

# CIC missense variants contribute to susceptibility for spina bifida

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## Abstract

Neural Tube Defects (NTDs) are congenital malformations resulting from abnormal embryonic development of the brain, spine, or spinal column. The genetic etiology of human NTDs remains poorly understood despite intensive investigation. CIC, homolog of the *Capicua* transcription repressor, has been reported to interact with ataxin-1 (ATXN1) and participate in the pathogenesis of spinocerebellar ataxia type 1. Our previous study demonstrated that CIC loss of function (LoF) variants contributed to cerebral folate deficiency by downregulating folate receptor 1 (FOLR1) expression. Given the importance of folate transport in neural tube formation, we hypothesized that CIC variants could contribute to increased risk for NTDs by depressing embryonic folate concentrations. In this study, we examined CIC variants from whole genome sequencing (WGS) data of 140 isolated spina bifida cases and identified 8 missense variants of CIC gene. We tested the pathogenicity of the observed variants through multiple in vitro experiments. We determined that CIC variants decreased FOLR1 protein level and planar cell polarity (PCP) pathway signaling in a human cell line (HeLa). In a murine cell line (NIH3T3), CIC loss of function variants down regulated PCP signaling. Taken together, this study provides evidence supporting CIC as a risk gene for human NTD.

## INTRODUCTION

Neural Tube Defects (NTDs) are severe congenital malformations in which the neural folds that ultimately give rise to the spine and brain failed to properly close during embryonic development. Spina bifida and anencephaly are the two most common subtypes of NTDs that affect the spinal cord and brain, respectively (Greene & Copp, 2014). Given their severity, NTDs are the leading cause of death in the first year of life. Standard of care for NTD varies greatly across nations. Including anencephaly, which is uniformly fatal, over 75% of NTD-affected births resulted in death before 5 years of age (Blencowe, Kanacherla, Moorthie, Darlison & Modell, 2018). In the industrialized nations, surgical and medical management has improved to the point that the majority of spina bifida affected infants will survive into adulthood (Oakeshott, Hunt, Poulton & Reid, 2010), but will suffer long-term associated disabilities and treatment costs remain major obstacles (Flores, Vellozzi, Valencia & Sniezek, 2014). The worldwide prevalence of NTDs ranges from 1 to 10 per 1000 births, varying widely among different countries and regions. The annual worldwide prevalence of NTD-affected birth was estimated to exceed 260,000 births (Blencowe et al. 2018; Au et al. 2010).

NTDs are complex malformations which have multifactorial etiologies, including genetic, environmental and dietary factors (Copp & Greene, 2013). There is both clinical and experimental evidence that maternal folate status is a nutritional modifier of NTD risks (Caffrey, McNulty, Irwin, Walsh & Pentieva, 2019). Folate molecules participate in one carbon metabolism to provide thymidine and purines for DNA synthesis and

s-adenosyl methionine (SAM), which is the universal methyl group donor required for multiple methylation reactions (Froese Fowler & Baumgartner, 2019). Up to 70% of NTDs may be folate-sensitive and can be prevented by folic acid supplementation prior to conception and continued in early pregnancy (Shlobin, LoPresti, Du & Lam, 2020). Based on this, the United States Food and Drug Administration (FDA) has required mandatory folic acid fortification of grain products including flour, bread, pasta, rice, and cereal in 1998. The United States Centers for Disease Control and Prevention (CDC) has also urged every woman who may become pregnant to obtain at least 400ug of folate every day to prevent NTDs occurrence.

Genetic factors are also considered to be significant contributors to the occurrence of NTDs in both human and mice (Copp & Greene, 2010; Wallingford, Niswander, Shaw & Finnell, 2013; Wilde, Petersen & Niswander, 2014; Greene, Stanier & Copp, 2009). Several studies using *Spotch* mouse mutant have shown that NTDs can be related back to their compromised ability to maintain cellular proliferation and an undifferentiation state of their neuroepithelium which hampers normal neurulation and leads to the failure of neural tube closure. Folic acid supplementation restores proper proliferation in the cranial neuroepithelium and compensated for the loss of *Pax3*, which prevents cranial NTDs and thereby rescues the normal phenotype (Sudiwala et al. 2019). Mutations in the *Vangl2* gene cause craniorachischisis through impaired *Vangl2* interaction with *Dvl1*, *Dvl2*, and *Dvl3* (Torban, Wang, Groulx & Gros, 2004). Furthermore, NTD caused by point mutation in the *Lrp6*<sup>Cd/Cd</sup> canonical WNT coreceptor are rescued both by folic acid and by inhibition of the WNT non-canonical pathway by RhoA inhibitors (Gray et al., 2013; Carter, Ulrich, Oofuji, Williams & Elizabeth Ross, 1999). To date, mutations in more than 300 genes have been reported to cause NTDs in mice (Wilde, Petersen & Niswander, 2014). Multiple signaling pathways, including the Wnt/planar cell polarity (PCP) pathway (Chen et al., 2018), sonic hedgehog (SHH) pathway (Murdoch & Copp, 2010) and mitochondrial folate metabolic pathway (Kim et al., 2018), have been reported to be involved in neural tube closure. However, only a few of these reported murine NTD genes have been positively associated with human NTDs, as the pattern of variants reported in human NTD patients supports a polygenic or oligogenic etiology (Copp & Greene, 2010).

CIC, homolog of the *Drosophila capicua* gene, was discovered in a screen for mutations affecting the antero-posterior pattern of *Drosophila* embryos (Bettegowda et al., 2011). It was found to function downstream of the receptor tyrosine kinase (RTK) pathways that includes the epidermal growth factor receptor, *Torso*, *Ras*, *Raf*, and mitogen-associated protein kinases (MAPKs), which are all related to embryonic pattern formation (Roch, Jiménez & Casanova, 2002). CIC can be transported into the nucleus through interactions with *Karyopherin Subunit Alpha 3* (KPNA3) and serves as a transcription repressor. In the absence of EGFR-ERK signaling, CIC binds to target promoters and/or enhancers and represses downstream genes, while activation of the pathway leads to CIC degradation and activation of genes normally repressed by CIC (Jiménez, Shvartsman & Paroush, 2012; Astigarraga et al., 2007).

Recurrent mutations in CIC were initially identified in oligodendroglioma; subsequently, several CIC aberrations were found in multiple types of cancers including medulloblastoma, breast cancer, and small blue round cell tumors (Wong & Yip, 2020; Huang et al., 2016). In mammals, CIC forms a transcriptional repressor complex with *ATXN1*. Gain of function of the complex underlies the pathogenesis of spinocerebellar ataxia type 1 (Lam et al., 2006), and a spectrum of neurobehavior phenotypes which include hyperactivity, impaired learning and memory, and abnormal maturation and maintenance of upper-layer cortical neurons (Lu et al., 2017). Our previous study demonstrated that CIC acts as a transcription activator of *FOLR1* and CIC loss of function contributes to the occurrence of cerebral folate deficiency through diminished *FOLR1* expression (Cao et al., 2021). Although previous studies have identified mutations in the human CIC gene are associated with neurobehavioral phenotypes, there are no reports of CIC mutations in NTD patients.

To better understand the genetic etiology of human NTDs, we analyzed whole genome sequencing (WGS) data on 140 cases with isolated spina bifida from the U.S. and detected eight rare missense CIC variants. Functional analysis indicated that CIC missense variants identified in NTD cases downregulated the *FOLR1* protein level and PCP signaling in *Hela* and *NIH3T3* cell lines. Overall, our results support for the first time that CIC variants potentially contribute to the etiology of human NTDs.

## MATERIALS AND METHODS

### Human subject

A total of 140 case samples with isolated spina bifida (myelomeningocele) which recently reported in our paper (Wolujewicz et al., 2021) were collected from California, USA and Doha, Qatar. Cases with no other anomalies, only minor anomalies or related anomalies were considered isolated.

### Editorial Policies and Ethical Considerations

The study was approved by institutional review board of Baylor College of Medicine, Stanford University, Weill Medical College of Cornell University and Hamad Medical Corporation. All samples were collected with written informed parental consent. CIC variants from gnomAD database were used as controls.

### Sequencing analysis of NTD samples

The WGS data were processed using standard pipelines, as described in the Broad Institute’s GATK Best Practices (Van der Auwera et al., 2013). Reads were aligned to the hg38 reference provided as part of the GATK Bundle using BWA (Li & Durbin, 2009). Variant calling was performed with GATK4 (Poplin et al., 2018) and joint genotyping was carried out on the whole cohort, followed by Variant Quality Score Recalibration (VQSR). Quality control (following standard practices such as obtaining sequencing metrics, per sample missing rate and level of heterozygosity), was done to check for DNA contamination and identify outliers, removing those samples with poor quality. Per-variant quality was also assessed and only variants with a “PASS” in the filter column were retained and annotated utilizing Ensembl Variant Effect Predictor (VEP) v.95 (McLaren et al., 2016). GnomAD (<https://gnomad.broadinstitute.org/>) database v2.1.1 was used as a reference to determine whether the variant is novel (allele frequency (AF) = 0) or is rare (AF < 0.001). Pathogenic effect prediction of all missense variants were performed using the online program SIFT (Sorting Intolerant From Tolerant; <https://sift.bii.a-star.edu.sg>). All parameters were set as per the software’s default settings. The localization of the variants in their protein domains was assessed by Uniprot (<http://www.uniprot.org/>). Gene lollipop structure was plotted using Lollipops program (Jay & Brouwer, 2016).

All eight CIC variants passed Bamfile checking (IGV) and were then validated by Sanger Sequencing. Variants lollipop plot was generated using the Lollipops software (Rothenberg et al., 2004).

### Plasmids

Wildtype EGFP-CIC plasmid and the R353X EGFP-CIC mutant plasmid were reported in our previous paper (Cao et al., 2021). Variants identified in NTDs were introduced into wildtype EGFP-CIC plasmid using site direct mutagenesis method by Genescript company.

**Cell culture and transfection** HeLa cells and NIH3T3 cells were grown in Dulbecco’s Modified Eagles’s Medium (DMEM, Sigma, D6429), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco) and 1X Antibiotic-Antimycotic (Gibco). NIH3T3 cells were cultured on 0.1% Gelatin (Gibco)-coated plate. Cell cultures were maintained at 37 in a humidified atmosphere containing 5% CO<sub>2</sub>. Transfection was performed at 50-70% confluency using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Medium was replaced with the culture medium without antibiotics prior to transfection. Further experiments were performed 24-48 hours after transfection.

### Immunofluorescence staining

HeLa cells for immunofluorescence staining were plated on glass bottom dishes. 24-48 hours after transfection, cells were fixed with pre-warmed 4% paraformaldehyde for 30 minutes at room temperature. Cells were permeabilized with 0.3% Triton X-100 in TBST for 20 minutes at room temperature, and then were blocked with 10% NGS in TBST for 1 hour at room temperature. FOLR1 antibody (1:100, Proteintech, 23355-1-AP) was diluted in blocking buffer and incubate overnight at 4. 14-16 hours later, cells were rinsed with PBS and incubated with secondary antibody (1:1000, Cell Signaling Technology, 8889S) diluted in blocking buffer for

1 hour in the dark. Cells were rinsed with PBS and allowed to air dry. A drop of antifade mounting medium with DAPI (Invitrogen, P36931) was added to the slides which were covered with a coverslip. Images were taken using a deconvolution microscope (Nikon T2).

### Western Blotting

Cells for western blotting were plated on 6-well plates (Corning). 48 hours post-transfection, HeLa cells and NIH3T3 cells were rinsed with ice-cold PBS, collected with cell scrapers and lysed with RIPA buffer (Thermo Scientific, 89900) with protease inhibitor (Sigma, 11836170001) for 30 minutes on ice, and were then centrifuged at 14000rpm for 20 minutes. The supernatant was transferred to a new tube to which SDS loading buffer was added and mixed before being boiled for 10 minutes at 95. Western Blotting was then performed as previously described. The proteins were immunoblotted with GFP antibody (1:1000, Santa Cruz Biotechnology, sc-9996), FOLR1 antibody (1:1000, Invitrogen, PA5-86666), GAPDH antibody (Cell Signaling Technology, 5174S), Vangl2 antibody (1:1000, Proteintech, 21492-1-AP), RhoA antibody (1:500, Santa Cruz Biotechnology, sc-418) and CIC antibody (Cat#: A301-204A, Bethyl, TX) overnight at 4. On the second day, the membrane was incubated with HRP-conjugated anti-rabbit antibody or anti-mouse antibody (1:5000, Cell Signaling Technology, 7074S and 7076S) for 1 hour at room temperature. Membranes were rinsed with ECL substrates (Thermo Scientific, 34580) and protein bands were detected using a BioRad Gel Doc XR+ Imaging System.

### Folate binding assay and folate quantification

HeLa cells were passaged and cultured in RPMI 1640 folate-free media (Sigma, R1145), supplemented with 10% FBS, 3.7g/L sodium bicarbonate, 1X glutamax (Gibco, 35050-061) and 1X Antibiotic-Antimycotic. Cells were transfected with wildtype and variant GFP-CIC plasmids when reaching 50% confluency. 36-48 hours later, culture medium was replaced with PBS supplemented with 2% FBS and 50nM folic acid (Sigma, F8758). Cells were cultured for 1 hour in the presence of FA, after which they were dissociated with 0.25% trypsin. Dissociated cells were resuspended in TBST with 1% ascorbic acid (pH 3.0-3.5) and incubated at 95 for 10 minutes. Supernatant was collected after being centrifuged for 10 minutes at 14000rpm. NaOH (1M) was added to collected supernatant at a ratio of 1:15 to reach a neutral pH value.

Bovine folate binding protein (50ng/ul, Sigma, F0504-1MG) was printed on a 96 well plate with 1ul in each well, and the plate was then sealed and incubated overnight at 4. On the second day, HRP-FA was 2-fold serially diluted to create a standard curve. Samples and HRP-FA were mixed at a ratio of 4:1. The printed 96 well plate was washed with TBST, and the mixed samples and standards were added into the wells and incubated for 1 hour prevented from light. The plate was washed with TBST, and 50ul of ELISA substrate were added to each well. Signal intensities were imaged using Q-view equipment and quantified using the Q-view software.

### Statistical analysis

All data were analyzed with a Student's t-test, and P-values < 0.05 were considered statistically significant. Data are presented as mean +- SE of the mean. All data were analyzed using two-tailed t-test.

## RESULTS

### Rare missense variants in CIC are associated with human NTD risks

WGS was performed on DNA samples from 140 spina bifida cases. Eight rare missense variants (allele frequency < 0.001 in gnomAD) in CIC gene were identified in 140 individuals (Table 1). Seven of the eight missense variants were predicted to be damaging using Polyphen or SIFT software, indicating they are likely pathogenic mutations. Lollipop plots showed that none of the eight missense mutations locates in the HMG box region (Fig. 1A). All eight variants were evolutionarily conserved across multiple mammalian species (Fig. 1B). Among these missense variants, p. E1452D and p. M671V were absent in the gnomAD database, indicating that they are novel variants. Notably p. M671V is also predicted-to-be-damaging by both prediction software and locates in a highly conserved region. All eight variants were heterozygous in

the seven different infants with isolated spina bifida (myelomeningocele). Three CIC variants carriers were collected from California before folic acid fortification. One case carried biallelic CIC variants (p.R666H and p.R1205Q).

### **CIC missense variants affected protein expression levels but not localization**

To determine whether the variants affect CIC protein subcellular localization or their level of expression, functional analyses were performed on the CIC variants. As shown in Fig. 2A, wildtype GFP-CIC was primarily located in the nucleus as expected. All eight variants were also located in the nucleus, indicating that none of these variants affected the cellular localization of CIC protein. Interestingly, we noticed that some of the mutants showed a weaker nuclear staining under the same exposure conditions. We speculated that the expression level of CIC may be affected by the missense mutations. We subsequently performed western blotting assays to investigate the effect of these variants on the CIC protein levels. As shown in Figure 2B, GFP-CIC protein of all eight variants were less abundant compared to the wildtype ( $p_i0.05$ ), which demonstrated that the missense variants may decrease CIC protein expression or potentially affect protein stability.

### **CIC missense variants affected FOLR1 protein level and cellular folate concentration**

We have previously found that CIC is capable of binding to FOLR1 promoter region, thereby, regulating its transcription (Cao et al., 2021). FOLR1 is a critically important transport molecule involved in the uptake of folates into the cells and plays a crucial role in neural tube closure. In mice, *Folr1* knock-out lead to 100% penetrant spina bifida. In humans, functional disruption of FOLR1 causes cerebral folate deficiency syndrome (CFD), while increased titers of FOLR1 autoantibody in maternal serum was reported to be associated with both NTDs (Rothenberg et al., 2004; Cabrera, et al., 2008) and CFD (Ramaekers et al., 2005). We examined the effect of variant containing CIC on FOLR1 protein levels in HeLa cells. As presented in Figure 3A, wildtype GFP-CIC increased FOLR1 expression compared to the control pEGFP plasmid ( $p_i0.001$ ), while overexpression of all GFP-CIC mutants profoundly downregulated FOLR1 expression compared to wildtype( $p_i0.01$ ).

We further investigated the effect of the CIC variants on folate absorption ability in HeLa cells. As expected, wildtype CIC overexpression increased intracellular folate concentrations compared to the pEGFP control, while overexpression of CIC mutants reduced cellular folate content compared to wildtype ( $p_i0.05$ ) (Fig. 3C), indicating that CIC mutants can reduce folate binding and/or absorption ability by downregulating FOLR1.

### **CIC missense variants downregulated PCP pathway protein Vangl2 and RhoA**

The PCP signaling pathway has been shown to play an important role in neural tube closure in multiple previous studies (Copp, Greene & Murdoch, 2003; Wallingford, 2006; Wallingford, 2012; Murdoch et al., 2014). Genetic defects in the *Vangl2* gene have been strongly associated with NTDs in mouse models, and there is growing evidence implicating *Vangl* genes (*Vangl1* and *Vangl2*) in the genetic etiology of human NTDs (Kibar et al., 2007; Lei et al., 2010; Kibar et al., 2011; Tian et al., 2020). To investigate whether dysregulation of the PCP pathway also occurs in CIC variants associated spina bifida, we performed western blotting to examine the level of the PCP signaling pathway core protein *Vangl2* and PCP downstream protein *RhoA* using overexpression wildtype CIC and CIC mutants in HeLa cells. As shown in Fig. 4, *VANGL2* and *RhoA* protein levels in the wildtype CIC overexpression group were significantly increased compared to the pEGFP control ( $p_i0.01$ ), while *VANGL2* and *RhoA* protein levels in those groups with overexpressed CIC mutants were less abundant compared to wildtype ( $p_i0.05$  and  $p_i0.01$ , respectively). Protein levels of *RhoA* in each sample were consistent with those of *Vangl2*. We further investigated the effects of CIC LoF mutation on PCP pathway proteins *Vangl2* and *RhoA* in NIH3H3 cells that were overexpressed with wildtype CIC, CIC-R353X and pEGFP control. As depicted in Fig. 5, both *Vangl2* and *RhoA* showed increased protein levels in GFP-wildtype CIC transfection compared to EGCP1 basic control vector transfection, and decreased protein level in CIC-R353X group compared to WT( $p_i0.01$ ). These results support that CIC variants may also modify NTD risk through PCP signaling pathway.

## DISCUSSION

Previous studies have indicated that CIC interaction with ATXN1 contributes to the pathogenesis of spinocerebellar ataxia type 1 (Rousseaux et al., 2018). Disruption of CIC-ATXN1 complex results in hyperactivity, impaired learning and memory, and abnormal maturation and maintenance of upper-layer cortical neurons in *Emx1-cre* mice (Cao et al., 2021). Forebrain-specific deletion of CIC in *Foxg1-cre* mice caused abnormal increases in oligodendrocyte progenitor and immature oligodendrocytes populations (Yang et al., 2017). Brain-specific deletion of CIC compromised neuroblasts transition to immature neurons in mouse hippocampus and compromises normal neuronal differentiation (Hwang et al., 2020). While CIC deletion clearly impacts numerous aspects of neurodevelopment, ours is the first evidence indicating a potential relationship between CIC and neural tube development.

In the present study, we performed and support the first genetic association analysis between CIC variants and an increased risk for NTDs. We also explored the possible underlying mechanisms by which CIC could contribute to NTDs in humans. We initially focused on the folate receptors, as they are membrane proteins that mediate cellular uptake of folates. FOLR1 is important for neural tube closure during early embryogenesis, as inactivation of this gene leads to embryonic death and NTDs in mice which can be rescued by folate supplementation (Piedrahita et al., 1999). In humans, homozygous biallelic LoF FOLR1 mutations lead to extremely low 5-methyltetrahydrofolate level in the cerebrospinal fluid (CFD) but have not been observed to result in NTDs (Steinfeld et al., 2009). However, it has been reported that increased level of maternal serum FOLR1 autoantibody during pregnancy is associated with the occurrence of fetal NTDs (Rothenberg et al., 2004). Although evidence is lacking to show that isolated FOLR1 gene variants are associated with human NTDs, Saitsu (2017) found that altered spatial and temporal *Folr1* expression patterns in mice are associated with anterior neural tube closure, and this expression pattern is conserved between human and mice engineered to express a lacZ reporter transgene. Additionally, there have been studies of human folate transport genes that described 12 novel variants in FOLR1, FOLR2, and FOLR3 (Findley et al., 2017) found in NTD cases. This included four large insertion deletion variants in FOLR3 as well as a single stop gain variant. While far from being conclusive evidence, it is suggestive that FOLR1 abnormalities might be involved in a subset of human NTDs. According to our previous study of CIC and CFD, CIC could regulate FOLR1 expression through binding to its promoter region. In this study, we confirmed that CIC variants can decrease FOLR1 expression levels in human cell lines. While we previously found that CIC binding motifs lie within the human FOLR1 promoter region, they do not exist in the mouse *Folr1* promoter region.

Convergent extension (CE) is a crucial process during neural tube closure by which the neural plate undergoes narrowing along its mediolateral axis and extends along anteroposterior axis (Tada & Heisenberg, 2012). The progression of convergent extension is driven by planar polarized cell intercalation, which in turn is reported to be driven by subcellular processes including extension of mediolaterally directed cellular protrusions and shrinkage of mediolaterally oriented cell-cell junctions (Butler & Wallingford, 2018; Blankenship, Backovic, Sanny, Weitz & Zallen, 2006). The PCP signaling pathway is the most well-characterized regulator of cell intercalation (Butler & Wallingford, 2017). Several genes in the PCP pathway have been associated with NTDs (Humphries, Narang & Mlodzik, 2020). Over 300 genes are known to be causative of NTDs in mice, and many of them participate in the PCP pathway (Wang et al., 2018). Core PCP genes are highly conserved from invertebrates to mammals, among which Van Gogh (*Vang*), *Vangl1/2* in mammals, is the critical regulator for normal extension conversion. Other PCP genes include *FZD3/FZD6*, *CELSR1/CELSR2/CELSR3*, *DVL1/DVL2/DVL3*, *PRICKLE1/PRICKLE2/PRICKLE3/PRICKLE4* and *ANKRD6*. *Vangl2* was the first genetically mapped PCP gene through studying Loop-tail mutant mice. Homozygous *Vangl2* mutants cause craniorachischisis, the most severe type of NTD (Kibar et al., 2001), while *Vangl1* and *Vangl2* compound heterozygous mice also exhibit a craniorachischisis phenotype (Torban et al., 2008). Besides *Vangl2*, inactivation of *Dvl1* and *Dvl2*, *Celsr1*, and *Fzd3* or both *Fzd3* and *Fzd6* also lead to severe NTD phenotypes, primarily craniorachischisis and exencephaly in mice (Kibar et al., 2001; Wang et al., 2006; Ybot-Gonzalez et al., 2007; Curtin et al., 2003). In addition to these core PCP genes, some noncore PCP genes also exhibit severe NTD in mice when gene targeted, such as protein tyrosine kinase 7 (PTK7), scribbled PCP protein, the gene responsible for the circle tail mouse phenotype, *Scrib*, and dishevelled binding antagonist of beta-

catenin 1 (Dact-1) (Mohd-Zin, Marwan, Abou Chaar, Ahmad-Annuar & Abdul-Aziz, 2017). In humans, PCP genes have been examined in case–control association studies or directly sequenced in mutation screens (Juriloff & Harris, 2012). Vangl2 variants in human NTD samples were reported by Lei et al.(2010) and Kibar et al.(2011). In the present study, we found that all eight CIC variants lead to decreased Vangl2 protein levels in human cell lines, and the CIC LoF variant also limited Vangl2 protein levels in a murine cell line. We suspect this contributes to the observed NTD phenotype.

Acting downstream of Vangl2, RhoA plays a crucial role in regulating cell shape and movement (Oishi, Makita, Sato & Iiri, 2012). In this study, protein levels of Vangl2 and RhoA showed similar trends for different CIC variants among groups in both human and mouse cell lines. Our *in vitro* assays showed that CIC variants diminished both FOLR1 expression level and PCP signals, both of which could increase the likelihood of human NTDs. However, the relationship between inhibiting Folr1 and associated PCP pathway gene expression and the underlying mechanisms by which CIC affects Vangl2, remain unclear. Balashova, Visina and Borodinsky (2017) found that FOLR1 is enriched in the apical surface of the neural plate in *Xenopus laevis*, colocalized with C-cadherin and  $\beta$ -catenin, and that FOLR1 is necessary for neural plate cell apical constriction during *Xenopus* neural tube formation. The seemingly contradictory evidence emphasizes the importance of continued experimentation on this system.

In this study, we identified a novel gene contributing to human NTDs. We demonstrated the possible underlying mechanisms. Future studies are needed to further elucidate these cellular and molecular mechanisms, which will help us to better understand the genetic etiology of NTDs and develop effective strategies to prevent these severe birth defects.

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## COMPETING INTERESTS

R.H.F., B.W.J. and R.M.C. formerly held leadership positions with the now dissolved TeratOmic Consulting LLC. R.H.F. also receives travel funds to attend editorial board meetings of the Journal of Reproductive and Developmental Medicine published out of the Red Hospital of Fudan University. The other authors declare no competing interests.

## DATA AVAILABILITY

Data pertaining to specific variants generated during the downstream analyses, which support the findings of this study, are available upon request to the corresponding author (R.H.F). De-identified data will be made available upon request.

## AUTHOR CONTRIBUTIONS

X.H., X.C., Y.L., B.J.W and R.H.F. conceived and designed the experiments. Y.L., X.C. and V.A.P. generated and analyzed sequencing data. X.H., X.C., R.M.C., P.A.P.R and M.K performed cell-based functional analysis. W.Y., G.M.S., L.L.W., A.R., M.E.R., and R.H.F. identified NTD cases and acquired clinical data. X.H., X.C., Y.L., and R.H.F. drafted the manuscript, and all other authors assisted in editing.

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Table1 Information of the detected CIC rare missense variants in infants with NTDs

Figure 1 Identification of CIC rare missense variants in infants with NTDs. (A) Protein amino acid locus of CIC missense variants. (B) Amino Acid conservation of identified variants among different species.

Figure 2 Subcellular localization and protein abundance of CIC wildtype and CIC variants. (A) HeLa cells were transfected with mutated and wildtype constructs of GFP-tagged CIC and pEGFP backbone vector for 36h and were imaged under deconvolution microscope. Scale bar 5 $\mu$ m. (B) Western Blotting was performed in HeLa cells 48h after transfection. GAPDH was used as loading control. (C) Western Blotting was repeated for three times and student t-test was performed to compare the protein level between wildtype and mutant.

Figure 3 Overexpression of CIC mutants affected FOLR1 protein level and folate binding ability of HeLa cells. (A) HeLa cells were transfected with mutated and wildtype constructs of GFP-tagged CIC and pEGFP backbone vector for 48h, and western blotting was performed to quantify FOLR1 protein level in each group. GAPDH was used as loading control. (B) Western blotting was repeated for three times and Student's t-test was performed to compare the protein level between wildtype and mutant. (C) Folate of HeLa cells transfected with CIC wildtype and variants were collected and quantified in triplicates.

Figure 4 Overexpression of CIC mutants affected core PCP protein Vangl2 and its downstream protein RhoA in HeLa cells. (A) HeLa cells were transfected with mutated and wildtype constructs of GFP-tagged CIC and pEGFP backbone vector for 48h, and western blotting was performed to quantify Vangl2 and RhoA protein level in each group. GAPDH was used as loading control. (B) Western blotting was repeated for three times and Student's t-test was performed to compare Vangl2 and RhoA protein level between wildtype and mutant.

Figure 5 CIC loss of function diminished core PCP protein Vangl2 and its downstream protein RhoA in NIH3T3 cells. (A) NIH3T3 cells were transfected with CIC wildtype, CIC-R353X construct and pEGFP backbone vector for 48h, and Western Blotting was performed to quantify GFP-CIC, Vangl2 and RhoA

protein level in each group. GAPDH was used as loading control. (B) Western Blotting was repeated for three times and student t-test was performed to compare Vangl2 and RhoA protein level between wildtype and R353X.

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