Identifying Novel Genetic Causes of Primary Ciliary Dyskinesia

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Abstract

Primary ciliary dyskinesia (PCD) is a disease that affects the function of respiratory cilia that helps protect against airway infections. Diagnosis is difficult with current methods but genetic testing may expedite the process since PCD is hereditary, however the etiology of 30% of cases remains unknown. To identify more causes of PCD, whole exome sequencing was performed on patients identifying Spindly (spdl1) as a candidate. Analysis using zebrafish indicate spdl1 is expressed in motile ciliated tissues. Spdl1 deficiency results in embryos with ventral curvatures indicating cilia dysfunction. The defects can be rescued with 100pg of WT RNA but not RNA containing one of the mutations identified. Cilia orientation and range of motion was unaffected but motile cilia length was significantly increased in mutant neural tube floorplate (p<0.001) corresponding to a decrease in ciliary beat frequency (p<0.001) at the same time morphological phenotypes arise. The results implicate spdl1 in PCD etiology.
Acknowledgments

First and foremost, I would like to thank my supervisor Dr. Brian Ciruna for giving me the opportunity to be part of the lab. Your guidance and support for the past three years scientifically and beyond have been invaluable. I would also like to thank you for allowing me to attend two conferences and helping me develop the presentation, communication and networking skills useful for anything my future holds.

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Chapter 1

1 Introduction

1.1 The cilium

The cilium is an organelle found on cells in interphase that protrudes into the extracellular environment and fulfills a variety of functions depending on their classification. The first class is non-motile and is termed primary cilia. They are found on nearly all cells of a vertebrate organism and are capable of initiating signal cascades through sensation of modalities such as temperature, light and signaling molecules including Sonic Hedgehog. A secondary class of cilia is only present on tissues that are responsible for generating and maintaining fluid flow such as on cells lining the ventricles of the brain to move cerebral spinal fluid and on the upper and lower airway to move mucous and other debris up to the nose or mouth for expulsion. Additionally, motile cilia can be found in the transient laterality organ to help establish proper left-right patterning in the developing animal. Each type of motile cilia also has a characteristic beat pattern. For example, laterality organ cilia exhibit rotational motion whereas airway cilia typically show asymmetrical bending. These beat patterns are termed ciliary waveforms.

Ciliogenesis describes the process of building a cilium which begins near the cell membrane where the basal body is found (Fig. 1a). The basal body is composed of the mother centriole, its closely linked daughter centriole, cilia-specific appendage proteins and the pericentriolar matrix. Appendage proteins help the basal body anchor to the surface of the cell and can recruit other ciliary proteins to aid in ciliogenesis. The pericentriolar matrix is indispensable for ciliogenesis as it contains tubulin, the primary component of microtubules. Nine microtubule doublets originating from the basal body and arranged in a circle makes up the basic structure of the cilium. Between the basal body and the cilium body, called the axoneme, is the transition zone. The transition zone is recognizable by dense structures projecting from the sides of the basal body called transition fibers. This area acts as a gate to allow only ciliary proteins into and out of the organelle.
Figure 1. The cilium is a complex organelle composed of many proteins. A) Overview of the cilium showing the basal body as the nucleating center. This organelle is composed primarily of microtubules. The transition fibers denote the beginning of the transition zone which acts as a gating mechanism preventing non-ciliary proteins entry into the organelle. Proteins are moved into and out of the cilium through kinesin and dynein motors respectively. IFT-A and IFT-B complex together and cargo destined for the cilium are loaded onto the complex at the transition zone. Calcium channels and receptors for signaling molecules may be found on the ciliary membrane. B) Cross section of the three types of cilia found in vertebrates. Primary cilia have the simplest structure of only microtubule doublets. Nodal cilia have rotation movement thus possess outer dynein arms. Motile cilia are the most structurally complex, containing both inner and outer dynein arms, nexin proteins that connect each doublet to its neighbors and a central complex that provides an additional anchoring point for the doublets via radial spoke proteins.
Motile cilia are structurally distinct from primary cilia by addition of numerous functional proteins that allow for movement and increased stability\textsuperscript{8}. First, the basal body will not only nucleate the nine microtubule doublets, but two microtubule singlets that are found in the center of the structure. These central microtubules are aptly named the central complex and are the anchoring point for radial spoke proteins (Fig. 1b). Radial spoke proteins are a complex made of stalk and head components, connecting each microtubule doublet to the central complex. Secondly, each doublet is also connected to neighbor doublets by nexin proteins. Radial spoke and nexin proteins provide additional structural support that allows the organelle to move uniformly. Finally and most importantly, two arms of axonemal dynein are found on each doublet that will interact with the neighboring doublet to generate the force required for motility. Each arm is also specialized: the inner arm is responsible for the ciliary waveform while the outer arm establishes beat frequency, or how fast the cilium completes one cycle of movement\textsuperscript{12,13}. Interestingly, a structural difference is also observed between typical motile cilia found in the airway and those found in the laterality organ. The latter does not have nexin, radial spoke proteins and the central complex; nodal cilia only contains the outer dynein arms for force generation\textsuperscript{14,15}. The distinction between the types of cilia and the corresponding cilium structure suggests axonemal organization is regulated and important for cilium function.

Due to the transition zone and other gating proteins, not all molecules are permitted entrance into the cilium\textsuperscript{11}. Furthermore, the cilium does not contain necessary components for protein translation thus proteins must be synthesized before entering the organelle. In order to import ciliary proteins to the organelle, a transport system known as intraflagellar transport (IFT) is present\textsuperscript{16}. IFT is composed of a complex of IFT-A and B adaptor proteins which themselves are complexes of many proteins, the BBsome which helps stabilize the complex, and motor proteins Kinesin II for anterograde (cell-to-cilium) transport and Dynein 2 for retrograde (cilium-to-cell) transport\textsuperscript{16}. To import proteins into the cilium, IFT-A and B are loaded onto Kinesin near the basal body\textsuperscript{18}. Cargo destined for ciliary function interacts with the IFT adaptor proteins at the transition zone\textsuperscript{19}. At the tip of the cilium, proteins carried into the organelle are replaced with proteins ready for degradation and the IFT complex, including cargo is loaded onto Dynein 2 for entrance back into the cell\textsuperscript{16,18}. This is a simplistic explanation of IFT, as the exact mechanism behind IFT loading, cargo interaction and switch between anterograde and retrograde transport is still unknown.
The primary cargo of IFT appears to be tubulin dimers that make up microtubules, and other axonemal components\textsuperscript{19,20}. Thus, IFT is considered essential for ciliogenesis and maintenance of cilium length\textsuperscript{20}. Indeed, knockdown of IFT proteins such as IFT88 and Kinesin II itself in animal models led to shortened cilia or no cilia at all\textsuperscript{21,22,23}. IFT also has roles in signal transduction as it is required to mobilize receptors into the cilium\textsuperscript{24}. For example, photoreceptors which are a group of specialized primary cilia are dependent on IFT to bring light-sensitive receptors such as opsins into the outer segment for vision\textsuperscript{25,26}. IFT is also essential for Sonic Hedgehog signaling by mobilizing the ligand receptor Patched once it is bound to a ligand\textsuperscript{27}. Since Sonic Hedgehog and other signaling pathways are involved in the development of many systems, lack of IFT components result in widespread detrimental effects and multisystem diseases. Such diseases are termed ciliopathies and include syndromes such as Bardet-Biedel syndrome, Joubert syndrome and retinitis pigmentosa\textsuperscript{28,29,30}.

Traditionally, research on the structure and function of cilia was performed on the unicellular flagellate \textit{Chlamydomonas reinhardtii}, a bi-flagellated algae\textsuperscript{31}. Taking advantage of the fact that the flagellum is essentially a large cilium, this organism allowed scientists to study the ultrastructure of cilia as well as the mechanisms regulating ciliogenesis and cilia length\textsuperscript{23}. Furthermore, by studying the locomotion of this organism, researchers have begun to understand flow dynamics generated by the organelle\textsuperscript{32}. However, as the flagellate is unicellular, modeling ciliopathies is difficult. To address this concern, a number of groups have turned to other models such as sensory cilia in the neurons of \textit{C. elegans}, olfactory neurons in \textit{Drosophila} or respiratory cilia in the mouse to study primary or motile cilia function\textsuperscript{31}. The zebrafish however has gained significant traction in recent years in the study of both primary and motile cilia function due to a combination of obvious cilia-associated phenotypes and ease in mutant generation\textsuperscript{33,34}.

1.1.1 Zebrafish as a model to study cilia

The zebrafish, \textit{Danio rerio}, is a vertebrate that possesses many ideal characteristics for studying cilia function\textsuperscript{33}. It develops \textit{ex utero} allowing early observation on the effects of cilia dysfunction. Phenotypes associated with cilia dysfunction are observable as early as 2 days post fertilization (dpf) and include ventral body axis curvatures, dorsal axis curvatures and hydrocephalus\textsuperscript{33,35}. Kidney cysts may also be observed by 3dpf as well\textsuperscript{35}. These phenotypes can
be induced by morpholino-mediated knockdown of candidate genes or mutants can be generated with ease using CRISPR/Cas9 genome editing technologies\textsuperscript{36}.

This animal is a particularly powerful tool for motile cilia studies since cilia motility can be visualized in live zebrafish embryos providing biologically relevant flow and beat frequency data\textsuperscript{37}. These cilia are found in the olfactory placodes, otic vesicle (fish ear), and the cells lining the ventricles of the brain, neural tube and pronephric ducts. Motile cilia can be imaged live without markers or with the use of Arl13b-GFP, which is a GFP attached to a small GTPase of the Arf family that is associated with all cilia to observe cilia movement. Additionally, motile cilia can be found in the zebrafish laterality organ, called the Kupffer’s vesicle\textsuperscript{38}. Defects in Kupffer’s vesicle cilia can be imaged live as well using Arl13b-GFP or by investigating whether the embryo have laterality defects through in situ for \textit{cmle2}, \textit{lefty1/2}, \textit{fkd2} or \textit{ins}, marking the heart loop, left hindbrain and neural tube, liver and pancreas respectively\textsuperscript{35}. Finally, cilia can be imaged in fixed embryos by staining with anti-acetylated tubulin, which marks all stable microtubule structures including cilia.

The pronephric duct is the typical tissue used for motile cilia analysis as the cells lining this tissue not only produce motile cilia but are multi-ciliated thus can better mimic respiratory epithelia in humans\textsuperscript{31}. As well, analysis using motile cilia in the neural tube floorplate is common due to ease in imaging this location\textsuperscript{37}. By investigating motile cilia function in the pronephric duct or neural tube following genetic manipulation in zebrafish embryos, a number of genes have been characterized as causes of primary ciliary dyskinesia in recent years.

### 1.2 Primary Ciliary Dyskinesia

Primary ciliary dyskinesia (PCD) is a congenital disease that affects the function of motile cilia which are found on the cells lining the upper and lower airways\textsuperscript{5}. Motile cilia are essential in producing mucociliary clearance as the beating of cilia lining the tracts move mucus trapping foreign materials and microbes up to the mouth and nose to be expelled\textsuperscript{14}. Thus the function of motile cilia in the airway is important for protecting the lungs from infection. Sufferers of PCD therefore experience frequent lung and sinus infection as well as respiratory and nasal distresses throughout life\textsuperscript{5,14,39,40}. If improperly treated or untreated, PCD can cause bronchiectasis and widespread inflammation in the airway and lungs possibly leading to lung collapse\textsuperscript{41,42}. Congestion due to improper mucus movement can also lead to inflammation of the nasal
mucosa and sinus, facial pain and rhinorrhea which can all lead to decreased or absent sense of smell\textsuperscript{42}. PCD is also associated with chronic ear infections since as many as 80\% of children younger than 12 years old experience such symptoms possibly leading to hearing loss\textsuperscript{43}. Finally, since motile cilia are involved in establishing left-right asymmetry, approximately half of PCD patients also exhibit situs abnormalities which could result in cardiac complications\textsuperscript{44,45}. Due to the numerous systems this disease can affect, a diagnosis of PCD is often difficult\textsuperscript{46}.

In order to reach a diagnosis of PCD, it is recommended that a panel of three tests be performed on a patient who suffers from frequent respiratory distresses\textsuperscript{47}. The first test, and previously the “gold-standard” test for PCD is an analysis on the structure of motile cilia lining the airway epithelium using transmission electron microscopy (TEM)\textsuperscript{40,47}. However, good samples may be difficult to obtain since patients are frequently affected with airway infections. Swabbing to obtain airway epithelial cells can also be uncomfortable and distressing, particularly for young patients or if the technician is unskilled\textsuperscript{47}. Furthermore, there is increasing evidence that some PCD causes have no effect on the ultrastructure of motile cilia, thus will be undetected by TEM (Table 1). The second test is to perform motility assays on cilia from airway epithelia\textsuperscript{47}. This test, though critical in arriving at a PCD diagnosis, is difficult to perform since it requires sophisticated machinery to capture the quick movements of these cilia and a skilled technician familiar with cilia movement thus is not offered at every hospital or facility. A third test is to perform nasal nitric oxide measurements by a hemiluminesence analyzer\textsuperscript{48}. For currently unknown reasons, patients with PCD have low nasal nitric oxide values and a cut-off of <77nl/min has been reported to successfully detect PCD patients with 98-99\% accuracy\textsuperscript{48}. However, this test is dependent on how cooperative the patient is in performing the specific breathing technique thus cutoff values for children <5 years old are lacking.

This panel of tests clearly delay diagnosis and is not feasible in all facilities for every patient. Genetic testing is a proposed method of diagnosis that is expected to be more accurate and easier than current tests\textsuperscript{40,49}. Since PCD follows an autosomal recessive inheritance pattern, a diagnosis can be determined by genetic analysis on the patient only, or a child can be predicted to have PCD if both parents have mutations in genes associated with cilia function. However, because the cilium is such a complex organelle with many proteins involved in its function, only 34 genes have been identified to cause PCD and about 40-30\% of cases still have no known
genetic cause\textsuperscript{40} (Table 1). Therefore more research is required to uncover the etiology of PCD in order to use genetic testing as a future diagnostic tool and possible therapeutic avenue.
Table 1. Overview of currently known PCD causing genes including defects to cilia structure, effects on motility and conservation with *Chlamydomonas reinharditii*. Table adapted from Lucas (2015)\(^7\).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Motion defect</th>
<th>Chlamydomonas orthologue</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Missing outer dynein arm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNAH5</td>
<td>Immotile or stiff</td>
<td>Yes</td>
<td>Olbrich <em>et al.</em>, 2002.</td>
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<tr>
<td>DNAH6</td>
<td>Immotile or stiff cilia, sparse cilia</td>
<td>Yes</td>
<td>Li <em>et al.</em>, 2016</td>
</tr>
<tr>
<td>DNAH8</td>
<td>Unknown</td>
<td>Yes</td>
<td>Watson <em>et al.</em>, 2014</td>
</tr>
<tr>
<td>DNAI1</td>
<td>Immotile</td>
<td>Yes</td>
<td>Pennarun <em>et al.</em>, 1999</td>
</tr>
<tr>
<td>DNAI2</td>
<td>Unknown</td>
<td>Yes</td>
<td>Loges <em>et al.</em>, 2008</td>
</tr>
<tr>
<td>DNAL1</td>
<td>Uncoordinated, reduced beat frequency</td>
<td>Yes</td>
<td>Horvath <em>et al.</em>, 2005; Mazor <em>et al.</em>, 2011</td>
</tr>
<tr>
<td>NME8</td>
<td>Normal or immotile</td>
<td>Yes</td>
<td>Duriez <em>et al.</em>, 2007</td>
</tr>
<tr>
<td>CCDC103</td>
<td>Immotile or uncoordinated</td>
<td>Yes</td>
<td>Panizzi <em>et al.</em>, 2012</td>
</tr>
<tr>
<td>CCDC114</td>
<td>Immotile or stiff</td>
<td>Yes</td>
<td>Onoufriadis <em>et al.</em>, 2013</td>
</tr>
<tr>
<td>ARMC4</td>
<td>Immotile or reduced beat frequency</td>
<td>No`</td>
<td>Hjeij <em>et al.</em>, 2013</td>
</tr>
<tr>
<td>CCDC151</td>
<td>Immotile</td>
<td>Yes</td>
<td>Hjeij <em>et al.</em>, 2014</td>
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<td>Missing outer/inner dynein arm</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>DNAAF1 / LRRC50</td>
<td>Immotile</td>
<td>Yes</td>
<td>Loges <em>et al.</em>, 2009</td>
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<td>DNAAF2</td>
<td>Immotile</td>
<td>Yes</td>
<td>Omran <em>et al.</em>, 2008</td>
</tr>
<tr>
<td>DNAAF3</td>
<td>Immotile</td>
<td>Yes</td>
<td>Mitchison <em>et al.</em>, 2012</td>
</tr>
<tr>
<td>HEATR2</td>
<td>Immotile</td>
<td>Yes</td>
<td>Diggle <em>et al.</em>, 2014</td>
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<tr>
<td>LRRC6</td>
<td>Immotile</td>
<td>Yes</td>
<td>Kott <em>et al.</em>, 2012</td>
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<tr>
<td>ZMYND10</td>
<td>Immotile or stiff</td>
<td>Yes</td>
<td>Moore <em>et al.</em>, 2013</td>
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<td>SPAG1</td>
<td>Immotile</td>
<td>No</td>
<td>Knowles <em>et al.</em>, 2013</td>
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<td>C21orf59</td>
<td>Immotile</td>
<td>Yes</td>
<td>Austin-Tse <em>et al.</em>, 2013</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Abnormal</td>
<td>Reference</td>
</tr>
<tr>
<td>--------</td>
<td>--------------------------------------</td>
<td>----------</td>
<td>------------------------------------------------</td>
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<tr>
<td>DYX1C1</td>
<td>Immotile</td>
<td>No</td>
<td>Tarkar et al, 2013</td>
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<td></td>
<td><strong>Missing inner dynein arm and/or microtubule disorganization</strong></td>
<td></td>
<td></td>
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<tr>
<td>CCDC39</td>
<td>Hyperkinetic with reduced amplitude</td>
<td>Yes</td>
<td>Merveille et al, 2011; Blanchon et al, 2012</td>
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<tr>
<td>CCDC40</td>
<td>Hyperkinetic with reduced amplitude</td>
<td>Yes</td>
<td>Becker-Heck et al, 2011; Blanchon et al, 2012</td>
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<tr>
<td>CCDC65</td>
<td>Stiff, dyskinetic</td>
<td>Yes</td>
<td>Austin-Tse et al, 2013</td>
</tr>
<tr>
<td>CCDC164</td>
<td>Hyperkinetic with reduced amplitude</td>
<td>Yes</td>
<td>Wirschell et al, 2013</td>
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<tr>
<td></td>
<td><strong>CP defects</strong></td>
<td></td>
<td></td>
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<td>RSPH1</td>
<td>Low beat frequency or immotile</td>
<td>Yes</td>
<td>Kott et al, 2013</td>
</tr>
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<td>RSPH4A</td>
<td>Low beat frequency or immotile, circular movement</td>
<td>Yes</td>
<td>Castleman et al, 2008</td>
</tr>
<tr>
<td>RSPH9</td>
<td>Low beat frequency, circular movement</td>
<td>Yes</td>
<td>Castleman et al, 2008</td>
</tr>
<tr>
<td>RSPH3</td>
<td>Hyperkinetic</td>
<td>Yes</td>
<td>Jeanson et al, 2015</td>
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<tr>
<td>HYDIN</td>
<td>Uncoordinated and reduced motility</td>
<td>Yes</td>
<td>Olbrich et al, 2012</td>
</tr>
<tr>
<td></td>
<td><strong>Basal body localization</strong></td>
<td></td>
<td></td>
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<tr>
<td>CCNO</td>
<td>Reduced number of cilia</td>
<td>No</td>
<td>Wallmeier et al, 2014</td>
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<tr>
<td>MCIDAS</td>
<td>Reduced number of cilia, cilia present are immotile</td>
<td>No</td>
<td>Boon et al, 2014</td>
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<tr>
<td></td>
<td><strong>No defect/ non-specific defects</strong></td>
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<td></td>
</tr>
<tr>
<td>OFD1</td>
<td>Normal or uncoordinated and hyperkinetic</td>
<td>No</td>
<td>Budny et al, 2006</td>
</tr>
<tr>
<td>RPGR</td>
<td>Motile and immotile</td>
<td>No</td>
<td>Moore et al, 2006</td>
</tr>
<tr>
<td>DNAH11</td>
<td>Increased beat frequency, or immotile</td>
<td>Yes</td>
<td>Schwabe et al, 2008</td>
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To identify additional causes of PCD, our collaborators Dr. Raymond Kim and Dr. Stephen Scherer at The Center of Applied Genomics performed whole exome sequencing on patients and their families recruited by respirologists Dr. Sharon Dell at The Hospital for Sick Children and Dr. David Hall at St. Joseph’s Hospital. These patients possessed symptoms indicative of PCD but no mutations in known causative genes at the time of sequencing. Gene candidates were identified based on the following criteria:

1. **The segregation of the mutation:** As PCD is an autosomal recessive trait, genes were considered candidates at this step if the patient possessed two mutations in the same gene in *trans*. Each parent must carry one of the mutations identified in the patient. Unaffected siblings must carry no mutations or only one mutation that a parent also carried.

2. **How damaging the mutation is:** if the mutations identified result in a new stop codon and therefore a truncated protein, the mutation would be considered damaging. In cases where the mutation did not produce a likely truncated protein, the mutation would be analyzed using online programs such as Polyphen and SIFT. Both programs predict the degree of damage a mutation would have on the protein using slightly different parameters. Polyphen predicts the degree of damage of a point mutation based on the biochemistry of the resulting residue and returns a score between 0.0 for “benign mutations” with high confidence to 1.0 for probably “damaging mutations “with high confidence. A score between these numbers are considered “possibly damaging”, with low confidence. SIFT predicts the degree of damage of a mutation based on how often the point mutation occurs naturally and the level of conservation of the affected site. Scores ranges from 0 to 1 with a score of <=0.05 representing a damaging substitution while scores >0.05 are tolerated substitutions. If both mutations were considered damaging by Polyphen or SIFT, then the gene was considered a candidate for further characterization.

3. **Possible role in cilia:** If the gene has family members or related genes by protein domains or function already implicated in cilia disease, the candidate would be rated higher priority. Otherwise, the gene was chosen for a possible novel role in cilia biology.
Following the above pipeline, four genes were identified as candidates for further testing. The candidates are described below.

1.2.1 Radial spoke head 1 (*rsph1*)

Both mutations identified by our collaborators were predicted to result in truncated protein. One mutation was a point mutation at r.54C>A which introduces a new stop codon at p.Glu29X (Fig.2a) The second mutation was an insertion at r.288_289insU, resulting in a reading frameshift and a premature stop at position109 (p.Asp96ArgfsX109). Humans have two protein coding *rsph1* transcripts one 309 amino acids (aa) long and another 271 aa long; both transcripts include the exons affected by the mutations. The affected exons encode Membrane Occupation and Recognition Nexus (MORN) domains which are the only functional domains of this protein. While the exact function of the MORN domain is unknown, other proteins that possess this domain include junctophilins thus the domain is hypothesized to function as a linker between membrane and other proteins. Using NCBI BLAST of the human protein sequence against zebrafish database reveals that the protein sequences are 65% identical with both mutated residues located in highly conserved regions (Fig.2b).

*RSPH1* is a component of the radial spokes in the cilium structure that physically connects the microtubule doublets to the central complex thus stabilizing the organelle and allowing for proper cilia motility. Furthermore, *rsph1* was considered a strong candidate because other radial spoke proteins have already been implicated in PCD pathogenesis. Patients with mutations in *rsph4* and *rsph9* developed disorganized axonemes resulting in cilia with abnormal beat. As well, knock down of *rsph9* in zebrafish resulted in embryos with motile cilia that were unable to generate directional flow. It is therefore likely that *rsph1* mutations would also produce cilia with disorganized ultrastructure and unproductive flow. This hypothesis was proven correct when Kott *et al* (2013) demonstrated patients with the same mutations (r.54C> A) in *rsph1* have mispositioned central pairs or microtubule doublets and respiratory cilia from patients exhibit hyperkinetic beat due to reduced beating angle and distance traveled of ciliary tips. As this gene was confirmed as a cause of PCD, no further work was done on *rsph1* for this thesis.
Figure 2. Schematic of human mutations identified in *rsph1*. A) Both mutations affect the MORN domain of *rsph1*, both introducing premature stop codons within functional domains of the protein. Red arrows depict approximate location of mutation at transcript level and red stars indicate location of resulting stop. Protein domain locations are based on the SMART domain database. B) NCBI protein BLAST output of *rsph1* between human and zebrafish residue sequences. Boxed in red are the amino acids affected by the mutation.
1.2.2 Cylin O (ccno)

Two siblings were identified with homozygous mutations in this gene. The mutation was an nonsense point mutation at r.961G>A resulting in a premature stop at p.Gln321X of a 350 aa long protein (Fig.3a). Only one transcript is protein coding in humans and this mutation affects an exon that encodes a cyclin-like domain\textsuperscript{52}. Blasting the human protein against the zebrafish protein database indicates that the zebrafish protein sequence is 47% identical to the human sequence.\textsuperscript{54} (Fig.3b).

This gene was considered a good candidate because ciliogenesis does not occur until the cell is in G1/G0\textsuperscript{1}. Since many cyclins do control cell division processes, ccno may have a role in initiation or inhibition of ciliogenesis and regulate cilium elongation.\textsuperscript{57} For example, CP110, a protein that prevents elongation of cilia is regulated by ubiquitination by a complex involving CYCLIN F function\textsuperscript{58}. Degradation of this protein allows CEP290, a basal body associated protein to promote ciliogenesis\textsuperscript{39}. Furthermore, the basal body itself hints at the intricate relationship between ciliogenesis and cell cycle since this organelle is involved in both processes. We therefore considered ccno a good candidate for further analysis since the gene may have a role in ciliogenesis by affecting basal body function. This hypothesis was also confirmed during the beginning of this thesis when Wallmeier \textit{et al} (2014) demonstrated that patients with the same mutations identified in this study had fewer respiratory cilia than unaffected individuals\textsuperscript{60}. The epithelia of \textit{Xenopus} embryos are multiciliated and knockdown of ccno in these embryos resulted in epithelial cells with fewer motile cilia. Since this gene was published as a cause of PCD, further analysis on this gene and the mutation identified was not performed.
Figure 3. Schematic of the mutation identified in ccno A) Two patients homozygous for the mutation shown in exon 3 of cyclin O results in a premature stop near the end of the protein at a cyclin-like domain. The approximate location of the nucleotide change at the transcript level is indicated by the red arrow and the red star indicates position of the new stop. Protein domain predication is based on the SMART domain database. B) Protein homology between human and fish residue sequences. Boxed in red is the residue affected by the mutation.
1.2.3 Myotubularin related protein 11 (mtmr11)

One mutation found in this gene is a single nucleotide insertion within the first exon (r.15_16insC) which introduces a new stop at position 28 (p.Arg6AlafsX28) in the protein sequence (Fig.4a). The second mutation is point mutation at r.1339G>A, introducing a new stop at p.Gln447X which affects the myotubularin-phosphatase-like domain. This gene is found on chromosome 1 and has two protein coding transcripts one 709aa long and the other 640aa long. Both transcripts contain the exons affected by the mutations. The human protein sequence is 42% identical to the zebrafish sequence (Fig.4b).

Myotubularin proteins are a family of phosphatases that have particular affinity towards the 3’ phosphate of phosphatidylinositol 3-monophosphates (PtdIns3P) and phosphatidylinositol 3, 5 biphosphates (PtdIns(3,5)P2). PtdIns3P and PtdIns(3,5)P2 have roles in marking identities of subcellular membrane compartments, regulating membrane trafficking and endocytosis as well as acting as secondary messengers. In dephosphorylating PtdIns3P and PtdIns(3,5)P2, myotubularins are capable of controlling their turnover. Interestingly, it was found that PtdIns3P may have a role in regulating primary cilium length as knock out of phosphatidylinositol 3-kinases class II a results in loss PtdIns3P at the base of primary cilium in mouse embryonic fibroblast cells leading to shorter cilia. Importantly however, Mtmr11 contains an inactive phosphatase domain thus it is unlikely the molecule can regulate the activity of PtdIns itself. There is evidence that myotubularins with dead phosphatase domains can heterodimerize with active myotubularins, altering the type of PtdIns the active myotubularin targets. Therefore, mtmr11 may regulate cilia length by partnering with an active myotubularin; loss of functional mtmr11 could lead to longer cilia because the active myotubularin partner is no longer affecting PtdIns that function at the cillum.
Figure 4. Schematic of the two mutations identified in mtmr11. A) This gene has 17 transcripts and encodes two functional domains: a pleckstrin homology domain (blue) that allows the protein to bind to lipid molecules and the myotubulin-like phosphatase domain (orange) which allows myotubularins to dephosphorylate PtdIns3P and PtdIns(3,5)P2 molecules. Protein domains are predicted through the Superfamily database. The red arrows indicate approximate location of nucleotide change at the transcript level and the red stars indicate the location of premature stop resulting from the mutation. B) NCBI protein BLAST output indicating that the human protein sequence (query) is 42% similar to the zebrafish (subject) protein sequence. The first mutation is not conserved between the organisms (beginning of homology starts at position 45) but the second mutation is boxed in red and affects a conserved residue.
1.2.4 Spindle apparatus coiled coil protein 1 (*spdl1*)

Both mutations identified in *spdl1* were point mutations that were not predicted to produce truncated proteins but were however still predicted to be damaging based on Polyphen analysis\(^50\) (Fig. 5c). The first mutation is at r.616 C>U resulting in p.Asp206Asn and the second is at r.1541A>U leading to p.Leu514His affecting exon 4 and 10 respectively of an 11 exon long transcript (Fig. 5a). There are 8 protein coding transcripts for *spdl1* found in humans but only one transcript contains both exons affected by the mutations\(^52\). The first mutation affects 1 of 8 coiled-coil domains, the only identified functional domain in this gene. The human protein sequence is 47% identical to the zebrafish protein and while neither residue is conserved, the mutation within the coiled-coil domain affects a conserved charge which may alter the folding of the coiled-coil domain and therefore its function and ability to interact with other coiled-coil containing proteins\(^54\) (Fig. 5b).

*Spdl1* was initially identified using *Drosophila* embryonic cells probing for novel components involved in the spindle assembly checkpoint which is a process that prevents commencement of anaphase before all kinetochores are attached to the spindle and establishment of spindle poles\(^65\). Knockdown by RNAi of *spdl1* resulted in the inability of Dynein-Dynactin complexes to localize to kinetochores and thus unstable attachment of microtubules. The role *spdl1* plays in localizing dynein to the kinetochore is conserved among metazoans including humans\(^67,68\). Thus we hypothesized that SPDL1 may be a likely candidate for the etiology of PCD by possibly localizing Dynein to the cilium so that it may be loaded onto Kinesin, activated at the cilium tip and fulfill a function in retrograde IFT\(^16\). Dynein is also found in the axoneme of motile cilia thus an alternative hypothesis is that SPDL1 may localize axonemal dynein to the base of cilia for transport into the structure maintaining cilium motility\(^8\).
Two point mutations were identified by whole exome sequencing. Neither mutations result in protein truncation however the first mutation may alter function of the coiled-coil. Red arrows indicate approximate location of the mutations at the transcript level. Alignment of protein domains are predicted using the Ncoils database. NCBI protein BLAST output of relevant locations between human and zebrafish protein sequences. The protein sequences are 48% identical. Affected residues are boxed in red. The first mutation affects a residue where the charge is conserved. The second mutation affects a portion of the protein sequences that appears to have poor homology and the residue is not conserved. As both mutations are point mutations, analysis through SIFT and Polyphen was performed. The first substitution is not damaging by SIFT (<0.05) but possibly damaging by Polyphen. The second substitution is also tolerated by SIFT (<0.05) but considered probably damaging by Polyphen (>0.5).
1.3 Purpose

The aim of this project is to investigate whether the genes identified through whole exome sequencing of PCD patients are causes of PCD. To do this, the zebrafish will be used to determine where the candidates are expressed and whether the genes will affect cilia function using gene knockdown and knock-out approaches. How the candidates might affect cilia function will be investigated by characterizing the effects of gene knock out on cilia presence, range of motion, orientation, length and beat frequency. The mutations identified in the patient screen will also be analyzed by introduction into mutants and determining if the mutant product can rescue phenotypes. Since two of the candidates identified were already published before the onset of this study or during the early stages of investigation, most work focused on the candidate Spindly.
Chapter 2

2 Investigating the role of spdl1 in motile cilia function

2.1 Brief introduction and rationale

Primary ciliary dyskinesia (PCD) is a rare disease of motile cilia dysfunction affecting approximately 1/30000 children\(^7\). The disease is characterized by neonatal pneumonia and frequent respiratory infections through life that could lead to inflammation or fluid in the bronchioles or in severe situations, lung collapse\(^41\). Additional symptoms of the disease include inflammation of the sinuses, chronic otis media with effusion that could lead to partial hearing loss and situs abnormalities possibly resulting in cardiac complications\(^41,43,45\). Without the symptom of heterotaxy, diagnosis occurs on average by 6 years of age in Europe but diagnosis during late childhood or early adulthood is also common\(^46,70\). Late diagnosis is in part due to difficulty in identifying the disease since current methods involve a panel of three tests each requiring repetition, sophisticated equipment and skilled respirologists and technicians\(^40\). As PCD is an autosomal recessive disease, genetic testing was proposed as a simpler and quicker diagnostic tool, however only approximately 60-70% of cases have known genetic cause\(^39\). To narrow our gap in knowledge, collaborators at The Center of Applied Genomics in Toronto and respirologists at The Hospital for Sick Children and St. Joseph’s hospital performed whole exome sequencing on PCD patients with no mutations in genes known to cause PCD at the time of testing. Four candidates were identified for further characterization in PCD etiology using the zebrafish embryo as a model to study motile cilia function. Of the four candidates, two were published before the onset of this thesis as PCD causes, indicating our sample cohort and strategy to identify candidates is valid. One gene of the remaining candidates, Spindly, was chosen for in-depth characterization in PCD etiology.

Spindly (spdl1) was first identified through an RNA interference screen in *Drosophila* embryonic cells for additional components in the spindle assembly checkpoint\(^65\). Through analysis on dynein localization in Spdl1 deficient cells, Griffis *et al* (2007) determined that Spindly is essential for silencing the checkpoint by recruiting the dynein-dynactin complex to
the kinetochore. Once dynein is bound to the kinetochore, inhibition of anaphase by the mitotic checkpoint complex is removed allowing mitosis to proceed\textsuperscript{66}. Without Spindly, dynein did not localize to the kinetochores and cells were arrested at metaphase, leading to apoptosis. Spdl1 has since been studied in the nematode \textit{C. elegans} and in HeLa cells, revealing that the function of the protein in localizing dynein to the kinetochore to terminate the spindle assembly checkpoint is conserved\textsuperscript{67,68}.

Based on the observation that SPDL1 is capable of localizing dynein to the kinetochore and that mutations in \textit{spdl1} was identified in one of our PCD patient cohort, we hypothesized that SPDL1 may affect motile cilia function possibly by regulating intraflagellar transport (IFT). Dynein fulfills a role in IFT by mobilizing cilium components back into the cell to be degraded thus without IFT dynein, cilia may accumulate damaged or non-functional protein resulting in organelle dysfunction\textsuperscript{16}. Dynein is also part of the motile cilia structure, enabling motile cilia to beat to maintain fluid flow\textsuperscript{8}. Therefore, SPDL1 may also affect motile cilia by localizing by localizing dynein for incorporation into the cilium body to impart motion. SPDL1 dysfunction may result in improperly beat or immotile cilia respectively.

In the following sections, I demonstrate that Spdl1 is associated with cilia function in zebrafish. \textit{Spdl1} is expressed in motile ciliated tissues by 8 somites to 2 days post fertilization (dpf). Importantly, Spdl1-deficient embryos either through morpholino-mediated knockdown or CRISPR/Cas9-mediated genetic knock-out develop phenotypes associated with cilia dysfunction. Phenotypes from Spdl1 knock out embryos can be rescued by exogenous addition of WT \textit{spdl1} RNA but zebrafish \textit{spdl1} RNA containing one of the mutations identified in the whole exome sequencing screen was unable to rescue phenotypes at the same concentrations, suggesting the mutation results in non-functional Spdl1. Finally, analysis on motile cilia characteristics in \textit{spdl1} mutant zebrafish embryos at 2dpf demonstrates loss of protein result in longer neural tube floorplate motile cilia and reduced ciliary beat frequency.
2.2 Results

2.2.1 Spdl1 is expressed in motile ciliated tissues

To determine if Spdl1 might fulfill a function in motile cilia, it was necessary to first identify whether the gene is expressed in tissues where motile cilia are found in the zebrafish embryo. These tissues include the cells lining the ventricles of the brain, the olfactory placode, the otic vesicle, neural tube and pronephric duct. Motile cilia are also transiently expressed in the laterality organ, the Kupffer’s vesicle. In order to visualize spdl1 expression during zebrafish development using whole mount in situ hybridization (WISH), WT spdl1 cDNA was amplified from a zebrafish blastula stage cDNA library. Spdl1 was inserted into a pCS2 vector following Gateway cloning technology and sense and antisense probes were synthesized from this plasmid. It was found that spdl1 is present during early embryogenesis (Fig. 6A-B). This indicates that spdl1 transcripts are likely maternally contributed into the embryo. Spdl1 transcripts are present in the embryo throughout gastrulation as well (Fig. 6 C-D). By early segmentation periods, staining using the antisense probe was specific for neural tissues in the brain and a midline structure that may be the neural tube (Fig. 6 E-G). Notably, this expression pattern was not observed when embryos at the same stage were stained with the sense probe, which should not bind to spdl1 transcripts, suggesting the expression pattern is not due to probe trapping or non-specific binding. The neural tube and tissues surrounding the ventricles of the brain continue to express spdl1 until 3dpf when staining in the neural tube is not observed (Fig. 7). However, staining in the brain is still present in the embryos treated with the antisense probe compared to the sense probe. Importantly, motile cilia are found in the neural tube floorplate and cells lining the ventricles of the brain, thus the expression patterns observed from early somitogenesis to 2dpf supports the notion that spdl1 has a function in motile ciliated tissues.
Figure 6. Expression of spdl1 in WT embryos during early development and somitogenesis stages. A-B) 2 cell embryos treated with spdl1 antisense probe (A, B) or sense probe (A’, B’) and imaged laterally (A, A’) or dorsally (B, B’). C-D) Shield stage embryos treated with spdl1 antisense (C, D) or sense (C’, D’) probes and imaged laterally (C, C’) or dorsally (D, D’). Shield stage occurs during gastrulation. Expression patterns during 2 cell and shield stages demonstrate spdl1 transcript is maternally contributed. E-G) 8 somite stage embryos treated with spdl1 antisense (E-G) or sense (E’-G’) probes and imaged laterally (E, E’), dorsally (F, F’) and from anterior (G, G’). Staining shows spdl1 expression in neural epithelia of the developing brain and facial features (G) and a midline structure (F) where the neural tube is expected to form (arrows). Scale, 1μm.
Figure 2. *Spdl1* expression in WT embryos during larvae stages. A-C) 1 dpf embryos treated with *spdl1* antisense (A, B, C) or sense (A’, B’) probes suggest *spdl1* is expressed in the neural tube (A, B, arrows) and lining of the ventricles in the brain (C, arrowhead). D-G) Staining in 2 and 3dpf embryos suggest *spdl1* expression decreases with age in the neural tube (Arrows) but staining in the ventricles persist. A,D,F- lateral orientation; B,E,G-dorsal orientation; C- anterior; Bar = 1um.
2.2.2 Spdl1 deficient embryos develop phenotypes associated with cilia defects

Zebrafish embryos with dysfunctional cilia develop ventral body curvatures, hydrocephalus and kidney cysts\(^\text{37}\). Knowing that \textit{spdl1} is expressed in motile ciliated tissues in the zebrafish, it was necessary to elucidate whether knockdown of Spdl1 will result in cilia dysfunction. Gene knockdown can be achieved in zebrafish by introduction of morpholino into the embryo at the 1 cell stage. Morpholinos (MO) are small 18-25 bp long RNA-like molecules that are antisense for a transcript of interest\(^\text{36}\). Binding of the MO to the transcript can prevent translation or proper splicing of the transcript resulting in decreased protein levels or aberrant protein respectively. To explore the effect of Spdl1 knockdown during development, a translation-blocking MO was injected at increasing dosages (3ng, 4.5ng, 6ng) into 1 cell stage wild type (WT) embryos (Fig. 8). Embryos injected with the MO (morphants) developed phenotypes associated with cilia defects, notably, ventral body curvatures and hydrocephaly by 2dpf. Interestingly, dorsal curvatures, a less common phenotype associated with cilia dysfunction was also observed at 2dpf\(^\text{35}\).

Phenotypes increased in severity according to dosage, with death occurring in WT morphants at 6ng, which was the highest dosage tested. This may be due to non-specific activation of P53 apoptotic pathways which is common in MO mediated knockdown\(^\text{71}\). Apoptosis in the neural tube can lead to body curvatures suggesting that phenotypes observed in WT morphants might be due to P53 activation and not cilia dysfunction\(^\text{71}\). Therefore \textit{spdl1} translation-blocking MO was also injected into a \textit{p53}\(^{-/-}\) background to determine if phenotypes associated with cilia defect still occurred (Fig. 8). By 2dpf, \textit{p53}\(^{-/-}\) morphants developed ventral curvatures that increased in severity according to dosage, suggesting phenotypes observed in WT morphants were indeed due to Spdl1 knockdown. More importantly, these results further suggest a role for Spdl1 in motile cilia function since Spdl1 knockdown resulted in phenotypes associated with cilia dysfunction.
Figure 8. Effect of spdl1 morpholino (MO)-mediated knock-down in WT and p53^-/- embryos. A) WT morphants develop body axis curvatures and hydrocephalus by 2dpf. All phenotypes are associated with cilia dysfunction. P53^-/- morphants also developed dosage dependent ventral curvatures by 2dpf, suggesting cilia associated phenotypes are not due to non-specific activation of apoptosis, common in MO experiments. B) Quantification of phenotypes based on MO dosage. WT embryos did not survive treatment with 6ng MO.
2.2.3 *Spdl1* mutants were generated using CRISPR/Cas9 genome editing

Since transient knockdown suggests *Spdl1* might have a role in the function of motile cilia, mutants were generated to study the effects of *Spdl1* knock out during development. Additionally, it was recently observed that many phenotypes that occur after MO-mediated gene knockdown are a result of off-target effects, thus phenotypes observed in morphants described previously may not be due to *Spdl1* knockdown\(^7\). Generation of *spdl1* mutants would allow analysis specifically on *Spdl1* function. To create mutants, CRISPR/Cas9 genome editing was used. This technique involves the introduction of a guide RNA (gRNA), an 18-20 nucleotide long RNA that is complementary to the gene of interest\(^7\). Along with the gRNA, the mRNA for Cas9, an endonuclease that can bind to the gRNA is also injected. Once the gRNA binds to the complementary sequence in the genome, Cas9 is capable of introducing double stranded breaks in the DNA, which will activate endogenous DNA repair mechanisms, resulting in possible insertions/deletions in the genome. Three gRNA sequences complementary to *spdl1* were designed using the online tool CHOPCHOP targeting exons 2, 4 and 6\(^7\) (Fig. 9). Each guide was injected into the 1 cell stage of WT embryos with cas9 RNA to generate fish carrying *spdl1* mutations. Only embryos injected with guide 3 produced cutting in the genome, indicating mutants might be produced from this guide (Fig. 9). In fact, seven fish frameshift mutations in *spdl1* in their germline were identified.
Figure 9. Generation of spdl1 zebrafish mutants using CRISPR/Cas9 genome editing.

Three guides were designed using the online tool CHOPCHOP targeting exons 2, 4 and 6 and individually injected into WT zebrafish embryos (purple bars). Only the guide RNA targeting exon 2 was found to induce cutting (red star). Mutations generated using this guide was predicted to interrupt translation and proper function of at least one of the coiled coil domains, denoted by dark blue boxes. Locations of coiled-coil coding domains were predicted using N-coil database (Ensemble). Serendipitously, 7 founders were identified with frameshift mutations resulting in a new stop codon in exon 3 (red asterisks). These frameshift mutations also resulted in missense mutations until the new stop codon is reached (light blue text), suggesting that even if protein is made from the transcript, the resulting product will be non-functional. All founders were out-crossed with WT and heterozygous offspring from three of the founders were used in this thesis (pink four point star).
2.2.4 Zygotic mutants developed cilia associated defects

Stable F1 heterozygotes from three different alleles were crossed to produce homozygous mutants to determine if genetic Spdl1 knock out recapitulated phenotypes observed by artificial gene knockdown and produce phenotypes associated with cilia dysfunction. The alleles analyzed were a 2 bp, 7 bp and 14 bp deletion which all resulted in addition of a new termination codon in exon 3 (Fig. 9). Homozygous zygotic mutants from each allele were indistinguishable from WT siblings until 2dpf wherein mutants developed mild ventral body curvatures that resembled phenotypes from p53^-/- morphants at the lowest MO dosage (Fig. 10). As well, similar to p53^-/- morphants, no other phenotypes associated with cilia dysfunction were observed. Phenotypes occurred at near Mendelian ratios and where also genotyped to confirm status as mutants. Interestingly, by 3dpf, mutants developed dorsal body curvatures. This phenotype was unexpected but is also associated with cilia dysfunction, further suggesting Spdl1 has a role in cilia function. Another unexpected phenotype occurred in the eyes. At 2dpf, zygotic mutant eyes were smaller than WT siblings; this phenotype became more noticeable by 3dpf (Fig. 11). Additionally, the eyes of mutants were also rounder than WT siblings at 3dpf. The phenotype increased in severity by 4dpf and persisted until embryonic death. Spdl1^-/- embryos did not survive past 7dpf. As well, mutants developed paracardial edema by 3dpf, which may have contributed to embryonic lethality.

While some phenotypes were unexpected, all phenotypes are associated with cilia dysfunction. Ventral body axis curvature is the canonical cilia associated defect while dorsal curvature is associated with polycystin 1/2 (pkd1 / 2) mutants, a bona-fide cilia associated protein\(^{35}\). The eye phenotype was unexpected but may be associated with cilia dysfunction as the eyes contain photoreceptors, which are specialized primary cilia\(^{3}\). Embryonic lethality is common in cilia mutants as well. All phenotypes present in spdl1 mutants suggest Spdl1 may affect cilia function.
Figure 10. *Spdl1* zygotic mutants develop phenotypes associated with cilia dysfunction. At 2 dpf, embryos develop ventral body curvatures which is the canonical phenotype associated with cilia dysfunction. The phenotypes at this age also resemble *p53/-* morphants. At 3dpf, embryos developed dorsal curvatures, which is also a phenotype associated with cilia defects. This phenotype persists until embryonic death at 7dpf. Starting at 3dpf, paracardial edema can be observed, which increases in severity until death. Scale bar = 5um.
Figure 11. *Spdl1*−/− embryos develop small, abnormally shaped eyes. This phenotype can be observed beginning at 2dpf, progressing in severity until death at 7dpf. At 2dpf, photoreceptors are not developed thus failure to develop elongated eyes may be due to proliferation defects. Indeed, at 3dpf and 4dpf, mutant eyes maintain the small, rounded shape established at 2dpf rather than elongating and flattening. Scale bar = 5μm.
2.2.5 Confirmation of Spdl1 knock out in spdl1−/− embryos

Since spdl1 mutations result in premature stop, it was predicted that spdl1−/− embryos would have less spdl1 transcripts due to nonsense mediated decay. To test this, reverse transcription quantitative PCR (RT-qPCR) was performed using cDNA from pooled mutant and WT embryos at 2dpf. Embryos were categorized as mutant or WT based on presence of ventral curvature. Results suggest that the amount of spdl1 transcripts are reduced in mutants compared to WT siblings after normalization to gapdh expression (Fig.12 A). WISH was also performed on 2dpf and 3dpf spdl1−/− and WT sibling embryos to visually determine if mutants have lower levels of spdl1 transcripts (Fig.12 B). Staining in WT embryos with the antisense probe which is expected to bind to spdl1 transcripts demonstrated expression in the cells lining the ventricles of the brain but little to no expression in the neural tube. Treatment with the sense probe which should not bind to spdl1 transcript resulted in staining diffusely in the head suggesting that staining in the ventricle lining is specific. This observation was similar to the expression pattern observed previously in a pure WT background. However, staining with the antisense probe in zygotic mutants also demonstrated specific staining in the ventricle lining, suggesting either that the probe can still bind to remaining transcripts in mutants, or the probe is trapped in the head. Nonetheless, RT-qPCR confirmed spdl1 transcript levels are reduced, suggesting nonsense-mediated decay pathways were activated.

However, if in situ results are accurate and spdl1 transcripts are still present in zygotic mutants, it is unlikely that a functional protein will be produced as all alleles investigated are deletions leading to insertions of premature termination sequences in exon 3 of spdl1. Not only is the exon affected in an early portion of the gene, but the new termination codon is located upstream of a conserved Spindly motif that is required for Spdl1-Dynein interaction. Any protein produced in zygotic embryos will lack this motif and will most likely be rendered non-functional.
Figure 12. Confirmation of *spdl1* knock out in zygotic mutants. **A)** By quantitative reverse transcription PCR on one biological sample, there are less transcripts in *spdl1* mutants than in WT siblings at 2dpf after normalization to *gapdh*. Embryos were categorized as mutants or WT based on expression of ventral curvatures. **B)** WISH in zygotic mutants results in similar staining patterns as WT siblings in 2dpf embryos. This staining pattern is slightly different from treatment with the sense probe, suggesting probe trapping or *spdl1* antisense probe is capable of binding *spdl1* transcript fragments before transcripts can be degraded via nonsense mediated decay. Scale bar = 1um.
2.2.6 Spdl1−/− neural epithelia exhibited proper cell division during early somitogenesis stages

Studies in Drosophila embryonic cells, C. elegans and HeLa cells demonstrate Spindly protein performs a conserved function in silencing the spindle assembly checkpoint by localizing the Dynein-Dynactin complex to kinetochores which signals the end of metaphase\(^6\). Without SPDL1, mitosis stalled and in the case of Drosophila and C. elegans embryonic cells, apoptosis occurred due to prolonged arrest in metaphase. Additionally, SPDL1 deficiency resulted in chromosome misalignments and spindle pole orientation defects in HeLa cells\(^6\). Since no studies have been performed on Spdl1 function in zebrafish, we wanted to probe whether zebrafish Spdl1 also regulates cell division. Additionally, if the function of Spdl1 is conserved, the argument that phenotypes associated with cilia defects is due to Spdl1 absence which may be the cause of PCD phenotypes in the patient will be strengthened. Thus, cell division was analyzed in spdl1 mutants by labeling chromosomes in red with mRNA encoding H2B-RFP and cell membranes in green using mRNA encoding GAP43-GFP. Cell division in the neural keel and the developing neural tube was imaged in embryos at 12hpf or 8 somite stages in mutants and 12 somite stages in WT. The neural keel (8 somites) or neural tube (12 somites) was chosen for imaging for two reasons: firstly, WISH results suggest spdl1 is expressed in these tissues and secondly, the cells in the neural keel and developing neural tube will allow investigation on spindle pole orientation. These cells initially orient their spindle poles with the anterior-posterior axis of the neural keel and the metaphase plate perpendicular to the neural midline\(^7\). Later, dividing cells will orient the metaphase plate with the midline to divide along the medio-lateral axis. Thus, a defect in spindle pole orientation could manifest as a failure in spindle rotation. As mentioned above, spindle pole orientation defects could also lead to chromosome misalignments which could possibly result in chromosome breakage and micronuclei formation\(^7\). Spdl1−/− embryo neural keel cells appear to divide without spindle orientation defects, chromosome misalignments or micronuclei formation (Fig 13, Supplemental movie 1, 2). These results suggest Spdl1 function in cell division is not conserved in the zebrafish. Alternatively, the results may support the argument that Spdl1 is maternally contributed and cell divisions at 8 somite stages is too early to observe zygotic mutant Spdl1 function, if protein is produced by the mutant transcript.
Figure 13. *Spdl1* mutants at the 8 somite stage do not exhibit cell division defects. Cells membranes are marked in green and chromosomes are marked with H2B, in red. Embryos were imaged dorsally. A and P represent anterior and posterior respectively. A series of stills taken from a movie of cell division in a *spdl1*−/− embryo is presented in chronological order (numbers). The arrow points at cell of interest undergoing mitosis. 1) Cell appears to be in prophase. 2) Chromosomes are aligning on the metaphase plate and spindles poles appear to reorient in preparation for anaphase. 3) Cell is in metaphase. 4) Anaphase is underway. 5) Anaphase is almost completed and telophase appears to be beginning. 6) Mother and daughter cells have separated. During telophase, one cell divided out of the plane of view. Beginning at image 4, three other cells are preparing for mitosis, suggesting cell division is occurring properly in the whole organism.
2.2.7 Introduction of WT *spdl1* RNA rescued mutant phenotypes but not *spdl1* RNA with human mutation

As a final test to confirm cilia associated phenotypes are due to mutations in *spdl1*, WT zebrafish *spdl1* RNA was introduced into mutants to determine if cilia associated phenotypes can be rescued. Injection of 20, 30 and 40pg *spdl1* RNA into 1-cell stage embryos was performed. Only embryos injected with 20pg *spdl1* RNA survived with only a few embryos developing cyclopia, a common phenotype in RNA overexpression\textsuperscript{78}. With only 20pg of RNA, *spdl1*\textsuperscript{−/−} embryos failed to develop ventral and dorsal curvatures (Fig. 14). The eyes also appeared similar to WT siblings suggesting WT *spdl1* RNA was able to rescue mutant phenotypes. However, exogenous RNA was not able to rescue paracardial edema or embryonic death.

Since WT *spdl1* RNA can rescue mutant phenotypes, I next determined if *spdl1* RNA containing the human mutation resulting in p.Asp206Asn in SPDL1 protein was functional. The WT residue at the conserved site in zebrafish protein was changed to a codon encoding Asn via site-directed mutagenesis. 20 pg of mutant RNA was injected into mutant embryos at 1-cell stage and was unable to rescue cilia associated phenotypes (Fig. 14). This suggests that the human mutation is indeed deleterious.
Figure 14. *Spdl1* mutant phenotypes following injection of exogenous *spdl1* RNA. Results suggest mutant phenotypes can be rescued by re-introduction of WT *spdl1* RNA but not *spdl1* RNA containing one of the human mutations at the same dosage (20 pg). Proportion of embryos expressing phenotypes is based on 3 experiments for each RNA used. A total of 28 mutant embryos were injected with WT *spdl1* RNA while 47 mutant embryos were injected with *spdl1* containing one of the human point mutations identified.
2.2.8 Motile cilia analysis
All experiments thus far demonstrated that Spdl1 may have a role in motile cilia function as it is expressed in motile ciliated tissues, knockdown and knock-out results in embryos with phenotypes associated with motile cilia dysfunction which can be rescued by reintroduction of functional spdl1 RNA. To elucidate what effect Spdl1 has on motile cilia function, investigation of cilia function must be performed. In particular, four questions will be addressed: whether motile cilia are present in the neural tube, if cilia are positioned correctly, whether the lengths of cilia are affected and if mutant cilia have a motility defect. Motile cilia in the neural tube and the olfactory placode will be the focus of analyses because spdl1 is expected to be expressed in these tissues based on in situ hybridization results.

2.2.8.1 Spdl1 mutant neural tube floorplate cells produce motile cilia that are positioned correctly
Ventral body curvature is a phenotype associated with motile cilia dysfunction, but it is also a phenotype present when neural tubes are devoid of cilia as demonstrated in IFT88 zebrafish maternal zygotic mutants\(^{21}\). Furthermore, one hypothesis to how spdl1 might affect motile cilia function is that it may localize axonemal dynein to be incorporated in the cilium body, thus allowing the cilium to be motile. To investigate whether spdl1 embryos express motile cilia, embryos were injected with mRNA encoding Arl13b-GFP to mark cilia and Memb-RFP to mark cell membranes. The neural tube floorplate was imaged at 30hpf. Fig. 15 demonstrates that spdl1\(^{-/-}\) embryos still developed motile cilia, suggesting phenotypes are not due to absence of cilia but perhaps dysfunction in motion or fluid flow.

Fluid flow in the neural tube may be altered by improperly positioned floorplate cilia. In the WT neural tube floorplate, cilia are positioned asymmetrically wherein the basal body is anchored near the more posterior side of the cell. Each cilium also exhibits posterior tilt\(^{79}\). If the tilt or positioning of cilia are abnormal or non-uniform, unproductive flow may result possibly leading to curvatures observed. However, it appears floorplate cilia are both positioned properly and experience posterior tilt in spdl1 mutants, indicating that spdl1 does not affect basal body localization or mechanisms involved in cilia posterior tilt (Fig. 15).
Figure 15. *Spdl1*<sup>−/−</sup> embryos have posterior tilted motile cilia in the neural tube floorplate. Cilia are labeled with Arl13b in green and cell membranes are labeled in red. A and P represent anterior and posterior respectively. Embryos were imaged dorsally. In this orientation, motile cilia can be found on the side of the tube (alongside primary cilia) and in the middle of the tube (arrows). Motile cilia are differentiated from primary cilia in the neural tube as motile cilia create a blurred fan-like shape when image at a slow speed. Motile cilia in the neural tube are also tilted in towards the posterior of the organism, which is not affected in the mutant embryo.
2.2.8.2  Motile cilia in the neural tube are significantly longer in *spdl1* mutants

Alterations to cilia length may result in aberrant cilia movement and decreased fluid flow leading to ventral curvatures in zebrafish embryos\(^8^0\). It was also hypothesized that Spdl1 may regulate IFT somehow possibly by interacting with dynein. Aberrant IFT result in alterations to cilia length thus the length of neural tube floorplate cilia was investigated by staining fixed 2dpf embryos with anti-acetylated tubulin (Fig. 16). This antibody marks all stable tubule structures, including cilia. Average length was calculated and revealed that *spdl1*^−/−^ 2dpf embryos had significantly longer floorplate cilia than WT siblings at the same age (p<0.001; Fig.11). The length of cilia in the olfactory placode was also investigated since *spdl1* is also expressed in this region. Unlike in the neural tube, cilia length in *spdl1* mutants was similar to WT cilia lengths. The result suggests spdl1 has a role in regulating length in fluid flow propagating cilia supporting the hypothesis that Spdl1 is involved in regulating IFT.
Figure 16. Cilia in *spdl1*−/− neural tube floorplate are significantly longer than WT siblings. Embryos were fixed at 2dpf in 100% methanol and stained for the same time with mouse anti-acetylated tubulin and anti-mouse Alexa488 fluorophore. Average floorplate cilia length in WT is 3.62±1.09 um compared to 4.37±1.15 um in mutants. ‘*‘ indicates the difference was statistically significant by a 2-tail unpaired t-test (p<0.001). Since *spdl1* is expressed in the olfactory placode, cilia length was also investigated in this region. Average olfactory cilium length in WT is 4.67±1.76 um and average length in mutants is 4.75±1.62 um. This difference is not statistically significant (p=0.6484).
2.2.8.3 Mutant neural tube motile cilia exhibit reduced ciliary beat frequency compared to WT siblings

Since cilia length was increased in mutants compared to WT siblings in the neural tube floorplate, it is possible cilia motility may also be affected. Furthermore, the hallmark of PCD is reduced cilia motility thus it was necessary to determine if Spdl1 absence result in decreased cilia motility to confirm the gene is a cause of the disease. Videos of neural tube floorplate cilia in 30hpf and 2dpf spdl1−/− and WT sibling embryos were recorded using high speed videomicroscopy and ciliary beat frequency (cbf) was analyzed following methods described by Drummond (2009) (Supplemental movies 3-8). Briefly, kymographs of each motile cilium with visible from tip to base were made using ImageJ, generating an image of waves based on how the cilium was bisected (Fig. 17 B). Since each pixel between peaks represent a frame in the video, the distance between peaks was used as the time between each cycle of movement. The ratio of the distance between peaks to overall video length at 20fps results in a rate described by cycles / minute. Using this method, cbf of mutants and WT embryos at 30hpf was not significantly different (Fig.17). However, by 2dpf, the cbf in mutant embryos was significantly reduced (p<0.001). Importantly, the reduction of cilia motility coincides with the onset of ventral curvatures. Cbf in the olfactory placode was not different between mutants and WT siblings at 2dpf, suggesting that increase in length of cilia in spdl1 mutants is associated with reduced motility in the neural tube.

The range of motion of neural tube cilia was also investigated to explore the possibility that the amplitude of cilia waveform was affected, which could also lead to reduced fluid flow. Cilia were once again labeled with Arl13b-GFP and videos of cilia movement in the neural tube was captured using a confocal microscope. Due to the slower capture rate of confocal microscopy, moving cilia create a fan-like appearance. Range of motion can therefore be measured by calculating the angle between the most extreme edges of the fan. Since embryos were imaged dorsally, floorplate cilia can appear either oriented vertically on the side of the tube with posterior tilt, or horizontally down the center of the tube facing towards the posterior end of the animal (Fig.18). The range of motion of motile cilia in the neural tube was not significantly different between spdl1−/− and WT siblings a 30hpf. Since the stage imaged was before phenotype onset, this was not an unexpected result. Range of motion analysis could not be performed on older embryos as it is difficult to visualize Arl13b-gfp in embryos 2dpf or older.
Figure 17. Ciliary beat frequency (CBF) of neural tube floorplate (nt) and olfactory placode cilia at 30hpf and 2dpf. A) Average CBF in WT neural tube floorplate at 30hpf is 81.87±11.5 beats/min (n=10) and in stage-matched mutants CBF is 86.27±14.69 beats/min (n=8). This difference was not statistically significant (p=0.486). Average CBF in WT floorplate at 2dpf is 70.41±5.42 beats/min (n=13) while average CBF in mutants is 53.15±8.2 beats /min (n=13). CBF in mutants at 2dpf was significantly slower by a 2-tail unpaired t-test (p<0.001), denoted by ‘*’. The average CBF in 2dpf wt in the olfactory placode is 67.17±11.95 beats/min (n=7) and in mutants CBF is 67.17±11.95 beats/min (n=14). This difference was not statistically significant (p=0.2769). All averages are indicated by a diamond marker. B) Representative kymographs of neural tube motile cilia at 30hpf in mutant and WT siblings.
Figure 18. Range of motion in neural tube motile cilia in *spdl1* mutants compared to WT siblings. Range of motion was investigated by measuring the angle between the most extreme ends of cilia motion in both dorsal floorplate cilia and lateral floorplate cilia (schematic). Average angle of motion in WT cilia is 42±6.3° and 35.93±5.8° for dorsal and lateral cilia respectively. Average angle of motion in mutant cilia is 42.84±6.03° and 37.73±5.23° for dorsal and lateral cilia respectively. No significant differences were observed between WT and mutant cilia range of motion by unpaired 2-tail t-test.
2.3 Discussion

I have shown that spdl1 is expressed in motile ciliated tissues in zebrafish and embryos deficient in Spdl1 develop phenotypes associated with cilia dysfunction. These phenotypes can be rescued by reintroduction of WT zebrafish spdl1 RNA but not zebrafish spdl1 RNA containing one of the human mutations injected at the same concentrations. Analysis on motile cilia characteristics and function suggest Spdl1 may fulfill a function in regulating IFT since spdl1−/− embryos develop significantly longer neural tube floorplate cilia that beat with significantly reduced frequency at the same time ventral curvatures can be observed. These results further suggest Spdl1 dysfunction is indeed associated with PCD pathology. To date, this is the first time a molecular mechanism affecting motile cilia length has been implicated in PCD etiology, despite two case studies published 2-3 decades ago. This was the first time spdl1 was characterized in zebrafish thus analysis on spdl1 expression throughout development was also warranted. Through WISH, spdl1 was found as early as the 2 cell stage before zygotic transcription is activated, indicative of maternal contribution. Analysis of cell migration also supports the notion that Spdl1 is maternally contributed as mutant neural epithelia continued to divide properly despite predicted non-functional Spdl1 protein and nonsense-mediated decay of zygotic transcripts. Importantly, despite the fact that maternal mRNA is degraded during the mid-blastula transition, it is unknown how much maternal Spdl1 remains. The remaining maternal product may be enough to protect the embryo from cell division defects during early somitogenesis and the neural tube cilia from improper length maintenance at 30hpf. Perhaps by 2dpf, when phenotypes are observable, maternal Spdl1 has degraded or concentrations of the protein have diffused too much across all the cells of the organism to rescue effects of zygotic Spdl1 deficiency.

The results from WISH suggest spdl1 is expressed motile ciliated tissues by 1dpf. This expression pattern appears valid since the sense probe which should not bind to spdl1 transcripts does not exhibit the same staining pattern nor is the staining as robust as treatment with the anti-sense probe. However, by 2dpf, signal from the anti-sense probe is as weak as staining with the sense probe. Despite slightly different staining patterns, results at this stage of development
suggest both probes may be subjected to probe trapping or difficulty penetrating the embryo. This is a likely scenario, as mutant embryos treated with antisense probe also exhibit similar staining pattern and intensity to treatment with the sense probe. An alternative explanation for why staining in 2dpf spdl1Δ/Δ embryos was observed is that the antisense probe is binding to the remaining mutant transcripts before processing by nonsense-mediated decay.

However, even if transcripts persist in mutant embryos and are translated, it is unlikely the resultant protein is functional. The new termination codon introduced in all mutants is found in exon 3, which is upstream of a conserved Spindly motif. The motif is located at residues 235-269 in the fish protein whereas the three alleles characterized in this thesis result in new termination sequence at residues 119 (2bp), 108 (7bp) and 121 (14bp). It was found that point mutations in the Spindly motif rendered the protein unable to bind and interact with the Dynemin-Dynactin complex, but does not affect the localization of SPDL1 to the kinetochore itself. However, since SPDL1 was unable to interact with dynein, mitotic arrest resulted because SPDL1 was unable to terminate the spindle assembly checkpoint to signal the end of metaphase. Thus, even if protein is produced in zygotic mutants, Spdl1 will be non-functional since it will lack the Spindly motif.

The fact that the Spindly motif is essential for protein function raises an interesting question as to why spdl1 RNA containing the human mutation was unable to rescue mutant phenotypes. This transcript contains a point mutation that substitutes Glu at position 208 to Asn in the zebrafish protein representing the human mutation at Asp206Asn. Importantly, the resultant protein still contains a functional Spindly motif yet the protein appears non-functional as it was unable to rescue any mutant phenotypes. Since the substitution affects a coiled-coil domain, it may be possible the altered residue has prevented the ability of the protein to fold properly, inhibiting the formation of a coiled-coil. This structural motif requires interaction of key residues to fold component alpha helices together. The mutation investigated changes an acidic, negatively charged amino acid (Asp or Glu) to a neutrally charged, hydrophilic residue (Asn). This residue change may be strong enough to interrupt proper Spdl1 folding, preventing the Dynein localization motif from functioning. Furthermore, since coiled-coil domains are involved in protein interactions, the affected coiled-coil may independently prevent interaction between Spdl1 and Dynein.
Spdl1−/− embryos developed longer neural tube floorplate cilia than WT siblings at 2dpf, which further suggests Spdl1 contributes to IFT function as cilia elongation and length maintenance is in part regulated by IFT. However, the reported length may not be entirely accurate as each cilium length was measured by tracing the organelle on each confocal image from a z-stack from base to tip on a single plane of view. Since cilia protrude from the cell surface, the structures have a 3D orientation which was not considered in this analysis, but may nonetheless confound measurements, contributing to variability. For example, a cilium tilted such that it is closer to the cell membrane will appear longer than a cilium that is tilted only slightly past 90°. Since cilia in the neural tube floorplate and olfactory placode are motile, cilia can be fixed in any position and in a range of angles from the cell surface which would also contribute to length variability. These two considerations may explain the large range of cilia length values in the neural tube and olfactory.

Cilium length maintenance is hypothesized to follow a balance point model wherein length-independent disassembly mediated by IFT dynein counter-acted by length-dependent assembly via Kinesin. Indeed, Kinesin deficiency is known to result in shortened or absent cilia or flagella in mice, C. elegans and Chlamydomonas reinhardtii. Conversely, increased activity of anterograde IFT results in longer cilia. As well, zebrafish and mouse mutants in IFT88, a component of the IFT-B complex associated with anterograde transport never develop cilia. Less research has been performed on retrograde transport and reports are conflicting. Investigation on IFT Dynein dysfunction in the unicellular ciliate Tetrahymena thermophila suggests cilia are elongated when IFT dynein is knocked down. This observation was supported in a report analyzing a mutation in dync2li1, the light intermediate chain of Dynein 2 that helps stabilize the protein complex, identified through whole exome screening of neonates affected with short rib polydactyly syndromes. Fibroblasts from these cases exhibited hyperelongated cilia as a result of absent IFT dynein. However, a secondary report using human fibroblasts as well, found that cilia were shortened following dync2li1 knock down by siRNA. Since Spdl1 regulates location of Dynein function in mitosis and spdl1−/− embryos develop significantly longer motile cilia, results suggest Spdl1 has a role in maintaining the balance between anterograde and retrograde transport. Spdl1 may normally localize dynein to the base of cilium to eventually participate in IFT. These results may further support the finding that dysfunction in IFT dynein lead to elongated cilia.
However there is no evidence so far to suggest that Spdl1 interacts with IFT dynein or other retrograde IFT components. Based on STRING protein interaction database, Spdl1 physically interacts with Cytoplasmic dynein 1 components. To date, there is no evidence that this Dynein functions in the cilium except in photoreceptors, which are specialized primary cilia. Zebrafish embryos harbouring a loss-of-function mutation in cytoplasmic dynein 1 heavy chain 1 (dync1h1) develop small, round eyes similar to spdl1 embryos. Insinna et al (2010) determined that the eye phenotype was due to lack of photoreceptor outer segments, which was unusual as Cytoplasmic dynein 1 is not expected to function in the connecting cilium. By antibody staining however, the group confirmed that Dyn1h1 is normally found in the connecting cilium and mutants did not have this localization as transcripts were likely sent for nonsense mediated decay. Since the eye phenotype in spdl1−/− phenocopies dyc1h1 mutants, it is possible that the eye phenotype in spdl1 is due to abnormal outer segment morphology as a result of IFT defects.

Unexpectedly, spdl1−/− embryos developed dorsal body axis curvatures at 3dpf after exhibiting ventral curvatures at 2dpf. This observation may further suggest spdl1 mutation leads to Dynein1 mislocalization or dysfunction. Dorsal curvatures is a characteristic phenotype of embryos deficient in polycystin 1/2 (pk1/2) which forms a Ca^{2+} receptor-ion channel complex together. The complex is found on primary cilia and appears to play roles in Ca^{2+} signaling in response to flow dynamics. It was found that the dorsal curvature was due to increased accumulation of collagen in the notochord sheath from overproduction of vesicular components for collagen secretion. The trafficking of vesicles carrying proteins destined for secretion is dependent on dynein 1-dynactin complexes as the subunit of Dynactin, the p150 subunit, is able to interact with Sec 23 and Rab interacting proteins found on ER vesicles heading to the Golgi or late endosomes, respectively. Since Dynactin is another regulator of Dynein 1 activity, this posits a model wherein spdl1 normally brings a pool of dynein to the cilium to participate in IFT. Without spdl1 activity, there is more Dynein to participate in vesicular trafficking via Dynactin resulting in increased amount or rate of collagen post-translational modification and ultimately secretion into the extracellular matrix leading to dorsal curvatures by 3dpf. It is important to note that change in curvature is not likely due to reduced Ca^{2+} signaling caused by reduced fluid flow since other cilia mutants do not experience transformations in curvature axis.
Spdl1 mutant neural tube floorplate cilia also beat with reduced frequency suggesting that elongated cilia negatively impacts fluid flow. However, it is worth noting that CBF was variable regardless of embryo genotype, age or anatomical location. Variability was also present not only between animals but in different cilium within the same animal in the same tissue, raising the possibility that fluid flow was disrupted or only slightly altered since the force generated by a slower moving cilium may be compensated by the force generated from a nearby faster beating cilium. Sources of this variability include difficulty measuring CBF, small sample size, variable environment factors such as ambient temperature or time spent anesthetized, or could be naturally occurring precisely to protect against developmental defects. The observation that WT embryos at 2dpf have a smaller range of CBF values in the neural tube compared to 30hpf embryos may support the latter explanation suggesting that CBF stabilizes as the embryo grows past a critical stage of development.
Chapter 3

3 Future directions

3.1 More experiments to confirm Spdl1 knock-out

To further confirm that *spdl1* is expressed in the neural tube, it is possible to section stained embryos or perform RNA in situ hybridization on sectioned embryos. Based on the results presented, *spdl1* may be expressed in the neural tube, or it may be expressed in the lateral line which is also found on the midline of the organism. Since WISH results in older embryos and mutants were inconclusive and might suggest probe trapping or problematic probe penetration, RNA in situ hybridization can be performed on sectioned samples. Alternatively, smaller probes can be synthesized by using primers containing T7 or T3 promoters to amplify a portion of *spdl1*\textsuperscript{104}. The promoter sequences will allow sense and antisense probe synthesis. Smaller probes may penetrate samples better and allow visualization in whole embryos.

By RT-qPCR, mutants appear to have less *spdl1* transcripts than WT after normalization by *gapdh* at 2dpf. However, only one biological sample was investigated, therefore the procedure will need to be repeated to confirm mutants have less *spdl1* transcript than WT siblings at 2dpf. If an antibody for Spdl1 is available, Western blotting may also be performed at 2dpf to determine if Spdl1 protein is produced and if mutants possess less protein than WT siblings at the same age. Based on the fact that 2dpf mutants develop a phenotype that can be rescued by exogenous *spdl1*, I expect mutants will have less Spdl1 protein than WT siblings at 2dpf.

3.2 Is range of motion and fluid flow affected in 2dpf mutants?

Since mRNA injection of *arll3b-gfp* cannot be used to analyze cilia range of motion past 30hpf, *spdl1* mutants will be crossed into a *Tg(βact::Arl13b-GFP)* background to visualize cilia in older embryos as exogenous Arl13b-GFP can no longer be visualized by 2dpf\textsuperscript{79}.

Motile cilia in mutant neural tubes have reduced beat frequency however, it is unknown whether slower motility results in reduced fluid flow. Thus, to truly confirm mutations in *spdl1* can cause
PCD, it would be important to investigate whether fluid flow has been affected. To investigate flow dynamics in the neural tube may be possible however to inject fluorescent microbeads into the ventricles of live fish and monitor bead movement in the neural tube after a few minutes. A similar assay was developed by Gutzman and Sive (2009) wherein fluorescent dye was injected into the roofplate of the hindbrain in 24hpf embryos to better visualize brain ventricle development. Since there was no evidence that motile cilia in spdl1 mutant brains are dysfunctional, cerebral spinal fluid should still flow from the ventricles into the neural tube and it is only at the neural tube that fluid flow is possibly impaired. Thus, injection of microbeads into the ventricle should result in some beads entering the neural tube allowing for analysis of flow dynamics in the tube. If dysfunctional spdl1 does cause PCD, it will be expected that microbead movement will be slower in mutant neural tubes than WT siblings, suggesting fluid flow velocity is impaired.

3.3 Can human spdl1 RNA rescue mutant phenotypes?

I have demonstrated that WT zebrafish spdl1 RNA can rescue mutant phenotypes when injected into embryos at 1 cell stage, indicating that the phenotypes observed are indeed due to spdl1 knock out. However, the zebrafish spdl1 protein sequence is only 47% identical to the human sequence. In order to show that the possible cilia dysfunction in spdl1 knock out can translate to human disease, it is important to demonstrate human spdl1 RNA can rescue mutant phenotypes as well. If human spdl1 can rescue mutant phenotypes, it will suggest that despite only sharing 47% identity; the two proteins fulfill a similar function, further implicating human SPDL1 in cilia biology by demonstrating zebrafish cilia characteristics following Spdl1 dysfunction can also occur in humans.

If human SPDL1 can rescue zygotic phenotypes, it will also be possible to investigate the effect of the second spdl1 mutation identified in our patient screen. This mutation results in the substitution L514H in the human protein sequence and was predicted to be damaging with high confidence by Polyphen. However, the mutation was not investigated because this region of the human protein is not conserved in the zebrafish protein sequence. As PCD is an autosomal recessive disease, it will be important to demonstrate that both spdl1 alleles identified in the patient result in non-functional protein leading to PCD symptoms. Thus, it will be important to
demonstrate whether the L514H mutation can rescue phenotypes associated with cilia defects in the mutants.

3.4 Where is Spdl1 found in non-mitotic cells?

To date, all studies on Spdl1 focus on the protein’s role in cell division, with only one paper published indicating spdl1 mutations may lead to microcephaly\(^{106}\). Thus, it is of interest to determine where Spdl1 is localized in non-mitotic cells. More importantly, since I hypothesize Spdl1 localizes dynein1 to the base of cilium, it will be crucial to determine that Spdl1 does indeed localize at the base of cilium. Previously, injection of spdl1 RNA tagged with GFP at both the N-terminus and C-terminus resulted in embryos that did not survive through epiboly or 3-4 somite stages and when imaged, spdl1 was found only to have nuclear localization (Fig. 19). In order to visualize Spdl1 fusions, 50-75pg of RNA was injected, which may have contributed to lethality observed.

An alternative method to visualize subcellular localization of Spdl1 is to create transgenic zebrafish with a construct wherein Spdl1 tagged by either GFP at either the N-terminus or C-terminus is under the control of a biologically relevant promoter. Based on in situ and other results reported in this thesis suggesting Spdl1 affects motile cilia function, the foxj1a promoter may be a good candidate. Foxj1a is a transcription factor that regulates the expression of motile cilia associated genes and is only expressed in motile ciliated tissues in the zebrafish\(^{107}\). Thus, the proposed spdl1 construct will only allow visualization of Spdl1 in relevant tissues for understanding how the gene may lead to PCD. If the hypothesis that Spdl1 localizes dynein to the cilium to participate in IFT, it will be expected that Spdl1 will be observed near the basal body. This transgene will also allow visualization of Spdl1 localization at endogenous levels. Similarly, an antibody specific for zebrafish Spdl1 can be made to also visualize endogenous Spdl1 localization.
Figure 19. Spdl1 localization in WT embryos at 90% epiboly. 50pg of Spdl1 tagged with GFP at the N-term (GFP-Spdl1) or Spdl1 tagged at C-term (Spdl1-GFP) were injected into WT embryos at 1 cell stage. Spdl1 appears to be localized in the nucleus at this stage however embryos did not survive past this stage. Embryos that escaped early lethality died by 3 somites.
3.5 Is dynein localization and activity affected in spdl1\(^{-/-}\) embryos?

To substantiate the hypothesis that Spdl1 localizes Dynein 1 to the base of cilium, it is necessary to demonstrate that Dynein 1 is present normally in motile cilia and is mislocalized in mutants. The best hint that Spdl1 affects dynein 1 function in the spdl1 mutants is that the eyes phenocopy zebrafish mutants in cyclospasmic dynein 1 heavy chain 1 (dync1h1)\(^9\). Thus to first determine if Dynein 1 function is disrupted in spdl1 mutants, histology analysis should be performed on sections of the spdl1\(^{-/-}\) embryos at 2, 3, and 4 dpf to investigate whether photoreceptor morphology is altered and if photoreceptors resemble dync1h1 mutants. If Dynein 1 is mislocalized, it is expected that histology will reveal truncated photoreceptors and organelle disorganization similar to dync1h1 mutants. An antibody specific for Dync1h1 may also be used to stain for dynein 1 in the photoreceptors of WT and spdl1 mutant embryos. It is expected that no Dync1h1 will be present in the photoreceptor connecting cilium in mutants. Acetylated tubulin or a photoreceptor cell marker will also be used to better identify photoreceptors in eye cryosections. If a zebrafish Spdl1 antibody is available, co-staining with Spdl1 in photoreceptors will also support the hypothesis that Spdl1 localizes dynein 1 to the connecting cilium of photoreceptors.

If mutants do indeed have little or no Dync1h1 signal in the photoreceptors and have abnormal photoreceptor morphology, the whole fish embryo or thick section of the neural tube will be stained with anti-Dync1h1, answering two questions. Firstly, this experiment will determine whether Dynein 1 is normally present in motile cilia, as there is no evidence currently that this dynein participates in IFT outside of photoreceptors. Secondly, staining for Dync1h1 will also show whether dynein mislocalization in the neural tube leads to lengthened motile cilia and will strengthen the hypothesis that Spdl1 is required to bring dynein to the cilium to maintain cilium length. GFP-tagged Dync1h1 may also be made to visualize Dync1h1 localization \textit{in vivo} however, given that this motor protein is a complex of many different components, GFP may interfere with the interaction of these complexes.

The dorsal curvatures spdl1 mutants develop by 3dpf may also implicate Dynein 1 dysfunction since the phenotype is associated with increased collagen in the neural tube\(^{100}\). Collagen is a large protein that has many post-translational modifications requiring the trafficking of the
protein from the endoplasmic reticulum (ER) to the Golgi and finally into late endosomes for exocytosis\textsuperscript{101}. All these processes require the activity of Dynein 1 mediated by Dynactin, a regulator of Cytoplasmic dynein 1 activity. I hypothesized earlier that Spdl1 mutants develop dorsal curvatures because the pool of Cytoplasmic dynein 1 available to participate in collagen trafficking has expanded due to less Dynein 1 used in IFT without Spdl1 localization. This hypothesis implies that firstly, there is more collagen secreted into the neural tube in mutants by 3dpf, and secondly, that if ER/Golgi trafficking is blocked then dorsal curvature will be abolished. To test this hypothesis, \textit{spdl1} mutants at 3dpf will be stained for collagen II to determine if the notochord sheath contains more collagen than WT siblings thus possibly leading to dorsal curvatures. Mutants will also be treated with Brefeldin A (BFA), an antibiotic that can block vesicular trafficking between ER and Golgi by indirectly preventing formation of vesicles carrying proteins between Golgi compartments or Golgi to ER\textsuperscript{108,109}. Treatment with BFA at non-toxic levels was shown to rescue dorsal curvatures by preventing secretion of collagen into the notochord sheath in \textit{pkd1 / 2} mutants\textsuperscript{101}. Results from these experiments will further suggest dynein 1 activity is misregulated in \textit{spdl1} mutants if mutants do have increased collagen in the in the notochord and dorsal curvatures can be rescued by BFA treatment.

3.6 How else may Spdl1 affect motile cilia function?
In the case that \textit{spdl1} does not localize to the base of cilium and/ or that Dynein 1 is not associated with eye phenotypes, not mislocalized and not misregulated, a myc-tagged Spdl1 transgenic fish line can be made to perform co-immunoprecipitation to identify proteins Spdl1 interacts with in non-mitotic cells. Since there is no antibody specific for zebrafish Spdl1, an antibody for myc will be used to pull out Spdl1 protein interactions. Candidates can be confirmed by using a split-GFP complementation assay wherein WT embryos will be injected with \textit{spdl1}-GFP fragment A and candidate-GFP fragment B RNA\textsuperscript{110}. If the candidate physically interacts with \textit{spdl1} or is brought into close proximity with \textit{spdl1}, then the GFP fragments will complex together to fluoresce.
3.7 What are the characteristics of maternal zygotic spdl1 mutants?

Since spdl1 knock-out is embryonic lethal, it is currently unknown what phenotypes will arise in zebrafish lacking both functional zygotic and maternal Spdl1. Based on the reported role of Spdl1 in cell division, maternal zygotics are predicted to die early in embryogenesis, as proper cell division will be prevented. Creating maternal zygotics may also confirm the hypothesis that proper cell divisions observed in zygotic mutants was due to maternal contribution of protein.

Maternal zygotics can be generated through germline transplantation\textsuperscript{11}. Spdl1 mutant embryos (donor) will be labeled with nanos-gfp RNA to mark primordial germ cells and WT embryos (host) will be injected with as a morpholino targeting dead end, a gene that is found in germ plasm in the cell of zebrafish embryos to prevent the host from developing germ cells. Cells from the margin of donor embryos will be injected into the margin or animal pole of host embryos at the mid-blastula stage, since primordial germ cell precursors are found at the margin at this stage of development. Host embryos can be screened at 30hpf for GFP expression in the gonad mesoderm to determine if transplantation was successful. Crosses with female hosts will yield maternal zygotic embryos for analysis. As stated earlier, it is expected that maternal zygotic embryos will not develop since cell division may not proceed without error. This may manifest as infertility in the adult hosts, or early embryonic death in the host’s offspring.

3.8 Does mtmr11 contribute to PCD etiology?

In the patient whole exome sequencing screen, four gene candidates were identified for further characterization. Of the four, two genes were published as PCD causing genes before the onset of this thesis. This project focused on characterizing spdl1 and how it may cause PCD pathology. However, one last gene from our candidate list remains to be analyzed. Both mutations identified in myotubularin related protein 11 (mtmr11) were indels leading to frameshift mutations with premature termination sequences. Since both mutations result in addition of a premature stop codon in exons that are included in both alternative mtmr11 transcripts, the patient essentially has no functional Mtmr11 product.
I have performed some analysis on Mtmr11 in zebrafish embryos. Much like Spd11, no research has been done on this protein thus far. As such, I first determined where mtmr11 is expressed, probing to see if the gene is expressed in motile ciliated tissues. Based on Yang et al, mtmr11 is not highly expressed until 3dpf, thus I performed in situ hybridization on WT embryos at this age using sense and antisense probe for exons 3-6. The antisense probe was designed to bind to a fragment of mtmr11 because I encountered difficulty synthesizing the full cDNA transcript for generation of an mtmr11 plasmid and since in situ was done on 3dpf embryos, a small probe may improve probe penetration and reduce trapping. RNA in situ hybridization revealed that that mtmr11 is expressed diffusely in the head but not in other motile ciliated tissues (Fig. 20A). Injection of a translation-blocking morpholino against mtmr11 did not produce any phenotypes associated with cilia defects such as ventral curvatures until 3dpf, and only at a high dose of 12ng (Fig. 20 B). This phenotype was preceded by reduced twitch response and voluntary movement, which may indicate defects in muscle function. Indeed, mutations in myotubularin are associated with muscular dystrophies and late onset of body curvatures is possible in embryos with muscle defects. This observation suggests phenotypes observed in mtmr11 morphants is may not be due to cilia dysfunction and furthermore that this gene does not affect cilia function. Nonetheless, because the mutations identified in the human patient was very damaging, it may be possible that knock-down is not enough to result in a phenotype. Thus, CRISPR/Cas9 genome editing was used to create mutants in mtmr11, using a guide targeting exon 8 of a 17 exon transcript. Injected fish are currently being screened to identify potential founders.
Figure 20. Expression of *mtmr11* and effect of gene knock-down. A) Mtmr11 is expressed diffusely in the head in 3dpf embryos. B) Knock-down of Mtmr11 results in ventral body curvatures at 3dpf after injection of 12ng translation-blocking MO into 1cell embryos. Prior to curvatures, embryos exhibit reduced motility suggestive of a muscle defect.
Chapter 4

4 Methods and materials

4.1 Fish strains

All fish used in this study were housed in conditions that adhered to established zebrafish husbandry protocols approved by the Animal Care Committee at the Hospital for Sick Children. Due to facility and lab protocol changes, wild-type backgrounds differed for certain experiments. The zebrafish spdl1 was cloned from pooled TLAB embryos. All other experiments including in situ, WT morpholino injections and mutant generation were performed in a TuAB background. Morpholinos were also injected into p53 mutants (tp53<sup>dfl</sup>) which carries a single T>A point mutation resulting in a Met to Lys at residue 214 (ZIRC).

4.2 Plasmids

To generate spdl1 clones for in situ and mRNA synthesis, RNA was extracted from pooled embryos at high stage and cDNA was synthesized using SuperScript VILO cDNA synthesis kit (Invitrogen). Using Gateway technology (Invitrogen), primers were designed to amplify full length spdl1 transcripts for middle entry insertion into the pCS2 expression vector (plasmid 22423: pCSDEST, ADDGENE). Primers used: forward 5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCCACCATGTCCGATCTAGAGGATG-3’; reverse 5’-GGGGACCACTTTGTACAAAAAAGCAGGCTTCGCCACCATGTCCGATCTAGAGGATG-3’.

4.3 Whole mount in situ hybridization (WISH)

Standard protocols for in situ on zebrafish were followed. Spdl1 sense and antisense probes were synthesized from linearized spdl1 plasmid by in vitro transcription using a DIG RNA Labelling kit (Roche) and T7 (antisense) or T3 (sense) polymerase (Roche). Mtmr11 probes were generated following David and Wedlich (2001)<sup>104</sup> wherein T3 or T7 promoter sequence included in the 5’ end of primers specific for mtmr11 exon 3-6, producing a probe 353bp long. After staining, embryos were washed with 4% PFA to fix colour and then cleared with 100%
methanol to reduce non-specific staining before storage in 80% glycerol. Embryos were imaged in 80% glycerol on depression slides.

4.4 Morpholino and mRNA microinjections

Morpholino and mRNA were injected into embryos at 1-cell stage. Translation blocking morpholino for spdl1 targeted the first exon with sequence 5’ ATCTCATCCTCTAGATCGGACATCT3’ ordered from Gene Tools, LLC. 3ng, 4.5ng and 6ng of morpholino were injected into WT and p53−/− backgrounds. mRNA was synthesized from gateway vectors using mMessage mMachime SP6 transcription kit (Invitrogen) for in vitro transcription. 15pg memb-RFP, 8pg arl13b-GFP, 12pg gap43-GFP, 60pg H2A-mCherry, 75pg spdl1-GFP, 75pg GFP-spdl1, 20pg spdl1 and 20pg mutant-spdl1 were used for injections.

4.5 CRISPR-Cas9 mutagenesis

Mutants were generated following protocols from Chen and Wente labs. Guide RNAs were designed using CHOPCHOP web tool. A guide targeting exon 2 of spdl1 was designed with the following primers: Forward – 5’ TAGGAGCAGCAACAAACACTGC-3’; reverse – 5’AAACGCAGTGTTTGTTGCTGCT-3’. The RNA was made using the MEGA shortscript T7 kit (Invitrogen). Both guide RNA and RNA for Cas9 were injected into embryos at 1 cell stage. To genotype founders and mutants, primers were designed to flank the guide RNA site: Forward-5’TGTGTGTGCAGTAGATTTTCCCTT-3’ and Reverse – 5’CAGCAGGCACAAAAACACATTG-3’. The full length product is 426bp long. If the guide did not induce genomic cutting, digestion of the amplicon with BtsaI restriction enzyme will yield two fragments at 195bp and 231bp.

4.6 Reverse transcription and real-time PCR

RNA from pooled embryos at specific timepoints was obtained from trizol homogenization. cDNA was synthesized from extracted RNA with SuperScript VILO cDNA synthesis kit (Invitrogen). Quantitative reverse transcription PCR was performed on cDNA obtained from pooled spdl1 mutant and wt siblings at 2dpf with the following primers: Forward- 5’-GGAACTGCACAACAGATTGGA-3’; reverse – 5’-CCTTAGACTCTCCTCAGACATGG-3’. The product produced is 127bp long. Gapdh was amplified as a control using the following
primers: Forward- 5’- GGGCTGCAAAGGCTGAGG-3’; Reverse – 5’- TGGGGGTGGGGACACCGGAAG-3’.

4.7 Immunofluorescence

Embryos destined for immunofluorescence microtubule staining were grown in standard embryo media and 1.5% propylthiouracil (PTU) introduced at 30hpf to prevent pigment growth. On the day of fixation, 2dpf embryos were treated with tricane until immotile and a section of the tail immediately after the end of the yolk extension was sliced for genotyping. Embryos were fixed in individual Eppendorf tubes with 100% methanol and stored in -20°C for up to two weeks before staining. Microtubule staining was performed starting with rehydration using a methanol series. Embryos were blocked for 1 hr at room temperature with 2% BSA + 2.5% goat serum in TBS (blocking solution) and incubated with anti-mouse anti-acetylated tubulin antibody (1:200, Sigma) in blocking solution overnight at 4°C. The following day, embryos were washed with TBS for at least three hours, changing the washing media every hour and subsequently incubated with goat anti-mouse Alexa 488 secondary antibody (1:1000, Invitrogen) in blocking solution overnight at 4°C. Embryos were washed in TBS for at least another 3 hours before processing for imaging.

4.8 Confocal and cilia imaging

Live or fixed embryos were mounted in 1% low-melt agarose. Images were taken at 40x objective at 2x optical zoom (neural tube) or 1.5 optical zoom (brain and olfactory placode) using a Zeiss 710 laser scanning confocal microscope. Z stacks were taken at 1μm intervals. In live imaging to record motile cilia movement, the neural tube at somites 4-6 were imaged in 30hpf embryos whereas the neural tube at somites 9-12 were imaged in 2dpf embryos. As well, 2dpf embryos used for live cilia imaging were first treated with 40mmol 2,3-butanedione monoxime to temporarily cease blood flow before mounting in agarose. Three second long videos at 200-250fps (neural tube) or 300 fps (ventricles) were taken using Volocity (Perkin-Elmer).
4.9 Cilia length analysis
Using Volocity, the length of each floorplate cilium where ends were easily visible was traced manually with the line tool, making measurement objects from each line. Each image from a stack was analyzed making sure the length of the same cilium was not included more than once.

4.10 Ciliary beat frequency calculations
For analysis, these videos were exported as .avi files and slowed down to 20fps using Volocity and converted to the appropriate codec for Image J using ffmpeg tool. Ciliary beat was analyzed following established protocols from Drummond (2009)\(^3\). Each cilium that was in focus was analyzed by drawing a line bisecting its movement. A reslice was made creating a kymograph of movement across video length. The distance between each peak of the kymograph was measured and divided by total number of frames to achieve a beat frequency of beats/min.

4.11 Graphing and statistics
All graphing was performed on Microsoft Excel 2007. Statistical analysis was performed on Graphpad Quickcalcs online tool.
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Appendices

2.2.6 Supplementary movie 1. Cell divisions in developing neural tube in WT 12 somite stage embryos. H2B is labeled in red and cell membrane is labeled in green.

2.2.6 Supplementary movie 2. Cell division in neural keel of spdl1 mutant embryos. Embryos were imaged at 8 somite stage. H2B is labeled in red and cell membrane is labeled in green.

2.2.8.3 Supplementary movie 3. Cilia motility in the neural tube of 30hpf WT embryos.

2.2.8.3 Supplementary movie 4. Cilia motility in the neural tube of 30hpf spdl1-/- embryos.

2.2.8.3 Supplementary movie 5. Cilia motility in the neural tube of 2dpf WT embryos

2.2.8.3 Supplementary movie 6. Cilia motility in the neural tube of 2dpf spdl1-/- embryos.

2.2.8.3 Supplementary movie 7. Olfactory cilia in 2dpf WT embryos

2.2.8.3 Supplementary movie 8. Olfactory cilia in 2dpf spdl1 mutant.