficult to study in the lab. Some models reveal a potential for using fish; however, to date no candidate genes have been characterized that truly recapitulate human IS\(^{390,461}\).
Figure 3.4.1 *Ptk7* mutants model two different types of spinal deformity depending on timing of gene loss-of-function. Maternal *ptk7* is deposited in the egg and drives early development including Ptk7-dependent morphogenesis and segment patterning. Solid blue line represents *ptk7* mRNA and dotted blue line represents Ptk7 protein, which are sufficient to rescue early developmental defects in zygotic *ptk7* mutant zebrafish. Loss of maternal and zygotic *ptk7* (MZ*ptk7*) results in segmentation defects and vertebral malformations that recapitulates human CS. Zygotic loss of *ptk7* leads to late onset spinal curvature with no underlying vertebral malformations that recapitulates human IS.
CHD7, a chromodomain helicase DNA-binding protein, was associated with idiopathic scoliosis through linkage association\textsuperscript{403}. MO-mediated knock-down of zebrafish chd7 led to abnormalities in vertebral segmentation and mineralization\textsuperscript{404,405}, illustrative of a CS-like deformity. Similarly, MO knock-down of zebrafish gpr126, a gene associated with AIS in multiple human populations, led to shorter larvae and delayed vertebral ossification at 14 dpf\textsuperscript{302}, again revealing a CS-like model. The difficulty regarding MO knock-down and late stage analysis is that MOs only have short-term effects in the developing zebrafish embryo and any skeletal abnormalities observed are likely secondary to earlier stage embryological defects, which may not recapitulate IS pathogenesis. Therefore, to study candidate genes, the use of genetic loss or gain-of-function models is important. Improvements in genetic technologies for zebrafish should make this type of analysis more realistic in the future. Also, the observation that scoliosis readily develops in ptk7 mutant zebrafish suggests that forward genetic screens could also be used to identify novel susceptibility or modifying factors not yet explored in human populations.

MZptk7 and ptk7 mutant zebrafish display distinct spinal deformities that model CS and IS, respectively. Independent studies have reported an increased incidence of IS in the family members of individuals with CS\textsuperscript{406,450}, suggesting that certain genetic defects may predispose families to different types of spinal deformity. My results suggest that genetic factors involved in Wnt signaling may be involved in both CS and IS; whole exome sequencing of these types of families will be a useful way to identify possible similarities between two forms of debilitating human disease.

MZptk7 mutant zebrafish, models of disrupted PCP signaling (vangl2 and Xdd1) and models of activated Wnt/\(\beta\)-catenin signaling (Wnt8, LiCl, BIO), suggest that multiple arms of the Wnt signaling pathway influence vertebral segmentation and may affect CS pathology. Mutations in Wnt/\(\beta\)-catenin target genes like tbx6 were previously identified in CS patients\textsuperscript{275}, while multiple mouse models of deregulated Wnt signaling display severe segmentation defects\textsuperscript{95,255,261}, supporting a role for Wnt/\(\beta\)-catenin signaling in axial patterning. However, my analysis suggests a previously undescribed role for Wnt/PCP signaling in vertebral segmentation. A previous report suggests that somitogenesis is unaffected in PCP mutant zebrafish\textsuperscript{462}; however, I found that somite segmentation is grossly affected in vangl2 mutant embryos as well as in embryos over-expressing a dominant negative form of Dsh, Xdd1\textsuperscript{199}. PCP-dependent C\&E defects have previously been shown to affect patterning of axial and paraxial tissues\textsuperscript{463} and neural tube disorders (NTDs) have been shown to affect formation of surrounding vertebrae\textsuperscript{156,160}.
Therefore, it is possible that PCP-related C&E and/or neural tube defects indirectly influence segment patterning. Also, PCP-related morphological abnormalities (mediolateral expansion and AP compression) within the PSM may affect how the tissue experiences posterior Wnt morphogen gradients, leading to differential activation of the Wnt/β-catenin signaling pathway and gene patterning within the PSM. Similarities and differences between the Wnt/PCP and Wnt/β-catenin segmentation phenotypes will help uncover the relationship between two Wnt pathways and vertebral patterning throughout development.

Dr. Carol Wise identified a rare sequence variant in one AIS patient, which suggests that PTK7 may also contribute to IS in humans. This allele was also present in one parent of the proband, which may reflect variable phenotypic penetrance and/or other genetic and environmental influences. This is further evidenced by the fact that overexpression of PTK7<sup>P545A</sup> in wild-type zebrafish embryos did not induce embryological phenotypes reminiscent of MZptk7, suggesting that the P545A variant does not confer dominant negative activity. Rather, my results suggest that the P545A variant is a hypomorphic allele of PTK7 and increased exogenous protein at the plasma membrane may indicate that PTK7<sup>P545A</sup> is not trafficked effectively. Since endocytosis and turnover of Wnt-Fz complexes is an essential component of Wnt signal transduction<sup>44,464</sup>, further analysis of the P545A allele may help define the mechanistic role for Ptk7 in Wnt signal transduction. Ultimately, I believe that this work highlights the utility of zebrafish embryogenesis as a functional model to test disease associated PTK7 alleles <i>in vivo</i> and, in the future, large scale screening of PTK7 may further implicate PTK7 in IS pathogenesis in humans.

Thus, I have demonstrated that MZptk7 and ptk7 mutant zebrafish develop two distinct spinal deformities that model CS and IS, respectively. This suggests that two different types of spinal curvature may have a common genetic basis. By using ptk7 mutant zebrafish models, we hope to further define scoliosis disease pathogenesis and understand how spinal curvatures develop in humans.
Chapter 4

4. DISCUSSION AND FUTURE DIRECTIONS

4.1 Summary of Principal Findings

The main objective of my thesis was to characterize the function of Ptk7, a novel regulator of PCP signaling. Lu et al. (2004) found that Ptk7 mutant mice displayed developmental defects indicative of abnormalities in PCP signaling; however, the mechanistic implications of loss of Ptk7 were initially investigated, nor were potential roles for Ptk7 in alternative Wnt signaling pathways. Mass spectrometry data from Dr. Stephane Angers (Department of Pharmacy, University of Toronto) suggested that PTK7 interacted with the Wnt receptor Fz7 (unpublished). Based on these data, I decided to characterize the role of Ptk7 in both non-canonical Wnt/PCP and canonical Wnt/β-catenin signaling pathways during vertebrate embryogenesis using zebrafish as a model. I was able to define Ptk7 as an important potentiator of PCP signaling and as an inhibitor of Wnt/β-catenin signaling in the embryonic tail bud. Morpholino (MO)-based data from Xenopus was conflicting; however, through characterization of a complete loss-of-function genetic zebrafish model, I provided definitive evidence regarding Ptk7’s role throughout vertebrate embryogenesis. This work will contribute towards the understanding of Ptk7 and Wnt signaling pathways, which has important implications for human development and disease.

Maternal ptk7 gene product was sufficient to rescue the embryonic phenotypes that I observed in complete loss-of-function maternal zygotic (MZ) ptk7 mutant embryos. Zygotic ptk7 mutant zebrafish were completely viable; however, ptk7 mutant zebrafish developed severe forms of late onset spinal curvature. Therefore, using these mutants, I characterized what I believe to be an important genetic model of human idiopathic scoliosis (IS). Unlike their zygotic mutant counterparts, MZptk7 mutant zebrafish displayed vertebral abnormalities analogous to those in human patients with congenital scoliosis (CS). MZptk7 mutants displayed defects in embryonic segmentation, revealing an early role for ptk7 during axial patterning that I found may be linked to deregulated Wnt signaling. Using MZptk7 and ptk7 mutant zebrafish, I characterized two novel models of human CS and IS, respectively, and suggest that disrupted Wnt signaling may contribute to both types of spinal deformity.
4.2 Ptk7 and Wnt signal transduction

Ptk7 has been characterized in multiple contexts as a novel Wnt signaling regulator with roles in both non-canonical Wnt/PCP and canonical Wnt/β-catenin signal transduction. In canonical Wnt signaling, Wnt-Fz-LRP6 complexes recruit Axin away from the destruction complex that promotes proteasomal degradation of the transcriptional activator β-catenin. Non-canonical Wnt/PCP signaling controls cellular polarity through activation of Rho family small GTPases.

PTK7 was identified as a possible interactor of Fz7 in human cell lines (Stephane Angers, unpublished data), suggesting that PTK7 could modify Wnt signaling through interactions with Fz receptor complexes. To analyze Ptk7-Fz7 complex formation during zebrafish gastrulation, I decided to adapt cell lysis and immunoprecipitaion protocols to suit whole zebrafish embryos in order to test co-immunoprecipitation of Ptk7 and Fz7 in vivo. All of my analysis was performed during late gastrula stages (9-10hpf), when I expected both Wnt/PCP and Wnt/β-catenin signaling pathways to be active.

To extract Ptk7 from whole embryo lysates, I fused Ptk7 to affinity purification tags (2xStrepFlag-Ptk7, Ptk7-2xStrepFlag) and expressed these constructs in zebrafish embryos. I confirmed that tagged-Ptk7 was expressed and decided to look at protein modifications such as phosphorylation in purified samples (Figure 4.2.1). Using anti-phosphoserine and anti-phosphotyrosine antibodies, I detected only a weak signal at the molecular weight expected for Ptk7 (Figure 4.2.1), indicating that Ptk7 itself was not readily phosphorylated in vivo. However, several lower molecular weight phospho-proteins were detected by Western blot (Figure 4.2.1b), suggesting that Ptk7 may associate with and/or regulate phosphorylation of other signaling proteins during gastrulation.

To test for a possible biochemical interaction between Ptk7 and Fz receptors in vivo, I attempted to co-immunoprecipitate Ptk7 with Wnt receptor complexes. However, I found that Ptk7 did not readily co-IP with zebrafish Fz7a, even in the presence of Dsh (Figure 4.2.2), which was shown to be required for Fz7-Ptk7 complex formation in Xenopus. Mass spectrometry data from human cells also suggested an interaction between PTK7 and Fz7; however, I was unable to detect Ptk7-Fz7-Dsh complex formation in vivo during zebrafish gastrulation. This could be due to a number of technical complications involved with performing this type of analysis, so whether or not Ptk7-Fz complex formation is a mechanistic aspect of Wnt signal transduction during zebrafish gastrulation remains to be determined.
Biochemical evidence published by other groups suggests that Ptk7 interacts with canonical Wnt/β-catenin factors including LRP6, Wnt3a and Wnt8 ligands$^2,^4$. I found that Ptk7 functionally inhibits Wnt/β-catenin signal transduction during embryogenesis, suggesting that through association with the complex, Ptk7 somehow inhibits Fz-Wnt-LRP5/6 activity. This could be achieved by stabilizing the complex at the plasma membrane, since it is understood that receptor-ligand endocytosis is an essential component of Wnt signal transduction$^6$. Evidence to support this hypothesis revealed that loss of PTK7 decreases LRP6 levels in Xenopus embryos and that PTK7 expression stabilized LRP6$^4$. Ptk7 could function similarly to Dkk1 (a well-studied inhibitor of β-catenin signaling), which prevents LRP6-Fz interactions and endocytosis$^{465}$, thereby freeing Fz to interact with non-canonical Wnt ligands and co-receptors and induce PCP signal activation. Therefore, future experiments could involve analysis of the effect of Ptk7 expression/inhibition on LRP5/6 activation (phosphorylation), LRP 5/6 endocytosis, and/or β-catenin destruction complex dissociation through LRP5/6 recruitment of Axin to the plasma membrane. This could also have implications for cancer biology, where PTK7 is associated with cell invasiveness perhaps by mediating a transition from tumor proliferation to tissue metastasis, which is strongly associated with cancer progression and negative patient outcomes$^{449}$.

Ptk7 may not readily associate with Fz7 during zebrafish gastrulation; therefore, to identify other possible interactors of Ptk7 in vivo, I performed mass spectrometry analysis with Dr. Avais Daulat (Angers lab, Department of Pharmacy, University of Toronto). Affinity purification of zebrafish Ptk7 from embryonic lysates followed by mass spectrometry revealed >110 possible interacting proteins (Table 4.2.1); however, a large number of seemingly non-specific “hits” from our screen were removed, including yolk, histone and other nuclear peptides. This analysis was optimized to detect phosphorylated peptides and we did not detect any evidence of phospho-Ptk7, which supported the described western blot analysis where Ptk7 was not readily phosphorylated in whole zebrafish lysates. Therefore, like all other vertebrate orthologs, zebrafish Ptk7 is likely a pseudokinase.

Ptk7 was not found to interact with any well-known PCP factors; however, associationa with cytoskeletal proteins and cytoskeletal modifiers suggest that Ptk7 might associate with downstream effectors of the PCP pathway to directly modulate the cytoskeleton and induce polarity (Table 4.2.3). Rock2b, a Rho-associated protein kinase, is an interesting candidate for
Further investigation because of a previous report implicating rock2b in polarized cellular morphology within the Kupffer’s vesicle in zebrafish\textsuperscript{466}.

Ptk7 was affinity purified with a cytoskeletal calmodulin RhoGEF (Table 4.2.3), suggesting a possible relationship between Ptk7 and calcium signaling. In an alternative non-canonical Wnt pathway known as Wnt/Ca\textsuperscript{2+} signaling, Wnt ligands increase the frequency of intracellular calcium transients and modulate calcium-sensitive proteins including calmodulin-dependent protein kinase II (CamKII) and protein kinase C (PKC)\textsuperscript{418} (Figure 1.1.1). During development, Ca\textsuperscript{2+} signaling is important for dorsoventral patterning, tissue morphogenesis, the specification of myoblasts, as well as proliferation and differentiation of chondrocytes (reviewed in \textsuperscript{418}). The mechanism through which non-canonical Wnt ligands exert these effects is not well understood\textsuperscript{418}, making the relationship between Ptk7 and Wnt/Ca\textsuperscript{2+} signaling an interesting possibility for future investigation.

Further characterization of these potential interacting partners could provide additional insight into the mechanistic implications of Ptk7 expression on tissue morphogenesis. Interactions with extracellular ECM components and intracellular cytoskeletal effector proteins like Rho-associated kinase 2b could be explored biochemically and in live embryos. Defects in ECM assembly is observed in zebrafish loss-of-function PCP mutants\textsuperscript{188}, therefore, similar analysis could be performed in MZptk7 mutant embryos to determine how direct Ptk7-ECM association could influence ECM integrity and metabolism. Otk, the \textit{Drosophila} homolog of Ptk7 was shown to mediate intercellular adhesion\textsuperscript{228}. Given the functional importance of the Ptk7 extracellular domain and Ptk7-dependent cell non-autonomous regulation of PCP (Figure 2.3.7), the relationship between extracellular Ptk7, cell-cell and ECM-cell interactions could shed light on how Ptk7 relates to cell polarity and directed tissue migration during C&E movements.
Figure 4.2.1 Ptk7 is not readily phosphorylated in vivo. (a) Western blot analysis of immunoprecipitated Flag-tagged Ptk7 expressed in zebrafish embryos. Both N- and C-terminal fusion proteins were detected. (b) Western blot analysis of immunoprecipitated 2xStrepFlag-Ptk7 to detect phosphoserine and phosphotyrosine residues. Asterisks indicate lower molecular weight peptides that may be phosphorylated in complex with Ptk7.
Figure 4.2.2 Ptk7 does not co-immunoprecipitate with Wnt signaling complexes. Immunoprecipitation of Fz7a-myc and Dsh-myc from early gastrula stage (shield stage, 6 hpf) embryos co-injected with fz7a-myc, dsh-myc and 2xStrepFlag-ptk7. 2xStrepFlag-Ptk7 does not co-immunoprecipitate with Fz7a and Dsh.
<table>
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<th>PROTEIN</th>
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<th>COVERAGE (%)</th>
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<td>18</td>
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<td>13</td>
<td>16.5</td>
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<td>17.9</td>
</tr>
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<td>12.1</td>
</tr>
<tr>
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<td>9</td>
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</tr>
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<td>1</td>
<td>11</td>
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<td>7</td>
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<td>22</td>
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<td>5</td>
<td>15.2</td>
</tr>
<tr>
<td>syk Spleen protein tyrosine kinase</td>
<td>1</td>
<td>6</td>
<td>3.4</td>
</tr>
<tr>
<td>Muscle coflin 2</td>
<td>2</td>
<td>4</td>
<td>16.4</td>
</tr>
<tr>
<td>gstpl glutathione S-transferase pi</td>
<td>2</td>
<td>4</td>
<td>11.5</td>
</tr>
<tr>
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<td>3</td>
<td>4.9</td>
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<td>4</td>
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</tr>
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<td>3</td>
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<td>2.8</td>
</tr>
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<td>2</td>
<td>32.7</td>
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<td>2</td>
<td>10.4</td>
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<tr>
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<td>2</td>
<td>3.0</td>
</tr>
<tr>
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<td>2</td>
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</tr>
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<td>zgc:76887, similar to protein phosphatase 2</td>
<td>1</td>
<td>1</td>
<td>3.7</td>
</tr>
<tr>
<td>zgc:162322 (cadherin domains)</td>
<td>2</td>
<td>2</td>
<td>4.5</td>
</tr>
<tr>
<td>eldne Claudin e</td>
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</tbody>
</table>
4.3 Characterization of the etiopathogenesis of scoliosis in *ptk7* mutant zebrafish

Human and animal studies have led to diverse theories regarding the etiopathogenesis of IS. *Ptk7* mutant zebrafish readily develop three-dimensional spinal curvatures phenotypically similar to human IS, implicating *ptk7* as a scoliosis susceptibility gene in zebrafish. In the future, defining the pathogenesis of scoliosis in *ptk7* mutant zebrafish may help us understand IS progression in human patients.

Anatomical and/or functional abnormalities within the CNS are thought to contribute to spinal curvature in certain cases of human IS\(^301\). To test whether wild-type anterior CNS tissue could rescue *ptk7* scoliosis, I transplanted wild-type cells into the region fated to be the anterior CNS (the animal cap) of *ptk7* mutant embryos (Figure 4.3.1). I screened embryos at 24hpf for significant wild-type cell contribution within the head (Figure 4.3.1), and raised these chimeric animals to adult stages. All surviving wild-type/*ptk7* mutant chimeras developed moderate to severe spinal curvatures (n=12), suggesting that wild-type CNS is not sufficient to rescue scoliosis in *ptk7* mutants. This experiment does not provide definitive evidence that CNS abnormalities do not contribute to the *ptk7* mutant phenotype; however, it does suggest that the primary defect may lie somewhere outside of the brain.

Defects in bone growth and metabolism have been associated with late onset forms of scoliosis; however, mineralized bone staining during early stages of vertebral development suggest that there is no defect in the early bone growth of *ptk7* mutant vertebrae just prior to the onset of scoliosis (Figure 4.3.2). I also analyzed bone density in adults by measuring vertebral pixel intensities in microCT scan compressions (Figure 4.3.2). I did not observe any obvious differences between *ptk7* mutants and *ptk7/+* controls (Figure 4.3.2b), suggesting that overall bone density is not affected. Polymorphisms in genes involved in bone growth and metabolism (*GPR126, ER, IGF1*) have been associated with curve severity in humans\(^302,344,348,467\). Since *ptk7* mutant zebrafish display a range of phenotypic severities, it would be interesting to investigate whether bone density defects are associated with curve severity rather than curve onset in *ptk7* mutant zebrafish. This could be done using specific zebrafish transgenic lines that label osteoblast cell populations\(^286\), alizarin red staining and/or microCT analysis.

The distribution of inter-segmental vessels is known to play an important role in vertebral body patterning\(^287,468\) and vascularization is an important aspect of bone growth\(^469\). I considered
defects in vasculogenesis as a potential cause of spinal curvature since Ptk7 is known to affect VEGFR signal transduction and is required for the migration of endothelial cells in culture. I used a stable transgeneic zebrafish line that expresses GFP in vascular endothelial cells (Tg(Flk1::GFP)) to directly visualize intersegmental vessel formation in both MZptk7 and ptk7 mutant zebrafish. In MZptk7, intersegmental vessels form (Figure 4.3.3b), suggesting that vasculogenesis is not grossly affected with early loss of ptk7. In ptk7 mutants, I looked at the intersegmental vessels during embryo and larval stages and did not observe any obvious differences between ptk7 mutant and ptk7/+ controls (Figure 4.3.3c,d). This suggests that defects in formation of the intersegmental vessels are not the primary cause of the spinal defects in MZptk7 and ptk7 mutants; however, a closer look at tissue vascularization may provide some insight into disease progression with respect to bone remodeling, for example, in response to asymmetrical spinal loading or with curve severity.

Scoliosis is a common feature of a number of neuromuscular diseases, making the role of ptk7 in muscle development, axon guidance and neuromuscular connectivity important areas for future investigation. I analyzed the structural integrity of developing muscle in ptk7 mutant larvae using birefringence, where light is rotated as it passes through organized muscle sarcomeres. Normally, the skeletal muscle appears very bright when visualized between two polarized light filters (Figure 4.3.4). Muscle detachment or degeneration can be visualized by dark patches, while myofiber disorganization is evident by an overall reduction in birefringence or light intensity. I failed to observe either of these phenomena in mixed clutches containing ptk7 mutant larvae (Figure 4.3.4), suggesting that defects in structural muscle integrity do not contribute to scoliosis in my model. One might not expect Ptk7 to have a direct effect on skeletal muscle integrity, since in humans and mice, Ptk7 expression is not enriched in muscle tissue; however, neuromuscular connectivity or other factors associated with paraxial muscle function may be affected in ptk7 mutants, creating abnormal force on the spine and scoliosis. Therefore, in the future, it will be important to assess paraspinal muscle activity and neuromuscular connectivity in MZptk7 and ptk7 mutant zebrafish.

Melatonin deficiencies in animal models implicate a neuroendocrine basis for scoliosis progression; however, no decrease in circulating melatonin has been observed in humans. Instead, downstream signaling through G-protein coupled receptors appears to be impaired in a number of IS patients, suggesting an important link between neuroendocrine signal transduction and IS pathogenesis. Interestingly, cells isolated from IS patients respond
differentially to melatonin (inhibition of cAMP) through cellular complexes of melatonin receptor 2 (MT2), receptor of activated C kinase 1 (RACK1) and protein kinase C delta (PKCδ)\textsuperscript{340}. Based on their melatonin response profile, patients can be classified into distinct subgroups, which may allow for predictive analysis of at risk individuals\textsuperscript{341}. Since Ptk7 has been shown to bind and recruit RACK1 and PKCδ to the plasma membrane\textsuperscript{237}, it would also be interesting to determine the requirement for Ptk7-RACK1-PKCδ complex formation in melatonin signaling and scoliosis in zebrafish.
Figure 4.3.1. CNS replacement strategy to attempt rescue of *ptk7* scoliosis. **(a)** Transplant strategy whereby membrane RFP (memRFP) labeled wild-type (WT) cells are transplanted into the animal pole (AP) of *ptk7* mutant or *ptk7* heterozygous (*ptk7/+*) embryos at 4 hpf. **(b)** Chimeric embryos at 24 hpf. **(c)** Membrane RFP-labeled WT cells localized to the anterior CNS in WT/*ptk7* or *ptk7/+* chimeric embryos.

Figure 4.3.2. Bone density is not affected in *ptk7* mutant zebrafish. **(a,b)** Maximum intensity projections (MIP) in the cranio-caudal direction from high-resolution micro-computed tomography (microCT) of adult **(a)** *ptk7* heterozygous (*ptk7/+*) and **(b)** *ptk7* mutant zebrafish. Dorsal is up and ventral is down. **(c)** Quantification of maximum pixel intensity corrected for background intensity in order to infer approximate bone density in *ptk7/+* and *ptk7* mutant zebrafish. Maximum pixel intensities of the vertebrae were extracted from MIPs made from 10 sequential microCT slices. Three locations from trunk to tail were quantified and averaged for each fish.
Figure 4.3.3. Intersegmental vessels form in MZptk7 and ptk7 mutants. (a,b) Lateral view of 48 hpf Tg(Flk1:GFP) (a) wild-type (WT) and (b) MZptk7 mutant embryos. (c,d) Lateral views of larval stage (4.5mm) Tg(Flk1:GFP) (c) ptk7 heterozygous (ptk7/+ ) and (d) ptk7 mutant embryos.

Figure 4.3.4. Birefringence to assess body muscle integrity. (a) Bright field and (b) polarized light image of 3 dpf embryos from mixed ptk7/+ and ptk7 population. Light is rotated as it passes through organized muscle sarcomeres. Dark patches indicate muscle detachment and reduced light intensity suggest myofiber disorganization. No obvious dark patches or overall reduction in light intensity was apparent in ptk7/+ or ptk7 mutant zebrafish.
4.4 Defining the temporal relationship between *ptk7* and scoliosis

80% of IS is classified as adolescent idiopathic scoliosis (AIS) and appears in young adults around the onset of puberty. The disease is understood to develop over time in susceptible individuals and with no strong causative candidates, it is still unknown whether IS is linked to a congenital defect or if individuals are born normal and go on to develop abnormalities later on. I failed to observe any embryological abnormalities in zygotic *ptk7* mutant zebrafish; however, these mutants developed a fully penetrant form of late onset spinal curvature, suggesting that at some time point beyond early embryogenesis *ptk7* is required for spinal morphogenesis. Since *ptk7* mutants recapitulate aspects of human IS, *ptk7* mutants can be used in the future to better define the time point of IS onset.

MZ*ptk7* zebrafish are a complete loss-of-function mutant model that display early defects in embryogenesis, whereas zygotic *ptk7* mutant embryos display no early defects due to maternal gene rescue. I titrated a splice blocking MO (*ptk7*SPL MO) that also caused no embryological abnormalities (Figure 2.3.3.2), reasoning that since the effect of MO is transient, I could use *ptk7*SPL MO to determine whether early loss of zygotic *ptk7* expression leads to scoliosis later on. However, juvenile zebrafish that had been injected with *ptk7*SPL MO do not exhibit any evidence of spinal curvature at 3 months post fertilization (n=30), suggesting that embryonic loss of zygotic *ptk7* is not sufficient to drive the scoliotic phenotype I observe in genetic loss-of-function *ptk7* mutant zebrafish.

Genetic methods could be used in the future to define a time point(s) at which loss of *ptk7* affects spinal curvature in zebrafish. I built one heat-shock inducible *ptk7* transgene (Tg(hs::Ptk7GFP)) that could be used to test the efficiency of Ptk7 rescue at defined intervals prior to the onset of scoliosis in *ptk7* mutant zebrafish. Because Ptk7 overexpression had an effect on Wnt/PCP and Wnt/β-catenin signaling, one challenge with this approach may be later stage artifacts of ectopic Ptk7 overexpression. Thus, to temporally analyze the requirement for endogenous *ptk7*, new genome editing technologies could be used to generate conditional loss-of-function alleles of *ptk7*. For example, loxP sites could be integrated at the *ptk7* locus through homologous recombination. This approach has been used to target other locations in the zebrafish genome using TALENs or the CRISPR/Cas9 system\(^{472,473}\). Temperature and/or tamoxifen inducible Cre-recombinases are efficient in zebrafish\(^{472,473}\); therefore, Cre/loxP *ptk7*
Excisions could be used to temporally induce functional loss of *ptk7*. More simply, a loxP-flanked Ptk7 transgene could be inserted into the genome of *ptk7* mutant zebrafish and temporal Cre-mediated loxP recombination could remove Ptk7 to test when expression is required to rescue *ptk7* scoliosis.

Tissue specific Cre drivers could also be used to assess tissue specific requirements for Ptk7. Muscle, neuron, and/or vertebrae specific Cre-recombinase in conjunction with engineered loxP-containing zebrafish could define the tissue specific requirement for *ptk7* with respect to scoliosis onset, which would help define the cause of certain cases of human IS.

One important aspect of determining when and where Ptk7 is required will be analysis of larval and juvenile stage *ptk7* expression. I determined the embryonic expression pattern of *ptk7* through *in situ* hybridization and observed anterior CNS expression at 24 hpf, suggesting that Ptk7 may affect CNS development. Data from human and mouse also indicate that Ptk7 is enriched in the adult nervous system\textsuperscript{202,203}; however, to explore larval/juvenile stage *ptk7* expression, endogenous *ptk7* promoter from genomic DNA or a *ptk7*-sequence containing bacterial artificial chromosome (BAC) could be used to drive a GFP transgene for direct observation of tissue expression. Alternatively, homologous recombination using TALENs or the CRISPR/Cas9 system could be used to insert a fluorescent reporter into an exon at the *ptk7* locus. Insertions into C-terminal domains of Ptk7 could be the most effective, since I found that the C-terminal domain is not required for Ptk7-mediated Wnt signal transduction during embryogenesis.

These approaches would help define both the temporal and tissue specific requirement for *ptk7* with respect to scoliosis onset and help in the overall understanding of IS pathology.
4.5 Defining the relationship between Wnt signaling and IS

I determined that ptk7 is an important regulator of both non-canonical Wnt/PCP and canonical Wnt/β-catenin signaling. Additionally, disruption of both Wnt/PCP and Wnt/β-catenin signaling led to vertebral malformations, suggesting that both Wnt pathways can contribute to CS pathology. Ptk7 has been shown to have alternative roles in the VEGF, and Plexin/Semaphorin pathways227,240,470; therefore, it will be important to determine if deregulated Wnt signaling contributes to zygotic ptk7 spinal curvature and how this may relate to IS pathology.

To test for possible genetic interactions between ptk7 and the Wnt/PCP signaling pathway with respect to late onset spinal curvature, I generated ptk7/+;vangl2/+ and ptk7/+;glypican4/+ trans-heterozygotic zebrafish. In mixed populations I did not observe any evidence of late-onset spinal curvature; however, trans-heterozygous interactions are rare in zebrafish. Thus, ptk7;vangl2/+ and ptk7;glypican/+ zebrafish could be generated to test whether manipulation of the PCP pathway can modify ptk7 mutant scoliosis.

To determine if and when disruption of Wnt/PCP signaling induces scoliosis, I generated heat-shock inducible transgenic zebrafish that expressed Xdd1199, a dominant-negative form of Dsh that inhibits PCP signaling (Tg(hs::Xdd1iresGFP)). I heat-shocked chimeric founder (F0) generations and their stable F1 progeny between 24 hours and 6 days post fertilization; however, I was unable to recover adult transgenic progeny with spinal curvatures (Table 4.5.1). This suggests that disrupting PCP signaling within the first week of development is not sufficient to induce scoliosis in zebrafish. Future analysis should extended the experimental time point up to the average time of curve onset in ptk7 mutants.

I have shown that ptk7 can inhibit Wnt/β-catenin signaling during embryogenesis, so to analyze the relationship between ptk7 and activated Wnt/β-catenin signaling with respect to late onset spinal curvature, I generated ptk7 mutant zebrafish containing a heat-shock inducible Dickkopf1 transgene (Tg(hs::Dkk1GFP). I induced Dkk1 expression from 2-6dpf and reasoned that if activated Wnt/β-catenin signaling affects ptk7 spinal curvature, inhibition by Dkk1 might suppress the phenotype. Similarly, I tested the ability of the Wnt antagonist IWR-1 to rescue ptk7 scoliosis by treatment during the first week of development. None of the experimental ptk7 mutant zebrafish displayed significant phenotypic rescue (Table 4.5.2), suggesting that Wnt/β-catenin inhibition during the first week of development is not sufficient to rescue scoliosis in ptk7 mutant zebrafish.
To test the effect of activated Wnt/β-catenin signaling, I treated wild-type embryos with LiCl and BIO (Wnt agonists) throughout the first week of development. I also used heat-shock inducible Wnt8 transgenic zebrafish (Tg(hs::Wnt8GFP)) to induce wnt8 expression throughout the entire first month of development. In these experiments, I did not recover any adults with spinal curvatures (Table 4.5.2), suggesting that activated Wnt/β-catenin signaling was not sufficient to induce late onset spinal curvatures in zebrafish.

To address the possibility that disrupted Wnt signaling may contribute to IS, this analysis should be extended to cover all possible time points and, thereby, contribute to defining the timing of disease onset to help better understand disease pathogenesis in human IS patients.
Table 4.5.1 Summary of manipulations performed to test a possible role for PCP signaling in late-onset spinal curvatures in zebrafish. *Spinal curves that did not segregate with the experimental population were observed in this experiment and were attributed to background or environmental effects.

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<tr>
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<td>20</td>
</tr>
<tr>
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<td>20</td>
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<td>transgene-injected chimeric generation</td>
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</tr>
</tbody>
</table>

Table 4.5.2 Summary of manipulations performed to test a possible role for Wnt/β-catenin signaling in late-onset spinal curvatures in zebrafish. *Spinal curves that did not segregate with the experimental population were observed in this experiment and were attributed to background or environmental effects.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Time</th>
<th>Curve</th>
<th>Total Num. Fish</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg(hs::Wnt8GFP)</td>
<td>hs at 48hpf</td>
<td>NO</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>3-6dpf (hs 1x/day)</td>
<td>1*</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>7-14dpf (hs 1x/day)</td>
<td>NO</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>15-21dpf (hs 1x/day)</td>
<td>NO</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>21-25dpf (hs 1x/day)</td>
<td>NO</td>
<td>10</td>
</tr>
<tr>
<td>ptk7;Tg(hs::dkk1GFP)</td>
<td>hs at 48hpf</td>
<td>YES</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>3-6dpf (hs 1x/day)</td>
<td>YES</td>
<td>32</td>
</tr>
<tr>
<td>ptk7+/+;Tg(hs::dkk1GFP)</td>
<td>hs at 48hpf</td>
<td>NO</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>3-6dpf (hs 1x/day)</td>
<td>NO</td>
<td>35</td>
</tr>
<tr>
<td>LiCl (1M)</td>
<td>2-6dpf (continuous)</td>
<td>NO</td>
<td>50</td>
</tr>
<tr>
<td>LiCl (2M)</td>
<td>2-6dpf (continuous)</td>
<td>NO</td>
<td>50</td>
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<td>BIO (100µM)</td>
<td>2-6dpf (continuous)</td>
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<td>50</td>
</tr>
<tr>
<td>BIO (200µM)</td>
<td>2-6dpf (continuous)</td>
<td>NO</td>
<td>50</td>
</tr>
<tr>
<td>ptk7+IWR (500µM)</td>
<td>24-48hpf (continuous)</td>
<td>YES</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>2-6dpf (continuous)</td>
<td>YES</td>
<td>21</td>
</tr>
<tr>
<td>ptk7/++;IWR-1 (500µM)</td>
<td>24-48hpf (continuous)</td>
<td>NO</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>2-6dpf (continuous)</td>
<td>NO</td>
<td>29</td>
</tr>
</tbody>
</table>
4.6 Ptk7, Calmodulin and Ca$^{+2}$ Signal Transduction

Melatonin deficiencies in chicken and rodent models have traditionally been the most popular animal models of scoliosis. Despite significant differences between these animal models and human IS, this phenotype strongly points towards a relationship between melatonin signaling and IS pathogenesis. Defining the primary tissue(s) affected by melatonin deficiency is complicated by the fact that melatonin has diverse roles throughout the body and receptors are expressed on a variety of tissues$^{474}$. In addition, the role of melatonin in human IS pathogenesis is controversial because absolute levels of circulating melatonin are not consistently deregulated in human patients$^{334}$. Interestingly, more recent evidence suggests that rather than melatonin itself, downstream effectors of the melatonin signaling pathway could influence IS. Polymorphisms in the melatonin receptor 1B (MTNR1B) and calmodulin 1 (CALM1) genes are associated with both IS susceptibility and severity$^{346,352,354}$ in certain populations, so theoretically, functional changes in these genes could affect how melatonin signals are translated in a variety of tissues to affect IS pathogenesis in humans.

Calmodulin is a calcium-binding messenger protein that transduces calcium (Ca$^{2+}$) signals through interactions with various target proteins and can be inhibited through phosphorylation by melatonin and protein kinase C (PKC)$^{326}$. Calmodulin levels may be an important predictor of IS disease progression$^{349}$; however, the mechanism through which calmodulin signaling affects the spine is not well defined. The incidence of scoliosis in melatonin-deficient animal models and AIS disease associations with the CALM1 gene locus make the calmodulin/Ca$^{2+}$ axis an intriguing candidate for further study. Ca$^{2+}$ signaling has a number of important physiological roles that could influence spinal morphogenesis, for example, muscle contractions, neuronal transmission and/or cellular motility.

Non-canonical Wnt/Ca$^{2+}$ signaling is a less well studied branch of the Wnt signaling pathway; however, it is known that Wnt ligands can influence intracellular Ca$^{2+}$ release and Ca$^{2+}$-dependent signaling through the G protein-coupled activity of Fz (Figure 1.1.1). The relationship between Ptk7 and Ca$^{2+}$ signaling is unknown; however, known interactions between Ptk7 and upstream components like Fz and Wnt ligands$^{2,235}$ suggest that Ptk7 may affect Ca$^{2+}$ signal transduction in addition to Wnt/β-catenin and Wnt/PCP signaling depending on the cell context in vivo. Therefore, a future aim of this project will be to determine if Ptk7 plays a role in
Ca$^{2+}$ signaling, with the aim of identifying alternative Ptk7 pathways that may influence scoliosis in my models.

Several methods have been used in zebrafish to study Ca$^{2+}$ dynamics$^{475-477}$, with genetically encoded Ca$^{2+}$ indicators offering a non-invasive, tissue-specific method of tracking Ca$^{2+}$ transients during embryogenesis$^{478,479}$. Imaging global Ca$^{2+}$ dynamics in MZptk7 mutant embryos could be an initial test to determine if Ca$^{2+}$ signaling is affected in a complete loss-of-function ptk7 model. Biochemical analysis of an endogenous effector protein like calmodulin could also reveal how the Ca$^{2+}$ signaling pathway is affected in ptk7 mutants. To test Ca$^{2+}$ signaling and its contribution to IS pathogenesis, transgenic ptk7 mutant zebrafish expressing muscle, cartilage and/or bone specific Ca$^{2+}$ indicators could be analyzed. This may help us understand the phenotype and/or point to the tissue(s) primarily affected in ptk7 mutant zebrafish. Finally, calmodulin antagonists have been shown to reduce the rate and magnitude of scoliosis in chicken and mouse models$^{327,328}$; therefore, calmodulin inhibitors could be used prior to the onset of scoliosis in ptk7 mutant zebrafish to test the contribution or modifying potential of deregulated calmodulin in this disease model.

In conclusion, I have characterized a functional requirement for ptk7 from embryogenesis through to juvenile and adult stages in zebrafish and have defined a role for Ptk7 as an important regulator of both canonical Wnt/β-catenin and non-canonical Wnt/PCP signaling. Loss-of-function ptk7 mutants closely model human neural tube defects (NTDs), scoliosis phenotypes (CS and IS), and as a genetic tool, provide a framework to investigate the molecular pathogenesis behind these forms of human disease.
Chapter 5

5. METHODS

5.1 Zebrafish Strains

Established zebrafish husbandry protocols were adhered to for all strains used throughout the duration of the study, and all protocols were approved by the Animal Care Committee at the Hospital for Sick Children. The vangl2 mutant allele (tri(tk50f)) was chemically induced and contained a deletion in the vangl2 coding sequence. The glypican4/knypek (kny119) mutant allele was chemically induced and consists of a base pair change that leads to premature termination of mutant protein at amino acid 247. Chimeric zebrafish harboring a homozygous vangl2 or glypican4 mutant germline were generated according to previously published germline replacement strategy. Mating of MZvangl2 female and vangl2 heterozygous male zebrafish was used to obtain MZvangl2 mutant embryos and vangl2 heterozygous controls. Mating of MZvangl2 and MZknv female zebrafish with wild-type male zebrafish was used to obtain vangl2 and kny heterozygous embryos, respectively. Vangl2 heterozygous zebrafish were crossed to obtain vangl2 mutant embryos.

Tg(hsp70::Xdd1IRESGFP) zebrafish were generated using the zebrafish Tol2kit and Gateway compatible vectors containing Xdd1 to express fluorescently tagged Xdd1 under the control of the hsp70-inducible promoter. Transgenic vectors were injected into one-cell stage embryos and incorporated into the genome using Tol2-mediated transposition (F0). F1 embryos were screened to identify germ-line transposition from F0 zebrafish and grown-up to isolate stable transgenic lines. Tg(hsp70::wnt8GFP) zebrafish were used. Tg(hsp70::dkk1GFPw32) zebrafish were crossed to ptk7hsp9 mutan zebras to generate Tg(hsp70::dkk1GFP;ptk7 trans-heterozygous zebrafish. Female transheterozygotes were crossed to ptk7 mutant males to generate Tg(hs::dkk1GFP;ptk7 mutant and Tg(hs::dkk1GFP;ptk7/+ embryos. For all heat-shock experiments, embryos were raised at 25°C for several hours and heat shocked at 38°C for one hour followed by recovery at 28°C.

5.2 Generation of ptk7 Mutants

ZiFiT software (http://zifit.partners.org/ZiFiT/) was used to identify potential target sites in ptk7. Using the Oligomerized Pool Engineering (OPEN) system method, two selections (one for each half-site were made). A bacterial one-hybrid assay was used to test specificity of the
engineered arrays against a sequence-specific pH3U3 reporter vector expressed in USOΔhisΔpyrArpoZ cells\textsuperscript{431}. The zinc-finger cassette was inserted into pCS2+ expression vectors. The sequences for left and right zinc-fingers are listed in Table 5.1.2.

mRNA (80pg) for each ZFN was injected into one-cell stage embryos (F0). F1 genomic DNA was screened for mutations in ptk7 using a PCR-based assay and primers: Transmembrane (TM) site forward, 5′-AGCAAAACACAGACAGACATTCCCAACACA-3′ and reverse 5′-CCACCTCTCGCTCGTCTCCGTCTCG-3′; pseudokinase (PK) site forward, 5′-CGAAGGCGCTGAGGATGA-3′ and reverse, 5′-CAGAAAACGCATGAAGTGACCAGC-3′. SfaN1 endonuclease was used to screen for mutations at the PK target site.

5.3 Microinjections

EGFP-tagged Drosophila Prickle (GFP-Pk)\textsuperscript{154} and membrane-localized monomeric red fluorescent protein (membraneRFP)\textsuperscript{434} were previously described. MOs were obtained from Gene Tools, LLC, and were designed and diluted according to manufacturer’s instruction. A MO targeted against the 5′UTR of ptk7 (ptk7ATG MO; 5′CGCGTCTTCTCGTCCA TAAGCCCAT3′) was injected into one-cell stage embryos at 2-6ng to block translation of ptk7 and vangl1, respectively. A MO was designed to target the exon2/inton2-3 boundary of ptk7 (ptk7SPL MO; 5′ATGAAGGTAAACTCACACTTGATGT3′) to prevent splicing of ptk7 pre-mRNA and was injected at 10ng. Since MO has been shown to non-specifically activate the p53 signaling pathway\textsuperscript{429}, 10ng of p53 MO (5′GCGCCATTGCTTTGCAAGAATTG3′) was co-injected in all experiments to reduce tissue necrosis.

Zebrafish ptk7, fz7a, fz7b, fz2 were amplified from 24hpf wild-type cDNA made using oligo(dT)\textsubscript{12-18} primer (Invitrogen) and SuperscriptII (Invitrogen) as per manufacturer’s instructions. Ptk7 was cloned into pCS2+ using Gateway technology (Invitrogen). Ptk7\textsubscript{ΔICD}, ptk7egfrTM, ptk7\textsubscript{ΔECD} and ptk7ECD were amplified from full-length ptk7. Human PTK7 was amplified from MGC clone #30347652-containing plasmid (TCAG, SickKids, Toronto) and cloned into pCS2+ using Gateway technology (Invitrogen). PTK7\textsubscript{P545A} was amplified from wild-type PTK7. All cloning primer sequences are listed in Table 5.1.3.

The mMESSAGE mMACHINE System (Ambion) was used to make mRNA. All injections were performed at the one-cell stage.
5.4 Cell Transplantation

Cell transplants were utilized to generate chimeric embryos for immediate or larval-stage analysis. Cells were extracted from donor embryos using established techniques at mid-blastula stage (4hpf), since cells are multi-potent and adhere to each other less tightly at this stage. Approximately 50-100 cells were transplanted from donor embryos labeled with rhodamine dextran (0.5ng red 10000MW or 2.5ng of green 3000MW, Sigma) into unlabelled host embryos at mid-blastula stage (4hpf), unless otherwise noted. Cells were transplanted into the ventral domain of unlabelled gastrula-staged (6hpf) host embryos to make tail-bud chimeras as previously described\textsuperscript{82}. Tail-bud chimeras were screened at 24hpf on an Axio Imager.M1 (Zeiss) for mutant cell contribution in the embryonic tail and raised to larval stages for larval-stage analysis.

5.5 Whole-mount \textit{In Situ} Hybridization (WISH)

Antisense RNA probes were prepared by \textit{in vitro} transcription (DIG RNA Labeling Kit, Roche) in the presence of digoxigenin-11-UTP from linearized plasmids containing the open-reading frame of zebrafish \textit{snail2}\textsuperscript{484}, \textit{papc}\textsuperscript{485}, \textit{dlx3}\textsuperscript{486}, \textit{hgg1} (cb15 from Thisse ISH screen), \textit{no-tail (ntl)}\textsuperscript{487}, \textit{myol}\textsuperscript{488}, \textit{krox20}\textsuperscript{489}, \textit{chordin (chd)}\textsuperscript{74}, \textit{goosecoid (gsc)}\textsuperscript{75}, \textit{dharma/bozozok (boz)}\textsuperscript{490}, \textit{pax2.1}\textsuperscript{491}, \textit{emx1}\textsuperscript{492}, \textit{tbx6}\textsuperscript{493}, \textit{her7}\textsuperscript{494}, \textit{dlc}\textsuperscript{252}. Zebrafish \textit{ptk7}, \textit{ripply1}, \textit{mespb}, \textit{sox2} and \textit{Xirp2a} open reading frame sequences were cloned from 24hpf cDNA libraries (cloning primer sequences are listed in Table A2.1.3) made using oligo(dT)\textsubscript{12-18} primer (Invitrogen) and SuperscriptII (Invitrogen) as per manufacturer’s instructions. PCR products were inserted into pDONR221 donor vectors using Gateway technology (Invitrogen) and linearized using \textit{ApaI} restriction endonuclease.

Embryos were either fixed and flat-mounted in 80\% glycerol or cleared in 100\% methanol, mounted in benzylbenzoate:benzylalcohol (2:1) and imaged on an Axio Imager.M1 (Zeiss) compound microscope.

5.6 Reverse Transcriptase (RT) - PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) according to manufacturer’s recommendations. First-strand cDNA was made using SuperScript II reverse transcriptase (Invitrogen) and oligo(dT)\textsubscript{12-18} primer (Invitrogen). \textit{Pt}k7\textit{SPL} MO efficiency was verified using primers: forward1 (exon2), 5’-\textit{CTGCGGTGTGAAAGTAAAGCAA}-3’; reverse1 (intron2), 5’-\textit{TTTTGAGCTCTGCATTGGTG}-3’; reverse2 (exon3), 5’-\textit{GCCATCATGTCCCTCGATCTC}-3’.
Taq DNA Polymerase (NEB) was used to PCR amplify with the following PCR conditions: 98°C for 30s, 35 cycles of 98°C for 10s, 64°C for 30s, 72°C for 45s, followed by 72°C for 10 min.

5.7 quantitative RT-PCR (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen) according to manufacturer’s recommendations. First-strand cDNA was made using SuperScript II reverse transcriptase (Invitrogen) and oligo(dT)$_{12-18}$ primer (Invitrogen). For primer sequences, see Table 5.1.7. SYBR green (Applied Biosystems) was used according to manufacturer’s recommendations. All analyses were carried out in triplicate using a Light Cycler 480 (Roche) platform. Fifty amplification cycles were performed, with each cycle consisting of 95°C for 10 seconds, 60°C for 20 seconds and 72°C for 30 seconds. Amplification and dissociation curves generated by the Light Cycler 480 Software release 1.5.0 SP4 were used for gene expression analysis. Ct values were obtained for each gene and normalised to Gapdh. Fold change was calculated relative to wild-type expression according to the equation: $2^{-\Delta\Delta Ct}$. Standard error was calculated as standard deviation of the fold change according to the equation: $\text{stdev}_{\text{fold change}}=(\ln 2)(\text{stdev}_{\Delta\Delta Ct})(2^{\Delta\Delta Ct})$, where $\text{stdev}_{\Delta\Delta Ct}=\sqrt{(\text{stdev of reference})^2 + (\text{stdev of gene of interest})^2}$. All graphs are representative of two independent experiments with three technical replicates each. Statistical significance was calculated using Student’s $t$-test.

5.8 Cell Transfections and TopFlash Assay

Lentiviruses containing the superTopFlash β-catenin-dependent luciferase reporter (Firefly luciferase) and pRL-TK (Renilla luciferase) were produced and used to establish stable HEK293T Wnt-reporter lines. Cells were seeded on 24-well plates, followed by plasmid or siPTK7 (siGENOME SMARTpool siRNA D-003167-25, -11, -10, -9, PTK7)$_3$ transfection with polyethyleneimine (PEI). Media was replaced with a 1:1 mix of fresh DMEM:Wnt3A or DMEM:Control conditioned media. Cells were assayed 16 hours after stimulation, performed in accordance with the Dual Luciferase assay specifications (Promega) using an Envision 2103 Multilabel Plate Reader (PerkinElmer).

5.9 Alizarin red staining

Adult zebrafish were fixed for one week in 4% paraformaldehyde at 4°C followed by two, one hour washes with 0.1% PBST. Fish were stained with 0.01% Alizarin red (Sigma) in 1% KOH for one week and washed in PBST for 3 days (1 wash/day). Tissues were cleared by 0.5%
trypsin digestion for one day followed by multiple washes with 0.1% PBST. The skin was manually removed and the skeleton was imaged on a Leica MZ16 dissecting scope using AxioVision (Zeiss) software.

5.10 Calcein Staining
Zebradish larvae were incubated in 0.2% Calcein (Sigma), pH 7.5 for 15 minutes and washed twice in system water. Larvae were immobilized using 0.003% Tricaine (Sigma) and mounted in 3% methyl cellulose. Imaging was performed on an Axio Imager.M1 (Zeiss) compound microscope.

5.11 MicroCT
Adult zebrafish were fixed in 10% neutral-buffered formalin for one week and mounted in 1% low-melt agarose (Sigma) in a plastic vial. Specimens were scanned for one hour using SkyScan1172 high resolution Micro-CTanner (Bruker micro-CT, Belgium) with the X-ray power at 45kVp and 218uA. All three-dimensional Micro-CT data sets were reconstructed with 18µm isotropic resolution. The images were then analyzed using Amira software (TGS Inc., Berlin, Germany)

5.12 AIS PTK7 Variant Discovery
20 coding exons of PTK7 were PCR amplified from 96 AIS peripheral blood samples and sequenced by the Sanger method (oligonucleotide primer sequences and PCR conditions available on request). This identified four nonsynonymous changes. Two highly conserved nonsynonymous SNPs, SNPs rs34021075 and rs34865794, were genotyped by Taqman assay in 736 IS NHW cases and 740 NHW controls and tested for allelic association with AIS.

5.13 Drug Treatments
Embryos were incubated in 100µM LiCl (Sigma) or 10µM BIO (Sigma) from bud stage (10hpf) until 24hpf, washed in system water and grown to larval stages for vertebrae analysis. Embryos were incubated in 0.1M LiCl or 100µM BIO from late somite stages until 24hpf, washed in system water and fixed in 4% paraformaldehyde for WISH. Ptk7 mutant and heterozygous embryos and larvae were incubated in 100-200µM IWR-1 for the indicated time period, washed in system water and grown to adult stages.
5.14 Embryonic Cell Lysis and Western Blot Analysis

Zebrafish embryos were deyolked in deyolking buffer (55 mM NaCl, 1.8 mM KCl, 1.25 mM NaHCO₃) by pipetting up and down with a narrow tip so that the yolk was disrupted. Centrifugation at 300 g for 30 seconds was done to pellet embryonic tissue. The yolk-containing supernatant was discarded and lysis buffer (25mM Tris-HCL, 150mM NaCl, 2mM EDTA, 1% Igepal (NP-40)) with protease inhibitor cocktail was added at 1uL/embryo. Protein was denatured using β-mercaptoethanol buffer at 95°C for 10 minutes, and separated on an 8%-10% SDS-PAGE gel. Anti-phosphotyrosine (Millipore) and anti-phosphoserine (gifted from the Boulianne lab) were used to detect the presence of phosphopeptides. Anti-flag (Sigma) and anti-myc (Abcam) were used to both immunoprecipitate and detect protein on Western blots. Human PTK7 was detected using goat anti-human PTK7 antibody (LSBio). Mouse anti-actin (Sigma) was used as a loading control.

5.15 Confocal Imaging

Live embryos were immobilized on a coverslip in 1% agarose, and imaged using a Zeiss LSM 710 microscope. Z-stacks were collected and processed using ImageJ software.
<table>
<thead>
<tr>
<th>Sequence (5’→3’)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TM L1</strong></td>
<td>ggtaccgcctctccagtgctgcatttgcattgcaggaacttttcgacgaaccaggaagttggtgtgctgcataccctctca taceggtgaaaccacgctgatgtgatgcaatttcctgtgcgcagaacttgcgcgaacactctgtgaccaagagctgtgtgctgcggeggc acctaaaccacactgaggggatcc</td>
</tr>
<tr>
<td><strong>TM L2</strong></td>
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</tr>
<tr>
<td><strong>TM R1</strong></td>
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<tr>
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</table>

**Table 5.1.2.** Sequences for left (L) and right (R) zinc-fingers engineered for the transmembrane (TM) and pseudokinase (PK) target sites.
<table>
<thead>
<tr>
<th>Primer pair(s)</th>
<th>Forward 5'</th>
<th>Reverse 3'</th>
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<tbody>
<tr>
<td>ptk7</td>
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<td>TCAGACTTTGCTCTCGGAGGCAG-3'</td>
</tr>
<tr>
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<td>TTCGAGGGAATTGTAG-3'</td>
</tr>
<tr>
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<tr>
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**Table 5.1.3.** Cloning primer sequences.
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Table 5.1.7. Primer sequences for qRT-PCR.
References


Fenstermaker, A. G., Prasad, A. A., Bechara, A., Aldofo, Y., Tissir, F., Goffinet, A., Zou, Y. & Pasterkamp, R. J. Wnt/planar cell polarity signaling controls the anterior-


161 Robinson, A., Esculin, S., Doudney, K., Vekemans, M., Stevenson, R. E., Greene, N. D., Copp, A. J. & Stanier, P. Mutations in the planar cell polarity genes CELSR1 and SCRIB are associated with the severe open neural tube defects craniorachischisis Hum Mutat 33, 440-447 (2012).


165 Matthews, H. K., Marchant, L., Carmona-Fontaine, C., Kuriyama, S., Larrain, J., Holt, M. R., Parsons, M. & Mayor, R. Directional migration of neural crest cells is regulated


186


Qiu, X. S., Tang, N. L., Yeung, H. Y., Lee, K. M., Hung, V. W., Ng, B. K., Ma, S. L., Kwok, R. H., Qin, L., Qiu, Y. & Cheng, J. C. Melatonin receptor 1B (MTNR1B) gene


Clevers, H. & Nusse, R. Wnt/beta-catenin signaling and disease. Cell 149, 1192-1205 (2012).

Wang, Y. Wnt/Planar cell polarity signaling: a new paradigm for cancer therapy. Mol Cancer Ther 8, 2103-2109 (2009).


