

ORIGINAL ARTICLE



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

Alvin Chun Hang Ma¹, PhD*; Christopher Chun Yu Mak¹, PhD*; Kit San Yeung¹, PhD; Steven Lim Cho Pei¹, PhD; Dingge Ying, PhD; Mullin Ho Chung Yu, MSc; Kazi Md Mahmudul Hasan¹, MSc; Xiangke Chen¹, MSc; Pak Cheong Chow, MBBS, MPH; Yiu Fai Cheung¹, MD; Brian Hon Yin Chung¹, MD

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 10000.²⁻⁴ Cardiovascular malformations

Correspondence to: Yiu Fai Cheung, MD, Department of Paediatrics & Adolescent Medicine, Queen Mary Hospital, New Clinical Bldg, 102 Pokfulam Rd, Hong Kong, Email xfcheung@hku.hk or Brian Hon Yin Chung, MD, Department of Paediatrics and Adolescent Medicine, Queen Mary Hospital, New Clinical Bldg, 102 Pokfulam Rd, Hong Kong, Email bhychung@hku.hk

*Drs Ma and Mak contributed equally as first authors.

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.

© 2020 The Authors. *Circulation: Genomic and Precision Medicine* is published on behalf of the American Heart Association, Inc., by Wolters Kluwer Health, Inc. This is an open access article under the terms of the [Creative Commons Attribution Non-Commercial-NoDerivs](#) License, which permits use, distribution, and reproduction in any medium, provided that the original work is properly cited, the use is noncommercial, and no modifications or adaptations are made.

Circulation: Genomic and Precision Medicine is available at www.ahajournals.org/journal/circgen

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 mutations¹⁶ or homozygous variants in consanguineous families.¹⁷ Other examples include a consanguineous family with a homozygous deletion in the *WDR16* gene¹⁸; two affected brothers with a homozygous splice site mutation in the *CCDC11* gene¹⁹; 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 al³² 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

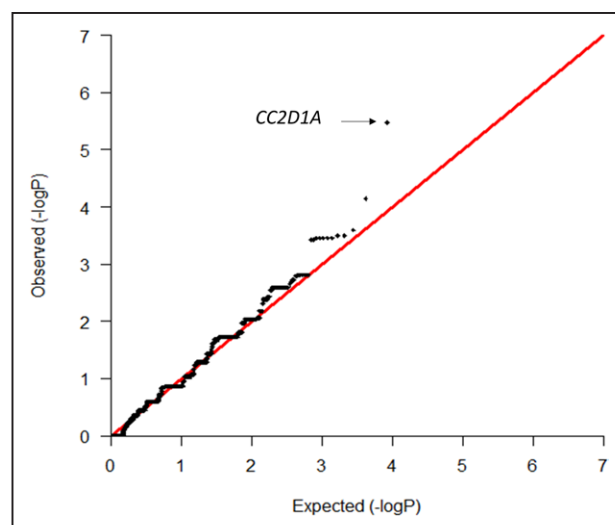


Figure 1. QQ plot of the P value derived from SNP-set Kernel Association Test by comparing the rare damaging mutations in cases and internal controls for all genes in cases or controls with at least one mutation.

Only the *CC2D1A* gene had a significant P value after Bonferroni correction.

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.

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 <i>CC2D1A</i>	Frequency	Odds ratio	95% CI	SKAT	Corrected
						P value	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 ($65.5 \pm 6.1\%$) 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 silico 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.

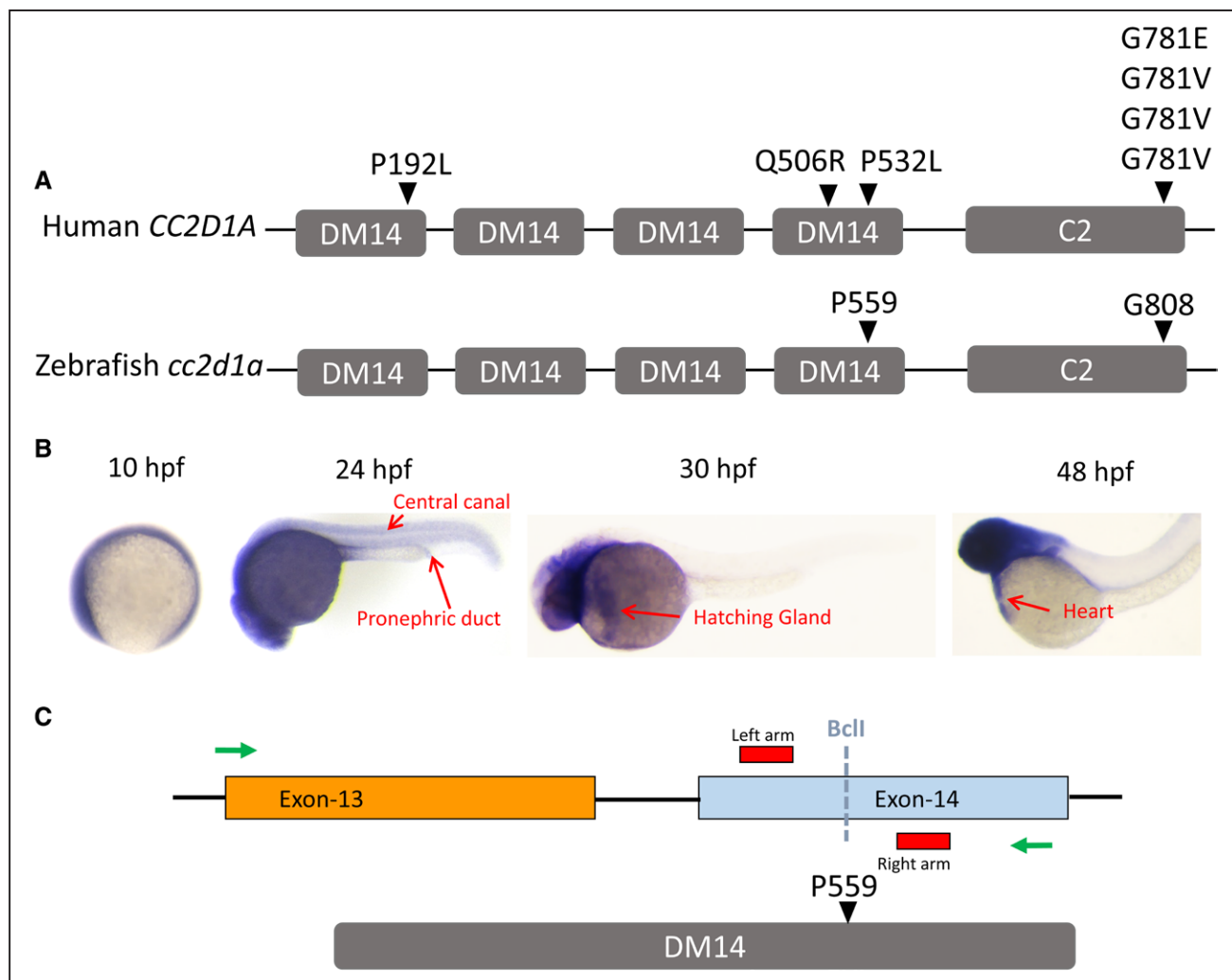


Figure 2. Corresponding mutations in orthologous *cc2d1a*, spatial expression pattern and TALEN design.

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

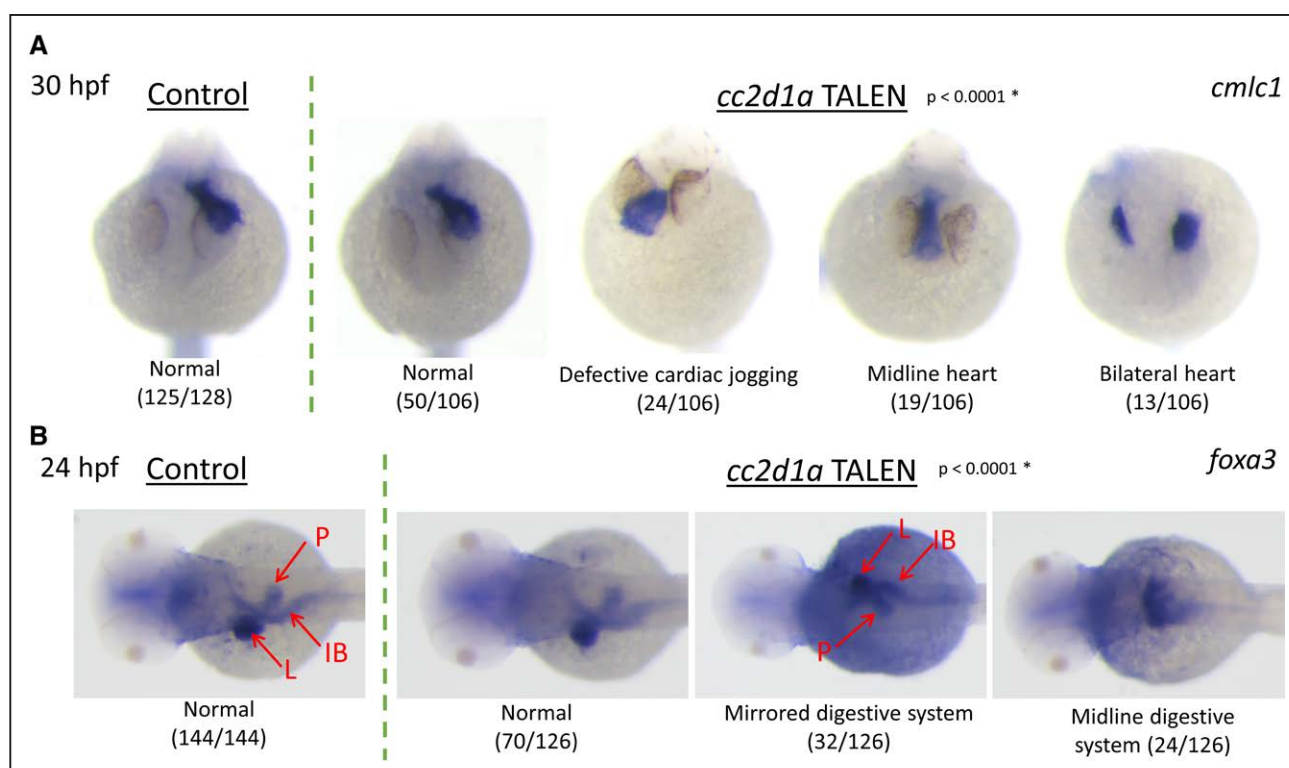


Figure 3. Laterality defects induced by TALEN (transcription activator-like effector nuclease)-induced *cc2d1a* mutation.

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 (\pm), 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 (lgs) 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

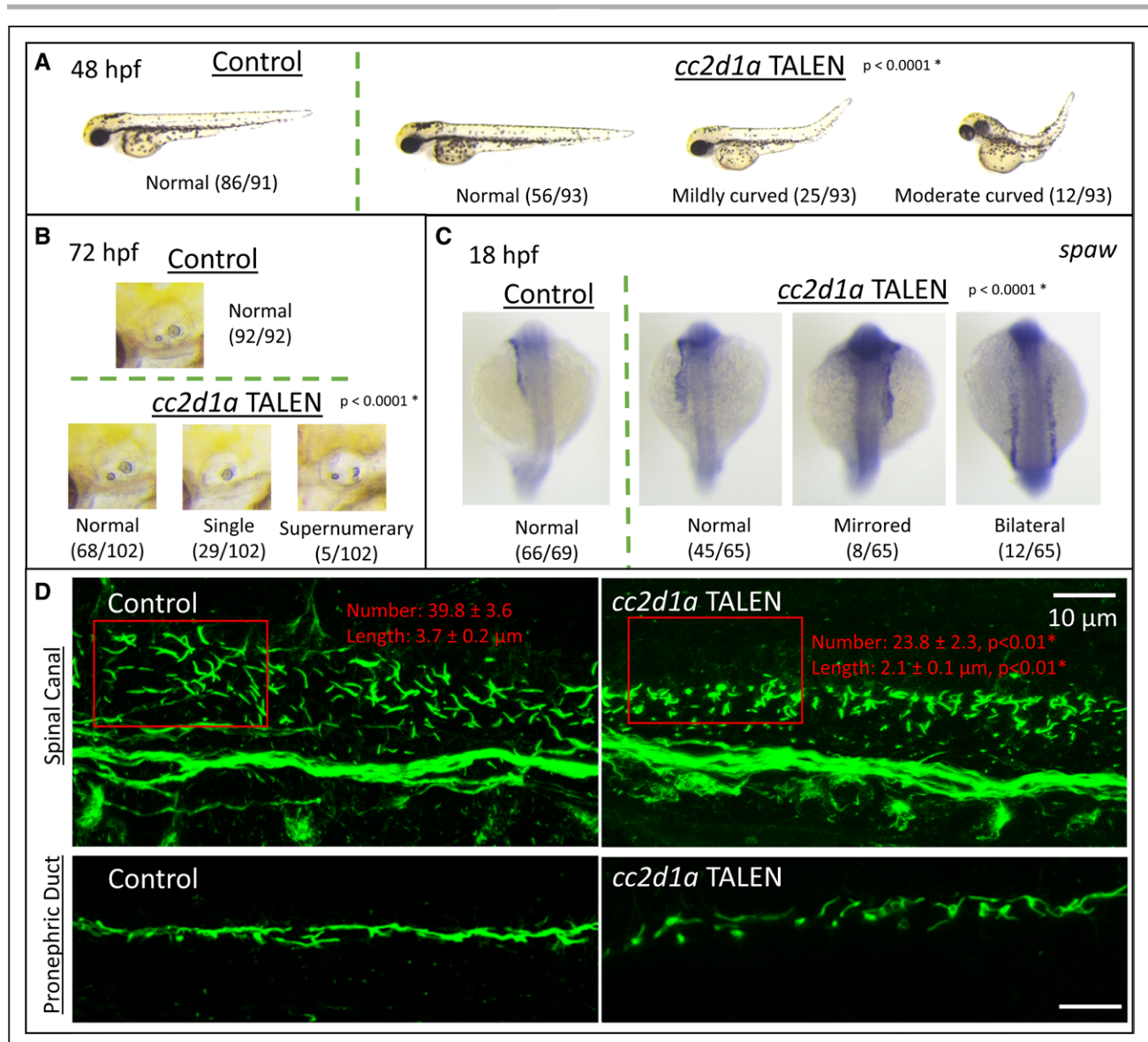


Figure 4. The effect of TALEN (transcription activator-like effector nuclease)-induced *cc2d1a* mutation associated with ciliary defects.

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 \pm 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 development⁵⁶ and postulated that cyanosis might be related to nervous system abnormalities. Drusenheimer et al⁵⁰ 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.

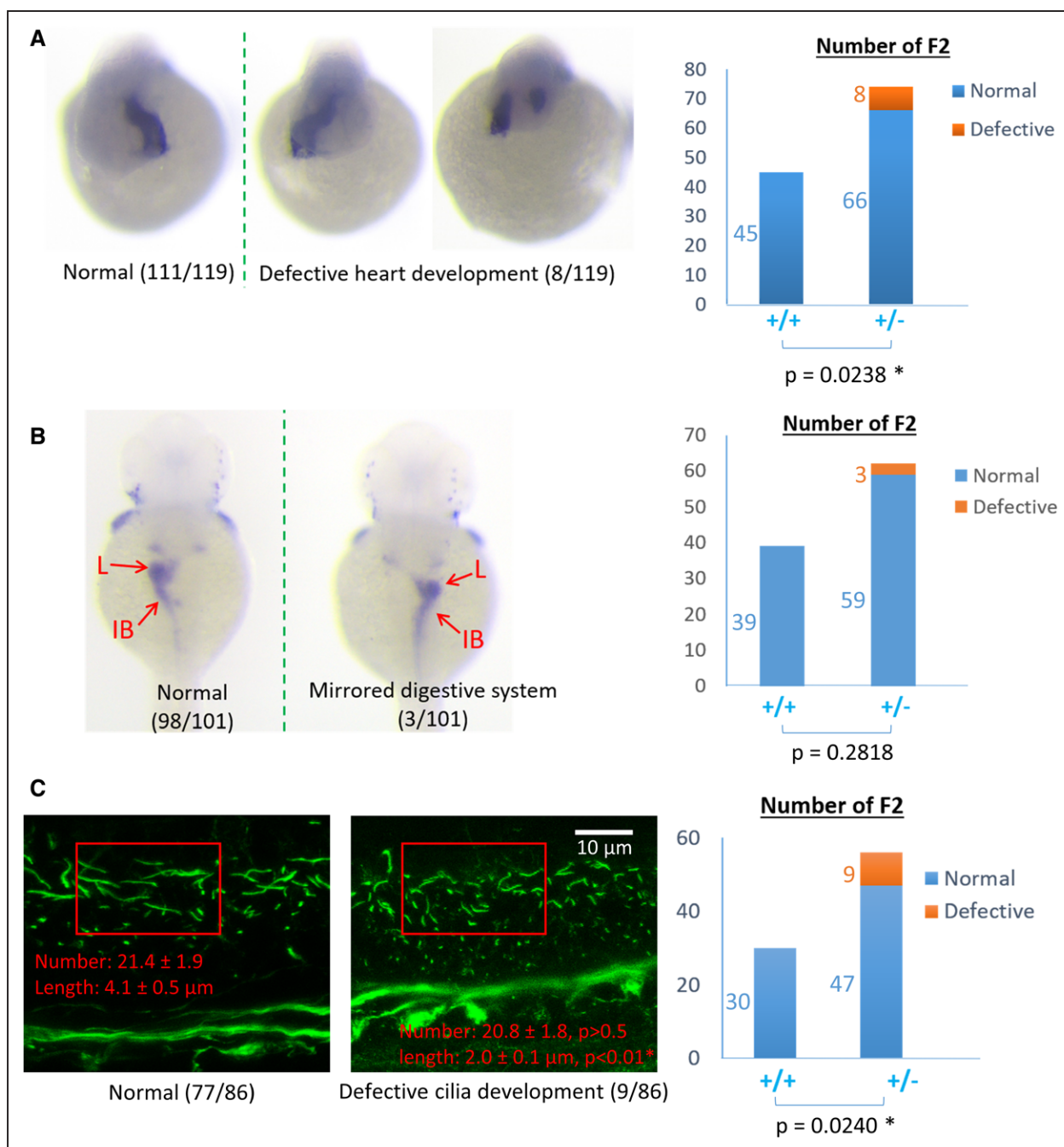


Figure 5. Heterotaxy and ciliopathy phenotypes observed in germline F2 heterozygous *cc2d1a*^{+/-} mutants.

A, Defective cardiac development observed in heterozygous *cc2d1a*^{+/-} 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*^{+/-} 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*^{+/-} 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*⁺ 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 non-syndromic 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.

Affiliations

Department of Health Technology and Informatics, The Hong Kong Polytechnic University, Hong Kong Special Administrative Region, China (A.C.H., K.M.M.H.). Department of Paediatrics and Adolescent Medicine, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong Special Administrative Region, China (C.C.Y.M., K.S.Y., S.L.C.P., D.Y., M.H.C.Y., P.C.C., Y.F.C., B.H.Y.C.). Laboratory of Neurodegenerative Diseases, School of Biomedical Sciences, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong Special Administrative Region, China (X.C.).

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.

REFERENCES

- Zhu L, Belmont JW, Ware SM. Genetics of human heterotaxias. *Eur J Hum Genet*. 2006;14:17–25. doi: 10.1038/sj.ejhg.5201506
- Maclean K, Dunwoodie SL. Breaking symmetry: a clinical overview of left-right patterning. *Clin Genet*. 2004;65:441–457. doi: 10.1111/j.0009-9163.2004.00258.x
- Shapiro AJ, Davis SD, Ferkol T, Dell SD, Rosenfeld M, Olivier KN, Sagel SD, Milla C, Zariwala MA, Wolf W, et al. Laterality defects other than situs inversus totalis in primary ciliary dyskinesia: insights into situs ambiguus and heterotaxy. *Chest*. 2014;146:1176–86. doi: 10.1378/chest.13-1704
- Lin AE, Krikov S, Riehle-Colarusso T, Frias JL, Belmont J, Anderka M, Geva T, Getz KD, Botto LD; National Birth Defects Prevention Study. Laterality defects in the national birth defects prevention study (1998–2007): birth prevalence and descriptive epidemiology. *Am J Med Genet A*. 2014;164A:2581–2591. doi: 10.1002/ajmg.a.36695
- Rigler SL, Kay DM, Sicko RJ, Fan R, Liu A, Caggana M, Browne ML, Druschel CM, Romitti PA, Brody LC, et al. Novel copy-number variants in a population-based investigation of classic heterotaxy. *Genet Med*. 2015;17:348–357. doi: 10.1038/gim.2014.112
- Sutherland MJ, Ware SM. Disorders of left-right asymmetry: heterotaxy and situs inversus. *Am J Med Genet C Semin Med Genet*. 2009;151C:307–317. doi: 10.1002/ajmg.c.30228
- Jacobs JP, Anderson RH, Weinberg PM, Walters HL III, Tchervenkov CI, Del Duca D, Franklin RC, Aiello VD, Beland MJ, Colan SD, et al. The nomenclature, definition and classification of cardiac structures in the setting of heterotaxy. *Cardiol Young*. 2007;17(suppl 2):1–28. doi: 10.1017/S1047951107001138
- Peeters H, Devriendt K. Human laterality disorders. *Eur J Med Genet*. 2006;49:349–362. doi: 10.1016/j.ejmg.2005.12.003
- Bhaskar J, Galati JC, Brooks P, Oppido G, Konstantinov IE, Brizard CP, d'Udekem Y. Survival into adulthood of patients with atrial isomerism undergoing cardiac surgery. *J Thorac Cardiovasc Surg*. 2015;149:1509–1513. doi: 10.1016/j.jtcvs.2015.01.038
- Guimier A, Gabriel GC, Bajolle F, Tsang M, Liu H, Noll A, Schwartz M, El Malti R, Smith LD, Klena NT, et al. MMP21 is mutated in human heterotaxy and is required for normal left-right asymmetry in vertebrates. *Nat Genet*. 2015;47:1260–1263. doi: 10.1038/ng.3376
- Mohapatra B, Casey B, Li H, Ho-Dawson T, Smith L, Fernbach SD, Molinari L, Niesh SR, Jefferies JL, Craigen WJ, et al. Identification and functional characterization of NODAL rare variants in heterotaxy and isolated cardiovascular malformations. *Hum Mol Genet*. 2009;18:861–871. doi: 10.1093/hmg/ddn411
- Peeters H, Voz ML, Verschueren K, De Cat B, Pendevel H, Thienpont B, Schellens A, Belmont JW, David G, Van De Ven WJ, et al. Ssn1 is a novel gene for left-right asymmetry and mediating nodal signaling. *Hum Mol Genet*. 2006;15:3369–3377. doi: 10.1093/hmg/ddl413
- Bamford RN, Roessler E, Burdine RD, Saplakoglu U, dela Cruz J, Splitt M, Goodship JA, Towbin J, Bowers P, Ferrero GB, et al. Loss-of-function mutations in the EGF-CFC gene CFC1 are associated with human left-right laterality defects. *Nat Genet*. 2000;26:365–369. doi: 10.1038/81695
- Ma L, Selamet Tierney ES, Lee T, Lanzano P, Chung WK. Mutations in ZIC3 and ACVR2B are a common cause of heterotaxy and associated cardiovascular anomalies. *Cardiol Young*. 2012;22:194–201. doi: 10.1017/S1047951111001181
- Kosaki K, Bassi MT, Kosaki R, Lewin M, Belmont J, Schauer G, Casey B. Characterization and mutation analysis of human LEFTY A and LEFTY B, homologues of murine genes implicated in left-right axis development. *Am J Hum Genet*. 1999;64:712–721. doi: 10.1086/302289
- Kaasinen E, Aittomäki K, Eronen M, Vahteristo P, Karhu A, Mecklin JP, Kajantie E, Aaltonen LA, Lehtonen R. Recessively inherited right atrial isomerism caused by mutations in growth/differentiation factor 1 (GDF1). *Hum Mol Genet*. 2010;19:2747–2753. doi: 10.1093/hmg/ddq164
- Marek-Yagel D, Bolkier Y, Barel O, Vardi A, Mishali D, Katz U, Salem Y, Abudi S, Nayshool O, Kol N, et al. A founder truncating variant in GDF1 causes autosomal-recessive right isomerism and associated congenital heart defects in multiplex Arab kindreds. *Am J Med Genet A*. 2020;182:987–993. doi: 10.1002/ajmg.a.61509
- French VM, van de Laar IM, Wessels MW, Rohe C, Roos-Hesselink JW, Wang G, Frohn-Mulder IM, Severijnen LA, de Graaf BM, Schot R, et al. NPHP4 variants are associated with pleiotropic heart malformations. *Circ Res*. 2012;110:1564–1574. doi: 10.1161/CIRCRESAHA.112.269795
- Ta-Shma A, Perles Z, Yaacov B, Werner M, Frumkin A, Rein AJ, Elpeleg O. A human laterality disorder associated with a homozygous WDR16 deletion. *Eur J Hum Genet*. 2015;23:1262–1265. doi: 10.1038/ejhg.2014.265
- Perles Z, Cinnamon Y, Ta-Shma A, Shaag A, Einbinder T, Rein AJ, Elpeleg O. A human laterality disorder associated with recessive CCDC11 mutation. *J Med Genet*. 2012;49:386–390. doi: 10.1136/jmedgenet-2011-100457
- Vetrini F, D'Alessandro LC, Akdemir ZC, Braxton A, Azamian MS, Eldomery MK, Miller K, Kois C, Sack V, Shur N, et al. Bi-allelic mutations in PKD1L1 are associated with laterality defects in humans. *Am J Hum Genet*. 2016;99:886–893. doi: 10.1016/j.ajhg.2016.07.011
- Grimes DT, Keynton JL, Buenavista MT, Jin X, Patel SH, Kyosuke S, Vibert J, Williams DJ, Hamada H, Hussain R, et al. Genetic analysis reveals a hierarchy of interactions between polycystin-encoding genes and genes controlling cilia function during left-right determination. *PLoS Genet*. 2016;12:e1006070. doi: 10.1371/journal.pgen.1006070
- Cast AE, Gao C, Amack JD, Ware SM. An essential and highly conserved role for Zic3 in left-right patterning, gastrulation and convergent extension morphogenesis. *Dev Biol*. 2012;364:22–31. doi: 10.1016/j.jydbio.2012.01.011
- Gebbia M, Ferrero GB, Pilia G, Bassi MT, Aylsworth A, Penman-Splitt M, Bird LM, Bamforth JS, Burn J, Schlessinger D, et al. X-linked situs abnormalities result from mutations in ZIC3. *Nat Genet*. 1997;17:305–308. doi: 10.1038/ng1197-305
- Fakhro KA, Choi M, Ware SM, Belmont JW, Towbin JA, Lifton RP, Khokha MK, Brueckner M. Rare copy number variations in congenital heart disease patients identify unique genes in left-right patterning. *Proc Natl Acad Sci USA*. 2011;108:2915–2920. doi: 10.1073/pnas.1019645108
- Auer PL, Nalls M, Meschia JF, Worrall BB, Longstreth WT Jr, Seshadri S, Kooperberg C, Burger KM, Carlson CS, Carty CL, et al; National Heart, Lung, and Blood Institute Exome Sequencing Project. Rare and coding region genetic variants associated with risk of ischemic stroke: the NHLBI Exome Sequence Project. *JAMA Neurol*. 2015;72:781–788. doi: 10.1001/jamaneurol.2015.0582

27. Auton A, Brooks LD, Durbin RM, Garrison EP, Kang HM, Korbel JO, Marchini JL, McCarthy S, McVean GA, Abecasis GR; 1000 Genomes Project Consortium. A global reference for human genetic variation. *Nature*. 2015;526:68–74. doi: 10.1038/nature15393
28. Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nat Protoc*. 2009;4:1073–1081. doi: 10.1038/nprot.2009.86
29. Adzhubei I, Jordan DM, Sunyaev SR. Predicting functional effect of human missense mutations using PolyPhen-2. *Curr Protoc Hum Genet*. 2013;Chapter 7:Unit7.20. doi: 10.1002/0471142905.hg0720s76
30. Kircher M, Witten DM, Jain P, O’Roak BJ, Cooper GM, Shendure J. A general framework for estimating the relative pathogenicity of human genetic variants. *Nat Genet*. 2014;46:310–315. doi: 10.1038/ng.2892
31. Shinohara K, Hamada H. Cilia in left-right symmetry breaking. *Cold Spring Harb Perspect Biol*. 2017;9. doi:10.1101/cshperspect.a028282
32. Nonaka S, Tanaka Y, Okada Y, Takeda S, Harada A, Kanai Y, Kido M, Hirokawa N. Randomization of left-right asymmetry due to loss of nodal cilia generating leftward flow of extraembryonic fluid in mice lacking KIF3B motor protein. *Cell*. 1998;95:829–837. doi: 10.1016/s0092-8674(00)81705-5
33. Nonaka S, Shiratori H, Saijoh Y, Hamada H. Determination of left-right patterning of the mouse embryo by artificial nodal flow. *Nature*. 2002;418:96–99. doi: 10.1038/nature00849
34. Grimes DT, Burdine RD. Left-right patterning: breaking symmetry to asymmetric morphogenesis. *Trends Genet*. 2017;33:616–628. doi: 10.1016/j.tig.2017.06.004
35. Santoriello C, Zon LI. Hooked! Modeling human disease in zebrafish. *J Clin Invest*. 2012;122:2337–2343. doi: 10.1172/JCI60434
36. Howe DG, Bradford YM, Eagle A, Fashena D, Frazer K, Kalita P, Mani P, Martin R, Moxon ST, Paddock H, et al. The Zebrafish Model Organism Database: new support for human disease models, mutation details, gene expression phenotypes and searching. *Nucleic Acids Res*. 2017;45:D758–D768. doi: 10.1093/nar/gkw1116
37. Zhao M, Zhao Z. CNVannotator: a comprehensive annotation server for copy number variation in the human genome. *PLoS One*. 2013;8:e80170. doi: 10.1371/journal.pone.0080170
38. Yang H, Robinson PN, Wang K. Phenolyzer: phenotype-based prioritization of candidate genes for human diseases. *Nat Methods*. 2015;12:841–843. doi: 10.1038/nmeth.3484
39. Stenson PD, Ball EV, Mort M, Phillips AD, Shaw K, Cooper DN. The Human Gene Mutation Database (HGMD) and its exploitation in the fields of personalized genomics and molecular evolution. *Curr Protoc Bioinformatics*. 2012;Chapter 1:Unit1.13. doi: 10.1002/0471250953.bi0113s39
40. Cordell HJ, Töpfer A, Mamasoula C, Postma AV, Bentham J, Zelenika D, Heath S, Blue G, Cosgrove C, Granados Riveron J, et al. Genome-wide association study identifies loci on 12q24 and 13q32 associated with tetralogy of Fallot. *Hum Mol Genet*. 2013;22:1473–1481. doi: 10.1093/hmg/dd552
41. Ma AC, McNulty MS, Poshusta TL, Campbell JM, Martínez-Gálvez G, Argue DP, Lee HB, Urban MD, Bullard CE, Blackburn PR, et al. FusX: a rapid one-step transcription activator-like effector assembly system for genome science. *Hum Gene Ther*. 2016;27:451–463. doi: 10.1089/hum.2015.172
42. Harrison MJ, Shapiro AJ, Kennedy MP. Congenital heart disease and primary ciliary dyskinesia. *Paediatr Respir Rev*. 2016;18:25–32. doi: 10.1016/j.prrv.2015.09.003
43. Austin-Tse C, Halbritter J, Zariwala MA, Gilbert RM, Gee HY, Hellman N, Pathak N, Liu Y, Panizzi JR, Patel-King RS, et al. Zebrafish ciliopathy screen plus human mutational analysis identifies C21orf59 and CCDC65 defects as causing primary ciliary dyskinesia. *Am J Hum Genet*. 2013;93:672–686. doi: 10.1016/j.ajhg.2013.08.015
44. Nakamura A, Arai H, Fujita N. Centrosomal Aki1 and cohesin function in separate-regulated centriole disengagement. *J Cell Biol*. 2009;187:607–614. doi: 10.1083/jcb.200906019
45. Chang CH, Lai LC, Cheng HC, Chen KR, Syue YZ, Lu HC, Lin WY, Chen SH, Huang HS, Shiau AL, et al. TBK1-associated protein in endolysosomes (TAPE) is an innate immune regulator modulating the TLR3 and TLR4 signaling pathways. *J Biol Chem*. 2011;286:7043–7051. doi: 10.1074/jbc.M110.164632
46. Chen KR, Chang CH, Huang CY, Lin CY, Lin WY, Lo YC, Yang CY, Hsing EW, Chen LF, Shih SR, et al. TBK1-associated protein in endolysosomes (TAPE)/CC2D1A is a key regulator linking RIG-I-like receptors to antiviral immunity. *J Biol Chem*. 2012;287:32216–32221. doi: 10.1074/jbc.C112.394346
47. Hadjighassem MR, Austin MC, Szweczyk B, Daigle M, Stockmeier CA, Albert PR. Human Freud-2/CC2D1B: a novel repressor of postsynaptic serotonin-1A receptor expression. *Biol Psychiatry*. 2009;66:214–222. doi: 10.1016/j.biopsych.2009.02.033
48. Ou XM, Lecomte S, Jafar-Nejad H, Bown CD, Goto A, Rogaeva A, Albert PR. Freud-1: a neuronal calcium-regulated repressor of the 5-HT1A receptor gene. *J Neurosci*. 2003;23:7415–7425.
49. Rogaeva A, Ou XM, Jafar-Nejad H, Lecomte S, Albert PR. Differential repression by freud-1/CC2D1A at a polymorphic site in the dopamine-D2 receptor gene. *J Biol Chem*. 2007;282:20897–20905. doi: 10.1074/jbc.M610038200
50. Drusenheimer N, Migdal B, Jäckel S, Tverikhina L, Scheider K, Schulz K, Gröper J, Köhrer K, Klein T. The mammalian orthologs of Drosophila Lgd, CC2D1A and CC2D1B, function in the endocytic pathway, but their individual loss of function does not affect notch signalling. *PLoS Genet*. 2015;11:e1005749. doi: 10.1371/journal.pgen.1005749
51. Martinelli N, Hartlieb B, Usami Y, Sabin C, Dordor A, Miquet N, Avilov SV, Ribeiro EA Jr, Göttlinger H, Weissenhorn W. CC2D1A is a regulator of ESCRT-III CHMP4B. *J Mol Biol*. 2012;419:75–88. doi: 10.1016/j.jmb.2012.02.044
52. McMillan BJ, Tibbe C, Drabek AA, Seegar TCM, Blacklow SC, Klein T. Structural basis for regulation of ESCRT-III complexes by Lgd. *Cell Rep*. 2017;19:1750–1757. doi: 10.1016/j.celrep.2017.05.026
53. Troost T, Jaekel S, Ohlenhard N, Klein T. The tumour suppressor Lethal (2) giant discs is required for the function of the ESCRT-III component Shrub/CHMP4. *J Cell Sci*. 2012;125:763–776. doi: 10.1242/jcs.097261
54. Basel-Vanagaite L, Attia R, Yahav M, Ferland RJ, Anteki L, Walsh CA, Olender T, Straussberg R, Magal N, Taub E, et al. The CC2D1A, a member of a new gene family with C2 domains, is involved in autosomal recessive non-syndromic mental retardation. *J Med Genet*. 2006;43:203–210. doi: 10.1136/jmg.2005.035709
55. Al-Tawashi A, Jung SY, Liu D, Su B, Qin J. Protein implicated in nonsyndromic mental retardation regulates protein kinase A (PKA) activity. *J Biol Chem*. 2012;287:14644–14658. doi: 10.1074/jbc.M111.261875
56. Zhao M, Raingo J, Chen ZJ, Kavalali ET. Cc2d1a, a C2 domain containing protein linked to nonsyndromic mental retardation, controls functional maturation of central synapses. *J Neurophysiol*. 2011;105:1506–1515. doi: 10.1152/jn.00950.2010
57. Li Y, Yagi H, Onuoha EO, Damerla RR, Francis R, Furutani Y, Tariq M, King SM, Hendricks G, Cui C, et al. DNAH6 and its interactions with PCD genes in heterotaxy and primary ciliary dyskinesia. *PLoS Genet*. 2016;12:e1005821. doi: 10.1371/journal.pgen.1005821
58. Rossi A, Kontarakis Z, Gerri C, Nolte H, Höpfer S, Krüger M, Stainier DY. Genetic compensation induced by deleterious mutations but not gene knockdowns. *Nature*. 2015;524:230–233. doi: 10.1038/nature14580
59. Natiq A, Elalaoui SC, Miesch S, Bonnet C, Jonveaux P, Amzazi S, Sefiani A. A new case of de novo 19p13.2p13.12 deletion in a girl with overgrowth and severe developmental delay. *Mol Cytogenet*. 2014;7:40. doi: 10.1186/1755-8166-7-40
60. Kennedy MP, Omran H, Leigh MW, Dell S, Morgan L, Molina PL, Robinson BV, Minnix SL, Olbrich H, Severin T, et al. Congenital heart disease and other heterotaxic defects in a large cohort of patients with primary ciliary dyskinesia. *Circulation*. 2007;115:2814–2821. doi: 10.1161/CIRCULATIONAHA.106.649038
61. Singla V, Reiter JF. The primary cilium as the cell’s antenna: signaling at a sensory organelle. *Science*. 2006;313:629–633. doi: 10.1126/science.1124534
62. Zaki MS, Sattar S, Massoudi RA, Gleeson JG. Co-occurrence of distinct ciliopathy diseases in single families suggests genetic modifiers. *Am J Med Genet A*. 2011;155A:3042–3049. doi: 10.1002/ajmg.a.34173
63. Trulioff A, Ermakov A, Malashichev Y. Primary Cilia as a possible link between left-right asymmetry and neurodevelopmental diseases. *Genes (Basel)*. 2017;8:48. doi: 10.3390/genes8020048