Circulation: Genomic and Precision Medicine

ORIGINAL ARTICLE

Monoallelic Mutations in *CC2D1A* Suggest a Novel Role in Human Heterotaxy and Ciliary Dysfunction

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BACKGROUND: Human heterotaxy is a group of congenital disorders characterized by misplacement of one or more organs according to the left-right axis. The genetic causes of human heterotaxy are highly heterogeneous.

METHODS: We performed exome sequencing in a cohort of 26 probands with heterotaxy followed by gene burden analysis for the enrichment of novel rare damaging mutations. Transcription activator-like effector nuclease was used to generate somatic loss-of-function mutants in a zebrafish model. Ciliary defects were examined by whole-mount immunostaining of acetylated α-tubulin.

RESULTS: We identified a significant enrichment of novel rare damaging mutations in the *CC2D1A* gene. Seven occurrences of *CC2D1A* mutations were found to affect 4 highly conserved amino acid residues of the protein. Functional analyses in the transcription activator-like effector nuclease–mediated zebrafish knockout models were performed, and heterotaxy phenotypes of the cardiovascular and gastrointestinal systems in both somatic and germline mutants were observed. Defective cilia were demonstrated by whole-mount immunostaining of acetylated α-tubulin. These abnormalities were rescued by wild-type *cc2d1a* mRNA but not *cc2d1a* mutant mRNA, strongly suggesting a loss-of-function mechanism. On the other hand, overexpression of *cc2d1a* orthologous mutations *cc2d1a* P559L and *cc2d1a* G808V (orthologous to human *CC2D1A* P532L and *CC2D1A* G781V) did not affect embryonic development.

CONCLUSIONS: Using a zebrafish model, we were able to establish a novel association of *CC2D1A* with heterotaxy and ciliary dysfunction in the F2 generation via a loss-of-function mechanism. Future mechanistic studies are needed for a better understanding of the role of *CC2D1A* in left-right patterning and ciliary dysfunction.

Key Words: cilia ■ exome ■ heterotaxy syndrome ■ isomerism ■ zebrafish

Heterotaxy, also known as situs ambiguous, is a class of human congenital disorders that are characterized by a failure to establish normal left-right (L-R) asymmetry and by the misplacement of one or more organs during embryonic development.[1] The phenotypic manifestation falls between the two extremes of situs solitus (normal) and situs inversus (complete mirror image of normal), resulting in the abnormal L-R positioning of visceral organs. The estimated incidence at birth is around 1 in 10 000.[2–4] Cardiovascular malformations

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The Data Supplement is available at https://www.ahajournals.org/doi/suppl/10.1161/CIRCGEN.120.003000.

For Sources of Funding and Disclosures, see page 705.

Nonstandard Abbreviations and Acronyms

CNV	copy number variant
TALEN	transcription activator-like effector nuclease
WISH	whole-mount in situ hybridization

are commonly associated with heterotaxy and account for ≈3% of all congenital heart defects.[5,6]

The two major subtypes of heterotaxy are left isomerism and right isomerism.[2] In left isomerism, structures normally found on the left side of the heart are found as mirror images on both sides of the L-R axis.[7] Typically, the heart has two long narrow atrial appendages associated with bilateral bilobed lungs and long hyparterial bronchi. Patients may also have pulmonary veins connecting to bilateral morphological left atria.[7] In right isomerism, both atrial appendages are typically pyramidal in shape and the lungs are trilobed with short eparterial bronchi. Right isomerism is commonly associated with the absence of the spleen, whereas left isomerism is associated with polysplenia.[8] Long-term survival of patients with right isomerism is poor, with only 22% of the patients surviving to 14 years of age.[9]

The genetic basis of heterotaxy has been studied for decades, yet it is still poorly characterized. Although most cases of heterotaxy are sporadic, Mendelian inheritance and familial occurrence have also been reported. Genes implicated in heterotaxy include components of the nodal signaling pathway, cilia or flagella associated protein-coding genes, and *ZIC3,* a member of the zinc finger family.[10] The nodal signaling pathway is essential for the activation of left side-specific gene expression in the developing embryo.[11] Embryos lacking nodal expression in the left lateral plate mesoderm exhibit multiple L-R patterning defects.[8] The first report of the mutations in the nodal signaling pathway was in a patient with heterotaxy and a de novo reciprocal translocation was found to disrupt the *SESN1* gene, which mediates nodal signaling.[12] Other studies reported several heterotaxy-related gene mutations in the nodal pathway, including *NODAL*, *CFC1*, *ACVR2B,* and *LEFTY2*, mainly with dominant inheritance and incomplete penetrance.[11,13–15] Examples of autosomal recessive inheritance have also been reported, such as the *GDF1* gene with compound heterozygous mutations[16] or homozygous variants in consanguineous families.[17] Other examples include a consanguineous family with a homozygous deletion in the *WDR16* gene[18]; two affected brothers with a homozygous splice site mutation in the *CCDC11* gene[19]; and nine patients with recessive mutations in the *MMP21* gene.[20] More recently, the *PKD1L1* gene has also been implicated in heterotaxy as homozygous loss of function mutations,[21] and the gene has been shown to regulate nodal signaling by acting downstream of nodal flow in knockout models of mice.[22] In addition, X-linked inheritance was reported in patients with heterotaxy,[14,23] and *ZIC3* was identified by linkage analysis in a large family with X-linked heterotaxy.[24]

Large-scale copy number variant (CNV) analyses using single-nucleotide polymorphism microarrays have led to the discovery of other heterotaxy-related genes including *BMP2* and *MNDA*.[5] Nevertheless, little overlap has been found between single-nucleotide polymorphisms and CNVs. Known heterotaxy mutations are only found in <10% to 20% of all cases when studying CNVs.[6,25] In addition to microarrays, next-generation sequencing technologies such as whole-exome sequencing and whole-genome sequencing have been used to identify recessive mutations such as in *MMP21* in patients with heterotaxy.[10] Taken together, the use of advanced genomic technologies, the availability of public variant databases, and in silico prediction tools have allowed further interrogation of the genetics of heterotaxy.[26–30]

Despite the heterogeneity of genetic causes, the role of cilia in the pathogenesis of heterotaxy is crucial and has a role in breaking the L-R symmetry. Unlike the 9+2 type immotile cilia found in the airway or brain with planar beating, the 9+0 type motile cilia present in the node cavity has a clockwise rotational movement.[31] Nonaka et al[32] showed that in murine, a leftward flow caused by a vortical motion of motile cilia (nodal flow) is related to L-R symmetry breaking; and further confirmed the role of fluid flow in L-R patterning by generating artificial flow of culture medium.[33] In cardiac development, asymmetries can be induced by defects in the nodal signaling pathway, which results in defective cardiac looping.[34]

In this study, we performed whole-exome sequencing on 26 patients with heterotaxy, which revealed significant enrichment of rare damaging mutations in the *CC2D1A* gene. We then functionally evaluated candidate mutations in vivo using a zebrafish model.[35,36] The knockout models of *cc2d1a* were found to have heterotaxy and ciliopathy phenotypes, which were rescued by wild-type *cc2d1a* mRNA, but not *cc2d1a* mutant mRNA. Our findings suggest an association of the *CC2D1A* gene with heterotaxy and ciliopathy via a loss-of-function mechanism.

METHODS

Detailed methods are available in the Data Supplement. Briefly, we performed exome sequencing in a cohort of 26 probands with heterotaxy followed by gene burden analysis for the enrichment of novel rare damaging mutations. TALEN (transcription activator-like effector nuclease) was used to generate somatic loss-of-function mutants in a zebrafish model. Ciliary defects were examined by whole-mount immunostaining of acetylated α-tubulin. The protocols used for all investigations were in conformance with the principles outlined in the Declaration of Helsinki. The subjects gave written informed consent. Ethical approval for involving human subjects was obtained from the Institutional Review Board of the University of Hong Kong and Hospital Authority of Hong Kong West Cluster (UW 12-211). Ethical approval for animal studies

was obtained from the Committee of the Use of Laboratory and Research Animals (3919-16, The University of Hong Kong, HK) and Animal Subjects Ethics Sub-Committee (16-17/23-HTI-R-GRF, The Hong Kong Polytechnic University, HK). The data that support the findings of this study are available from the corresponding author upon reasonable request.

RESULTS

Whole-Exome Sequencing and CNV Analysis

We performed whole-exome sequencing and CNV analysis on 26 patients with heterotaxy of whom 25 had right isomerism and one had left isomerism. The targets were enriched using Agilent SureSelect XT and sequenced by Illumina HiSeq 1500. After filtering the whole-exome sequencing data, we observed a total of 64 562 rare coding changes in all 26 patient samples. Affymetrix Genome-wide Human single-nucleotide polymorphism Array 6.0 was performed for CNV detection, which identified 49 CNVs with a size larger than 1 kbp after annotation by CNVannotator.[37]

Detection of Variants in Known Heterotaxy Genes

A list of candidate genes for heterotaxy disorders was generated from Online Mendelian Inheritance in Man, HGMD (The Human Gene Mutation Database) and Phenolyzer (phenotype-based gene analyzer) databases (Table I in the Data Supplement)[38,39] and also included all genes in the Nodal pathway. In the final candidate gene list, we discovered 11 rare coding changes. However, in silico prediction tools (SIFT [Sorting Intolerant From Tolerant], PolyPhen2 [Polymorphism Phenotyping v2], LRT [Likelihood Ratio Test] and CADD [Combined Annotation Dependent Depletion]) showed all these mutations were likely to be benign (Table II in the Data Supplement). In addition, none of the selected candidate genes overlapped with the 49 CNVs.

The Increased Burden of Rare Damaging Mutations in the *CC2D1A* Gene

We next examined whether genes were enriched for any of the rare damaging mutations in cases compared with controls. To determine the genes that showed significant enrichment in heterotaxy, SNP-set Kernel Association Test was performed in the 26 heterotaxy cases and in the 130 local controls with no known cardiac or laterality defects. For the 156 samples, all genes with at least one rare (minor allele frequency <0.03) damaging mutation (indicated by two out of four prediction tools) were selected (n= 8251). Among them, only one gene, *CC2D1A*, showed statistical significance (Figure 1). The derived *P* value for the enrichment in *CC2D1A* was 0.0379 after Bonferroni correction. To ensure that the same significance was observed in larger control sets, the analysis was repeated using data from the NHLBI GO Exome Sequencing Project (ESP 6500) and Exome Aggregation Consortium Browser (ExAC),[26] which also showed a similar statistical significance (Table 1). These results indicate a strong association between the rare damaging missense mutations in the *CC2D1A* gene and heterotaxy.

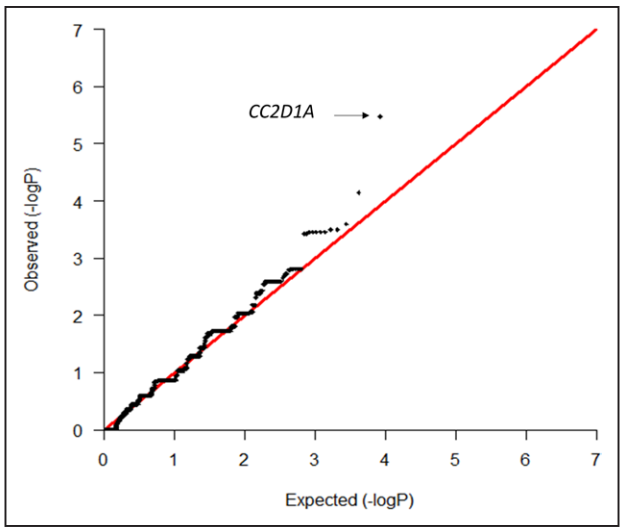

Figure 1. QQ plot of the *P* value derived from SNP-set Kernel Association Test by comparing the rare damaging mutations in cases and internal controls for all genes in cases or controls with at least one mutation.
Only the *CC2D1A* gene had a significant *P* value after Bonferroni correction.

Seven occurrences of the rare mutations in *CC2D1A* were identified in six out of the 26 cases, with one patient harboring two different mutations simultaneously (Table 2; Figure 2). Besides the one case with left isomerism, all of the other rare mutation cases were in patients with right isomerism (Table 2). All the mutations affected highly conserved amino acid residues, and the deleterious effects of the gene mutations were further supported by multiple lines of in silico evidence (Table 2). Among the six patients with *CC2D1A* mutations, parental DNA was only available in four of them. The mutations in these four patients were inherited from unaffected parent, suggesting variable expression and incomplete penetrance of *CC2D1A* in causing heterotaxy. As for the phenotype of the patients affected with *CC2D1A* mutations, two of them had dextrocardia (40% of all dextrocardia cases, n=5). Therefore, there may be an association between *CC2D1A* mutations and dextrocardia; however, a larger cohort will be required for a statistically significant observation (Table III in the Data Supplement).

Somatic *cc2d1a* Knockout Zebrafish Model Displayed Heterotaxy Phenotypes

To further elucidate the pathogenicity of these variants, we investigated the function of *CC2D1A* using a

Table 1. Mutation Burden Test of *CC2D1A* in Cases and in 3 Groups of Controls

Sample groups	Sample size	Samples with rare damaging missense mutations in *CC2D1A*	Frequency	Odds ratio	95% CI	SKAT *P* value	Corrected *P* value
Case	26	6	0.23				
Internal control	130	2	0.02	19.2	3.6–101.8	3.34×10^{-6}	3.79×10^{-2}
ESP6500 control	6525	74	0.01	26.1	10.1–67.0	3.81×10^{-8}	7.16×10^{-4}
ExAC control	61 486	936	0.02	19.4	7.8–48.4	1.97×10^{-7}	3.70×10^{-3}

The odds ratio refers to the ratio between the odds of cases with mutations and the odds of controls with mutations. SKAT indicates SNP-set Kernel Association Test.

zebrafish model by assessing the effects on vertebrate embryonic development. The *cc2d1a* gene is highly conserved between humans and zebrafish, consisting of four DM14 domains and a C2 domain (Figure 2A; Figure I in the Data Supplement). Three of the four candidate mutation spots, P192, P532, and G781, were conserved in zebrafish *cc2d1a*. During early zebrafish embryonic development, *cc2d1a* is expressed ubiquitously. At 24 hours post-fertilization (hpf), it is strongly expressed in the head with modest expression along the central canal and pronephric duct, whereas from 30 hpf onward, it is predominantly expressed in the head, hatching gland, and developing heart (Figure 2B).

We first generated a somatic loss-of-function mutant using TALEN[40,41] targeting exon 14 of *cc2d1a,* at residue P559 in the fourth DM14 domain (Figure 2C). Somatic mutagenic activity (65.5±6.1%) was quantified by restriction fragment length polymorphism assay,[40] and the frame-shifting deletions disrupting the fourth DM14 and C2 domains were confirmed by Sanger sequencing (Figure IIA and IIB in the Data Supplement). At the chosen dose, about 54% of somatic *cc2d1a* mutant displayed normal gross development and around 10% were severely deformed and excluded from subsequent analysis. Others exhibited mild to moderate deformities including curved ventral axis and cyclopia (Figure IIC in the Data Supplement).

Visceral organ development was examined by whole-mount in situ hybridization for *cmlc1* and *foxa3*. At 30 hpf, defective cardiac development including defective cardiac jogging of heart chambers, and midline and bilateral heart were observed in *cc2d1a* mutants ($P<0.0001$, Figure 3A and Figure IIIA in the Data Supplement). In addition, midline and mirrored digestive system were observed ($P<0.0001$), which recapitulates the phenotype in human heterotaxy (Figure 3B; Figure IIIB in the Data Supplement).

To examine potential gain-of-function in the identified *CC2D1A* mutations, we overexpressed zebrafish orthologous *cc2d1a* mutations (corresponding to human *CC2D1A* P532L and *CC2D1A* G781V) by injecting mRNA encoding *cc2d1a* carrying either P559L (*cc2d1a*^{P559L}) or G808V (*cc2d1a*^{G808V}) mutations into the cytoplasm of 1-cell stage wild-type zebrafish embryos (Figure 2A). There was no abnormal phenotype in either the cardiac or digestive system development, suggesting no gain-of-function or dominant-negative effects (Figure III in the Data Supplement).

To elucidate whether such mutations result in loss-of-function, we co-injected either wild-type *cc2d1a*, *cc2d1a*^{P559L} or *cc2d1a*^{G808V} mRNA with TALEN targeting *cc2d1a*. Only wild-type *cc2d1a* mRNA, but not those carrying orthologous mutations, partially rescued the defects in the cardiac ($P<0.0001$) and digestive systems ($P<0.0001$) because of the *cc2d1a* mutation (Figure III in the Data Supplement), which indicated a loss-of-function mechanism for these mutations.

Somatic *cc2d1a* Knockout-Induced Ciliopathy in Zebrafish

Heterotaxy has been well reported to be associated with primary ciliary dyskinesia.[42] Hence, we next examined cilia morphology and function in zebrafish *cc2d1a*

Table 2. Rare Damaging *CC2D1A* Mutations Discovered in Heterotaxy Patients

Patient No.	Mutations	In silco prediction				Case (n=26)		Control (n=130)		ESP6500 (n=6525)		ExAC (n=61 486)	
		SIFT	PolyPhen2	LRT	CADD	Count	Freq	Count	Freq	Count	Freq	Count	Freq
1	c.575C>T, p.(Pro192Leu)	D	D	D	D	1 (RI)	1.19%	0	0.00%	0	0.00%	4	0.00%
2	c.1517A>G, p.(Gln506Arg)	D	B	D	D	1 (RI)	1.19%	0	0.00%	0	0.00%	10	0.01%
3	c.1595C>T, p.(Pro532Leu)	D	B	D	D	1 (RI)	1.19%	0	0.00%	0	0.00%	0	0.00%
1, 4, 5	c.2342G>T, p.(Gly781Val)	D	D	D	D	3 (RI)	5.60%	1	0.29%	6	0.05%	147	0.12%
6	c.2342G>A, p.(Gly781Glu)	D	D	D	D	1 (LI)	1.19%	0	0.00%	0	0.00%	13	0.01%

CC2D1A transcript NM_017721.4. Homo sapiens (human) genome assembly GRCh37 (hg19). B indicates benign; CADD, combined annotation dependent depletion; D, deleterious; Freq, population frequency; LI, left isomerism; LRT, likelihood ratio test; PolyPhen2, polymorphism phenotyping v2; RI, right isomerism; and SIFT, sorting intolerant from tolerant.

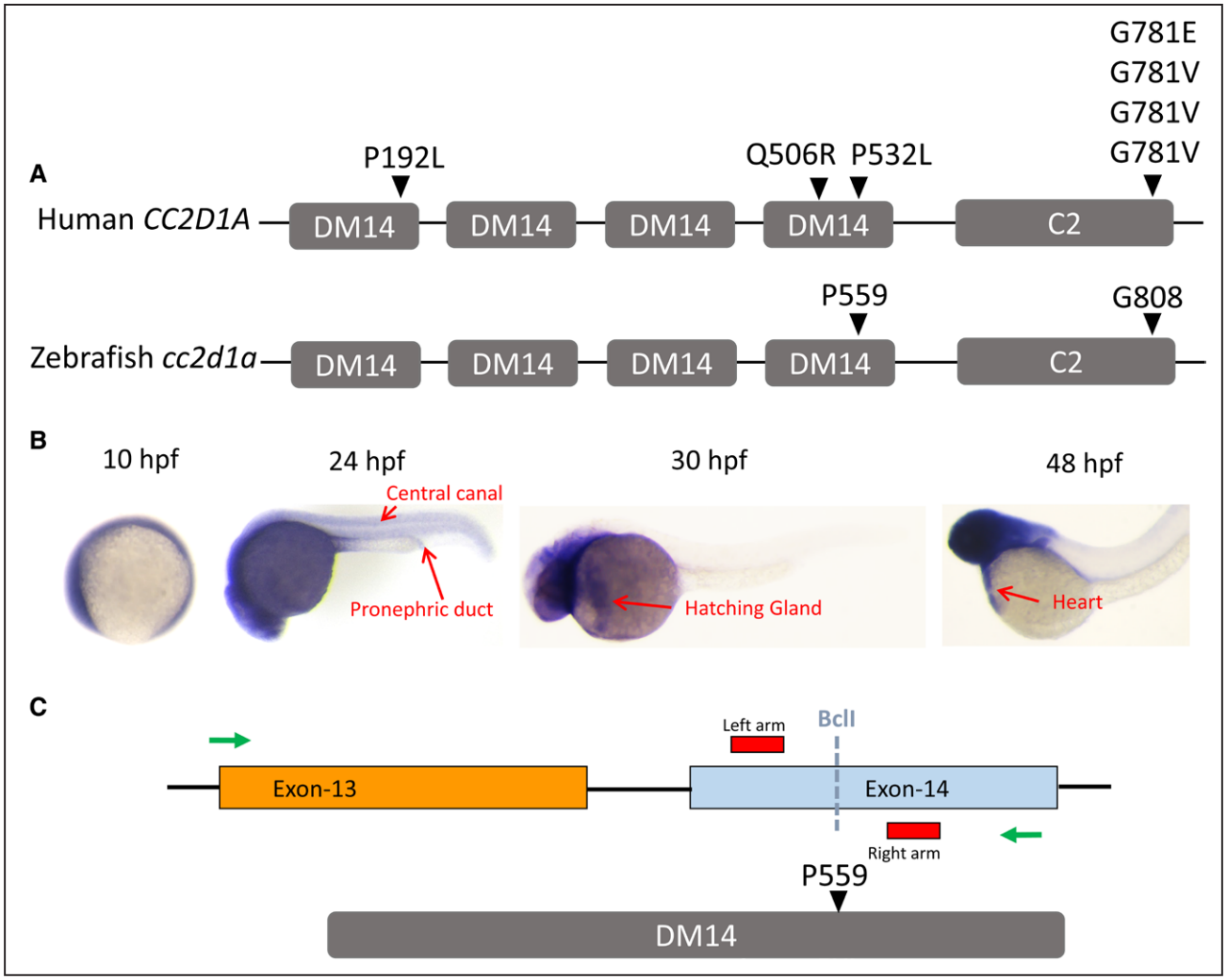

Figure 2. Corresponding mutations in orthologous *cc2d1a*, spatial expression pattern and TALEN design.
A, Human *CC2D1A* and zebrafish *cc2d1a* are highly conserved with 4 DM14 domains and a C2 domain. Spatial clustering of mutations reported in this cohort are shown. Seven occurrences of mutations in *CC2D1A* were found at four mutation sites. Three of the mutations were located in DM14 domains, and the remaining four were located in the C2 domain. Patient 1 harbored two mutations (P192L and G781V) simultaneously, hence a total of seven mutations were identified in six cases (see also Table 2). Zebrafish P559 (TALEN [transcription activator-like effector nuclease] target) and G809 residues are corresponding to human P523 and G781. **B**, Spatial expression pattern of *cc2d1a* during embryonic development as shown by whole-mount in situ hybridization. **C**, Diagram showing the TALEN pair (red boxes) targeting *cc2d1a* Exon-14, approximately at residue P559 in the fourth DM14 domain. Green arrows: primers for RFLP (restriction fragment length polymorphism) assay; dotted line: the endogenous BclI restriction site used in RFLP (restriction fragment length polymorphism) assay. hpf indicates hours post-fertilization.

mutant. Zebrafish ventral axis curves and otolith defects were examined, which are surrogate markers of ciliopathy during early embryonic development.[43] While most control displayed normal gross embryonic development, *cc2d1a* mutants were associated with curved ventral axis ($P<0.0001$, Figure 4A) and defective otolith development ($P<0.0001$, Figure 4B). In addition, abnormal mirror and bilateral expression of *spaw* (asymmetrical L-R marker) were also detected in *cc2d1a* mutants (Figure 4C). Similar to the results of the heterotaxy phenotypes, ciliopathy-associated phenotypes in *cc2d1a* mutant could be partially rescued by wild-type *cc2d1a* mRNA (Figure IV in the Data Supplement). In addition, whole-mount immunostaining of acetylated α-tubulin revealed cilia with defective conformation in *cc2d1a* mutant embryos along the spinal canal and pronephric duct at 24 hpf (Figure 4D). These results indicate the *cc2d1a* mutants had ciliopathy, likely via a loss-of-function mechanism.

Heterotaxy and Ciliopathy Were Observed in Germline *cc2d1a* Mutant With Reduced Penetrance

We next examined whether zebrafish carrying germline heterozygous *cc2d1a* mutants would also result in heterotaxy and ciliopathy. We identified a germline stable F1 carrying a frame-shift +7-bp mutation (*cc2d1a*$^{+7}$) in *cc2d1a* (Figure VA in the Data Supplement). The 7-bp

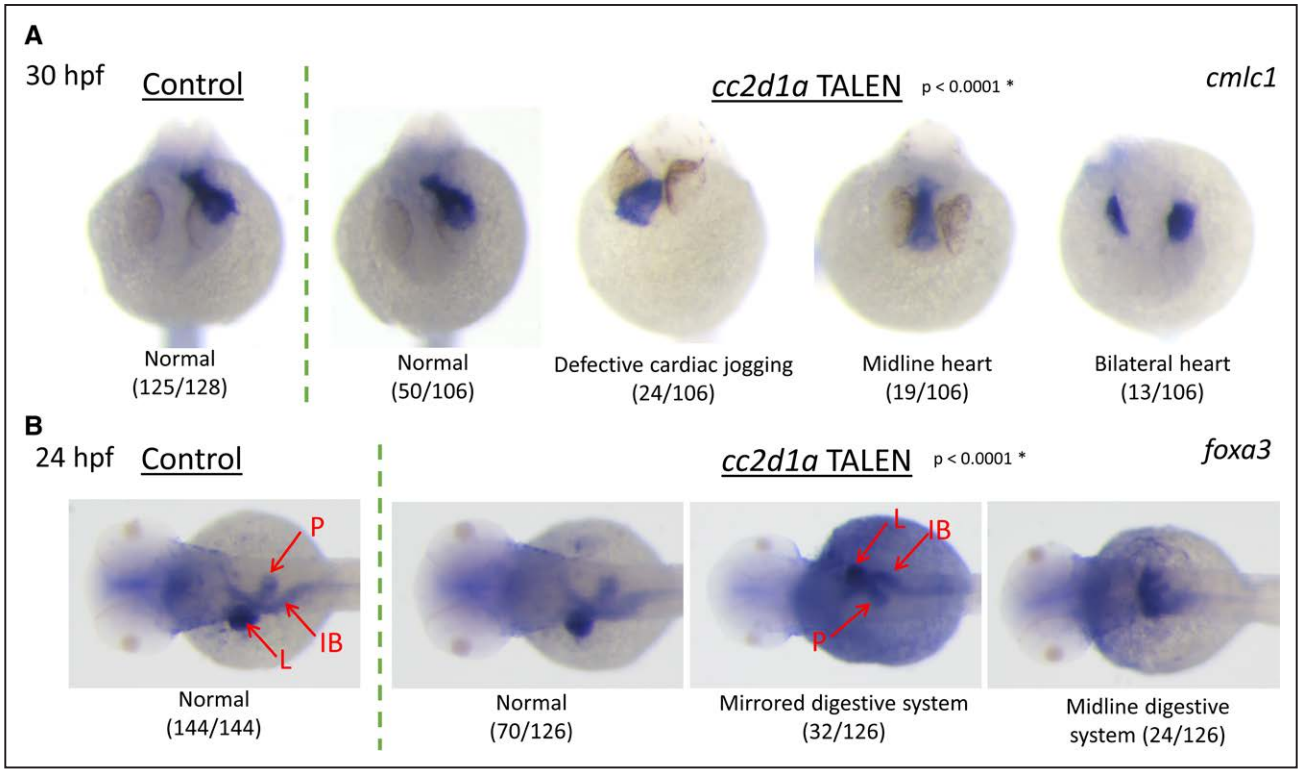

Figure 3. Laterality defects induced by TALEN (transcription activator-like effector nuclease)-induced *cc2d1a* mutation. **A**, cardiac development; (**B**) digestive system development. Number of embryos with each type of phenotype over the total number of embryos analyzed in ≥3 independent experiments is shown in brackets. hpf indicates hours post-fertilization; IB, intestinal bulb; L, liver; and P, pancreas. *Please refer to Figure III in the Data Supplement for comparison groups; $P<0.0125$ is considered as statistically significant (after correction for multiple testing).

insertion at the TALEN-targeting locus resulted in a premature stop and truncation of the *cc2d1a* protein before the fourth DM14 domain (Figure VB in the Data Supplement). The F1 *cc2d1a*$^{+7}$ mutants were in-crossed to produce F2 progeny. Although wild-type (+/+), heterozygous (±), and homozygous (−/−) siblings were generated (Figure VC and VD in the Data Supplement), all surviving homozygous mutants displayed severe early embryonic deformations and were therefore excluded from the subsequent analyses (Figure VC in the Data Supplement). Furthermore, heterozygous *cc2d1a*$^{+7}$ mutants, but not their wild-type siblings, displayed heterotaxy phenotypes including heart deformations (P=0.0238, Figure 5A) and perturbed cilia conformation (P=0.0240, Figure 5C). Mirrored digestive system was also observed but did not reach statistical significance (P=0.2818, Figure 5B). This suggested that *cc2d1a* is associated with heterotaxy and ciliopathy with reduced penetrance.

DISCUSSION

This study is the first to identify seven rare, damaging exonic missense variants of *CC2D1A* in six out of 26 (23%) patients with heterotaxy using whole-exome sequencing. The increased burden of mutations was statistically significant when compared with different control populations with an odds ratio ranging from 19.2 to 26.1. The mutations were located in the gene across three different domains. The P192L variant mapped to the first DM14 domain of the protein, the Q506R and P532L mapped to the fourth DM14 domain, and G781E and G781V mapped to the C2 domain (Figure 2). Human *CC2D1A* belongs to the evolutionarily conserved lethal giant discs (lgd) protein family. Members of this family contain four tandem repeats of the DM14 domain and one C2 domain. The human *CC2D1A* gene covers 37 kbp of genomic DNA on chromosome 19p13.12. It encodes a mRNA of 3715 bp and contains 31 exons. The functions of *CC2D1A* include centrosome cleavage,[44] regulation of signaling pathways, immune response,[45,46] synapse maturation,[47–49] and endocytic pathway regulation.[50–53] However, the function of *CC2D1A* during embryonic development and in the formation of the L-R axis is unclear.

In mouse embryos, expression of the gene has been shown in the embryonic ventricular zone and developing cortical plate.[54] However, *cc2d1a* deficiency in mice leads to cyanosis and breathing difficulties, resulting in death within minutes to hours after birth. Although no gross abnormalities of the heart or lung have been identified,[46,55,56] previous investigators could not rule out subtle

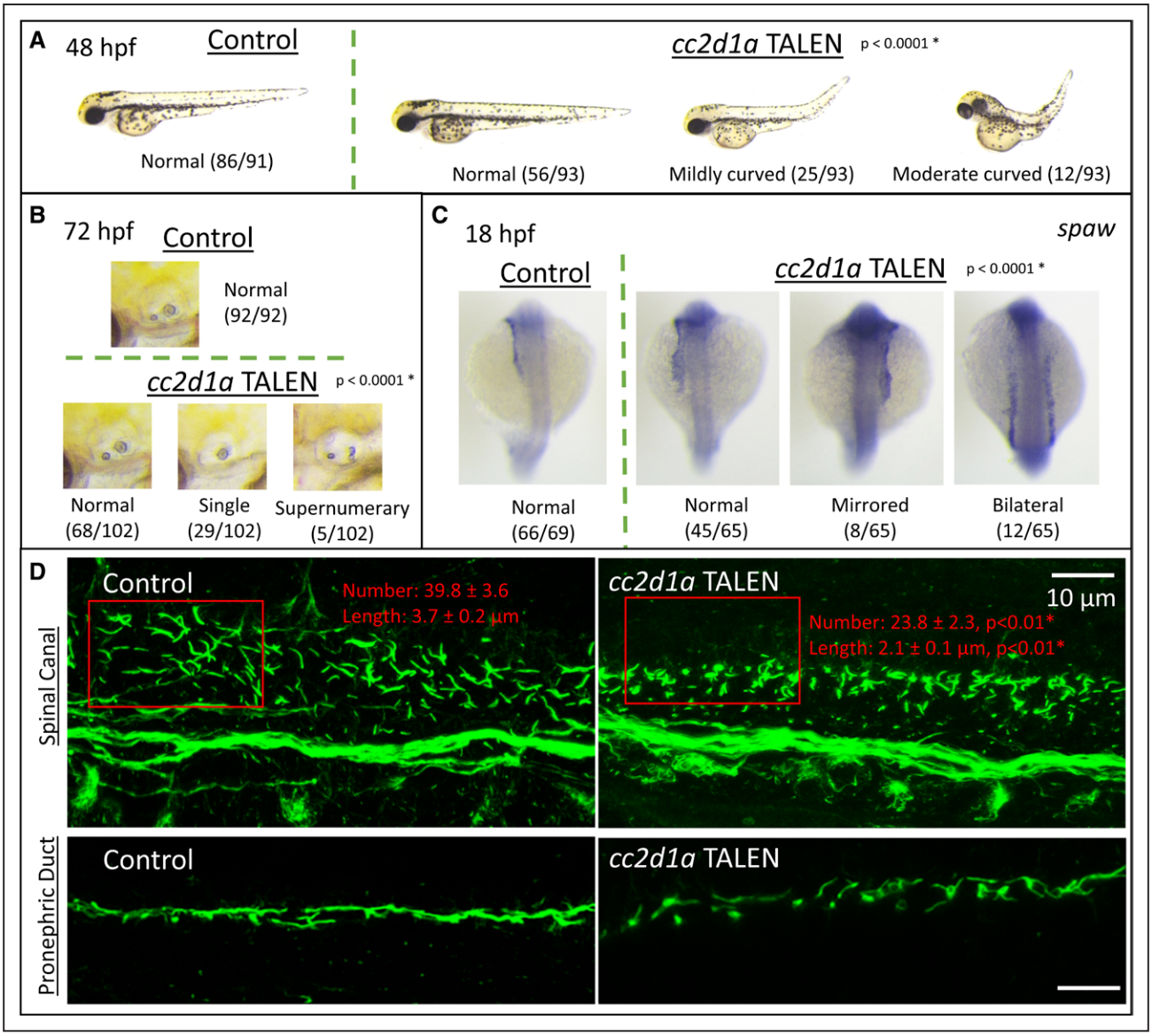

Figure 4. The effect of TALEN (transcription activator-like effector nuclease)-induced *cc2d1a* mutation associated with ciliary defects.

A, ventral body axis; (**B**) otolith development; (**C**) spaw expression; and (**D**) cilia conformation. Number of embryos showing each type of phenotypes over the total number of embryos analyzed in three independent experiments are shown in bracket. Number of cilia and cilia length were quantified within an equal area (red box) of control and *cc2d1a* mutant embryo. Three control and three mutant embryos were quantified in each of the three independent experiments and the average number±SEM were shown. *Please refer to Figure IV in the Data Supplement for comparison groups; $P<0.025$ is considered as statistically significant (after correction for multiple testing).

alterations to organ development[56] and postulated that cyanosis might be related to nervous system abnormalities. Drusenheimer et al[50] used a conditional knockout model to test this hypothesis by comparing *cc2d1a*-deficient mice with brain-specific *cc2d1a* mutants. All (8/8) of the *cc2d1a*-deficient mice had breathing difficulties and were cyanotic after birth, whereas only one-third (4/12) of the brain-specific conditional knockout mice showed abnormal phenotype. It is, therefore, possible that the cyanosis after birth may be related to non-neurological conditions, probably cardiac or respiratory-related abnormalities.

We used the zebrafish model to elucidate the functional impact of the *cc2d1a* mutations in relation to heterotaxy. This model is widely used in the study of genes that are developmentally crucial and embryonically lethal in mammalian models such as heterotaxy-related genes.[10,57] In particular, zebrafish embryos obtain oxygen from the culture medium by simple diffusion to compensate for major respiratory defects. Unlike the mouse knockout model, zebrafish embryos with TALEN-induced somatic *cc2d1a* knockout are more likely to survive for phenotypic analysis.

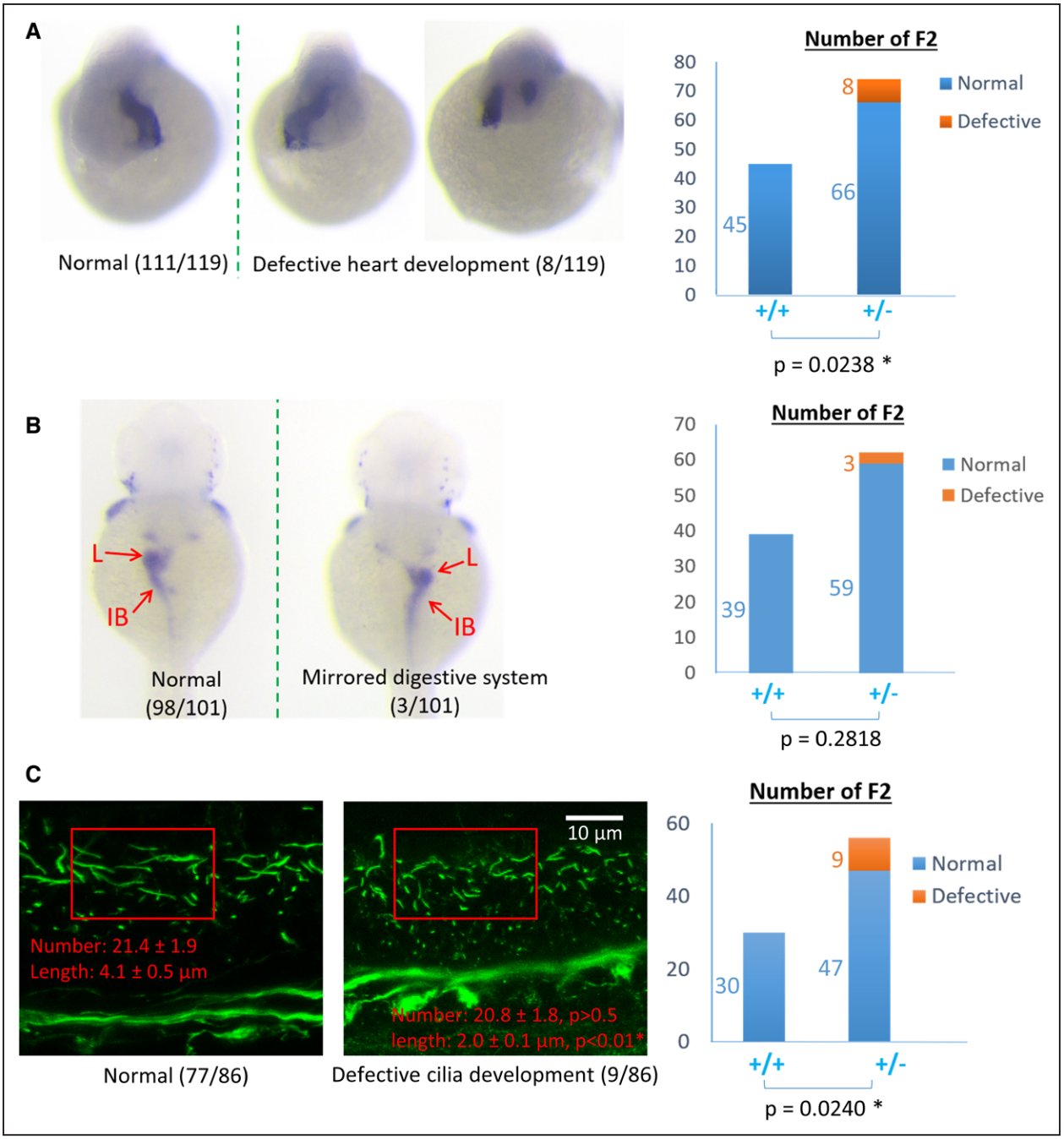

Figure 5. Heterotaxy and ciliopathy phenotypes observed in germline F2 heterozygous *cc2d1a*$^{+7}$ mutants.
A, Defective cardiac development observed in heterozygous *cc2d1a*$^{+7}$ mutant zebrafish embryos at 30 hours post-fertilization (hpf). Number of embryos with each type of phenotype over the total number of F2 embryos analyzed in three independent experiments is shown in brackets. The number of wild-type (+/+) and heterozygous (±) F2 embryos with normal and defective cardiac development in three independent experiments are presented in the graph. **B**, Defective digestive system development observed in heterozygous *cc2d1a*$^{+7}$ mutant zebrafish embryos at 30 hpf. Number of embryos with each type of phenotype over the total number of F2 embryos analyzed in ≥3 independent experiments is shown in brackets. The number of wild-type (+/+) and heterozygous (±) F2 embryos with normal and defective digestive system development in ≥ 3 independent experiments are presented in the graph. **C**, Cilia with defective conformation observed in heterozygous *cc2d1a*$^{+7}$ mutant zebrafish embryos at 24 hpf. Number of embryos with each type of phenotypes over the total number of F2 embryos analyzed in three independent experiments is shown in brackets. Number of cilia and cilia length were quantified within an equal area (red box) in embryos with normal and defective cilia development. Five normal and five defective embryos were quantified and the average number±SEM were shown. The number of wild-type (+/+) and heterozygous (±) F2 embryos with normal and defective cilia conformation in three independent experiments are presented in the graph. IB indicates intestinal bulb; L, liver; and P, pancreas. *For statistical comparison, wild-type (+/+) is compared against heterozygous (±); $P<0.05$ is considered as statistically significant.

Our knockout zebrafish model with *cc2d1a* mutations exhibited obvious heterotaxy and ciliopathy phenotypes, providing additional evidence of the important role of *cc2d1a* in L-R axis formation during embryonic development. In our zebrafish mutant model, we found only a proportion of somatic mutant embryos had heterotaxy, possibly because TALEN could only induce somatic *cc2d1a* mutations in 65% of the zebrafish. We found that wild-type *cc2d1a* mRNA could partially rescue these phenotypes but not with mRNA of orthologous mutations. Furthermore, overexpression of *cc2d1a* orthologous mutations did not produce corresponding phenotypes, indicating the abnormal phenotype was because of a loss-of-function rather than gain-of-function or dominant-negative effects of *cc2d1a*.

Similar heterotaxy (mirrored heart and digestive system) and ciliopathy phenotypes were observed in germline heterozygous *cc2d1a* mutant carrying a 7-bp frame-shift insertion, which further confirmed the specificity of the TALEN-mediated *cc2d1a* targeting. Although the number of heterozygous F2 was roughly double that of the wild-type siblings, the number of homozygous F2 was significantly lower than expected, which could be explained by the early embryonic lethality of homozygous *cc2d1a*$^{+7}$ mutant. In fact, the percentage of homozygous F2 progeny genotyped at early embryonic stages (6 hpf) was around 20% (data not shown). All the remaining homozygous mutants were severely deformed, whereas wild-type and heterozygous F2 were grossly normal, demonstrates the crucial but undescribed role of *cc2d1a* during embryonic development. Compared with somatic mutants, the penetrance of heterotaxy and ciliopathy in *cc2d1a* mutants was greatly reduced. Although this could be potentially explained by genetic compensation observed in the stable deleterious mutant,[58] the lower mutational burden in F2 heterozygous mutant (uniformly 50%) compared with mosaic somatic mutants (65%) might also contribute to the reduced penetrance. Nevertheless, the somatic and germline zebrafish *cc2d1a* mutants provide a unique in vivo model for mechanistic studies of the role of *cc2d1a* in heterotaxy and embryonic development.

To date, the only human disease reported to be associated with *CC2D1A* is autosomal recessive nonsyndromic intellectual disability (Online Mendelian Inheritance in Man: 608443). In 9 consanguineous families with nonsyndromic intellectual disability, biallelic mutations were identified in the *CC2D1A* gene causing complete deletion of exons 14-16, resulting in the truncation of the fourth DM14 domain and the C2 domain of the protein.[54] Personal communication with Dr L Basel Vanagaite, the first author of this study, confirmed that none of the patients or the carrier parents had laterality defects. Natiq et al reported two patients who harbored larger deletions in the *CC2D1A* gene, with 19p13.2-p13.12 deletions resulting in moderate to severe developmental delay. These deletions overlapped *CC2D1A* and other Online Mendelian Inheritance in Man genes, but there was no mention of any laterality defects.[59] Interestingly, a *de novo* deletion of 19p13.13-13.12 was reported in a patient with dextrocardia (in ClinVar accession no: RCV000051051.4 https://www.ncbi.nlm.nih.gov/clinvar/27202273/). Although dextrocardia is well described in heterotaxy disorders, further details of this patient were not available. To the best of our knowledge, our study is the first to suggest an association of *CC2D1A* with heterotaxy.

The underlying mechanism of the exonic missense variants in *CC2D1A* and development of heterotaxy remains speculative, but our results from the whole-mount immunostaining suggest that ciliary dyskinesia likely plays an important role. The idea of laterality defects because of underlying cilia dysfunction is not new, as ≈12% of individuals with primary ciliary dyskinesia present with heterotaxy,[3] and 50% of primary ciliary dyskinesia patients develop situs inversus.[60] The involvement of cilia is also a plausible explanation for the impact of *CC2D1A* on both intellectual disability and heterotaxy. This is because ciliary disorders are associated with diverse phenotypes, from polycystic kidney disease to neural tube defects and retinitis pigmentosa.[61] The co-occurrence of distinct ciliopathy manifestations within families also suggests the possibility of genetic modifiers.[62] As proposed by Trulioff et al,[63] defects in ciliary proteins may be associated with both neurodevelopmental disorders and visceral asymmetry. These possible mechanisms should be considered when interpreting the reduced penetrance and multiple genotype-phenotype correlations of *CC2D1A*.

Although the association between *CC2D1A* and the nervous system has been established in mouse models, the subtle cardiac laterality defects may have remained undetected because of early lethality. With the use of our zebrafish model, we were now able to establish a novel association of *CC2D1A* with heterotaxy and ciliary dysfunction. Future mechanistic studies will be required for a better understanding of the role of *CC2D1A* in left-right patterning and ciliary dysfunction.

CONCLUSIONS

Using zebrafish model, we were able to demonstrate a novel association of *CC2D1A* with heterotaxy and ciliary dysfunction via a loss-of-function mutation. This is an important finding as cardiac and gastrointestinal phenotypes were previously not observed in mouse studies, likely because of early lethality. Our findings also suggest that *CC2D1A* is associated with both intellectual disability and heterotaxy involving ciliary dysfunction. Future mechanistic studies are needed for a better

understanding of the role of *CC2D1A* in heterotaxy and ciliary dysfunction.

ARTICLE INFORMATION

Received March 26, 2020; accepted November 5, 2020.

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Acknowledgments

We thank all patients for providing blood and DNA samples for this study. The zebrafish maintenance was supported by the Zebrafish Core Facility (FCF, LKS Faculty of Medicine, HKU, HK SAR, China) and the Fish Model Translational Research Laboratory (HTI, PolyU, HK SAR, China). Confocal imaging was performed in University Research Facility, Life Sciences (ULS, PolyU, HK SAR, China) with the help of Dr Clara Hung. A.C.H.M. designed and conducted most of the experiments on the zebrafish model. Drs Mak and Chow processed and analyzed the clinical samples and data. Dr Chow recruited the patients in this study. Drs Mak, Ying, Yeung, and Pei performed the whole-exome sequencing and genetic analysis. X. Chen and K.M.M. Hasan performed microinjection, genotyping, whole-mount immunostaining of cilia in zebrafish embryos. Drs Cheung and Chung oversaw and supervised this project. The article was written by Drs Ma, Mak, Pei, and Chung and was agreed by all coauthors.

Sources of Funding

This study was supported by the University of Hong Kong Seed Fund for Basic Research (Project code: 201711159132) and the Society for the Relief of Disabled Children.

Disclosures

None.

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