A NOVEL ZEBRAFISH MUTANT REVEALS NEW INSIGHT INTO THE REGULATION OF CILIA MOTILITY AND BODY AXIS FORMATION

by

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A THESIS

Presented to the Department of Biology
and the Robert D. Clark Honors College
in partial fulfillment of the requirements for the degree of
Bachelor of Science

May 2022

An Abstract of the Thesis of

Samuel Craig for the degree of Bachelor of Science
in the Department of Biology to be taken June 2022

Title: A Novel Zebrafish Mutant Reveals New Insight into the Regulation of Cilia Motility and Body Axis Formation

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Primary Thesis Advisor

Motile cilia are responsible for critical functions in development, including left-right patterning and cerebrospinal fluid flow. Their motility depends on the assembly of outer dynein arms: ATPases which power ciliary beating. Defects in dynein arm function occur in Primary Ciliary Dyskinesia, a disorder affecting 1:15,000–30,000 human births. Daw1 is a cytoplasmic protein thought to be required for cilia beating by controlling import of dynein arms into cilia. Here, I use zebrafish as a model to understand Daw1 function during development and growth. I characterize *daw1*^{b1403} mutants, a new *daw1* mutant line harboring a 2-amino acid deletion in a conserved region of the protein generated by CRISPR mutagenesis. Defects associated with motile cilia dysfunction in *daw1*^{b1403} mutants, including otolith abnormalities, left-right patterning defects, and abnormal body axis curvature are observed. Surprisingly, *daw1*^{b1403} mutants exhibit recovery of body curve defects later in development. Consequently, we hypothesize that Daw1 is not essential for cilia motility per se, but only for timely onset of beating over developmental timescales. To support this, live imaging of the central canal showed that the beating of motile cilia is abrogated on the

first day post-fertilization (dpf) in *daw1*^{b1403} mutants but recovered to an indistinguishable level from sibling controls by the second dpf.

Lastly, collaborators based in the United Kingdom have identified patients from two families with homozygous Daw1 mutations that present with *situs* defects. Here, I show that upon injection of Daw1 mRNA containing the human mutations, *daw1*^{b1403} mutants fail to rescue, suggesting that these mutations encode a null protein, and that the defects present in human patients, like our mutants, are the result of Daw1 abrogation.

Importantly, this Daw1 model of delayed cilia motility and body straightening provides an opportunity to study how early embryos can sense, or correct, shape deformations, which is an exciting and relatively unknown aspect of developmental morphogenesis. Ultimately, understanding these processes may help inform our treatments of congenital disorders.

Acknowledgements

I have many people to thank in support of my research. First, the Grimes lab has been a home to me, and as part of it I discovered my love of developmental biology and science. Here, I have gained experience in testing hypotheses, conducting research independently, and sharpening both writing and presentation skills. Through these, I was able to develop my abilities to professionally communicate, critically think, and navigate both successes & challenges in research. I will always have gratitude for Professor Daniel Grimes (my Primary Thesis Advisor), Zoe Irons (my graduate mentor), and other members of the Grimes Lab, including Beth Bearce, Katie Fisher, and Gabriel Luna, for helping me grow as an individual and develop these skills. I feel lucky to have been surrounded by mentors who were so invested in my success as a student and person.

I'd also like to thank Professor Carol Stabile, my CHC representative, and Professor Adam Miller, my second reader, for their time and contribution to this capstone project of my undergraduate career.

Lastly, I would also like to thank my family. My parents, Jennifer and Phillip Craig, are my role models for persistence, hard work, and dedication to making the world a better place. They have helped guide and support me in all my endeavors and were the catalyst for love of science and research. My appreciation and gratitude for them are beyond words. My sisters, EllaKathryn and LucyWest Craig, have always been willing to listen to me explain my research, and pushed me to do my best. I love them dearly.

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Introduction

Intracellular Communication is Important for Development:

Development of multicellular organisms requires the precise positioning of cells in time and space to form a functional body. Developmental Biology investigates the mechanisms underlying this process. How does an entire person form from a single cell? How do cells communicate and organize themselves to assume a final morphology? What occurs in disorders of development that lead to the "species-typical" morphology being unattainable?

Throughout the growth of multicellular bodies, communication between cells is of utmost importance for the correct development to occur. Communication informs cells of their developmental purpose: if cells are unable to communicate, how are they to "know" what their function in forming the body is? Signaling mediates this cellular "awareness" of location and function (Gilbert, 2002). Disorders of communication between cells is thus the basis for many developmental pathologies.

Intracellular Communication:

Cells communicate with each other through many different means. They can communicate with neighbors through direct contact, known as juxtracrine signaling. They can also communicate via secreted factors in which one cell secretes a chemical signal that travels in the extracellular environment and is received by a receptor on or in the signal receiving cell (paracrine signaling). Chemical-based

signals (proteins and small molecules) are well studied, and the details of their pathways are well studied (e.g. Wnt and Hedgehog) (Gerhart, 1999). My work has focused on another type of intracellular signaling: hydrodynamic signaling involving fluid flows. This involves a signal-sensing cell (or large group of cells) generating an extracellular fluid flow within the organism which is then sensed by a flow-sensory cell. The hydrodynamic force of flow could be sensed directly, or flow could be responsible for moving chemical signals between sending and receiving cells. Fluid flows in this context are observed throughout many systems, including within the reproductive tracts, kidneys, and in the spinal canal and brain ventricles (Kramer-Zucker et al., 2005; Koyama et al., 2019; Olstad et al., 2019; Thouvenin et al., 2020).

Motile Cilia Generate Fluid Flows Upon Which Sensory Cilia Respond:

Cilia are small hair-like projections that protrude from the plasma membrane into extracellular space and are critically important organelles involved in fluid flow signaling. Broadly, there are two categories of cilia: sensory and motile. Sensory cilia, like their name implies, act as cellular "antennae": they convey extracellular signals through transduction machinery, including cilia-localized receptors, ion channels and enzymes that are part of molecular pathways (Oh et al., 2012) (Figure 1). Sensory cilia are usually present singly on cell surfaces. For a long time, these so-called "primary cilia" were thought to be vestigial. However, in the late 1990s and early 2000s, genetic experiments in the mouse linked both flow sensation in left-right patterning and Hedgehog signaling in the neural tube to primary cilia, showing they are critical for flow-based and chemical-based signaling (Nonaka et

al., 1998; Takeda et al., 1999, Huangfu et al., 2003). Primary cilia are also critical for sensory functions, including transduction of smells, light, mechanical forces, and hearing (Oh et al., 2012; Grati et al., 2015; Sreekumar et al., 2019).

Motile cilia, on the other hand, act to generate the flow of extracellular fluid. Motile cilia are organized as an axoneme, with 9 doublets of microtubules forming an outer circle as well as an inner doublet (Figure 1) (Ishikawa et al. 2021). The main difference between motile cilia and primary cilia is that motile cilia contain dynein arms, large motor complexes visible by electron microscopy, which power ciliary beating (King 2016; Oda et al. 2014). These dynein arms are transported into cilia through the Intraflagellar Transport (IFT) complex, a group of proteins that transports ciliary components along the axoneme and is required for the construction and maintenance of cilia (Kozminski et al. 1993; Lechtreck 2015). The movement and transport of dynein arms into the cilium is thus critical for motile cilia action.

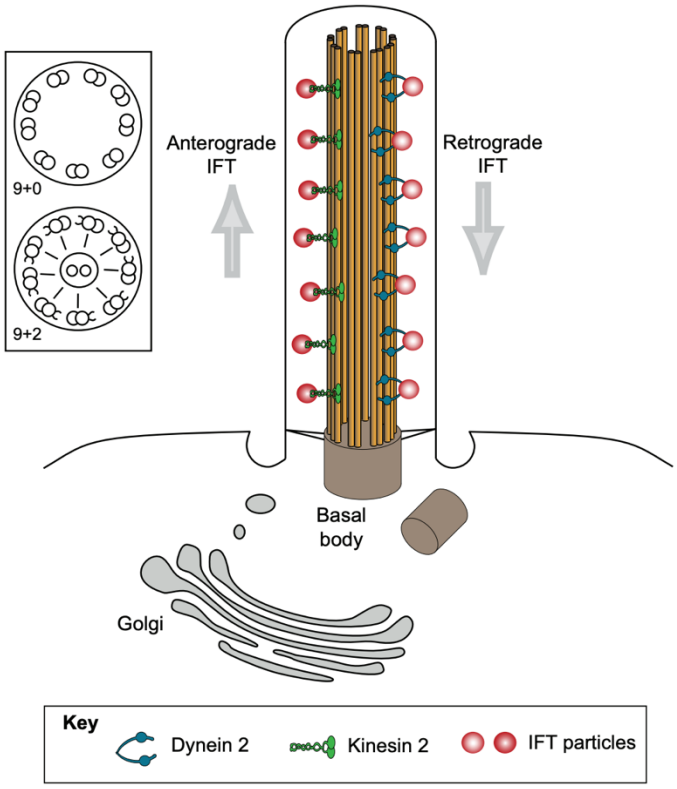

Figure 1: Structure and components of primary and motile cilia.

Motile cilia are composed of a ring of 9 doublet microtubules, with a central pair (9+2). Primary cilia contain this ring but are without a central pair (9+0). In motile cilia, dynein arms (blue) connect the doublets. Intraflagellar transport (IFT) particles are shown in red, and the motor protein kinesin in green. Source: Norris and Grimes, 2012.

Motile Cilia in Disease:

Motile cilia and the flows they generate are critical for many homeostatic processes. In humans, defects in motile cilia cause Primary Ciliary Dyskinesia (PCD), a disorder affecting 1:15,000–30,000 human births (Mitchison and Valente 2017; Wallmeier et al. 2020). In patients with PCD, motile cilia are missing dynein arms, and are thus unable to beat, or beat aberrantly, with reduced force (Figure 2). Complications arising from this disease, including abnormal positioning of organs,

infertility, and failure to clear mucus in the lungs, attest to the diverse functions that motile cilia have across systems.

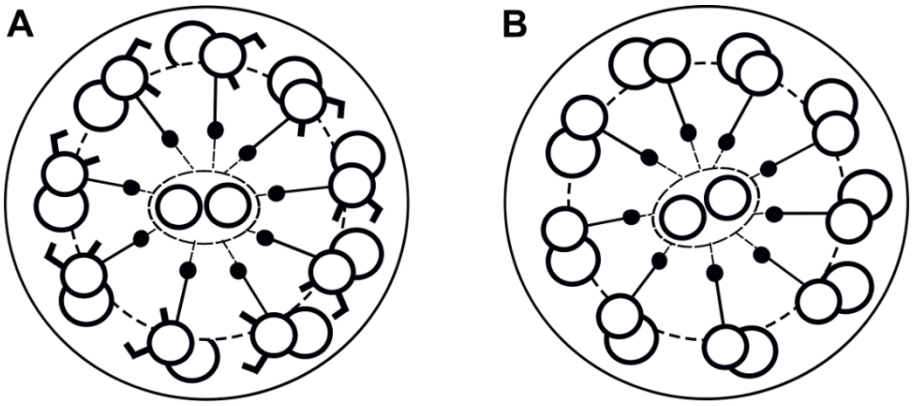

Figure 2: Cross section of motile cilia in a healthy patient (A) and in a patient with PCD (B).

In the patient with PCD, motile cilia are missing outer dynein arms. Due to this, motile cilia are unable to hydrolyze ATP and beat. Source: AboutKidsHealth, 2022.

Motile Cilia Have Varied Timescales for Action in Different Contexts:

In different systems, motile cilia function over different timescales. For example, mucus clearance in the lungs is the result of action by hundreds of motile cilia, and occurs over the entire lifespan of a human, with slow turnover of these ciliated cells (Bustamante-Marin and Ostrowski, 2017). By contrast, establishment of Left/Right (L/R) asymmetry (the asymmetric development of internal organs), which also requires cilia, occurs over only a narrow range of early developmental time (day 7.75-8 in a mouse embryo) (Little and Norris 2021; Komatsu et al., 2013). Here, 200-300 motile cilia in the L/R organizer, which is termed the ‘node’, are angled such that their stroke pushes fluid in the left/posterior direction (Okada et al., 2005). Combined fluid flow in this direction breaks symmetry in the vertebrate

embryo (Nonaka et al., 1998, Okada et al., 1999, Takeda et al., 1999). This asymmetric fluid flow biases the distribution of Nodal, such that greater amount of this protein is present on the left-hand side of the embryo (Figure 3) (Nonaka et al., 1998, Takeda et al., 1999). Nodal then activates transcription of persistence factors that are only expressed on the left side of the developing embryo, which establishes an asymmetric development plan (Nonaka et al., 1998, Takeda et al., 1999). If the original fluid flow were reversed, Nodal would ultimately be expressed on the *right* instead of *left* side, and the organs would develop on the opposite side (Nonaka et al., 2002). As such, motile cilia are critical during a short window of development but also long-term for lung homeostasis.

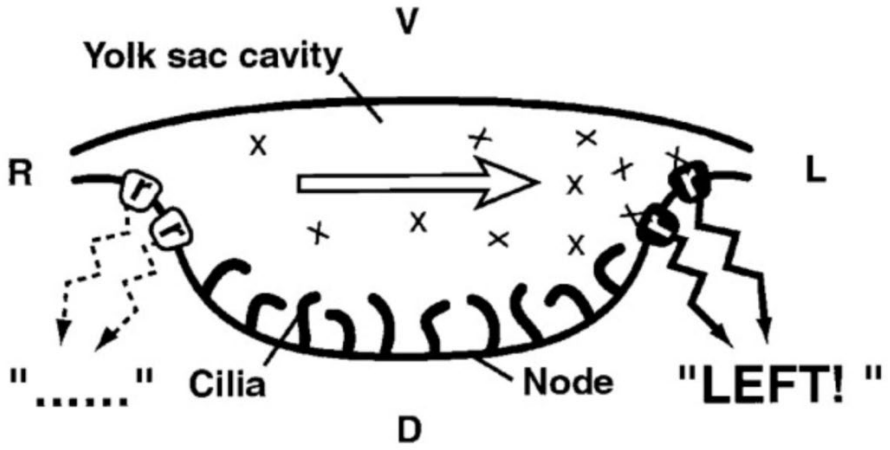

Figure 3: Diagram 7A from Nonaka et al., 1998.

In the yolk salk, Nodal (X) is carried by leftward-generated fluid flow initiated by motile cilia. Nodal concentrates on the left side of the node, and its binding to receptors (R) triggers expression of genes that establish an asymmetric development plan. R = rostral, L = lateral, D = dorsal, V = ventral.

The roles of cilia in L/R patterning and the lungs show that motility is required over vastly different timescales in these different contexts. In the former, motility is needed rapidly for a developmental patterning event that takes only a few hours; in the latter, motility is required throughout life. As such, the rapidity with which cilia start beating after growing from cells is likely to be more important in some contexts (such as the L/R organizer), where the motile cilia function over a short period. However, the mechanisms governing the timely onset of beating throughout these situations are not well understood. Investigating factors that regulate cilia beating onset is thus potentially important, as regulation of the onset of beating may be a critical point of control.

One such factor has recently been defined by research performed in *Chlamydomonas*, a unicellular green alga. ODA16, a cytoplasmic protein, mediates contact between outer dynein arms and an IFT complex protein (IFT46) (Figure 4) (Dai et al. 2018; Hou and Witman 2017; Taschner et al. 2017). As stated above, the IFT complex transports ODAs into the cilium. As such, abrogation of ODA16 (previously known as Wdr69) was shown to lead to a decrease in transport of outer dynein arms into the ciliary body, which resultantly led to defects in motility (Ahmed and Mitchell 2005; Ahmed et al. 2008). However, the function of this protein in the motile cilia of a *vertebrate* body has been poorly investigated in the literature.

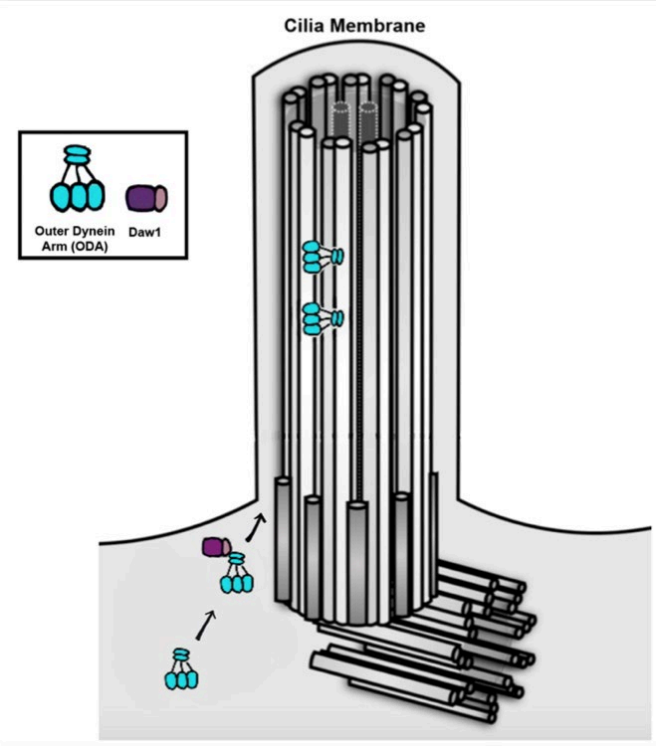

Figure 4: Diagram of ODA16 (Daw1) function within a motile cilium.

Here, ODA16 (purple) functions to guide outer dynein arms (blue) into the cilium.

Motile Cilia in Zebrafish:

This research will focus on the function of ODA16 in the development of a vertebrate body. To do this, I will use zebrafish: an excellent model organism for this work as it is possible to watch some of their earliest morphological changes live during the first few days of development. During early development, zebrafish embryos begin curled around the yolk and slowly straighten over the first day post fertilization until achieving a characteristic straight body axis (Gilbert, 2002). As the larva grows to adulthood, this straight body axis is usually maintained to form similarly straight adult fish.

In zebrafish, there are several characteristic complications of defective motile cilia. One (held in common with humans) is Left/Right patterning defects: in which organs develop in the opposite orientation within the body (Grimes et. al, 2016). This occurs when motile cilia are defective during early development (~11 hours post-fertilization) in the Kupffer's Vesicle, which is the L/R organizer of zebrafish (Figure 5). As such, cilia motility mutants develop with abnormal laterality of the heart. This is known as abnormal directionality of heart jogging (Figure 6).

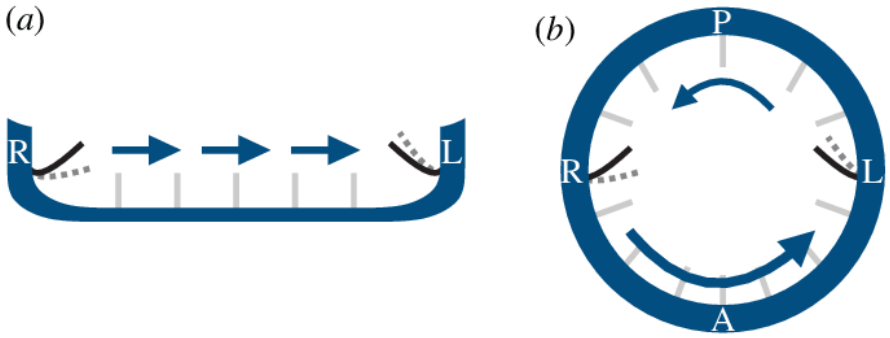

Figure 5: L/R organizers in mice and zebrafish are the node (A) and kupffer's vesicle (B) respectively.

In both, the beating of motile cilia drives asymmetric fluid flow that informs the development of an asymmetric body plan. Source: Cartwright et al., 2019

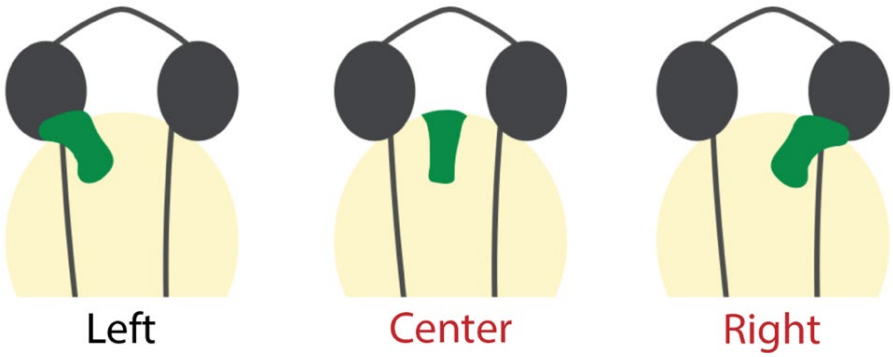

Figure 6: Heart laterality defects are common in cilia motility mutants.

In wild-type zebrafish, the normal phenotype is development of the heart on the left. In cilia motility mutants, L/R patterning defects are common. Prominent among these is abnormal laterality of the heart, in which the organ develops in the center or right.

Motile cilia in the central canal of the zebrafish are also responsible for the flow of cerebrospinal fluid (CSF), and when this doesn't occur, abnormal ventral curves form as the axial straightening process fails, which has been shown to be characteristic of cilia motility mutants (Figure 7) (Grimes et. al, 2016).

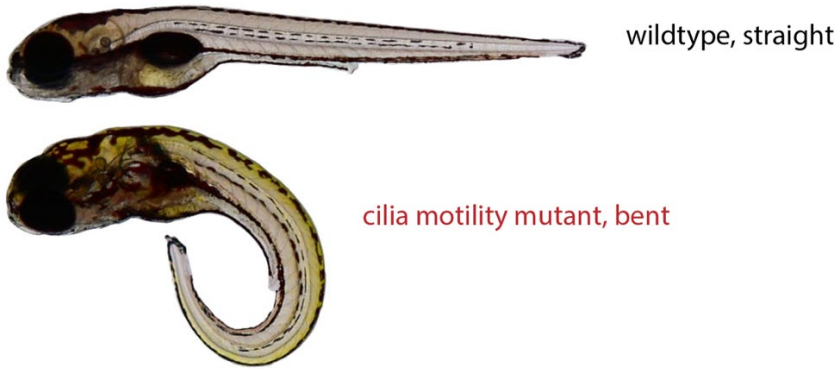

Figure 7: Cilia motility mutants develop with abnormal body axis curvature.

In wildtype zebrafish, the body axis develops a "straight" morphology. In cilia motility mutants the body axis develops curled, with failure to straighten.

Another reliable indicator of motile cilia dysfunction is otolith abnormalities (Stooke-Vaughan et al., 2012). Otoliths are biomineralized structures necessary for the sensation of sound, acceleration, and gravity in the zebrafish ear. Motile cilia in the otic vesicle beat and disperse the precursor particles that ultimately form otoliths. When these motile cilia are dysfunctional, these precursor particles aren't properly dispersed and end up fusing together or floating around. Two otoliths are the typical phenotype for wildtype zebrafish, while in mutants with defective motile cilia, otoliths are untethered or fused together (Figure 8).

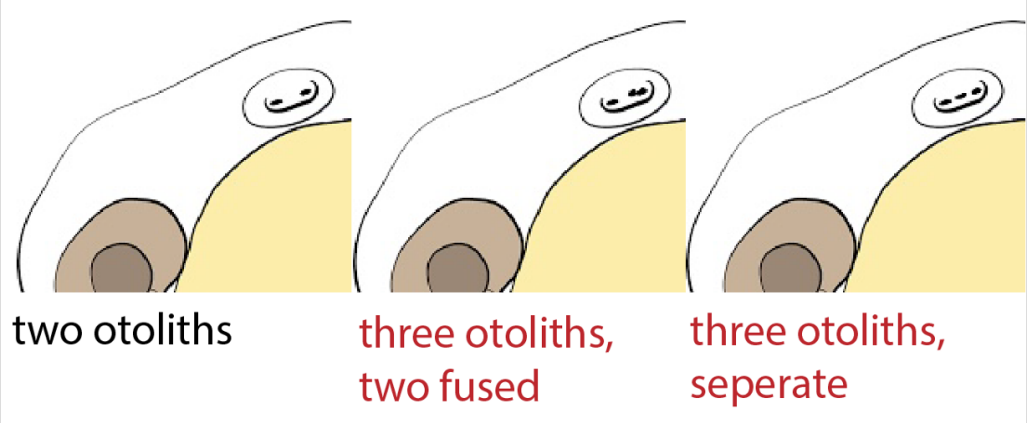

Figure 8: Cilia motility mutants develop with abnormal otoliths.

The normal, "wildtype" number of zebrafish otoliths is two, one anterior and one posterior. In cilia motility mutants, zebrafish often develop with three otoliths (two fused), or three otoliths (all separate).

My Work:

The zebrafish homologue of ODA16 is Daw1. For this investigation, we generated a new Daw1 mutant line, *daw1*^{b1403}. This mutant contains a 2-amino acid deletion in a conserved region of the protein, generated through CRISPR mutagenesis: a technology that allows for the mutation of specific genes. Because the

deletion affects a highly conserved region of the protein, I predicted this would perturb Daw1 function and disrupt cilia motility.

Another focus of my project will be in characterizing the role of Daw1 in human disease. To address whether Daw1 might be involved in human ciliopathy, I collaborated with human geneticists to investigate mutations in the human analog of Daw1. Will these patients present with L/R defects characteristic of cilia defects early in development? I will be investigating this question.

Question/Hypothesis:

Ultimately, my hypothesis is that Daw1 is important for cilia motility, and that Daw1 is resultantly important for the homeostatic processes in which motile cilia play a role.

Finally, these experiments will allow us to study the regulation of how motile cilia start beating, and the physiological consequences of perturbing this "timely onset" in a vertebrate body. By investigating the regulatory mechanisms of motile cilia, and the axial curvature defects that are produced upon their perturbation, I'm also interested in how embryos may be able to sense or correct early shape deformations, which is an exciting and relatively unknown aspect of developmental morphogenesis. Ultimately, understanding these processes may also help inform our understanding of congenital disorders.

Methods

Zebrafish:

Zebrafish (*Danio rerio)* embryos were obtained from natural matings and were of the AB strain. Incubation at 28°C proceeded fertilization. The zebrafish mutant lines used were *cfap298*^{tm304} (Jaffe et al. 2016) and *daw1*^{b1403}. Experiments followed approval by the University of Oregon Institutional Animal Care and Use Committee and the guidelines of the International Association for Assessment and Accreditation of Laboratory Animal Care.

gRNA Synthesis and CRISPR/Cas9 Somatic Mutagenesis:

A look-up table (Wu et al., 2018) provided the 4 gRNA oligos (containing target sites for the gRNA and adaptor sequences) for *daw1*^{b1403} and *cfap298* somatic mutagenesis.

daw1^{b1403} oligo sequences:	5'/TAATACGACTCACTATAGGTCACCTGCTCGAC ACAGGGTTTTAGAGCTAGAAATAG C/3'; 5'/TAATACGACTCACTATAGGGCGGTGCTGTTAC CCGTAGTTTTAGAGCTAGAAATAGC/3'; 5'/TAATACGACTCACTATAGGGTCTATTATAGGT ATGCCGTTTTAGAGCTAGAAATAGC/3' 5'/TAATACGACTCACTATAGGCTGCTTGCGTATA GGTGTGTTTTAGAGCTAGAAATAGC/3'

cfap298 oligo sequences:	5'/TAATACGACTCACTATAGGTTCTCTTCAACAC TACGGGTTTTAGAGCTAGAAATAGC/3'; 5'/TAATACGACTCACTATAGGGCTCCACAATCTG ATCATGTTTTAGAGCTAGAAATAGC/3'; 5'/TAATACGACTCACTATAGGCATTCTTATTGGA TCATGGTTTTAGAGCTAGAAATAGC/3' and 5'/TAATACGACTCACTATAGGTCTCTGGCAGGTG CGCCCGTTTTAGAGCTAGAAATAG C/3'.

Synthesis of gRNAs in multiplex: first, equimolar concentrations of the top strand oligos were mixed (10µl each). 1 µL of this was mixed with 0.5 µL Phusion DNA polymerase, 25 µl 2x Phusion buffer +dNTPs, 22.5 µl nuclease-free water, and 1 µl of the bottom strand ultramer (10 µM) of sequence:

5'/AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTT ATTTTAACTTGCTATTTCTAGCTCTAAAAC/3'

Oligos were annealed and extended using the thermocycler. The cycling proceeded at 98°C (2 min), 50°C (10 min), 72°C (10 min), 4°C (hold).

The next step was to purify the assembled oligos (DNA Clean & Concentrator-5 kit, Zymo Research), and then subject them to *in vitro* RNA synthesis (HiScribe T7 High Yield RNA Synthesis kit). After treatment with 2 µl TURBO DNase (ThermoFisher Scientific), the RNA solution was then purified (RNA Clean & Concentrator kit). Eluted gRNA was aliquoted and stored at -80°C. For use in mutagenesis, 1000 pg of gRNA + 1600 pg/nl Cas9 was injected into embryos at the single cell stage.

Larval Body Curve Quantification:

Through use of a Leica S9i stereomicroscope, lateral images of zebrafish larvae were taken daily at 24-hour intervals over the course of 5 days of development. Angle measurements (θ) were performed in ImageJ after double blinding, and following Schindelin et al., 2012.

Otolith Scoring: 24 h.p.f. embryos were dechorionated using forceps and subsequently anesthetized with tricaine. Through use of a Leica S9i stereomicroscope, the otic vesicle was viewed, and otolith formation scored as either 2 otoliths, 3 otoliths (the posterior two fused, PF), or 3 otoliths (all separate, S). Representative images of otolith categories were captured using a Leica THUNDER Imager.

Scoring of Heart Position: Through use of a Leica S9i stereomicroscope, 24 h.p.f. embryos were viewed from a ventral angle and heart laterality was scored as either left, center, or right.

Results

Generation of *daw1*^{b1403} Mutant Line:

To investigate the role of Daw1 in motile cilia within a vertebrate context, we generated a novel zebrafish mutant line, *daw1*^{b1403}. Through Cas9/CRISPR mutagenesis, a deletion of amino acids A141 and I140 was made in the Daw1 protein. These amino acids are conserved across many species, including within rats, *Chlamydomonas*, and humans (Figure 9A). They are both located in the second of eight beta-transducin (WD-40) repeats (Figure 9A). These eight repeats form a beta propeller, in which the two amino acids are centrally located (Figure 9B). As the ODAs interact with Daw1 via this beta propeller, I predicted that these deletions would perturb the function of this protein, and thus lead to abrogation of ODA import.

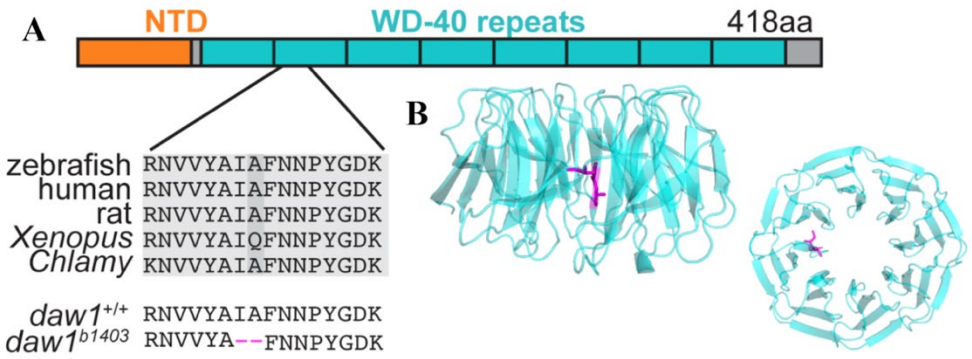

Figure 9: Diagram of the Daw1 protein.

(A) Schematic of the Daw1 amino acid (AA) sequence. NTD = N-terminal, blue = beta-propellor domain. The AA sequence surrounding CRISPR-mediated deletion of I140 and A141 is shown. These amino acids are conserved across several model organisms. (B) A rendering of the X-ray structure for Daw1 in humans (Protein Data Bank: 5NNZ). Highlighted are the two amino acids, located centrally in the beta propellor.

daw1^{b1403} Mutants Present with High Frequency of Left-Right (L/R) Patterning Defects:

As I've introduced, complications of defective motile cilia in zebrafish include L/R patterning defects, otolith abnormalities, and failure to straighten the body axis. An investigation into whether abrogation of Daw1 leads to these complications would shed light into the function of this protein in motile-cilia dependent processes in a vertebrate context.

First, I determined whether *daw1*^{b1403} mutants present with L/R patterning defects. One such defect is abnormal laterality of the heart. This is where the heart develops abnormally in the center or right of the embryo, instead of on the left, which is the normal phenotype for wild-type zebrafish. My hypothesis was that *daw1*^{b1403} mutants, like other cilia motility mutants, would present with high frequency of heart jogging defects. Heart placement was scored at 23 hpf and categorized as either 'left', 'center', or 'right' in three clutches of homozygous *daw1*^{b1403} mutants, as well as wild-type controls (Figure 10A). At this stage of development, heart placement is apparent, and the embryo hasn't developed a significant amount of pigment, making quantification easy.

As observed from the ventral view, in the wild-type controls, over 96% had correct left placement (Figure 10B). In *daw1*^{b1403} mutants, however, only 30% of all hearts were found to be jogging left. In 47% and 23% of mutants, hearts also developed on the center and right sides, respectively, while in siblings these percentages were 3% and 0% (Figure 10B). A chi-squared test produced a P value of $4.84E^{-21}$ (far below the critical value of 0.05), meaning that the differences between these groups is statistically

significant. Thus, *daw1*^{b1403} mutants developed with a significantly higher frequency of L/R patterning defects.

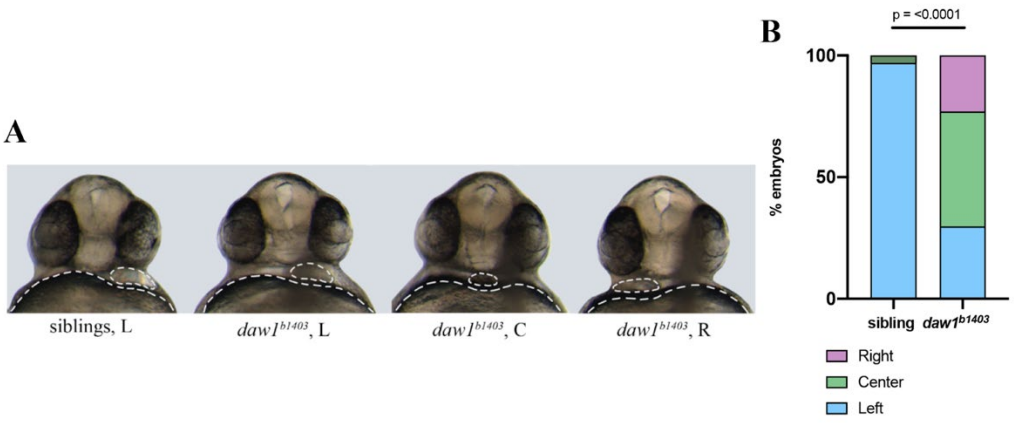

Figure 10: Characterization of heart laterality in *daw1*^{b1403} mutants.

(A) Ventral view of 24 hpf fish. Heart location is shown as a dotted oval, while the yolk salk is outlined as a dotted line. From left to right: image of sibling fish with normal development of heart on the left, followed by *daw1*^{b1403} fish with left, center, and right development. (B) Quantification of heart laterality in *daw1*^{b1403} mutants. Heart development was randomized in *daw1*^{b1403} mutants (n = 91 mutants and 98 sib controls; P = 4.84E-21, chi square test).

daw1^{b1403} Mutants Present with High Frequency of Otolith Abnormalities:

To determine whether cilia-associated defects were present in other contexts, I investigated the development of otoliths in *daw1*^{b1403} mutants. Motile cilia in the otic vesicle of a developing zebrafish beat and disperse the precursor particles that ultimately form otoliths. When these motile cilia are dysfunctional, these precursor particles aren't properly dispersed and end up fusing together or floating around, which is visible by the end of 1 dpf (Stooke-Vaughan et al., 2012). My hypothesis was that I would view a significantly higher frequency of otolith abnormalities in *daw1*^{b1403} mutants compared to siblings. Otoliths were scored in 3 clutches of mutants and siblings

at 24 hpf. I scored otic vesicles as containing either 2 otoliths, 3 otoliths (the posterior two fused, PF), or 3 otoliths (all separate, S) (Figure 11A).

A high frequency of otolith abnormalities was present by *daw1*^{b1403} mutants: 54% of siblings presented with the wild-type phenotype of 2 otoliths, while this was only 14% in the mutants (Figure 11B). 45% of siblings developed with 3 otoliths (2 fused), and only 2% developed with 3 separate, while these percentages for the mutants was 66% and 21%, respectively (Figure 11B). Chi-square analysis provided a P value of 1.31E^{-12}. As such, *daw1*^{b1403} mutants developed with a significantly higher proportion of abnormal otoliths than siblings.

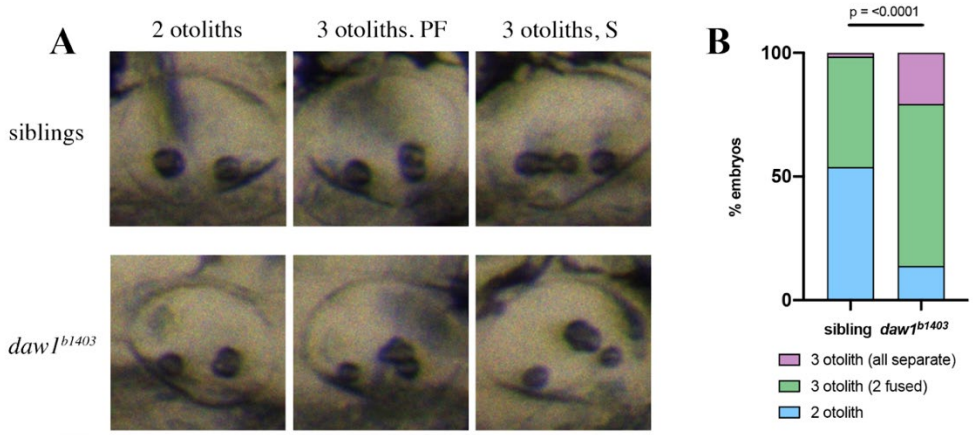

Figure 11: Characterization of otoliths in *daw1*^{b1403} mutants.

(A) Images of the otic vesicle (oval shape) in 24hpf zebrafish. Representative images are shown for the 3 categories of otolith formation in siblings and *daw1*^{b1403} mutants: 2 otoliths, 3 otoliths (the posterior two fused, PF), or 3 otoliths (all separate, S). (B) Otolith abnormalities (being 3, PF or 3, S) are at a much higher frequency in *daw1*^{b1403} mutants (n = 116 mutants and 132 sib controls; P = 1.31E^{-12}, chi square test).

***daw1*^{b1403} Mutants Fail to Straighten the Body Axis on the First Day, but Surprisingly Straighten by 5 dpf:**

Development of the straight body axis is also under the influence of motile cilia. I found that during the first day post fertilization, *daw1*^{b1403} embryos exhibit the characteristic failure of axial straightening (Figure 12). Thus, the presence of L/R defects, otolith abnormalities and failure to straighten the axis all support my hypothesis that Daw1 is required for cilia motility in zebrafish, as it is in Chlamydomonas.

However, I then made the interesting observation that *daw1*^{b1403} embryos gradually recover from their curves such that they are straight by 3-5 days of age (Figure 12). This was surprising, as it is believed that cilia motility is absolutely required for straightening, and other motile cilia mutants don't straighten at all (Jaffe et al. 2016).

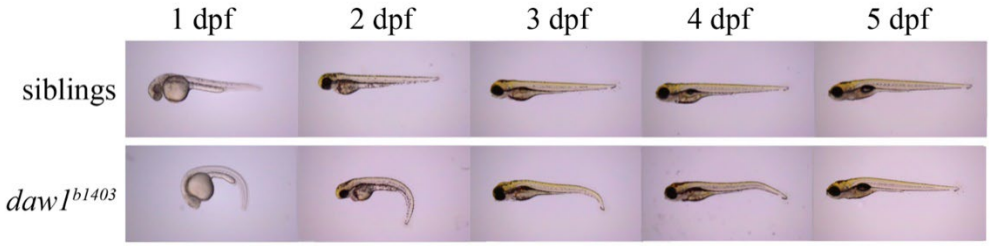

Figure 12: Lateral view of the first 5 days of development for siblings and *daw1*^{b1403} mutants.

The first 5 days of development were imaged for siblings and *daw1*^{b1403} mutants. Representative images were selected that displayed the majority phenotype of the clutch in terms of axial curvature.

Intrigued by this finding, I quantified the body angle (Figure 13 for reference) for three clutches of *daw1*^{b1403} mutants over the first 5 days of development. On the first day, the majority of *daw1*^{b1403} mutants were quite curved, with an average ventral angle

of 120.5 ± 20.2° (mean ± SD) in comparison to the wild-type of 185.5 ± 5.8° (Figure 13). Over the next four days, however, the *daw1*^{b1403} mutants gradually straightened. By 5 dpf, the average ventral angle of *daw1*^{b1403} embryos was 177.8 ± 12.1°, and that of the siblings was 190.4 ± 0.8° (Figure 13). As such, failure to straighten the body axis was only temporary for the *daw1*^{b1403} mutants, as they were able to correct early curvature. Again, this is contrary to what we would expect with a cilia motility mutant: we would expect the body to remain curved throughout development.

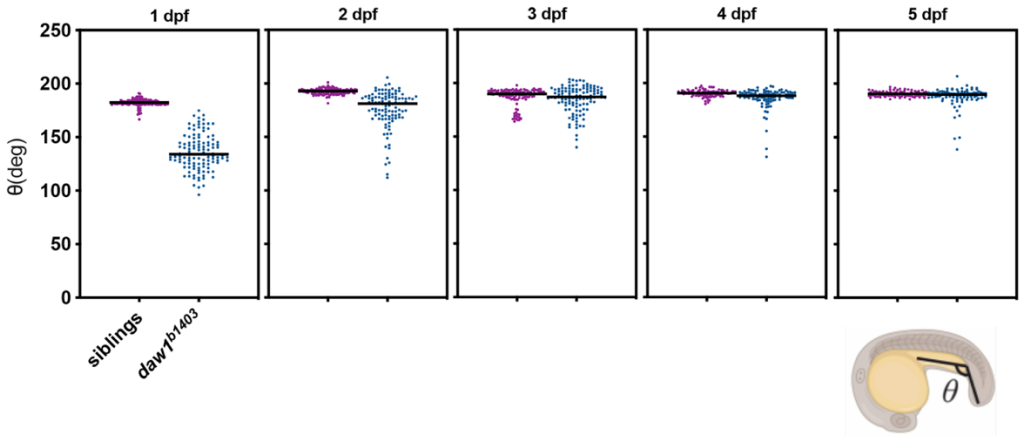

Figure 13: Superplot of body angle, θ, measurements for the first 5 days of sibling and *daw1*^{b1403} mutant development.

Each individual dot is an angle measurement for a single embryo, while the black bars represent clutch averages. The angle being measured is represented by a schematic in the lower right of the figure.

This surprising result suggests that Daw1 is not absolutely required for cilia motility in zebrafish but may only be needed for the timely onset of beating, with delayed onset leading to delayed axis straightening. In this model, ODAs can still be imported into cilia without Daw1, but it occurs more slowly (Figure 14B). However, as I describe above, the *daw1*^{b1403} mutation is a 2-amino acid deletion and not necessarily a

complete loss of function mutation. So, it's possible that this is a hypomorphic allele, which would mean that the Daw1 protein product, while mutated, still retains partial function. This could explain why straightening occurs with a delay (Figure 14C). In this model, Daw1 would still function in ODA import, but would also be slowed down by the hypomorphic Daw1 mutant version.

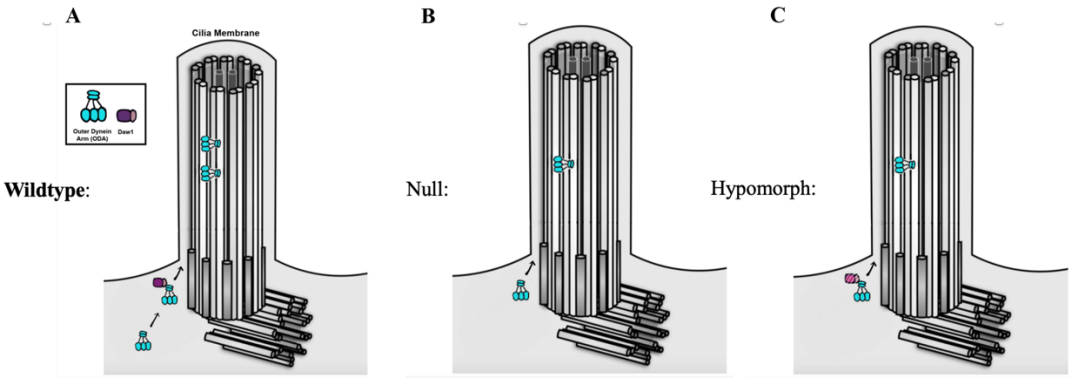

Figure 14: Different models for the function of Daw1 in *daw*^{b1403} mutants.

(A) In wildtype fish, Daw1 (purple) functions to robustly import outer dynein arms (blue) into the cilium. (B) In this model, Daw1 is null. Here, Daw1 would not be absolutely required for cilia motility in zebrafish but may only be needed for the timely onset of beating, with delayed onset leading to delayed axis straightening. (C) In this model, Daw1 is a hypomorph. Here, Daw1 would still function in ODA import, but would also be slowed down by the hypomorphic Daw1 mutant version.

Daw1 Somatic Mutants Phenocopy *daw1*^{b1403} Mutants:

To distinguish these possibilities, I wanted to knockout *daw1* completely and assess whether body straightening can occur at all. To do this, I used high doses of CRISPR-Cas9 mutagenesis to knock out the Daw1 gene, creating G0 somatic mutants, termed "crispants". In crispants, CRISPR-Cas9 creates a mosaic effect, with different

mutations throughout the Daw1 locus in different cells. This is in comparison to germline mutants like *daw1*^{b1403_3} fish, where the mutations in all cells are the same.

If the somatic mutants present phenotypes like the *daw1*^{b1403} mutants, this would support model 14B, where the mutation encodes a null protein (the absence of a functional protein product). If this was the case, then I would know Daw1 isn't necessary for cilia motility over the long term. If the somatic mutants remained curled throughout the first 5 days, I would know that our *daw1*^{b1403} mutant was hypomorphic and partially functional, which would support the model in Figure 14C.

For characterization of these crispants, a positive control, *cfap298*, was included alongside the *daw1*^{b1403} and sibling populations. *cfap* stands for cilia and flagella associated protein, and this gene encodes an ODA assembly factor that is absolutely required for cilia motility (Austin-Tse et al. 2013; Jaffe et al. 2016). As such, *cfap298* mutants develop with severe ventral curvature. If the *daw1* crispants develop with ventral curvature like the positive control, this would support a model where the Daw1 protein in *daw1*^{b1403} mutants is hypomorphic (leading to delayed onset of cilia motility and straightening) (Figure 14C). If they develop curved and subsequently straighten, however, this would provide strong evidence for a model in which the Daw1 protein in *daw1*^{b1403} mutants is null (Figure 14B).

First, as was the case in the *daw1*^{b1403} mutant, Daw1 crispants presented with significantly greater frequency of otolith abnormalities and heart jogging defects compared to siblings (Figure 15).

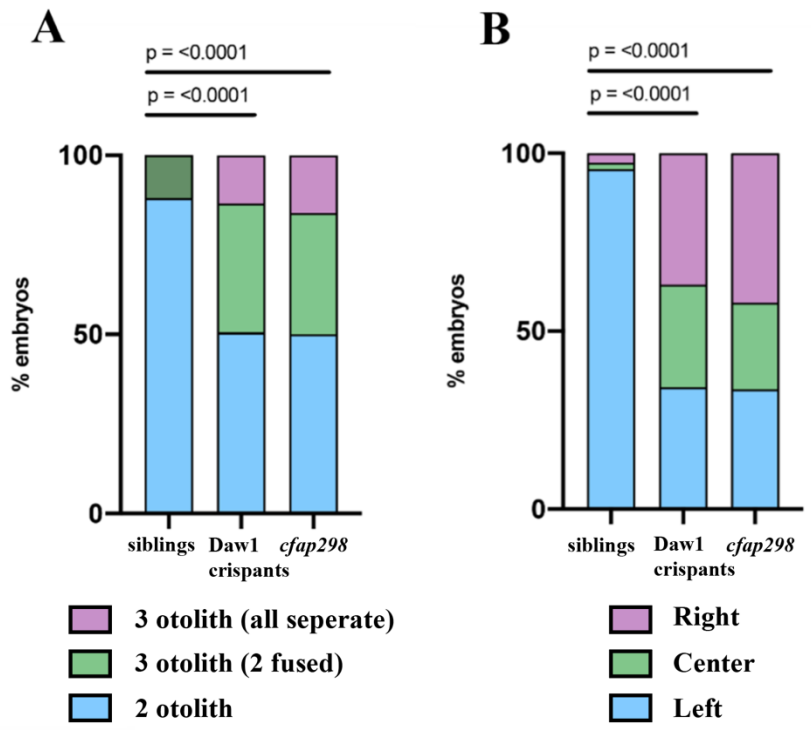

Figure 15: Quantification of otolith formation (A) and heart laterality (B) in siblings, *daw1*, and *cfap298* crispants.

(A) Otolith formation following the same categorization as in figure 11 was quantified for the three populations of fish. A significantly higher percentage of abnormalities (3, PF and 3, S) were observed in *daw1* and *cfap298* crispant populations. (B) Heart laterality was scored in all three populations. *daw1* and *cfap298* crispant fish presented with a significantly greater percentage of center and right development.

Next, I quantified body angles for these three populations over the first 5 days of development. As can be observed in Figure 16, both the *daw1* and *cfap298* crispants present with a much lower average ventral angle than the wild-type population. However, over the 5 days of development, the *daw1* crispants straightened. By the fifth day 91.2 ± 6.1% of *daw1* crispants had recovered, while only 13.7 ± 16.7% *cfap298* controls had done the same. This additional loss-of-function approach supports the

model in which the Daw1 protein in *daw1*^{b1403} mutants is null and that late-onset axial straightening occurs in the absence of Daw1 function.

As the crispants present with similar heart jogging and otolith defects to the positive control, but develop straight, this also supports the notion that timely onset of cilia motility is more critical to first two processes than the latter in early development, and that disruption of this onset leads to heart and otolith but not straightening defects as the embryo grows.

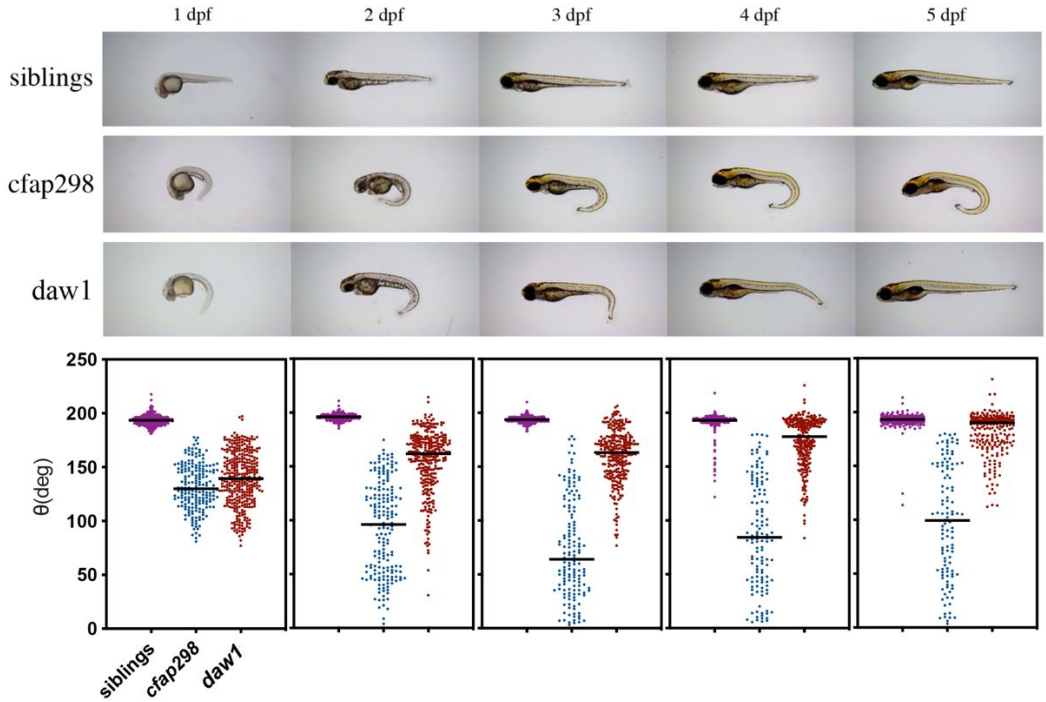

Figure 16: Representative images and angle quantifications of the first 5 days of development for siblings, *daw1*, and *cfap298* crispants.

Fish were imaged on their lateral side. Representative images were selected based on the predominating phenotype (in terms of axial curvature) for respective populations on each day of development. Each dot = an angle measurement for an individual fish, and black bars = averages.

Studies in Chlamydomonas suggested that Daw1 is essential for cilia motility by controlling the import of dynein motors. This work reveals that, in zebrafish, Daw1 is not essential for motility *per se*, but only for the timely onset of cilia beating across developmental timescales.

daw1^{b1403} Mutants have a Delayed Onset of Cilia Motility:

To test this idea, the Grimes lab directly imaged beating motile cilia within the central canal to observe whether fish that lack Daw1 demonstrate early cilia motility defects, and if so, whether these defects recover by later points. First, the expected length and pattern of cilia were encountered in the mutants (data not shown, Beth Bearce). The motility of cilia was next assessed through rapid DIC imaging, and the data were visualized using temporal image correlation spectroscopy (TICS). In a control visualized at 25 hpf, there was a lot of oscillating movement (17.1 ± 5.8 Hz) created by beating of the motile cilia (Figure 17A). When we imaged *daw1*^{b1403} mutants, however, motile cilia appeared static, and the cilia that *were* beating were doing so at much slower and inconsistent frequency (7.4 ± 2.7 Hz) (Figure 17A). These were both corroborated by measurement of the density of moving cilia (Figure 17A')

However, at later time points (43 hpf, 52 hpf) where *daw1*^{b1403} mutants have begun to straighten, cilia motility in both mutants and controls appears much more similar (Figure 17C-D'). Motility was significantly reduced in *daw1*^{b1403} mutants at 25 hpf, but by 52 hpf had recovered to a level that was not statistically different from wildtype (Figure 17D).

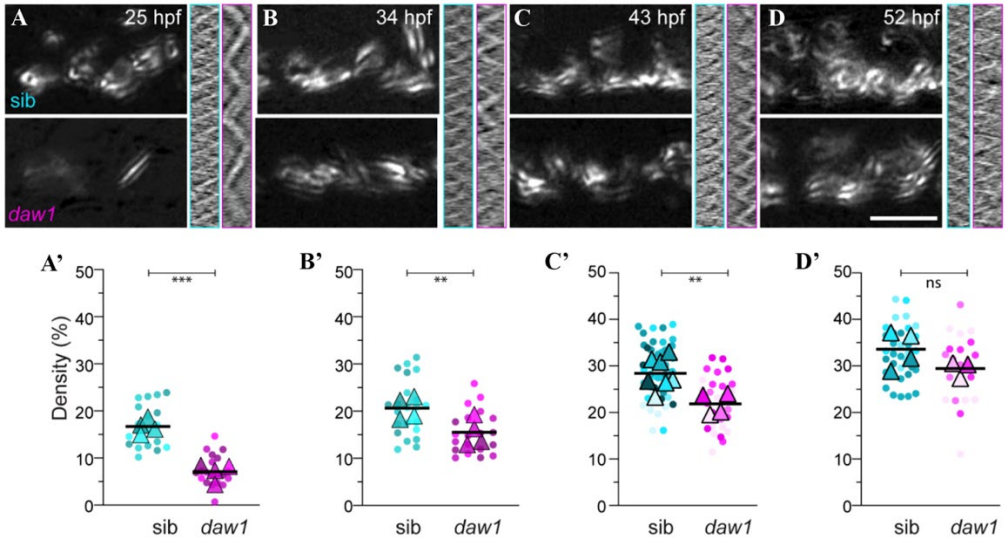

Figure 17: Measurement of cilia motility and density of moving cilia.

(A-D) Cilia motility in the central canal of zebrafish. Imaged using TICS at 4 different developmental time points. (A'-D') Visualization of the proportion of the central canal covered by cilia movement. Black lines show means, triangles show averages of data from individuals, and dots represent distinct regions of interest within an individual fish. Unpaired t tests provided statistical comparison. ***P < 0.001, **P < 0.01, *P < 0.05, ns – not significant. Scale bars: F-I; 5 µm.

This means that while *daw1*^{b1403} might be necessary for early initiation of motile cilia, it isn't required during later timepoints, but perhaps only for the onset of beating. Ultimately, this data supports my hypothesis that Daw1 facilitates onset of cilia motility, and thus timely axis straightening.

Daw1 Mutations in Human Patients:

Our Daw1 model also finds representation in human patients. Collaborators based in the United Kingdom (Institute of Biomedical and Clinical Science, RILD Wellcome Wolfson Centre, University of Exeter Medical School), have been studying human patients homozygous for *DAW1* mutations. Two families were identified that

have mutations in the coding sequence for DAW1, and present with mild respiratory PCD symptoms and situs abnormalities. Like our *daw1*^{b1403} mutant: these mutations (Asn143Asp, and Trp119Cys) appear in a conserved region of the protein. As with our *daw1*^{b1403} mutant, we were curious to determine if these mutations lead to only a weakly-functioning protein (that being hypomorphic) or a completely null allele. If the alleles were hypomorphic, I hypothesized that overexpression of Daw1 mRNA harboring human mutations in our *daw1*^{b1403} mutants could perhaps axial straightening.

To test this, Daw1 mRNA containing the two of the mutations found in humans: Trp119Cys and Asn143Asp were injected into *daw1*^{b1403} embryos at the single cell stage. What I discovered was that overexpression of mRNA containing these mutations did *not* rescue the phenotype while overexpression of WT Daw1 mRNA did rescue (Figure 18). Through angle quantifications conducted 1 dpf, our *daw1* mutants injected with both types of mutant mRNA did not rescue, and on average presented with significant body curvature. *daw1*^{b1403} mutants injected with WT *daw1* mRNA, however, *did* rescue their curvature. These results confirm that the human mutations encode a null Daw1 protein, and so demonstrates that the underlying ciliopathy in these patients is likely caused by these *DAW1* mutations. Our *in vivo* modeling using the zebrafish therefore implicates loss of the Daw1 function as a cause of PCD and L/R patterning defects in humans.

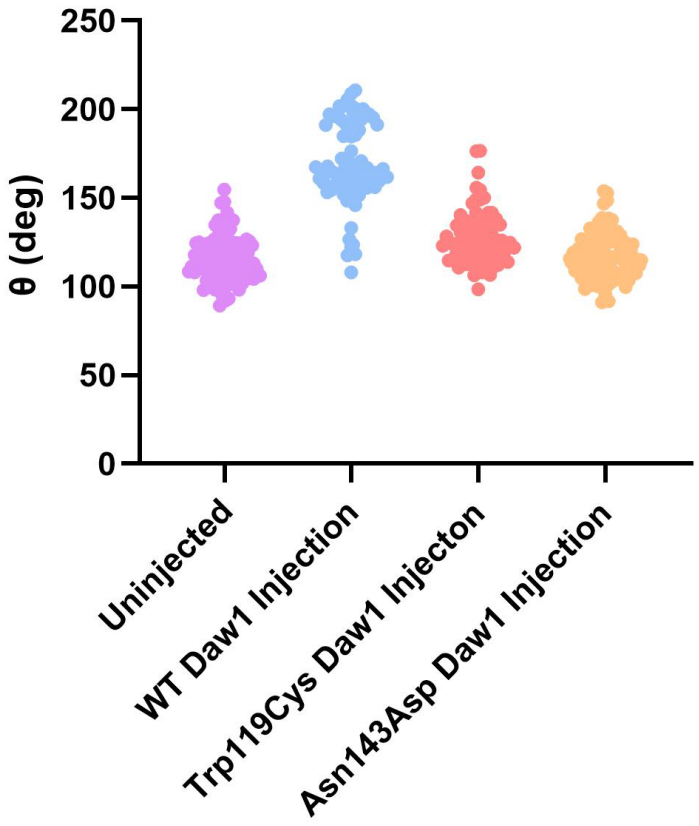

Figure 18: *daw1*^{b1403} rescue experiments using Daw1 mRNA with human mutations.

Bars represent the average ventral angle of respective populations after 5 days of development. While injection of WT Daw1 mRNA rescued axial curvature for *daw1*^{b1403} fish, injection of mRNA containing human mutations did not.

Discussion

Investigations into the function of ODA16, the homologue of Daw1 in Chlamydomonas, suggested that ODA16 is essential for the onset of cilia motility by controlling the import of outer dynein arms into the cilium (Ahmed and Mitchell 2005; Ahmed et al. 2008). However, the role of this protein in motile-cilia dependent processes within a vertebrate context has been little explored. The purpose of this investigation was therefore to examine this role using zebrafish, a popular vertebrate model organism.

To investigate the necessity of Daw1 in developmental processes that are dependent upon motile cilia in zebrafish, including L/R patterning, otolith formation, and axial straightening, a new Daw1 mutant line was generated (*daw1*^{b1403}) (Grimes et al., 2016; Stooke-Vaughan et al., 2012). My hypothesis was that Daw1 is important for cilia motility, and that Daw1 is therefore important for the homeostatic processes in which motile cilia play a role. Results in support of this hypothesis would include L/R patterning defects (heart jogging), otolith abnormalities, and failure to straighten the body axis during early development (1-5d.p.f.) in the *daw1*^{b1403} mutants.

Upon characterization of heart laterality and otolith formation in *daw1*^{b1403} mutants, I encountered significantly higher levels of defects/abnormalities than in sibling controls. This was in support of my hypothesis: heart jogging defects and otolith abnormalities are to be expected in cilia motility mutants. These results suggested that Daw1 was essential for cilia motility and subsequently the developmental processes dependent upon them.

Characterization of axial straightening in *daw1*^{b1403} mutants, however, produced results that are inconsistent with this hypothesis. On the first day post-fertilization, *daw1*^{b1403} mutants develop curved, with a very low ventral angle. Surprisingly, however, *daw1*^{b1403} mutants straightened over the next few days of development to the point that their ventral angle was not significantly different from sibling controls. These results diverged from my hypothesis: cilia motility mutants classically fail to straighten the body axis, which suggested that Daw1 is not, in fact, essential for cilia motility.

As the *daw1*^{b1403} mutation is a deletion of only two amino acids, my next idea was that this straightening could be the result of a partially functioning, hypomorphic Daw1 protein. In this model, Daw1 works, but in a reduced capacity, to import dynein arms (explaining the delayed straightening). The alternative model would be that Daw1 is non-functional in these mutants, and thus not necessary for cilia motility. To distinguish these possibilities, I generated somatic mutants of Daw1 through use of CRISPR-Cas9. If these mutants (which harbor many mutations in Daw1 in a somatic mosaic fashion) phenocopy *daw1*^{b1403} mutants, this would suggest that the Daw1 protein in *daw1*^{b1403} is not functional. Alternatively, if crispants exhibited more severe defects like failing to straighten, this would suggest that the Daw1 protein *daw1*^{b1403} is hypomorphic.

Upon characterization, the crispants were shown to phenocopy *daw1*^{b1403}: with significantly higher frequency of otolith abnormalities and heart jogging than control injected fish, as well as delayed onset of straightening over the first 5 days of development. This confirmed my earlier findings that Daw1 is not necessary for the axial straightening, though it is essential to ensure straightening occurs in a timely

fashion. I interpreted this to mean that Daw1 is not needed for cilia motility of cilia *per se*, but only for the timely onset of beating, with delayed onset leading to delayed straightening.

This idea was supported by subsequent experiments performed by the Grimes lab which directly compared cilia motility in the central canal of *daw1*^{b1403} zebrafish to that of siblings. What these demonstrated was that cilia motility onset in *daw1*^{b1403} mutants is, in fact, delayed. While there were not statistically different numbers and lengths of motile cilia between the mutants and controls, the density of moving cilia and the frequency of beating was significantly lower in *daw1*^{b1403} mutants than siblings at 25 h.p.f. By 52 h.p.f., however, motility was completely restored to the point of statistical insignificance between mutant and control populations. This strongly supports the notion that while Daw1 might not be *necessary* for cilia motility, it *is* necessary for the timely onset of beating.

In processes that require rapid onset of beating after cilia emerge from cells, significant perturbation would be expected based on these findings. This provides an explanation for why some complications of defective motile cilia are observed in *daw1*^{b1403}, whereas others are not. L/R patterning and otolith formation, for example, both occur over a very short window of time in early development. Motile cilia in the node and otic vesicle both emerge and regress within the first few days of development, functioning over a short window to establish L/R asymmetry and the initial formation of otoliths (Whitfield, 2020; Little and Norris 2021). Axial straightening, however, is a process that proceeds over many days of development, in which cilia in the central canal beat to drive the flow of extracellular cerebrospinal fluid and straighten the body

and then maintain the straightness of the spine (Grimes et al., 2016). Serious perturbation to the first two processes but not the latter would thus be expected in *daw1*^{b1403} mutants and, indeed, this is what we see.

This model, in which homeostatic processes dependent upon timely onset of cilia beating are perturbed by lack of Daw1 function, can also be used to explain the *situs* defects present in the human patients that have mutations in *DAW1*. As previously described, L/R patterning, but not mucus clearance in the lungs or axial straightening, is dependent upon timely initiation of cilia beating. Complications of the first, but not latter two would thus be expected in human mutants of *DAW1*. Indeed, this is what is observed. Patients with *DAW1* mutations presented with L/R patterning defects (abnormal heart laterality), but not complications of respiration. This unusual ciliopathy presentation can be explained by our findings in zebrafish: L/R abnormalities are present because DAW1 is needed for the rapid onset of beating and function of these cilia whereas lung defects are mild or absent because small delays in the onset of beating would not be expected to significantly impact lung homeostasis over the long term.

Lastly, the ability of *daw1*^{b1403} mutants to sense, or correct, shape deformations, is of great importance. It could be expected that *daw1*^{b1403} mutants, unable to straighten their body axis due to defective motile cilia on the first day of development, might be committed to this morphology. Rather than remain curved, however, Daw1 mutants subsequently straighten when cilia motility is restored. Thus, given the necessary factors and conditions, these zebrafish embryos can "sense" their apparent malformation, and correct themselves to achieve a "target morphology" – their species-

specific “correct” anatomy. Significantly, this contributes to an understanding of the process of developmental not as a fixed sequence of events that if not met results in pathology, but rather a flexible process where embryos endeavor to reach a goal state or morphology.

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