Rare coding variants in axonemal dynein heavy chain genes are associated with adolescent idiopathic scoliosis

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Abstract

Adolescent idiopathic scoliosis (AIS) is the most common pediatric musculoskeletal disorder worldwide, characterized by atypical spine curvatures in otherwise healthy children. Human genetic studies have identified candidate genes associated with AIS, however, only a few of these genes have been shown to recapitulate adult-viable scoliosis in animal models. To further define susceptibility loci for AIS, we performed whole exome sequencing on a cohort of 195 Han Chinese AIS patients and 229 healthy controls. We identified members of the axonemal dynein family associated with both sporadic and familial AIS. We demonstrate that disruption of the *dynein axonemal heavy chain 10 (dnah10)* gene results in recessive adult-viable scoliosis in zebrafish. These *dnah10* mutant zebrafish display reduced ependymal cilia beating and a disassembly of the Reissner fiber in the hindbrain and spinal canal, concurrent with the onset of body curvatures. Altogether, these results demonstrate that mutations in axonemal dynein genes are linked with human AIS and suggest that ependymal cell cilia function plays an essential role in maintaining spine alignment in humans.

Introduction

Adolescent idiopathic scoliosis (AIS) is a common pediatric disorder characterized by atypical three-dimensional curvatures, with rotation of the spinal column (Cheng, Castelein et al. 2015). The prevalence of AIS is approximately 1.0–5.0% among children worldwide (Zhang, Guo et al. 2015, Yilmaz, Zateri et al. 2020). Approximately 10% of AIS patients suffer severe physical and mental issues due to progressive spine curvature (Cheng, Castelein et al. 2015). Analysis of familial scoliosis and the recognition of a high concomitance of scoliosis in twin-studies demonstrate that AIS is a multifactorial disease with a strong genetic predisposition (Hadley Miller 2000, Wise, Gao et al. 2008). Hypothesis-free genome wide association study (GWAS) analyses from different ethnic backgrounds have mapped a range of candidate loci and implicated several candidate genes associated with this disorder, including *LBX1*, *BNC2*, *GPR126*, *AJAP1* and *PAX1* (Takahashi, Kou et al. 2011, Kou, Takahashi et al. 2013, Ogura, Kou et al. 2015, Sharma, Londono et al. 2015, Zhu, Xu et al. 2017). Recent, exome sequencing studies has identified additional rare variants associated with AIS in different genes, including within *POC5*, *HSPG2*, *AKAP2*, *SLC39A8* and *FLNB* (Baschal, Wetthey et al. 2014, Patten, Margaritte-Jeannin et al. 2015, Li, Li et al. 2016, Haller, McCall et al. 2018, Jiang, Liang et al. 2020). Additional analysis of exome data have identified an enrichment of potentially deleterious variants in *FBN1/2*, extracellular matrix (ECM) and cilia-associated genes in AIS patients (Buchan, Alvarado et al. 2014, Haller, Alvarado et al. 2016, Baschal, Terhune et al. 2018). Overall, these data suggest that AIS is a complex polygenic disease with significant genetic heterogeneity. Therefore, additional genetic studies are needed to expand our understanding of the genomic architecture of AIS.

Recent studies in the zebrafish model system to functionally test the role of human AIS associated genes, including for *poc5*, *slc39a8*, and *mapk7* show scoliosis phenotypes when knocked out in zebrafish (Patten, Margaritte-Jeannin et al. 2015, Gao, Chen et al. 2017, Haller, McCall et al. 2018). In addition, forward genetic studies in zebrafish have identified several mutant zebrafish lines displaying adult-viable scoliosis without vertebral patterning defects, which resemble the pathology of human AIS (hereafter called scoliosis) (Buchan, Gray et al. 2014, Hayes, Gao et al. 2014, Grimes, Boswell et al. 2016, Zhang, Jia et al. 2018, Vesque, Anselme et al. 2019, Rose, Pompili et al. 2020, Troutwine, Gontarz et al. 2020). Altogether, these studies support a model where defects in the physiology of the spinal cord central canal including: motile cilia motility dysfunction and

alterations in cerebrospinal fluid (CSF) flow, disruption of the Reissner fiber (RF), and urotensin neuropeptide signaling to the dorsal musculature are antecedents of scoliosis in zebrafish (Bagnat and Gray 2020).

The RF, is a glycoprotein-rich extracellular thread, which stretches from the third cerebral ventricle down the central canal in most chordate lineages (Munoz, Kahne et al. 2019). Dysfunction of motile cilia in the central canal of the spine leads to disrupted RF assembly and severe ventral ward body curvatures (Cantaut-Belarif, Sternberg et al. 2018). Importantly, the continuous assembly of the RF during larval stages of development in zebrafish is essential for maintaining spine stability in larval zebrafish (Rose, Pompili et al. 2020, Troutwine, Gontarz et al. 2020) and the assembly of the RF appears to require the function of motile cilia components, including *kinesin family member 6 (kif6)* (Troutwine, Gontarz et al. 2020) and *protein kinase 7a (ptk7a)* (Rose, Pompili et al. 2020). Finally, loss of CSF flow and RF assembly can alter the expression of urotensin II-related neuropeptides in the central canal (Zhang, Jia et al. 2018, Vesque, Anselme et al. 2019, Lu, Shagirova et al. 2020), which has been shown to signal to the dorsal musculature to help maintain a straight body axis in larval zebrafish (Zhang, Jia et al. 2018). Altogether, analysis of scoliosis in zebrafish have begun to elucidate a robust pathway for control of spine morphogenesis. However, there remains a lack of evidence linking the pathogenesis of scoliosis in these zebrafish models to genetic studies of human AIS.

In this study, we report a whole exome sequencing (WES) on 195 sporadic AIS patients and 229 in-house controls to identify novel AIS-associated mutations enriched with variants predicted to be deleterious. We inferred variants in ten genes encoding a variety of dynein axonemal heavy chain proteins. Using CRISPR/Cas9 targeted mutagenesis, we functionally verified that a frameshift-truncating mutation in the *dynein axonemal heavy chain 10 (dnah10)* gene resulted in adult-viable scoliosis in zebrafish. High-speed time-lapse confocal imaging of motile cilia within the spinal cord central canal demonstrated alterations in motile cilia beating. Using an endogenous RF-labeling *scospondin-GFP^{ut24}* knock-in zebrafish line, we demonstrated a range of RF assembly defects concurrent with the onset and severity of spine curvatures in *dnah10^{ut28}* mutant zebrafish. To our knowledge, this is the first study to associate rare variants of several axonemal dynein gene with human AIS, with relevant functional testing and demonstration that *dnah10* is essential component of motile cilia dynamics and spine morphogenesis in zebrafish. This study provides key evidence that alterations in ependymal cell motile cilia function may underlie AIS in humans.

Materials And Methods

AIS and control subjects

This study was approved by the Ethics Committee of Xiangya Hospital of Central South University (Changsha, China) and all participants and their legal guardians involved in this project have signed a written consent form. A total of 195 unrelated Han Chinese AIS patients were recruited from Xiangya Hospital. The in-house control cohort consisted of 229 healthy Han Chinese subjects from The Han Chinese Genomes Database (PGG.Han), a population genome database to serve as the central repository for the genomic data of the Han Chinese genome database (<https://www.hanchinesegenomes.org/HCGD/index>) (Gao, Zhang et al. 2020). The inclusion criteria for AIS were as follows: (1) AIS diagnosis for each case was confirmed by three certified senior spine surgeons. Patients with congenital vertebral malformations, connective tissue diseases, skeletal developmental delay or other scoliosis related syndromes were excluded; (2) Cobb angle > 10°; (3) No second-degree family members of AIS cases were diagnosed as scoliosis or inherited diseases. The mean Cobb angle of these AIS patients was

41.88 ± 18.35°. The female-male ratio was 4 (156 females vs. 39 males), which following the natural sex distribution of AIS population (Table S1).

The second exome sequencing cohort consists of 28 IS families enrolled as previously described (Baschal, Wethey et al. 2014). A diagnosis of IS required a standing anteroposterior spinal radiograph showing $\geq 10^\circ$ curvature by the Cobb method with pedicle rotation, and no congenital deformity or other co-existing musculoskeletal disorder. Pedigrees were created using PedigreeXP.

Whole exome sequencing and data preprocessing

Genomic DNA was extracted from the venous peripheral blood samples of AIS cases. Exome sequencing within sporadic AIS patients was performed on BGISEQ-500 sequencing platforms (BGI Group, Wuhan, China) according to the manufacturer's protocol. After high-throughput sequencing, the raw data of each case were generated and stored in the FASTQ format. Clean data were kept by removing reads including sequencing adapters, low-quality (base quality ≤ 20) and uncertain base (N > 10%). We mapped clean reads to the human reference genome (hg19/GRCh37) using the Burrows-Wheeler Aligner (BWA; Version 0.7.10) and removed the duplicate reads marked by Picard tools (Version 1.117). Then we used the Genome Analysis Toolkit (GATK; Version 3.6) to perform local realignment, Base Quality Score Recalibration (BQSR) and variants calling (Using HaplotypeCaller and output in a genomic variant call format [GVCF] file).

Exome sequencing within 28 IS families was conducted and filtered as described previously. Briefly, 3-6 affected individuals were sequenced per family using the Illumina TruSeq exome kit and sequenced with a 2 x 100 bp run on the Illumina HiSeq 2000 at the University of Colorado Denver Genomics and Microarray Core facility. Reads were aligned to the Hg38 genome and variants were identified with FreeBayes.

Joint calling, quality control and variants filtration

We performed joint calling in all sporadic samples (195 AIS and 229 controls) using GATK. To generate an accurate calling result, We performed variant-level quality control (QC). we filtered out the variants with low coverage due to uneven coverage of WES by remove variants which missing rate more than 20% in AIS. To further filter out low-confidence variants, Variant Quality Score Recalibration (VQSR) from GATK was performed. In this step, we select the filtering indicators including read depth, strand bias, mapping quality, read position bias. To further identify the AIS-related candidate variants, we used the following filters: (1) exclude high frequency SNPs (MAF ≥ 0.01) in an in-house control dataset of Han Chinese, Han Chinese in Beijing (CHB) and Southern Han Chinese (CHS), from 1000 Genomes Project (1000G) dataset; (2) Variants with highest frequency in AIS cohort among AIS, in-house control, CHB and CHS cohorts were reserved; and (3) Variants classified as "HIGH" or "MODERATE" impact by Variant Effect Predictor (VEP) were kept. All the filtered deleterious candidate variants were subjected to gene-burden analysis.

For the exome data from 28 IS families. Variants were retained that were present in all sequenced family members, had an allele frequency of $\geq 5\%$ within ExAc, and were scored as damaging by at least one predictive functional algorithm (SIFT, Polyphen2, LRT, MutationTaser).

Sanger sequencing

DNA from additional affected and unaffected family members was Sanger sequenced across the variants of interest detected through exome sequencing. Primers were designed using Primer3 and obtained from Integrated DNA Technologies. PCR was conducted in 20 µL reactions with 10 µL Premix D (Epicentre Biotechnologies), 0.2 µL Taq Polymerase (Sigma), 20 ng genomic DNA, and 20 µM Forward and Reverse Primers. PCR reactions were run with a touchdown PCR protocol on a SimpliAmp Thermocycler (Fisher Scientific). Annealing temperatures were set with the delta function to begin at 65°C and decrease by 0.3 °C each cycle, ending at 55 °C. PCR reactions were cleaned up for Sanger sequencing using ExoSAP-IT Express (Thermo Fisher). Sanger sequencing was performed by Quintara Biosciences and chromatograms were analyzed using the CodonCode Aligner v9.0 (CodonCode Corporation, <https://www.codoncode.com/index.htm>).

Gene-burden analysis and functional analyses

To perform gene-burden analysis, we determined two indicators in gene-level, The samples carrying proportion of harmful variation and deleterious variant count per sample, in AIS cases and in-house controls by the candidate variants. Considering sequence discrepancy may lead to loci missing, we corrected the sample size for each gene using the equation below.

$$N_{cor} = \sum_{i=1}^m n_i \cdot \frac{AF_i}{\sum_{i=1}^m AF_i}$$

Where m denotes number of variants, n indicates observable sample size, and AF represents derived allele frequency. The gene-level samples carrying proportion of harmful variation generated by dividing the corrected sample size by counting samples that contain deleterious variants for each gene. Gene-level deleterious variant count per sample was calculated as the number of deleterious variants in one gene divide corrected sample size in this gene. To avoid the bias of gene length, we correct these two indicators by dividing them by length. A two-sided Fisher's exact test was performed with deleterious variant count per sample in two datasets. The difference was estimated between deleterious variant proportions in the two datasets. Further, we used a systematic functional annotation approach, including Gene Ontology (GO) analysis, KEGG pathway, gene family enrichment analysis and Protein Annotation Through Evolutionary Relationship (PANTHER) classification system to interpret these AIS-associated genes (Mi, Muruganujan et al. 2019). Gene family annotation information was based on HGNC database (HUGO Gene Nomenclature Committee).

Zebrafish husbandry

All *in vivo* experiments were performed following approved Institutional Animal Care and Use Committee (IACUC) protocols. All zebrafish procedures were approved by the Animal Studies Committee at the University of Texas at Austin (AUP-2018-00342). Animals were maintained and raised at 28.5 °C with 14/10 hours of light-dark periodicity.

Skeletal staining

Animals were fixed in 10% formalin overnight, then incubated in 100% acetone overnight, and stained in Bone/Cartilage Stain (59.5% Ethanol, 5% Acetic Acid, 0.015% Alcian Blue and 0.005% Alizarin Red) solution at 37 °C overnight. Solution and soft tissue were cleared with 1% KOH until only stained skeletal tissue was left. After

clearing, specimens were further moved to 25%, 50% and 80% glycerol. Zebrafish and stained skeletal specimen were imaged using an Olympus SZX2-ILLT microscope (Olympus, Tokyo, Japan).

CRISPR-mediated zebrafish F0 screen

To rapidly test whether these AIS candidate genes may be involved in spine development, we used a CRISPR/CAS9 screening approach by injecting four guide RNAs to redundantly target the same genetic loci to generate thorough gene disruption (Wu, Lam et al. 2018). In brief, sgRNAs were synthesized in vitro with T7 RNA Polymerase (New England Biolabs, Ipswich, MA, USA) and purified by Zymo RNA Clean & Concentrator kit (Zymo, Irvine, CA, USA). A mixture of 5 μ M of Cas9 protein (Integrated DNA Technologies, Coralville, IA, USA) and 31 μ M of sgRNA (25 μ M each) was made and incubated for 5 minutes at room temperature. About 500 pL of the mixture of Cas9 and sgRNAs was injected into the yolk of 1-cell stage zebrafish embryos. Embryos were raised and screened at 3- and 5-days post fertilization (dpf). A total of six genes of interest, including *dnah1*, *dnah3*, *dnah7l*, *dnah7*, *dnah9* and *dnah10*, were screened using this method. Oligos and gRNA target sequences are listed in Table S2.

Identification of *dnah10* mutation

To preserve and isolate the *dnah10* mutation, we outcrossed the F0 scoliosis zebrafish with AB wild-type fish to generate F1 zebrafish. Then we in-crossed the F1 zebrafish and generated scoliosis F2 zebrafish. One scoliosis F2 zebrafish was chosen and outcrossed with wild-type and other transgenic zebrafish for further mutation identification and functional analyses. To determine the mutation of the scoliosis *dnah10* mutant, genomic DNA from heterozygous F3 (scoliosis F2 outcross with wildtype) zebrafish was extracted from tissue samples using 50 mM NaOH according to the protocol. DNA segments around four gRNA targets in the *dnah10* gene were amplified and Sanger sequenced (PCR primers listed in Table S3). Variants were identified using ApE (Version 0.55) and annotated using SnpEff (Version 4.3T).

Whole mount in situ hybridization

Whole mount in situ hybridization was performed to detect *dnah10* expression according to a previously published protocol (Thisse and Thisse 2008). For the generation of *dnah10* probes, a ~1.2kb fragment of the *dnah10* transcript (ENS DART00000087708.6) was amplified from cDNA of AB wild-type (PCR primers see Table S3). The fragment of *dnah10* was then cloned into a pGEM-T Easy Vector (Promega) and validated by Sanger sequencing. The template of *dnah10* was generated by Apal restriction enzyme cutting of the recombinant plasmid. The antisense DIG-labeled RNA probe of *dnah10* was synthesized with T7 RNA polymerase.

In vivo imaging and immunofluorescence

The transgenic zebrafish strains *scospondin-GFP^{ut24}* and Tg (Foxj1:Arl13b-GFP) were created as previously described (Konjikusic, Yeetong et al. 2018, Troutwine, Gontarz et al. 2020). Tg (Foxj1:Arl13b-GFP); *dnah10^{ut28}* and *scospondin-GFP^{ut24};dnah10^{ut28}* lines were generated by cross homozygous *dnah10^{ut28}* to each transgenic zebrafish and incrossing of their progeny to generate the homozygote. For live imaging, larvae were anaesthetized in 0.16% tricaine and embedded in 1% low-melt agarose. Time-lapse videos (from 10 s to 10 mins) were acquired at 0.1 to 33 Hz imaging frequency. Videos were cropped and edited by Adobe Premiere Pro (Version 12.0). Reissner fiber and floor plate immunofluorescence staining was performed as previously described (Troutwine, Gontarz et al. 2020). In brief, samples were fixed, permeabilized and incubated with primary

antibodies. After washing and incubation with secondary antibodies, specimens were fixed and filmed immediately. Confocal images and videos were taken using a Nikon Ti2E / CSU-W1 spinning disc confocal system. Images were analyzed and reconstructed using Fiji (Schindelin, Arganda-Carreras et al. 2012). ROIs were manually adjusted on max intensity Z-projection.

Ciliary movement analysis

All the videos for ciliary movement analysis were acquired at 33 Hz frequency. The range of ciliary motion was observed in a maximal intensity Z projection of 10 s time-lapse confocal video. Cilia beat frequency was calculated by Fiji (Schindelin, Arganda-Carreras et al. 2012). We drew a one-pixel width straight line across the cilia to create kymographs to obtain the time trajectory of cilia (Figure S5). Kymographs generated a wave diagram for each cilium, and the beat frequency was calculated by pixels for one second (33 pixels for 33 Hz video) divided by pixels for one beat cycle. Cilia were sorted into “immobile cilia” and “motile cilia” categories according to their kymograph result (Figure S5).

Statistical analysis

Statistical methods used for the gene-burden analysis was described previously. The ciliary data analyses were performed using Fiji (ImageJ) and GraphPad Prism (Version 8.0.2). Changes between the groups were examined using Student's t test. $P < 0.05$ was considered significant.

Results

Identification of loci associated with AIS in humans using gene-burden analysis

To identify variants associated with AIS susceptibility, we enrolled AIS patients selected for surgery at the Xiangya hospital Changsha, China. We performed WES on a cohort of 195 Han Chinese AIS patients and 229 Han Chinese in-house controls. The WES data of 229 control samples were randomly selected from deep-sequencing datasets (~ 30-80x) (n = 319) of PGG.Han genomic database. We identified a total of 481,961 variants after joint calling and quality control. To further identify the high-confidence variants, we used the following filters: (1) exclude high frequency SNPs ($MAF \geq 0.01$) identified from in-house controls and two external Han Chinese population datasets, Han Chinese in Beijing (CHB) and Southern Han Chinese (CHS), and from the 1000 Genomes Project (1000G) database; (2) Variants with highest frequency in AIS cohort among AIS, in-house control, CHB and CHS cohorts were reserved; and (3) Variants predicted as highly or moderately damaging by the Variant Effect Predictor (VEP) were kept. After filtering, a total of 42,967 candidate variants were subjected to further analysis.

We performed gene-burden analysis (see Methods) of candidate variants in all genes. The gene-level deleterious variant frequency and gene-level deleterious variant count per sample were calculated for the AIS and in-house controls, respectively. A candidate gene with enriched deleterious variants was identified if (i) there was a significant difference of deleterious variant frequency in two groups as calculated using the chi-squared test; and (ii) the deleterious variant count of AIS samples was ≥ 0.05 compared to in-house controls, considering that the chi-squared test was not applicable for some genes without deleterious variants in the controls. Altogether, we identified 256 candidate genes associated with AIS (Table S4) after gene-burden analysis.

Variants in dynein axonemal heavy chain genes are significantly enriched in AIS patients

To define the biological function of our candidate AIS susceptibility loci, we performed enrichment analysis on our set of 256 inferred genes. The most enriched gene family was “dynein, axonemal” ($P = 3.66 \times 10^{-10}$) (Fig. 1A; Table 1, S5), implicating 10 genes associated with AIS in our cohort. An orthogonal enrichment analysis showed that genes encoding microtubule-binding motor proteins, were the most enriched protein class in the 256 candidate genes (Fig. 1B; Table S6). Finally, KEGG pathway enrichment analysis of our candidate genes showed the most significance for the “ECM-receptor interaction” ($P = 7.49 \times 10^{-14}$) (Fig. 1C; Table S7). Intersection of all three gene enrichment analyses showed an overlap of 10 dynein axonemal heavy chain genes common to Protein Class Ontogeny and Gene family enrichment analyses (Fig. 1D); however, there were no common candidate genes identified in the KEGG pathway enrichment analysis (Fig. 1D).

Table 1
Gene burden analysis of genes in dynein, axonemal gene family

Gene	VC in AIS	Damaging (h/m)	Adj VC in AIS (n = 195)	Fre (%)	AV in AIS	Adj VC in cont (n = 229)	Fre (%)	AV in cont	Fre dif (AIS - Control)	Pvalue
DNAH9	30	2/28	31	15.90%	0.169	2	0.87%	0.009	15.02%	2.09E-09
DNAH3	24	1/23	34	17.44%	0.210	7	3.06%	0.039	14.38%	5.87E-07
DNAH1	31	0/31	40	20.51%	0.374	13	5.68%	0.148	14.84%	5.82E-06
DNAH14	26	5/21	61	31.28%	0.374	37	16.16%	0.188	15.12%	0.000314
DNAH17	40	1/39	121	62.05%	1.103	102	44.54%	0.773	17.51%	0.00043
DNAH7	31	2/29	67	34.36%	0.692	44	19.21%	0.424	15.15%	0.000558
DNAH10	24	2/22	29	14.87%	0.179	11	4.80%	0.048	10.07%	0.000679
DNAH11	24	1/23	74	37.95%	0.436	57	24.89%	0.284	13.06%	0.004385
DNAH6	10	0/10	51	26.15%	0.267	34	14.85%	0.148	11.31%	0.004951
DNAH12	18	1/17	53	27.18%	0.626	39	17.03%	0.410	10.15%	0.013071
P value is calculated using Fisher's exact test. Adjusted variants count see method. VC, variants count; h/m, high / moderate; Adj, adjusted; Fre, frequency; AV, average variant; Cont, control; Fre dif, frequency difference; AIS, adolescent idiopathic scoliosis.										

Next, we sorted all significantly enriched Gene Ontology (GO) terms into two categories, the axonemal dynein gene family and ECM-receptor interaction associated. The GO terms consisted of at least one gene common to both intersecting analyses. We found that 64 of 88 GO terms, including most of the top enriched terms, were associated with the dynein, axonemal gene family. However, only 37 GO terms contained genes from the ECM-receptor interaction pathway (Fig. 1E; Table S8). Altogether, these results suggest that variants in dynein axonemal heavy chain (*DNAH*) genes including *DNAH1*, *DNAH3*, *DNAH5*, *DNAH7*, *DNAH8*, *DNAH9*, *DNAH10*, *DNAH11*, *DNAH12*, *DNAH14*, and *DNAH17*, may be associated with AIS susceptibility in humans (Table 1, 2 and S5, 11).

We next analyzed DNAH variants detected in a second exome sequencing cohort of 28 IS families, five families of which were previously published (Baschal, Terhune et al. 2018). Low frequency variants (minor allele frequency $\leq$ 5%) passing filtering were present within all sequenced members of the family (3–6 individuals sequenced per family) and were predicted to have damaging functional consequences (see Methods). Two variants within *DNAH1* and *DNAH6* were detected within two families, and one variant in *DNAH7*, *DNAH8*, *DNAH14* and *DNAH17* were each detected within one family. All variants were missense except for *DNAH7*, which was a splice site variant. One family possessed variants in both *DNAH1* and *DNAH6* (Family A)(Table S9).

We next performed Sanger sequencing of each variant within additional family members affected and unaffected with IS for which DNA was available. Pedigrees and corresponding genotypes are provided in Fig. 2. Of 8 variants, 6 variants segregated with the IS phenotype within the families. The variants that did not validate were within *DNAH7* (Family E) and *DNAH17* (Family F). The remaining variants segregated with the scoliosis phenotype, with the exception of an unaffected individual in Family A (II:9) who possessed the *DNAH6* variant of interest and an affected individual who did not possess the *DNAH8* variant of interest (III:6). Unaffected males III:1 in Family B and III:1 in Family D are obligate carriers who possessed the variants of interest, but are expected to as they have children affected with scoliosis.

A C-terminal truncating mutation of *dnah10* causes scoliosis in zebrafish

Because several dynein axonemal gene family were enriched in our cohort of human AIS patients, we next sought to functionally test which axonemal dynein genes were involved in spine stability in zebrafish (Fig. 1D). Of these, six zebrafish *dnah* genes, with clear human homologues, were screened using a multiplexing CRISPR/Cas9 approach (Wu, Lam et al. 2018), employing four independent gene specific guide RNAs injected with Cas9 protein (Table S2). Systematic screen of phenotypes at 3-, 5-days post fertilization (dpf) showed that targeting mutagenesis to *dnah1* and *dnah10* resulted in viable larval fish with atypical body curvatures (Fig. 3A, B, Table S10). However, at 30 dpf only *dnah10* targeted F0 zebrafish displayed adult-viable scoliosis (Fig. 3C).

We next sought to generate a stable genetic mutant of *dnah10* by targeted CRISPR/Cas9 mutagenesis to the region encoding the microtubule binding domain of the Dnah10 protein. To identify a stable mutant line, we outcrossed F0 CRISPR/Cas9-injected zebrafish with scoliosis to expediently isolate deleterious *dnah10* mutations in F1 families. We next incrossed siblings within distinct F1 families to screen for heterozygous carriers as shown in the schematic (Fig. 3D). Progeny from one of these crosses showed recessive scoliosis in adult zebrafish (Fig. 3E). Finally, we bottlenecked a single mutation from an individual F2 male *dnah10* mutant zebrafish. From this progeny we identified a single homozygous frameshift variant in *dnah10* (c.10304_10307del [p.N3435SfsX20]) that is predicted to generate a frameshift and premature stop codon 20-amino acids after N3435 in exon 60 of the *dnah10* loci (Fig. 3F, S3), which was assigned a specific allele *dnah10*^{ut28}. Longitudinal analysis of the progression of body axis curvature phenotypes from crossing homozygous *dnah10*^{ut28/ut28} mutants showed the penetrance of scoliosis was approximately 79% at 30 days post fertilization (dpf) (n = 165; Fig. 3G).

To determine the *dnah10* expression pattern at different developmental stages, we cloned a ~ 1.2 kb piece of the *dnah10* (ENS DART00000087708.6) cDNA to generate an immunolabeled riboprobe and assayed at a variety of developmental stages. Whole-mount *in situ* hybridization showed *dnah10* expression at the 8–10 somite stage (12 hours post fertilization [hpf]) in a ubiquitous expression pattern. At 24 hpf, *dnah10* expression was diffuse throughout the body but with more robust staining within the brain and pronephric tubules. After 48 and 72 hpf, *dnah10* expression was found to be much more localized within the brain and pronephric tubules (Fig. 3H).

Dnah10 function is required for ependymal cilia motility in the ventricular system

Dnah10 (called DHC1 in *Chlamydomonas*) functions as an inner arm dynein motor of motile cilia, leading to reduced motility when mutated in *Chlamydomonas* (Myster, Knott et al. 1997, Kamiya and Yagi 2014). *DNAH10* knockdown in the *Trypanosoma brucei* flagellate caused severe motility defects of cilia, without obvious ultrastructural defects of the axoneme (Zukas, Chang et al. 2012). To determine whether *dnah10*^{ut28/ut28} mutant zebrafish show alterations in the development or motility of motile cilia in the central canal we crossed it to a transgenic *Tg(Foxj1a:Ar13B-GFP)*^{ut22} strain, which labels motile ependymal cilia in the brain and central canal of zebrafish (Konjikusic, Yeetong et al. 2018), without affecting the body axis (Fig. 4A). Analysis of the central canal in fresh-fixed animals showed no difference in the density of Arl13B-GFP labeled *foxj1a* expressing motile cilia comparing wild-type (WT) (Fig. 4C, E) and homozygous *dnah10*^{ut28/ut28} mutant transgenic fish (Fig. 4D, E) (67.1 ± 5.0 vs 66.5 ± 5.3 cilia in 200 μm , $n = 15$).

Confocal imaging using slow acquisition speed of Arl13B-labeled motile cilia in the central canal of living zebrafish produce a characteristic fan-like appearance, as the result of blurring demonstrating the range and direction of ciliary motion (Borovina, Superina et al. 2010). This same fan-like shape was observed in WT *Tg(Foxj1a:Ar13B-GFP)*^{ut22} zebrafish (Fig. 4G). In contrast, *dnah10*^{ut28/ut28}; *Tg(Foxj1a:Ar13B-GFP)*^{ut22/+} transgenic mutant zebrafish showed fewer fan-shaped cilia (Fig. 4H), which establishes that cilia motility in *dnah10*^{ut28/ut28} mutants is reduced. Next, we analyzed cilia motility using high-speed (33 Hz) spinning disc confocal time-lapse imaging, which consistently revealed rapid beating of cilia in WT *Tg(Foxj1a:Ar13b-GFP)* transgenic animals (Video S1). In contrast, *dnah10*^{ut28/ut28}; *Tg(Foxj1a:Ar13B-GFP)*^{ut22/+} transgenic mutant zebrafish displayed a consistent reduction in central canal cilia motility (Video S2). Kymographic time trajectory analysis of several WT *Tg(Foxj1a:Ar13b-GFP)* motile cilia showed a distinctive waveform pattern for central canal cilia, while *dnah10*^{ut28/ut28}; *Tg(Foxj1a:Ar13B-GFP)*^{ut22/+} mutants displayed severely reduced waveform (Fig. 4I). Interestingly, we observed that the loss of central canal cilia motility in *dnah10*^{ut28/ut28} mutants was at times regionally variable within the same animal at 3 dpf. For example, we would sometimes observe reduced or absent central canal cilia motility in a more anterior region, while the more posterior region could display typical motility, in the same individuals (Fig. 4J, J' and Video S3). To assess which region of the ventricular system was most consistently susceptible to a reduction of motile cilia defects, we imaged several ($n = 32$) maternal-zygotic (MZ)*dnah10*^{ut28/ut28}; *Tg(Foxj1a:Ar13B-GFP)*^{ut22/+} mutant larvae (generated by *dnah10*^{ut28/ut28} $\times$ *dnah10*^{ut28/ut28}; *Tg(Foxj1a:Ar13B-GFP)*^{ut22/+} mutant cross) at defined regions of the ventricular system including: (i) the hindbrain ventricle, and within the central canal both at (ii) the vent/cloaca; and (iii) at the tip of the tail (Fig. 4K). When assessed at 3dpf, the majority of these mutant zebrafish exhibited dysfunctional cilia motility in the cerebral ventricle portion of the central canal ($n = 23$; 72%) (Fig. 4K), while cilia motility within the central canal, at vent or in the tail, were less affected in aggregate at this time. To evaluate whether the *dnah10*^{ut28/ut28} mutation affected overall cilia beat frequency, we sorted high-speed confocal imaging (33 Hz) of MZ*dnah10*^{ut28/ut28}; *Tg(Foxj1a:Ar13B-GFP)*^{ut22/+} into two categories, "immobile cilia" and "motile cilia". As expected, the analysis of beat frequency of "immobile cilia" was zero (Fig. 4L, S4B). Similar analysis of beat frequency of the seemingly normal "motile cilia" in *dnah10*^{ut28/ut28} mutants was reduced (6.2 ± 1.6 Hz, $n = 22$) compared with WT transgenic zebrafish (7.4 ± 2.3 Hz, $n = 27$), however this reduction was not significant at 3 dpf (Fig. 4L). Similar analysis of the beat frequency of motile cilia of WT *Tg(Foxj1a:Ar13B-GFP)*^{ut22/+} transgenic zebrafish found no significant

differences in cilia beat frequency when comparing all three different locations of the hindbrain ventricle and within the central canal at vent and tail tip (n = 9; each region) (Figure S4D).

After imaging, we reared all individuals (n = 32) for longitudinal analysis of the onset body axis curvatures during larval development. We observed that the majority of *MZdnah10^{ut28/ut28}; Tg(Foxj1a:Arl13B-GFP)^{ut22/+}* larvae displayed body curvature and scoliosis phenotypes by 28 dpf (90.4%; n = 19) (Fig. 4M), which was similar to our observations of incomplete penetrance of scoliosis observed in *MZdnah10^{ut28/ut28}* mutants from independent clutches (Fig. 3G). Altogether, these results demonstrated that *dnah10* is important for the motility of motile cilia in the brain and central canal, albeit expressed in stochastically in specific regions at 3dpf and that this loss of central canal motile cilia beating is associated with the onset and progression of body curvature and scoliosis phenotypes in zebrafish.

***dnah10* is required for maintaining Reissner Fiber assembly**

The Reissner fiber (RF) has recently been shown to play an important role morphogenesis of a straight body axis during embryonic development (Cantaut-Belarif, Sternberg et al. 2018) and its continued assembly during larval development is essential for the maintenance of a straight spine in zebrafish (Rose, Pompili et al. 2020, Troutwine, Gontarz et al. 2020). To test whether motile ciliary defects in *dnah10^{ut28/ut28}* mutant zebrafish are also associated with RF disassembly, we first utilized immunofluorescence using antiserum raised against bovine RF (AFRU) in *dnah10^{ut28/ut28}* mutant and WT zebrafish at 1, 5, and 10 dpf. As expected, WT zebrafish displayed the formation of a straight RF at all developmental timepoints (Figs. 5A-C). In contrast, the organization of the RF in *dnah10^{ut28/ut28}* mutants at 1 dpf was already observed to be defective (Fig. 5A). The diffuse and discontinuous AFRU staining in *dnah10^{ut28/ut28}* mutants at 1 dpf shows that the Reissner material is synthesized and secreted from the Sub-Commissural Organ (SCO), but is able to form a tightly polymerized RF within the central canal. By 5 and 10 dpf, we consistently observed more severe RF disassembly or the complete absence of a RF in *dnah10^{ut28/ut28}* mutants, in contrast to the obvious RF structure in the WT animals (Fig. 5B and C). Categorical analysis of the RF disassembly demonstrated a high degree of heterogeneity at 3 dpf (n = 25) including: (i) the formation of Reissner material as a bolus or punctate (n = 9, 36%); (ii) an intact RF which appeared distorted (n = 3, 12%); (iii) disassembly of the fiber but with diffuse RF material filling the central canal (n = 8, 32%); or a complete absence of RF staining in the central canal (n = 5, 20%) (Fig. 5D and E). These results, together with our analysis of cilia motility, demonstrate that the defects in RF correlated with the onset of cilia dysfunction in *dnah10^{ut28/ut28}* mutants and suggests that the heterogeneity of RF disassembly may be related to the heterogeneity of cilia motility defects outlined above (Fig. 4K).

To facilitate the visualization of RF dynamics in *dnah10^{ut28/ut28}* mutants we crossed these to the *scospondin-GFP^{ut24}* gene fusion knock-in zebrafish line (Troutwine, Gontarz et al. 2020). *scospondin-GFP^{ut24}* embryos display a fluorescently labeled RF, which continually moves in a rostral-caudal direction (Fig. 6A, A'; Video S4). In contrast, the RF moved in a disorganized manner in *scospondin-GFP^{ut24};dnah10^{ut28/ut28}* mutant embryos (Fig. 6B, C; Video S5). Analogous to RF defects demonstrated in fixed, AFRU immuno-stained *dnah10^{ut28/ut28}* mutant animals (Fig. 5), we observed heterogenous RF disassembly patterns *scospondin-GFP^{ut24};dnah10^{ut28/ut28}* mutants *in vivo* (Video S6 and Fig. 6H) including: (i) the formation of bolus or punctate Reissner material filling the central canal (n = 5, 17%) (Fig. 6D); (ii) the complete absence of fiber or Reissner material in the central canal

(n = 4, 14%) (Fig. 6E); (iii) absence of a fiber with only diffuse Reissner material filling the central canal (n = 14, 48%) (Fig. 6F); or (iv) a continuous RF surrounded with diffuse Reissner material (n = 6, 21%) (Fig. 6G). We observed heterogeneity in the ability of the RF to move along the central canal including moving in non-linear, meandering patterns (Fig. 6J), or slowly traversing in a rostral direction, or simply not moving within the central canal (Fig. 6L, M and Video S7). Altogether, these data demonstrate that alterations in the assembly of the RF and its typical rostral-caudal dynamics are caused by the loss of *dnah10*-dependent cilia motility lining the brain and central canal.

Discussion

In this study, we report exome sequencing results from 195 AIS patients. We identified a significant association of variants in axonemal dynein heavy chain genes in AIS patients compared to controls. An exome sequencing study in multigenerational families with idiopathic scoliosis also implicated components of the microtubule cytoskeleton including *DNAH6* and *DNAH8* (Baschal, Terhune et al. 2018). We generated a stable *dnah10*^{ut28/ut28} mutant zebrafish line, which models aspects of AIS including late-onset, atypical three-dimensional spine curvatures without patterning defects. We go on to demonstrate that *dnah10*^{ut28/ut28} mutants display heterogeneous defects in cilia motility throughout the brain and spinal canal, which is consistent with heterogeneous RF disassembly within the central canal and with the onset of body curvatures and adult-viable scoliosis. Altogether these results suggest that mutations in axonemal dynein genes may be involved in AIS susceptibility in humans.

Recent WES and GWAS have identified several candidate genes associated with AIS. However, only a few of these candidate genes have been verified in animal models. Of the 28 reported AIS candidate genes previously identified in genetics studies, only four genes (*GPR126*, *MAPK7*, *POC5* and *SLC39A8*) have provided robust validation in an animal model (Karner, Long et al. 2015, Patten, Margaritte-Jeannin et al. 2015, Gao, Chen et al. 2017, Haller, McCall et al. 2018). Forward genetic screens in zebrafish have identified numerous scoliosis phenotype-causing genes, but few of these genes correspond to candidate genes implicated in human AIS (Boswell and Ciruna 2017, Liu and Gray 2018). Previous studies have also demonstrated that defects in ependymal cell motile cilia formation or beating after disruption of ciliary components (*i.e. ptk7a*, *kif6*, *cfap298*) (Buchan, Gray et al. 2014, Hayes, Gao et al. 2014, Grimes, Boswell et al. 2016) and the subsequent disassembly of the RF as in *scospondin* mutants (Rose, Pompili et al. 2020, Troutwine, Gontarz et al. 2020) are key initiating factors causing scoliosis in zebrafish. Here, we extend this model demonstrating an association of several axonemal dynein genes in human AIS and that disruption of one of these genes, *dnah10*, leads to scoliosis in zebrafish. Thus supporting a model where disruption of ependymal cell cilia beating in the hindbrain and spinal canal may underlie the pathogenesis of human AIS.

The most significantly enriched gene family of the 256 genes filtered using the gene-burden test was the axonemal dynein family. Axonemal dyneins are components of the inner and outer arms of motile cilia (Kamiya and Yagi 2014). In humans, variants in *DNAH1*, *DNAH9*, *DNAH11*, *DNAH17* have been implicated in ciliary dyskinesia and spermatogenic failure (www.omim.org). *DNAH10* has been associated with heterotaxy syndrome a congenital disorder characterized by malformation of left-right body asymmetry and associated with defective motile nodal cilia (Grimes and Burdine 2017). A previous study showed that disruption of *dnah10* by an early terminating truncating mutation caused heart looping defects and randomization of left-right patterning genes (Liu, Cao et al. 2018). In contrast, we observed no obvious heterotaxy phenotypes in *dnah10*^{ut28/ut28} mutant

zebrafish reported here, most likely due to the hypomorphic nature of the C-terminal truncating mutation we generated, which could allow for residual protein function during early embryogenesis. In support of this model, we also consistently observed incomplete penetrance of scoliosis, and heterogeneous defects in cilia motility and RF disassembly in *MZdnah10^{ut28/ut28}* mutant animals where both the egg and sperm should only have mutant forms of the Dnah10 protein during embryogenesis. In addition, during the multiplex CRISPR/Cas9 screening of the *dnah10* locus using four guide RNAs we consistently observed animals displaying severe curled-tail down, larval lethal phenotypes, which is commonly associated with cilia defects in zebrafish. While, in the same injected clutch we also recovered milder body curvature and adult-viable scoliosis phenotypes associated with the stable *dnah10^{ut28/ut28}* mutants reported here. A similar variance in the severity of body curvature phenotypes was observed between *scospondin* hypomorphic (Troutwine, Gontarz et al. 2020) and null (Cantaut-Belarif, Sternberg et al. 2018) mutant zebrafish and again in the comparison of the loss of maternal versus zygotic Ptk7a function (Hayes, Gao et al. 2014) are established in zebrafish. Altogether, this demonstrates that hypomorphic mutations in otherwise essential genes can display a range of body curvature phenotypes in zebrafish, which should inform the characterization of future human genetics studies of AIS.

Indeed, seminal human genetics studies have demonstrated that increased polygenic burden of rare variants across extracellular matrix genes in human AIS (Haller, Alvarado et al. 2016). Our current analysis of the C-terminal truncating *dnah10^{ut28/ut28}* mutant zebrafish may suggest that milder mutations in axonemal dynein gene contribute to increase susceptibility of AIS, in contrast more deleterious mutations in the same gene may cause more severe motile cilia disorders attributed to primary cilia dyskinesia in humans and animal models. Interestingly, the incidence of scoliosis is higher in primary cilia dyskinesia patients (Knowles, Daniels et al. 2013) and there is a strong association between organ symmetry and the direction of the spine curvatures reported in heterotaxic *situs inversus* patients, suggesting that left-right patterning has a key role in the regulation on spine morphogenesis (Schlösser, Semple et al. 2017). Further testing of this model of hypomorphic mutations underlying AIS by targeted genome editing of candidate human alleles of axonemal dynein genes associated with AIS is warranted.

We demonstrate that deleterious variants even with mild effect size in axonemal dynein genes may contribute to AIS susceptibility. However, replication of these variants in larger, multi-ethnic cohorts is necessary to help elucidate the inheritance pattern of these variants and to identify other genetic interactions and susceptibility loci for AIS. In addition, to axonemal dynein genes we also found by KEGG pathway analysis that ECM-receptor interaction related genes, which included three collagen genes (*COL6A2*, *COL6A3*, *COL6A5*, and *COL6A6*) were associated in our patient cohort. Another genetic study using WES performed in a European descent AIS cohort found that rare variants in *COL6A3* were enriched in 435 AIS cases (Haller, Alvarado et al. 2016). We also identified two previously reported AIS candidate genes, *HSPG2* and *CELSR2*, among our 256 candidate genes (Baschal, Wetthey et al. 2014, Einarsdottir, Grauers et al. 2017).

In conclusion, our study has identified a significant accumulation of variants in axonemal dynein genes in Han Chinese AIS patients (Table S11) and showed that a C-terminal truncating mutation of *dnah10* in zebrafish led to an adult-viable scoliosis phenotype by affecting motile cilia physiology and RF assembly. Further studies are needed to determine whether other components of motile cilia physiology may be involved in susceptibility of AIS in humans.

Declarations

Author contributions:

Conceptualization: H.Z., R.S.G.

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Figures

Figure 1

Enrichment analysis of AIS associated variants implicate dynein axonemal heavy chain genes. (A) Gene family enrichment analysis of 246 AIS associated genes. Significantly enriched gene families ranked by the gene ratio and $-\log_{10}(P\text{-value})$ on the vertical axis. The horizontal axis represents the gene ratio and the dot sizes represent the number of candidate genes in each gene family. The color of the circle represents $-\log_{10}(P\text{-value})$. (B)

AIS families with variants in dynein axonemal heavy chain genes. Pedigrees for IS families showing genotypes at the variant of interest, provided in the upper left corner. Variants were found through exome sequencing of 3-5 members of each family (see methods for sequencing and filtering details), and additional family members were genotyped at the locus through Sanger sequencing. Circles indicate females; squares indicate males; filled in shapes indicate affected with a scoliosis curvature > 10°; ? indicates unknown scoliosis status. Curvature measurements with a degree (including 0°) have been verified by x-ray; conf. neg (negative) or pos (positive) measurements have been verified by physician but not x-ray. When two scoliosis degrees are provided, second measurement indicates secondary curvature.

Figure 3

CRISPR/Cas9 screening and mutagenesis of candidate dnah genes implicates dnah10 for scoliosis in zebrafish. (A) Incidence of curved body phenotype (red bar) in F0 CRISPR/Cas9 injected embryos for axonemal dynein heavy chain genes after screening at 3 and 5 dpf (days post fertilization). The number of injected embryos screened is displayed above. (B) F0 phenotype of a subset of dnah10 guide RNA-pool injected zebrafish larvae (5 dpf). Scale bar: 1 mm, WT SL= 4.6 mm. (C) F0 scoliosis phenotypes in brightfield (left panels) and Alcian blue/ Alizarin red stained skeletal preparation (right panels) at ~30 dpf. Scale bar: 1 mm, WT SL=12.7 mm. (D) Schematic of workflow used to identify stable inheritance of a microtubule binding domain targeted C-terminal truncation mutation in dnah10 from F0 phenotypic zebrafish. Larva and adult zebrafish with scoliosis phenotypes are represented by curved bodies. (E) Brightfield (left panels) and Alcian blue/ Alizarin red stained skeletal preparations (right panels) showing the scoliosis of F2 dnah10 mutant zebrafish at ~ 30dpf. Scale bar: 1mm. (F) Schematic of Danio rerio Dnah10 protein and location of the dnah10ut28 C-terminal truncation allele. Each box represents a conserved domain. MT, microtubule-binding domain. (G) Longitudinal survey of the incidence of body axis and spine curvature, a variety of crosses to address maternal effect of the phenotype in

dnah10 mutants including: progeny from Maternal Zygotic (MZ)dnah10ut28 crosses; progeny from heterozygous dnah10u28x/+ x homozygous dnah10ut28/ut28 mutant crosses from both male and female homozygous dnah10ut28 mutants; and progeny from heterozygous dnah10ut28/+ incrosses. n= 165 (black dot), 120 (green square), 110 (red triangle) and 126 (gray triangle). Analysis complied from > 3 independent clutches. (H) Whole-mount in situ hybridization using a dnah10 riboprobe, showing expression specific expression in the brain and pronephric tubules during larval stages of development (>24 hpf). Scale bar: 0.25 mm.

Figure 4

dnah10ut28/ut28 mutants display impaired motility of monocilia lining the brain and central canal. (A and B) Images of larval zebrafish of Tg(Foxj:Ar13b-GFP) or Tg(Foxj:Ar13b-GFP); dnah10ut28/ut28 (5 dpf). Scale bar: 1 mm. (C and D) Z projection of maximal intensity of lateral section confocal images of Tg(Foxj:Ar13b-GFP) or Tg(Foxj:Ar13b-GFP); dnah10ut28/ut28 larvae (3 dpf) showing mono cilia (green) in the central canal. Scale bar: 50 μ m. Schematic shows the imaging position at vent. (E) Quantification of cilia in a 200 μ m lateral section of

maximal Z projections of a confocal stack from larval fish as described in (C and D). (n=15, mean $\pm$ SD). (F) Schematic shows the imaging position of each larvae for ciliary motion analysis. (G and H) Maximal intensity Z-projections of Tg(Foxj:Arl13b-GFP) (G) or Tg(Foxj:Arl13b-GFP); dnah10ut28/ut28 (H) larvae (3 dpf) showing the range of cilia motion outlined with white dotted lines. Yellow dotted lines represented the recording range of kymograph analysis in (I). d, v, r and c represent dorsal, ventral, rostral and caudal, respectively. (I) Time projection of 10 seconds at 33 Hz is recorded by confocal for the same area in (F, G, and H). Cilia movement is by a line scan (yellow dotted line in G and H) and kymograph analysis in ImageJ. Time increases left to right. (J-J'') Z projection of maximal intensity of lateral section confocal images of Tg(Foxj:Arl13b-GFP) or Tg(Foxj:Arl13b-GFP); dnah10ut28/ut28 larvae (3 dpf) showing mono cilia (green) in the central canal. Scale bar: 50 μ m. Schematic shows the imaging position at vent. White and yellow dotted line rectangles (J' and J'') are enlarged portions of the indicated region in (J). Range of cilia motion is depicted by the dotted line. Scale bar: 50 μ m. (K) Schematic represents the imaging position for subsequent cilia defect statistics. Distribution of immotile cilia in the central canal around the cerebral ventricle, vent and tail from 32 cilia defect mutant larvae (Number, percentage). (L) Beat frequencies calculated from a 10 second movie collected at 33 Hz sample from Tg(Foxj:Arl13b-GFP) or Tg(Foxj:Arl13b-GFP); dnah10ut28/ut28 larvae (3 dpf) (n = 27, 22 and 22; mean $\pm$ SD, see Method for cilia beat frequency calculation). (M) Longitudinal analysis of body curvature and scoliosis phenotype over developmental time for the 32 cilia defect mutant zebrafish imaged in K.

Figure 5

Mutation in *dnah10^{ut28}* leads to Reissner fiber (RF) disassembly (A-D) Merged images of Z projection maximal intensity of lateral section confocal images of fixed WT and *dnah10^{ut28}/ut28* larvae mutant zebrafish staining with AFRU (Green) and ZO-1 (Purple) at 1 (A), 5 (B) and 10 (C) dpf. (D) Representative images of the heterogeneous phenotypes of disassembly of the RF starting at 5 dpf. Schematic shows the imaging position. Scale bar = 50 μ m (A-C) and 25 μ m (D). (E) Quantification of the heterogeneous phenotypes of disassembly of the RF at 5dpf (n=25). Total number and percentage for each classification is depicted. WT, wildtype; RF, Reissner fiber.

Figure 6

Heterogenous disassembly of Reissner fiber in vivo in *dnah10^{ut28/ut28}* mutant zebrafish. (A-C') Inverted grayscale images of Z projection maximal intensity of lateral section confocal images from *sospondin-GFP^{ut24}* and *sospondin-GFP^{ut24};dnah10^{ut28/ut28}* mutants at 3dpf. Reissner fiber in the rostral (A and B-B') and caudal regions (A' and C-C') are shown. Enlarged portions from white dotted line rectangles in B and C are shown in B' and C', respectively. Scale bar: 50 μ m. (D-G) Z projection maximal intensity of confocal images from *sospondin-GFP^{ut24}* and *sospondin-GFP^{ut24};dnah10^{ut28/ut28}* mutants shows the heterogeneity of Reissner fiber disassembly, including bolus (D), absence (E), diffuse (F) and Reissner fiber surrounded by diffused Reissner materials (G). We classified 29 mutants with Reissner fiber defect into these four classifications and show that the number and percentage in each class (E). Scale bar: 50 μ m. CC, central canal; RF, Reissner fiber; FP, floor plate. (I-M) Inverted grayscale images of single frames from 5-minute time-lapse confocal dataset of *sospondin-GFP^{ut24}* and *sospondin-GFP^{ut24};dnah10^{ut28/ut28}* at 3dpf. The time point of each frame is indicated as min:sec (minute: second) in the lower right-hand side of each panel. Moving Reissner fiber/material are marked by red arrowheads (J and M) and the motion patterns are depicted by black arrows in the box above each panel. Scale bar: 50 μ m.

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