Mono-allelic Mutations in CC2D1A Suggest a Novel Role in Human Heterotaxy and Ciliary Dysfunction

Running title: Ma et al., Novel role of CC2D1A in heterotaxy

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Abstract:

**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 (TALEN) 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 \textit{CC2D1A} gene. Seven occurrences of \textit{CC2D1A} mutations were found to affect four highly conserved amino acid residues of the protein. Functional analyses in the TALEN-mediated zebrafish knock-out models and identified heterotaxy phenotypes of the cardiovascular and gastrointestinal systems in both somatic and germline mutants. Defective cilia were identified by whole-mount immunostaining of acetylated α-tubulin. These abnormalities were rescued by wild-type \textit{cc2d1a} mRNA, but not \textit{cc2d1a} mutant mRNA, strongly suggesting a loss-of-function mechanism. On the other hand, over-expression of \textit{cc2d1a} orthologous mutations \textit{cc2d1a} P559L and \textit{cc2d1a} G808V (orthologous to human \textit{CC2D1A} P532L and \textit{CC2D1A} G781V) did not affect embryonic development.

**Conclusions** - Using a zebrafish model, we were able to establish a novel association of \textit{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 \textit{CC2D1A} in left-right patterning and ciliary dysfunction.

**Key words:** heterotaxy; CC2D1A; ciliary dysfunction; left-right asymmetry
Nonstandard Abbreviations and Acronyms

CNV, copy number variant
LPM, left lateral plate mesoderm
OMIM, online mendelian inheritance in man
PCD, primary ciliary dyskinesia
RFLP, restriction fragment length polymorphism
SNP, single-nucleotide polymorphism
TALEN, transcription activator-like effector nuclease
WES, whole-exome sequencing
WGS, whole-genome sequencing
WISH, whole-mount in situ hybridization

Introduction

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. 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. Cardiovascular malformations are commonly associated with heterotaxy and account for approximately 3% of all congenital heart defects.

The two major subtypes of heterotaxy are left isomerism and right isomerism. 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. 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. 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. Long-term survival of patients with right isomerism is poor, with only 22% of the patients surviving to 14 years of age.

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 signalling pathway, cilia or flagella associated protein-coding genes, and ZIC3, a member of the zinc finger family. The nodal signalling pathway is essential for the activation of left side-specific gene expression in the developing embryo. Embryos lacking nodal expression in the left lateral plate mesoderm (LPM) exhibit multiple L-R patterning defects. The first report of the mutations in the nodal signalling pathway was in a patient with heterotaxy and a de novo reciprocal translocation was found to disrupt the SESN1 gene, which mediates nodal signalling. 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. Examples of autosomal recessive inheritance have also been reported, such as the GDF1 gene with compound heterozygous mutations or homozygous variants in consanguineous families. Other examples include a consanguineous family with a
homozygous deletion in the WDR16 gene\textsuperscript{18}; two affected brothers with a homozygous splice site mutation in the CCDC11 gene\textsuperscript{19}; and nine patients with recessive mutations in the MMP21 gene\textsuperscript{20}. More recently, the PKD1L1 gene has also been implicated in heterotaxy as homozygous loss of function mutations\textsuperscript{21} and the gene has been shown to regulate nodal signalling by acting downstream of nodal flow in knockout models of mice\textsuperscript{22}. In addition, X-linked inheritance was reported in patients with heterotaxy\textsuperscript{14,23}, and ZIC3 was identified by linkage analysis in a large family with X-linked heterotaxy\textsuperscript{24}.

Large-scale copy number variant (CNV) analyses using single-nucleotide polymorphism (SNP) microarrays have led to the discovery of other heterotaxy-related genes including BMP2 and MND\textsuperscript{2}\. Nevertheless, little overlap has been found between SNPs and CNVs. Known heterotaxy mutations are only found in less than 10\%-20\% of all cases when studying CNVs\textsuperscript{6,25}. In addition to microarrays, next-generation sequencing technologies such as whole-exome sequencing (WES) and whole-genome sequencing (WGS) have been used to identify recessive mutations such as in MMP21 in patients with heterotaxy\textsuperscript{10}. Taken together, the use of advanced genomic technologies, the availability of public variant databases, and \textit{in silico} prediction tools have allowed further interrogation of the genetics of heterotaxy\textsuperscript{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\textsuperscript{31}. Nonaka et al showed that in murine, a leftward flow caused by a vortical motion of motile cilia (nodal flow) is related to L-R symmetry.
breaking;\textsuperscript{32} and further confirmed the role of fluid flow in L-R patterning by generating artificial flow of culture medium\textsuperscript{33}. In cardiac development, asymmetries can be induced by defects in the nodal signalling pathway which results in defective cardiac looping\textsuperscript{34}.

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

\textbf{Methods}

Detailed methods are available in the Supplemental Material. 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. Transcription activator-like effector nuclease (TALEN) was used to generate somatic loss-of-function mutants in a zebrafish model. Ciliary defects were examined by whole-mount immunostaining of acetylated \textit{\alpha}-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.
(CULATR, 3919-16, The University of Hong Kong, HK) and Animal Subjects Ethics Sub-Committee (ASESC, 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 WES 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 WES data, we observed a total of 64,562 rare coding changes in all 26 patient samples. Affymetrix Genome-wide Human SNP Array 6.0 was performed for CNV detection, which identified 49 CNVs with a size larger than 1 kbp after annotation by CNVannotator.

Detection of variants in known heterotaxy genes

A list of candidate genes for heterotaxy disorders was generated from OMIM (Online Mendelian Inheritance in Man), HGMD (The Human Gene Mutation Database) and Phenolyzer (Phenotype-based gene analyzer) databases (Supplementary Table S1), 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, PolyPhen-2, LRT, CADD) showed all these mutations were likely to be benign (Supplementary Table S2). 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 to controls. To determine the genes that showed significant enrichment in heterotaxy, SKAT 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= 8,251). 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 [http://exac.broadinstitute.org/](http://exac.broadinstitute.org/))\textsuperscript{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 harbouring two different mutations simultaneously (Table 2 and 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 \textit{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 \textit{CC2D1A} mutations, two of them had dextrocardia (40% of all dextrocardia cases, n=5). Therefore, there may be an association between \textit{CC2D1A} mutations and dextrocardia, however, a larger cohort will be required for a statistically significant observation (Supplementary Table S3).

\textbf{Somatic \textit{cc2d1a} knock-out zebrafish model displayed heterotaxy phenotypes}

To further elucidate the pathogenicity of these variants, we investigated the function of \textit{CC2D1A} using a zebrafish model by assessing the effects on vertebrate embryonic development. The \textit{cc2d1a} gene is highly conserved between humans and zebrafish, consisting of four DM14 domains and the C2 domain (Figure 2A and Supplementary Figure S1). Three of the four candidate mutation spots, P192, P532 and G781, were conserved in zebrafish \textit{cc2d1a}. During early zebrafish embryonic development, \textit{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 onwards, it is predominantly expressed in the head, hatching gland, and developing heart (Figure 2B).

We first generated a somatic loss-of-function mutant using transcription activator-like effector nuclease (TALEN)\textsuperscript{40,41} targeting exon 14 of \textit{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 (RFLP) assay\textsuperscript{40}, and the frame-shifting deletions disrupting the fourth DM14 and C2 domains were confirmed by Sanger sequencing (Supplementary Figure S2A and S2B). At the chosen dose, about 54% of somatic \textit{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 (Supplementary Figure S2C).

Visceral organ development was examined by WISH for *cmle1* 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 Supplementary Figure S3A). In addition, midline and mirrored digestive system were observed (p < 0.0001), which recapitulates the phenotype in human heterotaxy (Figure 3B and Supplementary Figure S3B).

To examine potential gain-of-function in the identified *CC2D1A* mutations, we over-expressed zebrafish orthologous *cc2d1a* mutations (corresponding to human *CC2D1A* P532L and *CC2D1A* G781V) by injecting mRNA encoding *cc2d1a* carrying either P559L (*cc2d1a*<sup>P559L</sup>) or G808V (*cc2d1a*<sup>G808V</sup>) 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 (Supplementary Figure S3).

To elucidate whether such mutations result in loss-of-function, we co-injected either wild-type *cc2d1a*, *cc2d1a*<sup>P559L</sup> or *cc2d1a*<sup>G808V</sup> 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) due to the *cc2d1a* mutation (Supplementary Figure S3), which indicated a loss-of-function mechanism for these mutations.
**Somatic cc2d1a knock-out induced ciliopathy in zebrafish**

Heterotaxy has been well reported to be associated with primary ciliary dyskinesia. Hence, we next examined cilia morphology and function in zebrafish cc2d1a mutant. Zebrafish ventral axis curves and otolith defects were examined, which are surrogate markers of ciliopathy during early embryonic development. 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 (asymmetric 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 (Supplementary Figure S4). 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 (Supplementary Figure S5A). The 7-bp insertion at the TALEN-targeting locus resulted in a premature stop and truncation of the cc2d1a protein before the fourth DM14 domain (Supplementary Figure S5B). The F1 cc2d1a+7 mutants were in-
crossed to produce F2 progeny. Although wild-type (+/+), heterozygous (+/-) and homozygous (-/-) siblings were generated (Supplementary Figure S5C and S5D), all surviving homozygous mutants displayed severe early embryonic deformations and were therefore excluded from the subsequent analyses (Supplementary Figure S5C). Furthermore, heterozygous cc2d1a+/- 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 to 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, regulation of signalling pathways, immune response, synapse maturation and endocytic pathway regulation. 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. 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, previous investigators could not rule out subtle alterations to organ development and postulated that cyanosis might be related to nervous system abnormalities. Drusenheimer et al. used a conditional knock-out 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 knock-out 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. In particular, zebrafish embryos obtain oxygen from the culture medium by simple diffusion to compensate for major respiratory defects. Unlike the mouse knock-out model, zebrafish embryos with TALEN-induced somatic cc2d1a knock-out are more likely to survive for phenotypic analysis.
Our knock-out zebrafish model with \textit{cc2d1a} mutations exhibited obvious heterotaxy and ciliopathy phenotypes, providing additional evidence of the important role of \textit{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 \textit{cc2d1a} mutations in 65\% of the zebrafish. We found that wild-type \textit{cc2d1a} mRNA could partially rescue these phenotypes, but not with mRNA of orthologous mutations. Furthermore, over-expression of \textit{cc2d1a} orthologous mutations did not produce corresponding phenotypes, indicating the abnormal phenotype was due to a loss-of-function rather than gain-of-function or dominant-negative effects of \textit{cc2d1a}.

Similar heterotaxy (mirrored heart and digestive system) and ciliopathy phenotypes were observed in germline heterozygous \textit{cc2d1a} mutant carrying a 7-bp frame-shift insertion, which further confirmed the specificity of the TALEN-mediated \textit{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 \textit{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 \textit{cc2d1a} during embryonic development. Compared with somatic mutants, the penetrance of heterotaxy and ciliopathy in \textit{cc2d1a} mutants was greatly reduced. Although this could be potentially explained by genetic compensation observed in the stable deleterious mutant\textsuperscript{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.

So far, the only human disease reported to be associated with *CC2D1A* is autosomal recessive non-syndromic intellectual disability (ID) (OMIM: 608443). In nine consanguineous families with non-syndromic ID, bi-allelic 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. 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 harboured larger deletions in the *CC2D1A* gene, with 19p13.2-p13.2 deletions resulting in moderate to severe developmental delay. These deletions overlapped *CC2D1A* and other OMIM genes, but there was no mention of any laterality defects. 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 due to...
underlying cilia dysfunction is not new, as approximately 12% of individuals with primary ciliary dyskinesia (PCD) present with heterotaxy⁵, and 50% of PCD 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 pigmentosa⁶¹. The co-occurrence of distinct ciliopathy manifestations within families also suggests the possibility of genetic modifiers⁶². As proposed by Trulioff *et al.*, defects in ciliary proteins may be associated with both neurodevelopmental disorders and visceral asymmetry⁶³. 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 due to 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.

**Conclusion**

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 due to 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 \textit{CC2D1A} in heterotaxy and ciliary dysfunction.

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\textbf{Disclosures:} None

\textbf{References:}

1. Zhu L, Belmont JW, Ware SM. Genetics of human heterotaxias. \textit{Eur J Hum Genet}. 2006;14:17-25. doi:10.1038/sj.ejhg.5201506

2. Maclean K, Dunwoodie SL. Breaking symmetry: a clinical overview of left-right patterning. \textit{Clin Genet}. 2004;65:441-57. doi:10.1111/j.0009-9163.2004.00258.x

3. 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. \textit{Chest}. 2014;146:1176-86. doi:10.1378/chest.13-1704

4. Lin AE, Krikov S, Riehle-olorusso T, Frias JL, Belmont J, Anderka M, Geva T, Getz KD, Botto LD, National Birth Defects Prevention S. Laterality defects in the national birth defects prevention study (1998-2007): birth prevalence and descriptive epidemiology. \textit{Am J Med Genet A}. 2014;164A:2581-91. doi:10.1002/ajmg.a.36695

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5. 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;17:348-57. doi:10.1038/gim.2014.112

6. Sutherland MJ, Ware SM. Disorders of left-right asymmetry: heterotaxy and situs inversus. Am J Med Genet C Semin Med Genet. 2009;151C:307-17. doi:10.1002/ajmg.c.30228

7. Jacobs JP, Anderson RH, Weinberg PM, Walters HL, 3rd, 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

8. Peeters H, Devriendt K. Human laterality disorders. Eur J Med Genet. 2006;49:349-62. doi:10.1016/j.ejmg.2005.12.003

9. 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;149:1509-13. doi:10.1016/j.jtcvs.2015.01.038

10. 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;47:1260-3. doi:10.1038/ng.3376

11. Mohapatra B, Casey B, Li H, Ho-Dawson T, Smith L, Fernbach SD, Molinari L, Niesch 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;18:861-71. doi:10.1093/hmg/ddn411

12. Peeters H, Voz ML, Verschueren K, De Cat B, Pendeville H, Thienpont B, Schellens A, Belmont JW, David G, Van De Ven WJ, et al. Sesn1 is a novel gene for left-right asymmetry and mediating nodal signaling. Hum Mol Genet. 2006;15:3369-77. doi:10.1093/hmg/ddl413

13. 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;26:365-9. doi:10.1038/81695

14. 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

15. 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;64:712-21. doi:10.1086/302289

16. Kaasinen E, Aittomaki 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;19:2747-53. doi:10.1093/hmg/ddq164

17. 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

18. 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;110:1564-74. doi:10.1161/CIRCRESAHA.112.269795

19. 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;23:1262-5. doi:10.1038/ejhg.2014.265

20. 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;49:386-90. doi:10.1136/jmedgenet-2011-100457

21. 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 Med Genet A*. 2016;182:987-993. doi:10.1002/ajmg.a.61509

22. 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

23. 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.ydbio.2012.01.011

24. 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;17:305-8. doi:10.1038/ng1197-305

25. 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 U S A*. 2011;108:2915-20. doi:10.1073/pnas.1019645108

26. Auer PL, Nalls M, Meschia JF, Worrall BB, Longstreth WT, Jr., Seshadri S, Kooperberg C, Burger KM, Carlson CS, Carty CL, et al. Rare and Coding Region Genetic Variants Associated With Risk of Ischemic Stroke: The NHLBI Exome Sequence Project. *JAMA Neurol*. 2015;72:781-8. doi:10.1001/jamaneurol.2015.0582.

27. Genomes Project C, Auton A, Brooks LD, Durbin RM, Garrison EP, Kang HM, Korbel JO, Marchini JL, McCarthy S, McVean GA, et al. A global reference for human genetic variation. *Nature*. 2015;518:52:68-74. 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;4:1073-81. 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*. Jan 2013;Chapter 7:Unit7 20. doi:10.1002/0471142905.hg0720s76

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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;46:310-5. 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;95:829-37. 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;418:96-9. 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;122:2337-43. 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;12:841-3. 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. Sep 2012;Chapter 1:Unit1 13. doi:10.1002/0471250953.bi0113s39

40. Cordell HJ, Topf 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;22:1473-81. doi:10.1093/hmg/dds552

41. Ma AC, McNulty MS, Poshusta TL, Campbell JM, Martinez-Galvez 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;27:451-63. 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, Gilberti 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;93:672-86. doi:10.1016/j.ajhg.2013.08.015

44. Nakamura A, Arai H, Fujita N. Centrosomal Aki1 and cohesin function in separase-regulated centriole disengagement. *J Cell Biol*. 2009;187:607-14. doi:10.1083/jcb.200906019

45. Chang CH, Lai LC, Cheng HC, Chen KR, Syue YZ, Lu HC, Lin WY, Chen SH, Huang HS, Shiu 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;286:7043-51. doi:10.1074/jbc.M110.1164632

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;287:32216-21. doi:10.1074/jbc.C112.394346

47. Hadjighassem MR, Austin MC, Szewczyk B, Daigle M, Stockmeier CA, Albert PR. Human Freud-2/CC2D1B: a novel repressor of postsynaptic serotonin-1A receptor expression. *Biol Psychiatry*. 2009;66:214-22. doi:10.1016/j.biopsych.2009.02.033

48. Ou XM, Lemonde 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;23:7415-25.

49. Rogaeva A, Ou XM, Jafar-Nejad H, Lemonde S, Albert PR. Differential repression by freud-1/CC2D1A at a polymorphic site in the dopamine-D2 receptor gene. *J Biol Chem*. 2007;282:20897-905. doi:10.1074/jbc.M610038200

50. Drusenheimer N, Migdal B, Jackel S, Tveriakhina L, Schulz K, Hering R, Kohrer 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, Miguet N, Avilov SV, Ribeiro EA, Jr., Gottlinger 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, Jaeckel 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(Pt 3):763-76. 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;43:203-10. 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;287:14644-58. 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;105:1506-15. 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, Holper S, Kruger M, Stainier DY. Genetic compensation induced by deleterious mutations but not gene knockdowns. Nature. 2015;524:230-3. 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;115:2814-21. 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;313:629-33. 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:3042-9. 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. doi:10.3390/genes8020048
Table 1. Mutation burden test of \textit{CC2D1A} in cases and in three groups of controls.

| Sample groups       | Sample size | Samples with rare damaging missense mutations in \textit{CC2D1A} | Frequency | Odds ratio | 95% Confidence interval | SKAT p value  | Corrected p value |
|---------------------|-------------|---------------------------------------------------------------|-----------|------------|------------------------|---------------|-------------------|
| Case                | 26          | 6                                                             | 0.23      |            |                        |               |                   |
| Internal Control    | 130         | 2                                                             | 0.02      | 19.2       | 3.6, 101.8             | 3.34E-06      | 3.79E-02          |
| ESP6500 Control     | 6525        | 74                                                            | 0.01      | 26.1       | 10.1, 67.0             | 3.81E-08      | 7.16E-04          |
| ExAC control        | 61486       | 936                                                           | 0.02      | 19.4       | 7.8, 48.4              | 1.97E-07      | 3.70E-03          |

The odds ratio refers to the ratio between the odds of cases with mutations and the odds of controls with mutations.

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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=61486) |
|-------------|----------------------------|----------------------|-------------|-----------------|------------------|---------------|
|             |                            | SIFT PolyPhen2 LRT CADD | Count | Freq. | 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% |

B: Benign, D: Deleterious, LI: Left isomerism, RI: Right isomerism, Freq.: Population frequency

*CC2D1A* transcript NM_017721.4. Homo sapiens (human) genome assembly GRCh37 (hg19).
Figure Legends:

**Figure 1.** QQ plot of the p values derived from SKAT 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.

**Figure 2.** (A) Human *CC2D1A* and zebrafish *cc2d1a* are highly conserved with four 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 harboured two mutations (P192L and G781V) simultaneously, hence a total of seven mutations were identified in six cases (see also Table 2). Zebrafish P559 (TALEN 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 assay; dotted line: The endogenous BclI restriction site used in RFLP assay.

**Figure 3.** The effect of TALEN-induced *cc2d1a* mutation on (A) cardiac development and (B) digestive system development. Number of embryos with each type of phenotype over the total number of embryos analysed in ≥ 3 independent experiments is shown in brackets. L: Liver; IB:
Intestinal Bulb; P: Pancreas. * Please refer to Supplementary Figure S3 for comparison groups; p<0.0125 is considered as statistically significant (after correction for multiple testing).

**Figure 4.** The effect of TALEN-induced cc2d1a mutation on (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 Supplementary Figure S4 for comparison groups; p<0.025 is considered as statistically significant (after correction for multiple testing).

**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 hpf. Number of embryos with each type of phenotype over the total number of F2 embryos analysed in 3 independent experiments is shown in brackets. The number of wild-type (+/+) and heterozygous (+/-) F2 embryos with normal and defective cardiac development in 3 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 analysed in ≥ 3 independent experiments is shown in brackets. L: Liver; IB: Intestinal Bulb; P: Pancreas.
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 analysed in 3 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 3 independent experiments are presented in the graph.

* For statistical comparison, wild-type (+/+) is compared against heterozygous (+/-); p < 0.05 is considered as statistically significant.
**A**

30 hpf

**Control**

- Normal (125/128)
- Normal (50/106)
- Defective cardiac jogging (24/106)
- Midline heart (19/106)
- Bilateral heart (13/106)

**cc2d1a TALEN**

$\text{p < 0.0001}^*$

**cmlc1**

**B**

24 hpf

**Control**

- Normal (144/144)
- Normal (70/126)

**cc2d1a TALEN**

$\text{p < 0.0001}^*$

**foxa3**

- Mirrored digestive system (32/126)
- Midline digestive system (24/126)
A) Normal (111/119) vs. Defective heart development (8/119)

B) Normal (98/101) vs. Mirrored digestive system (3/101)

C) Normal (77/86) vs. Defective cilia development (9/86)

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Number of F2

- Normal: 45, Defective: 66
  - p = 0.0238 *

- Normal: 39, Defective: 59
  - p = 0.2818

- Normal: 30, Defective: 47
  - p = 0.0240 *