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***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 the cerebral folate deficiency syndrome 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*

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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.

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.

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.

Keywords

CIC; NTDs; PCP signaling pathway; FOLR1

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 (Iskandar & Finnell, 2022; Greene & Copp, 2014). Given their severity, NTDs are the leading cause of death in the first year of life. Standard of care for NTDs 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, Kancherla, 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 (Iskandar & Finnell, 2022; 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 beginning 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 (Iskandar & Finnell, 2022; Copp & Greene, 2010; Wallingford, Niswander, Shaw & Finnell, 2013; Wilde, Petersen & Niswander, 2014; Greene, Stanier

& Copp, 2009). Several studies using the Splotch 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^{Cd/Cd}* 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 & 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 anteroposterior 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). 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°C in a humidified atmosphere containing 5% CO₂. 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 incubated overnight at 4°C. 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°C. 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°C. 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.

Real-time quantitative PCR

Total RNA of cells was extracted using TRIzol reagent as previously described, and RNA concentrations were adjusted to 100ng/ul. Reverse transcription PCR was performed using High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, 4368813),

and real-time quantitative PCR was performed using SYBR GREEN PCR Master Mix (ThermoFisher Scientific, 4309155). Primers were synthesized by Integrated DNA Technologies (IDT). *GAPDH* forward primer sequence: CTCCTCCTGTTCGACAGTCA, reverse primer Sequence: CAATACGACCAAATCCGTTG; *ETV4* forward primer sequence: CAGTGCCTTTACTCCAGTGCC, reverse primer sequence: CTCAGGAAATTCCGTTGCTCT; *ETV5* forward primer sequence: TCAGCAAGTCCCTTTTATGGTC, reverse primer sequence: GCTCTTCAGAATCGTGAGCCA; *FOLR1* forward primer sequence: GCTCAGCGGATGACAACACA, reverse primer sequence: CCTGGCCCATGCAATCCTT.

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°C 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°C. 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 \pm 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). 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.Glu1452Asp and p.Met671Val were absent in the gnomAD database, indicating that

they are novel variants. Notably p.Met671Val 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.Arg666His and p.Arg1205Gln).

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. Transfection efficiency of different *CIC* plasmids was controlled by relative quantification of GFP DNA amount in cells. As presented in Supplementary Figure 1, the relative GFP/GAPDH DNA quantification demonstrated that there was no transfection efficiency difference between different *CIC* expression plasmids. As shown in Figure 2B, GFP-*CIC* protein of all eight variants were less abundant compared to the wildtype ($p < 0.05$), which demonstrated that the missense variants may decrease *CIC* protein expression or potentially affect protein stability.

To further explore the mechanism that these missense variants decrease *CIC* protein expression, cycloheximide (CHX), a protein synthesis inhibitor, was used to explore the protein stability. Cells were treated with 10 μ g/ml CHX and DMSO, respectively, for 24 hours and WB was performed. As presented in Supplementary Figure 2, less mutant *CIC* protein was detected compared with wildtype *CIC* protein after CHX treatment, indicative that the missense variants may decrease *CIC* protein amount through affecting protein stability.

We also performed RT-qPCR on *ETV4* and *ETV5*, which are well-known *CIC* target genes. As shown in Supplementary Figure 3, compared with wildtype *CIC*, overexpression of mutant protein significant increased *ETV4* and *ETV5* gene expression, indicated that these variants were loss of function variants.

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* inactivation leads to 100% penetrant exencephaly. 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 *CIC* variants on *FOLR1* protein

and mRNA levels in HeLa cells. As presented in Figures 3A and 3D, wildtype GFP-CIC increased FOLR1 expression compared to the control pEGFP plasmid ($p < 0.001$), while overexpression of all GFP-CIC mutants profoundly downregulated FOLR1 expression compared to wildtype ($p < 0.01$).

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 ($p < 0.05$) (Fig. 3C), indicating that *CIC* mutants can reduce folate binding and/or absorption ability by downregulating FOLR1.

In our study, one case carried biallelic *CIC* variants (p.Arg666His and p.Arg1205Gln). To further identify whether biallelic *CIC* mutations could cause more severe functional loss than single mutation, we co-transfected *CIC*-WT and *CIC*- p.Arg666His, *CIC*-WT and *CIC*- p.Arg1205Gln, *CIC*- p.Arg666His and *CIC*- p.Arg1205Gln, respectively, and performed RT-QPCR and western blot analysis. As seen in Supplementary Figure 4, compared with single *CIC* variant, biallelic *CIC* variants overexpression had lower FOLR1 gene expression, which demonstrated that these variants might contribute to susceptibility for spinal bifida.

***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 ($p < 0.01$), while *VANGL2* and *RhoA* protein levels in those groups with overexpressed *CIC* mutants were less abundant compared to wildtype ($p < 0.05$ and $p < 0.01$, 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*-p.Arg353Ter and pEGFP control. As depicted in Fig. 5, both *Vangl2* and *RhoA* showed increased protein levels in GFP-wildtype *CIC* transfection compared to EGPC1 basic control vector transfection, and decreased protein level in *CIC*- p.Arg353Ter group compared to WT ($p < 0.01$). These results support the idea that *CIC* variants may also modify NTD risk through impacting the 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, Saito (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 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 during early development. Other PCP genes include *FZD3/FZD6*, *CELSR1*, *DVL1/DVL2/DVL3*, *PRICKLE1/PRICKLE2/PRICKLE3* 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 & Iri, 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 β -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 the etiology of 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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. Newly reported CIC variants p.M671V and p.E1452D have been submitted to LOVD database. URLs for the two variants are as follows: p.M671V: <https://databases.lovd.nl/shared/individuals/00416321>; p.E1452D: <https://databases.lovd.nl/shared/individuals/00416322>

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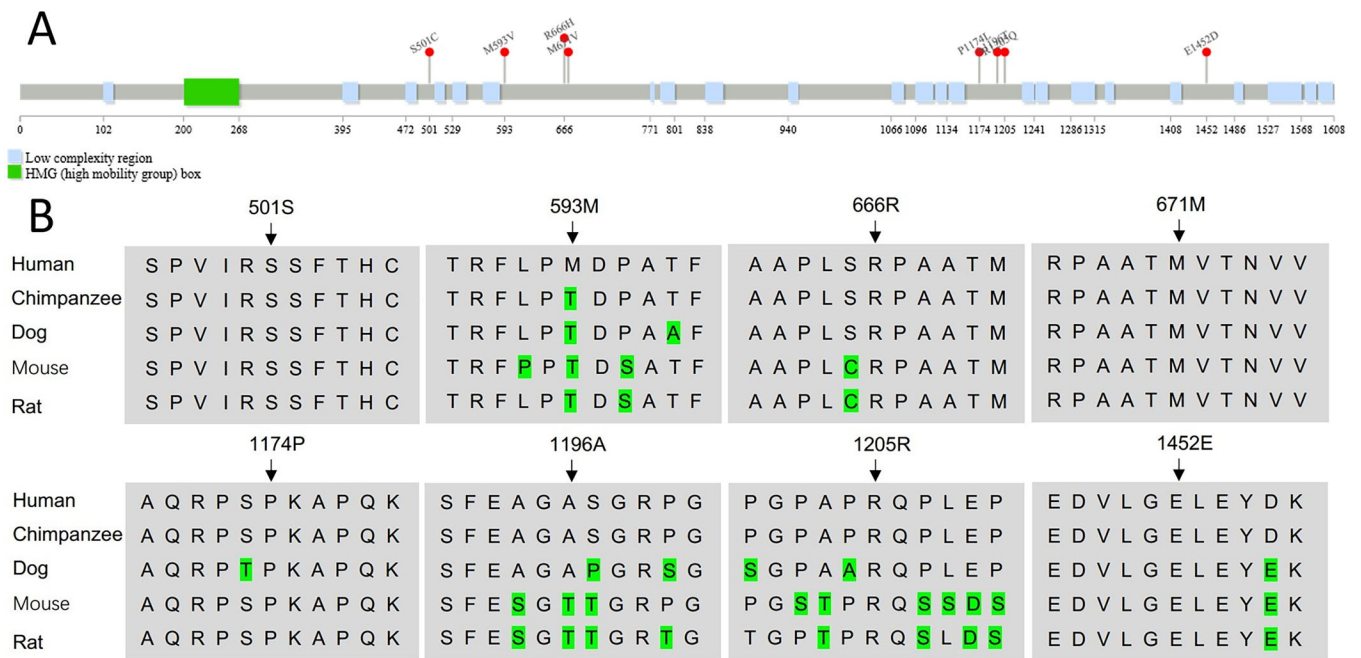


Figure 1. Identification of CIC rare missense variants in infants with NTDs. (A) Protein amino acid locus of CIC missense variants. (B) Amino Acid conservation of identified variants among different species.

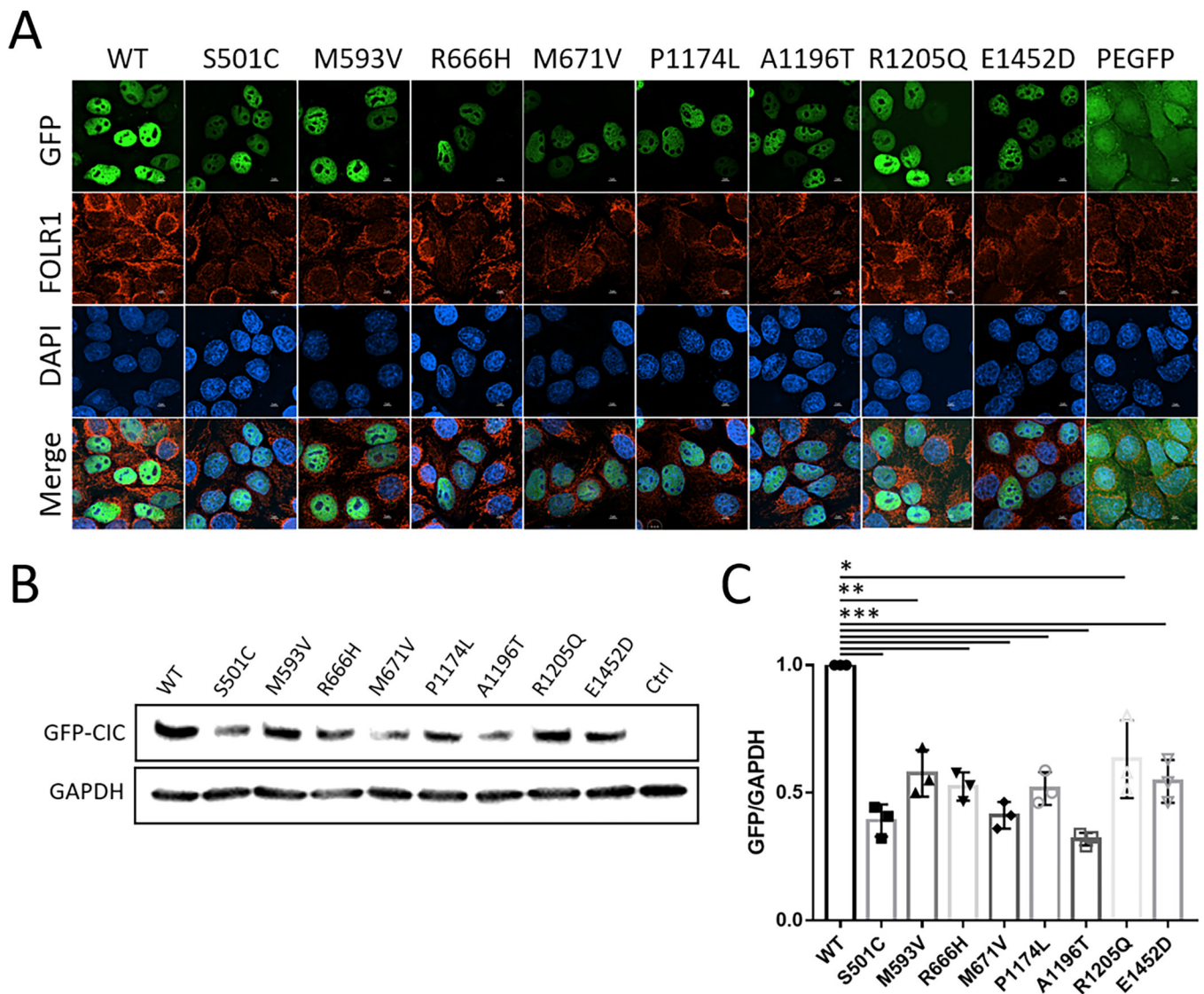


Figure 2. Subcellular localization and protein abundance of CIC wildtype and CIC variants. (A) HeLa cells were transfected with mutated and wildtype constructs of GFP-tagged CIC and pEGFP backbone vector for 36h and were imaged under deconvolution microscope. Scale bar 5um. (B) Western Blotting was performed 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.

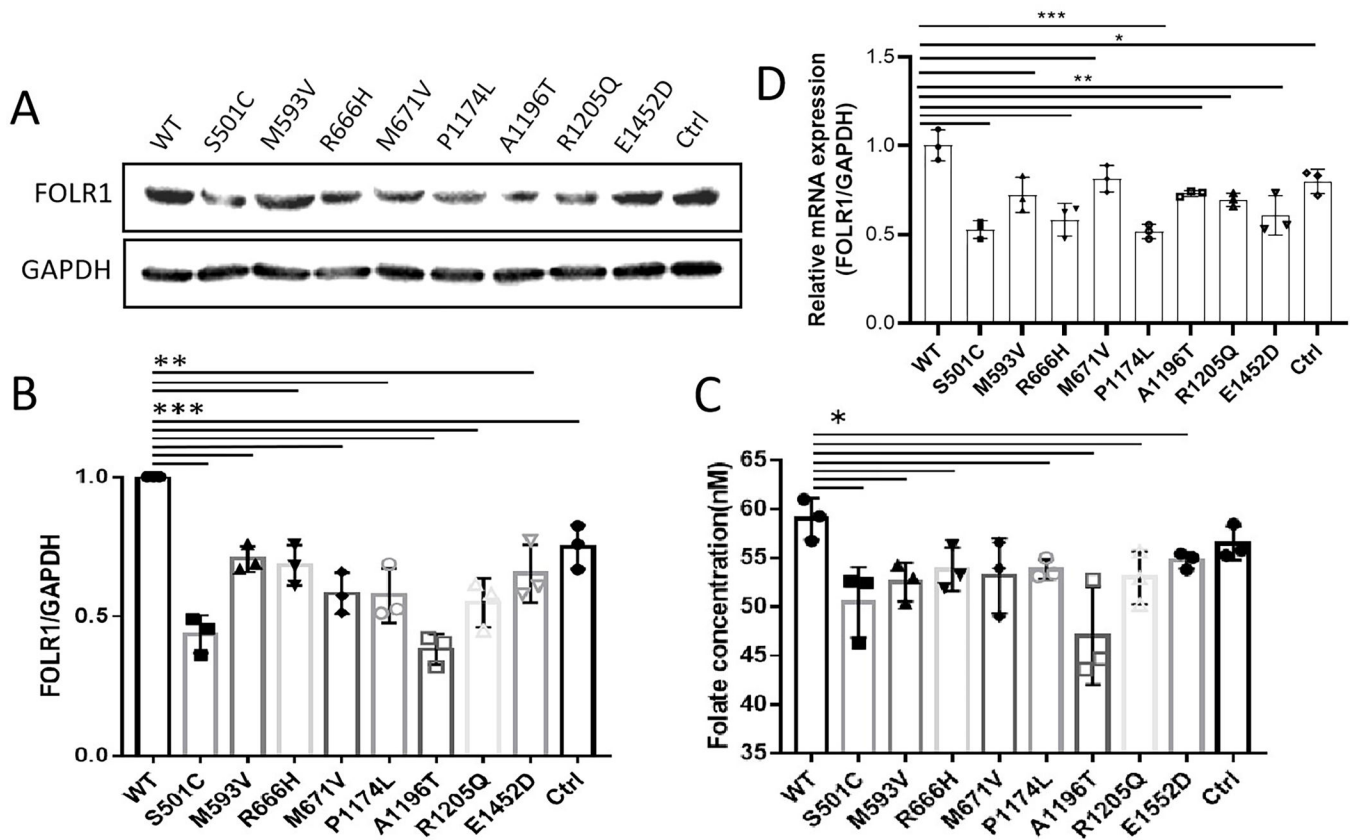


Figure 3.

Overexpression of CIC mutants affected FOLR1 protein level and folate binding ability of HeLa cells. (A, D) HeLa cells were transfected with mutated and wildtype constructs of GFP-tagged CIC and pEGFP backbone vector for 48h, and western blotting and RT-PCR was performed to quantify FOLR1 mRNA and 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.

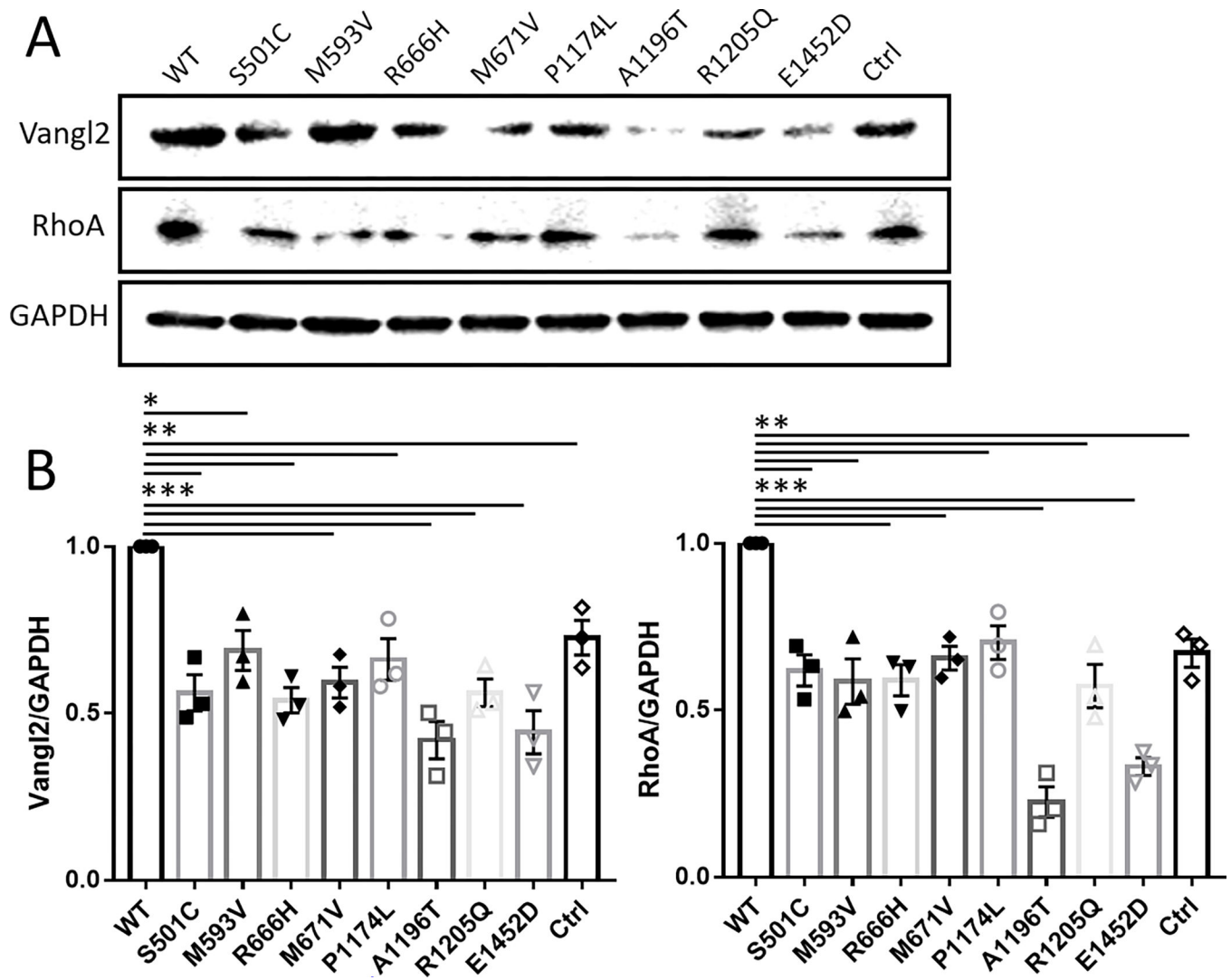


Figure 4.

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.

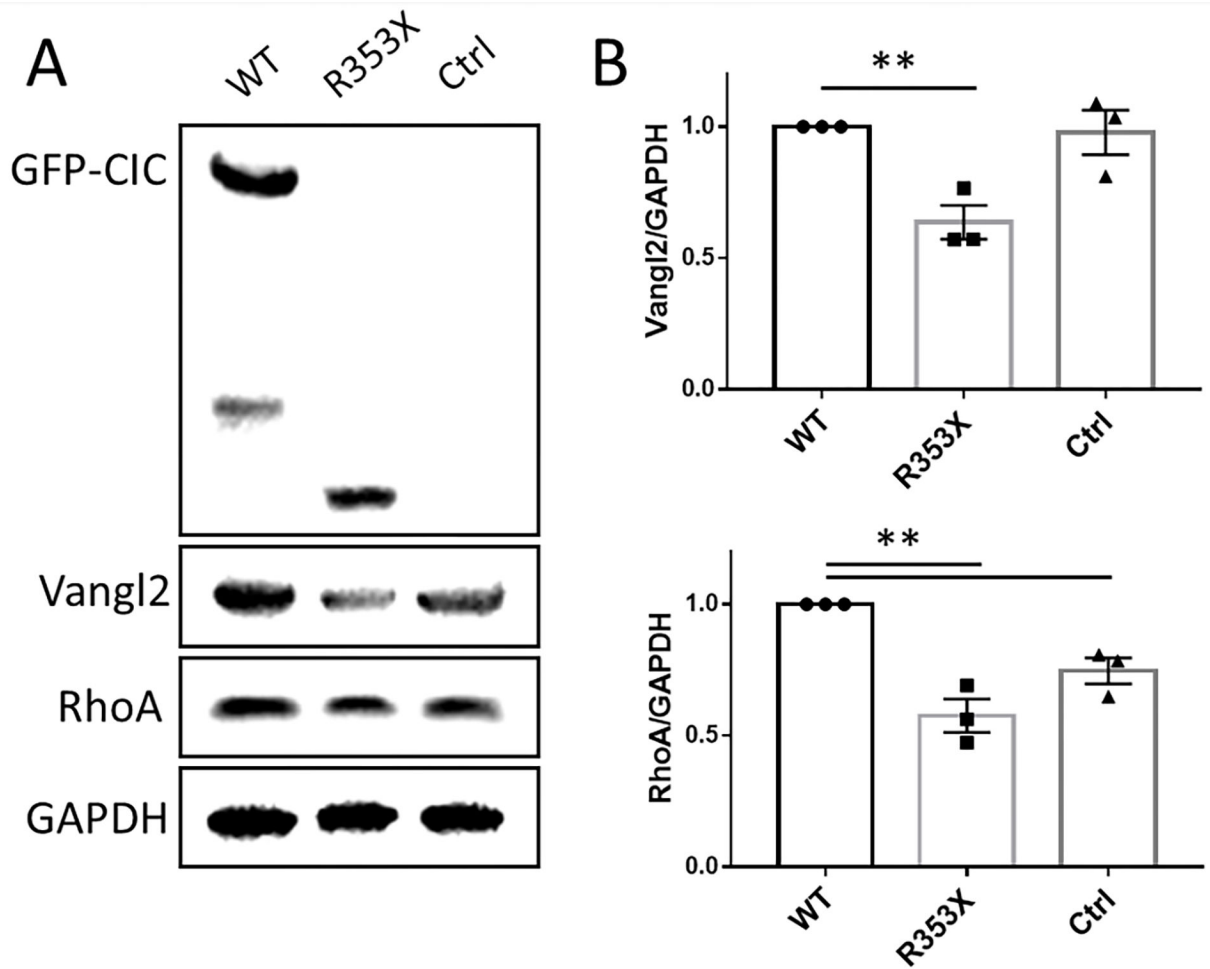


Figure 5.

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 p.Arg353Ter 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 p.Arg353Ter.

Table 1Information of the detected *CIC* rare missense variants in infants with NTDs

Patient	Sex	Nucleotide Change(hg19)	HGVSc	HGVSp	Amino Acid Change	gnomAD Frequency
1	Male	chr19:42794422:C>G	ENST00000575354.2:c.1502C>G	ENSP00000458663.2:p.Ser501Cys	p.S501C	3.98E-06
2	Male	chr19:42794697:A>G	ENST00000575354.2:c.1777A>G	ENSP00000458663.2:p.Met593Val	p.M593V	6.04E-05
3	Male	chr19:42794917:G>A	ENST00000575354.2:c.1997G>A	ENSP00000458663.2:p.Arg666His	p.R666H	2.56E-05
4	Female	chr19:42794931:A>G	ENST00000575354.2:c.2011A>G	ENSP00000458663.2:p.Met671Val	p.M671V	0
5	Male	chr19:42797159:C>T	ENST00000575354.2:c.3521C>T	ENSP00000458663.2:p.Pro1174Leu	p.P1174L	0.000216
6	Male	chr19:42797224:G>A	ENST00000575354.2:c.3586G>A	ENSP00000458663.2:p.Ala1196Thr	p.A1196T	0.000107
7	Male	chr19:42797252:G>A	ENST00000575354.2:c.3614G>A	ENSP00000458663.2:p.Arg1205Gln	p.R1205Q	2.14E-05
8	Female	chr19:42798784:G>C	ENST00000575354.2:c.4356G>C	ENSP00000458663.2:p.Glu1452Asp	p.E1452D	0

WGS was performed on DNA samples from 140 spina bifida cases. Eight rare missense variants in *CIC* gene were identified in 140 individuals.