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Rare deleterious *PARD3* variants in the aPKC-binding region are implicated in the pathogenesis of human cranial neural tube defects via disrupting apical tight junction formation

Xiaoli Chen¹, Yu An², Yonghui Gao^{1,3}, Liu Guo⁴, Lei Rui⁵, Hua Xie¹, Mei Sun⁶, Siv Lam Hung⁷, Xiaoming Sheng⁷, Jizhen Zou⁸, Yihua Bao¹, Hongyan Guan⁹, Bo Niu¹⁰, Zandong Li⁵, Richard H. Finnell¹¹, James F. Gusella^{6,12}, Bai-Lin Wu^{2,7,13,*}, and Ting Zhang^{1,*}

¹Beijing Municipal Key Laboratory of Child Development and Nutriomics, Capital Institute of Pediatrics, Beijing, 100020, China

²Children's Hospital and Institutes of Biomedical Science, Shanghai Medical College of Fudan University, Shanghai, 200032, China

³Institute of Acu-moxibustion, China Academy of Chinese Medical Sciences, Beijing, 100700, China

⁴Department of Neurology, Affiliated Children's Hospital of Capital Institute of Pediatrics, Beijing, 100020, China

⁵State Key Laboratories for Agrobiotechnology, China Agricultural University, Beijing, 100193, China

⁶Center for Human Genetic Research, Massachusetts General Hospital, Boston, MA, 02114, USA

⁷Department of Laboratory Medicine, Children's Hospital Boston, Boston, MA, 02115, USA

⁸Department of Pathology, Affiliated Children's Hospital of Capital Institute of Pediatrics, Beijing, 100020, China

⁹Department of Integrated Early Childhood Development, Capital Institute of Pediatrics, Beijing, 100020, China

¹⁰Department of Biotechnology, Capital Institute of Pediatrics, Beijing, 100020, China

¹¹Dell Pediatric Research Institute, Department of Nutritional Sciences, The University of Texas at Austin, Austin, Texas 78723 USA

¹²Department of Genetics, Harvard Medical School, Boston, MA, 02115, USA

¹³Department of Pathology, Harvard Medical School, Boston, MA 02115, USA

Abstract

*Correspondence to: zhangtingcv@126.com or bai-lin.wu@childrens.harvard.edu.

Disclosure statement

The authors declare that they have no conflict of interest.

Increasing evidence that mutation of planar cell polarity (PCP) genes contributes to human cranial NTD susceptibility prompted us to hypothesize that rare variants of genes in the core apical–basal polarity (ABP) pathway are risk factors for cranial NTDs. In this study, we screened for rare genomic variation of *PARD3* in 138 cranial NTD cases and 274 controls. Overall, the rare deleterious variants of *PARD3* were significantly associated with increased risk for cranial NTDs (11/138 vs. 7/274, $p<0.05$, OR=3.3). These NTD-specific variants were significantly enriched in the aPKC-binding region (6/138 vs. 0/274, $p<0.01$). The East Asian cohort in the ExAC database and another Chinese normal cohort further supported this association. Over-expression analysis in HEK293T and MDCK cells confirmed abnormal aPKC binding or interaction for two *PARD3* variants (p.P913Q and p.D783G), resulting in defective tight junction formation via disrupted aPKC binding. Functional analysis in human neural progenitor cells and chick embryos revealed that *PARD3* knockdown gave rise to abnormal cell polarity and compromised the polarization process of neuroepithelial tissue. Our studies suggest that rare deleterious variants of *PARD3* in the aPKC-binding region contribute to human cranial NTDs, possibly by disrupting apical tight junction formation and subsequent polarization process of the neuroepithelium.

Keywords

cranial NTDs; *PARD3*; rare deleterious variants; apical tight junction formation

Introduction

Human neural tube defects (NTDs) are common, severe, and costly birth defects that arise between the third and fourth week of embryogenesis due to the partial or complete failure of neural tube closure (NTC). Worldwide, approximately 0.5–2 out of every 1,000 pregnancies are affected by NTDs, but the global prevalence varies considerably (Feuchtbaum, et al., 1999; Lary and Edmonds, 1996; Mitchell, 2005). China has a high incidence of NTDs, with approximately 2.74 per 1,000 pregnancies reported in some regions (Xiao, 1989). Shanxi Province in China has the highest known prevalence of NTDs (13.87/1,000 (Li, et al., 2006), making it particularly worthwhile to investigate the causality of NTDs in Chinese populations.

The neural tube is derived during early embryogenesis from a sheet of multi-potential epithelial cells. In order to form a closed lumen, the neuroepithelial cells must adopt the correct three-dimensional morphology through cell migration and remodeling. The establishment and maintenance of cell polarity is a crucial step in neuroepithelium morphogenesis (Doudney and Stanier, 2005). There are two polarity pathways, the planar cell polarity (PCP) pathway and the apical–basal polarity (ABP) pathway (McCaffrey and Macara, 2009b). The PCP pathway is required for convergent extension (CE) movements in *Xenopus* gastrulation (Wolff and Rubin, 1998) and is essential for normal NTC in vertebrates (Torban, et al., 2004). Known perturbations of this pathway result in a high prevalence of NTD-affected embryos in both *Xenopus* and mouse models (Kibar, et al., 2001; Ueno and Greene, 2003; Wallingford, et al., 2000). Recently, identification of rare mutations in a series of core PCP genes has established that genetic defects in the PCP pathway confer risk for NTDs in humans (Allache, et al., 2012; Bosoi, et al., 2011; De

Marco, et al., 2012; Kibar, et al., 2009; Kibar, et al., 2011; Kibar, et al., 2007; Lei, et al., 2013; Lei, et al., 2010; Robinson, et al., 2012; Seo, et al., 2011; Shi, et al., 2012). In particular, the high yield of genetic variants in subjects with cranial NTDs implies a major role for disruption of the PCP pathway in severe forms of NTDs (Robinson, et al., 2012).

The ABP pathway is also important for the acquisition of the cell shape by regulating tight junctions. A subset of ABP pathway components governs the apical constriction of epithelial cells, and converts the columnar cell into a wedge-shaped cell which is essential for NTC (Fan, et al., 2004; Martin-Belmonte and Mostov, 2008; McCaffrey and Macara, 2009b). ABP is mediated by three interacting protein complexes (Pard3, Crumbs and Scribble). Of these, Pard3 is the most important, as it can influence the other ABP complexes via aPKC. Pard3 was first identified in *C. elegans*, and is highly conserved among species, containing a conserved N-terminus and 3 PDZ domains that bind aPKC, Pard6, and Pten (Fan, et al., 2004). Recent studies have demonstrated that Pard3 controls the asymmetric neural progenitor divisions during zebrafish neurulation (Tawk, et al., 2007). Pard3 depletion in zebrafish results in hydrocephalus (Hong, et al., 2010), a phenotype often seen in association with human NTDs. In humans, *PARD3* (MIM#606745) intragenic microdeletions and missense mutations have been identified at high frequency in human epithelial cancer cell lines (Rothenberg, et al., 2010). We previously reported a significant association between common SNPs of *PARD3* (rs2496720, rs2252655, rs3851068, and rs118153230) and NTDs, and this association was more significant for cranial rather than spinal defects (Gao, et al., 2012). Given the importance of *PARD3* in neuroepithelial morphology, we hypothesized that rare *PARD3* genomic variants are risk factors for human cranial NTDs. Therefore, we screened for rare copy number variation (CNV) and single nucleotide variant (SNV) of *PARD3* in 138 cranial NTD cases and 274 matched controls. The functional consequences of the NTD-specific *PARD3* variants on the ABP complex and tight junction formation were investigated *in vitro*. Finally, we studied the polarity phenotype of human neural progenitor cells and the epithelial polarization behavior in chick embryonic neural tube after *PARD3* knock-down to explore the pathogenic mechanism involved in NTDs.

Materials and Methods

Recruitment of study cohorts

138 NTD-affected cases were enrolled in multiple local county hospitals in Shanxi Province. There were 42 cases with craniorachischisis and 96 cases with anencephaly. 274 embryos that were terminated for non-medically related reasons in same geographical regions were enrolled and used as the matched controls (Chen, et al., 2010). The distributions of gestational age and sex were similar between the case and control groups (Supp. Table S1). 800 healthy, non-malformed Chinese children were recruited from kindergartens during their annual routine physical checkups, and they were all found to be free of NTDs and other structural malformations.

The study protocol was reviewed and approved by the Ethics Board of Capital Institute of Pediatrics and Fudan University. Informed consent was obtained from all pregnant women and the children's guardians for participation in this study.

Detection of genomic copy number variant of *PARD3*

The 244K array-CGH platform (Agilent Technologies Inc., Palo Alto, CA) was performed to detect *PARD3* CNVs in 100 cases and 100 controls using our previous method, of which 51 cases and 75 controls have been reported as part of a previous mixed-NTD association study (Chen, et al., 2013). For the remaining test samples, CNVs were detected using a modified multiplex ligation-dependent probe amplification method (Supp. Table S2) (Zhang, et al., 2015).

Sequencing and locus-specific genotyping of *PARD3*

The promoter/coding region (25 exons) and the exon-flanking 50–100 bp of the introns of *PARD3* (NM_019619.3) and its isoform (NM_001184793.1) were screened for SNVs (Supp. Table S2). 100 cases and 100 controls were screened by Sanger sequencing (ABI Prism 3700 Genetic Analyzer). The remaining test samples were screened by massively parallel sequencing after multiplex PCR panels (200–270bp).

For previously reported SNV, variants with minor allele frequencies (MAF) $\geq 0.5\%$ in any public database were not considered as rare variants. For novel SNV, the variant was not considered to be a rare variant if it co-occurred in both control and case cohorts with frequency $\geq 1\%$. Only the rare variants in coding or splicing regions were collected for functional predictions using a series of *in silico* analysis programs.

800 normal Chinese children were genotyped using the Sequenom MassARRAY System to assess the frequency of rare NTD-specific SNVs (Supp. Table S2).

Expression construction, mutagenesis and cell transfection followed by immunofluorescence staining, immunoprecipitation and western blotting

Pk-myc-*PARD3* (4062bp), pCMV-*aPKC*-3flag (1764bp) and GFP-*PARD3* were purchased (Addgene) or re-constructed by double enzyme digestion (Hind III, Xba I). Mutations were created using Site-Directed Mutagenesis (Agilent, Santa Clara, CA) and verified by DNA sequencing.

HEK293T cells and MDCK II cells (ATCC, Manassas, VA) were grown in minimum Eagle's medium supplemented with 10% fetal bovine serum and antibiotics. 70–90% confluence was achieved one day before transient transfection experiments. Myc-*PARD3*, GFP-*PARD3* and 3flag-*aPKC* were transiently transfected into HEK293T and MDCK II cell lines successfully by Lipofectamine 2000 or Lipofectamine Plus (Invitrogen, Grand Island, NY). After 48 h transfection, the MDCK cells were washed, fixed for further immunofluorescence (IF) staining, and HEK293T cells were washed, collected for immunoprecipitation (IP) or western blotting experiments.

Low calcium switch assay in MDCK cells

MDCK cells transfected with myc-*PARD3* plasmid were pre-incubated overnight in minimum essential medium for suspension culture (SMEM, Invitrogen, Carlsbad, CA) containing low Ca_{2+} (5 μM) and 5% FBS (Rajasekaran, et al., 2001). The medium was

replaced with normal DMEM and continued incubation for 30min to allow tight junction formation.

***PARD3* knockdown by siRNA transfection in human neural progenitor cells**

Normal human neural progenitor cells (NPCs, derived from iPSC clone 8330-8) were a gift from Dr. Stephen J. Haggarty's Lab (derived from Coriell GM08330 fibroblasts and characterized as described) and cultured using standard methods (Sheridan, et al., 2011). *PARD3* eSiRNA (SG118942) and siRNA universal negative control (SIC001, Sigma, St. Louis, MO) were transfected into NPCs at 60% confluence via X-tremeGENE esiRNA Transfection Reagent (Roche, Indianapolis, IN). After 48h transfection, NPCs were washed for IF staining.

***cPard3* knockdown by *in ovo* shRNA-induced lentiviral infection in chick**

Chick *Pard3*-specific (*cPard3*) shRNAs with a GFP tag were packaged in HEK293 cells that were transiently transfected using a lentiviral expression plasmid package (GeneChem Biotechnology Co. Shanghai, China). The dosage and knockdown efficiency of three shRNA supernatants was determined in infected chick embryo neuroepithelial cells (Supp. Table S3). Embryos were staged according to HH stages (Hamburger and Hamilton, 1992). The fertilized chick eggs were incubated in a humidified incubator at 37°C for 46–48h, enabling the embryos to develop to stage HH12–14. One microliter of viral suspension diluted by DMEM was micro-injected into the neural tube using a glass micropipette (40–50 mm) under a dissecting microscope (Zhang, et al., 2012). The eggs were sealed and incubated another 48h–8 days, to allow for the complete development of the nervous system prior to capturing images. Non-injected eggs were used to evaluate the toxicity of shRNA supernatant, and lentiviral supernatant with GFP, which was used as the control. 20 eggs were microinjected for each group. Embryos at the HH34–HH38 stage were collected for phenotype evaluation.

Only embryos with high GFP expression were used for histopathology sections, immobilization and IF experiments. The tissue was transverse sectioned (10–15um) at the same level of the rostrocaudal axis of the spinal cord and tail according to the somite location for anti-*Pard3* staining, and imaged using the Zeiss LSM-410 confocal microscope, scale bar with 10–25um was used for image capture.

Animal welfare and experimental procedures conformed to the Institutional Guidelines of the Care and Use of Laboratory Animals at the China Agricultural University (Beijing, China). Other information was listed in the Supp. Materials and Methods.

Results

Rare intragenic *PARD3* deletion resulted in decreased *PARD3* expression levels in a subject with cranial NTD

Only one intragenic 139kb deletion (hg19, chr10:34835589-34975192, Supp. Fig S1A), published previously (Chen, et al., 2013) was detected in a cranial NTD case. No *PARD3* deletions were detected in 274 matched controls and our internal clinical array-CGH

database (Children's Hospital Boston). Long-range PCR from kidney, heart, and muscle and skin tissue were consistent with this lesion being a germline microdeletion. It is worth noting that this singleton *PARD3* deletion has similar coordinates to one reported pathogenic deletion (Patient 256704, chr10:34813019-34994077, Supp. Fig S1A) in the Database of Chromosomal Imbalance and Phenotype in Humans Using Ensembl Resources (DECIPHER). Patient 256704 was diagnosed with autism, delayed speech and language development, and intellectual disability. In the Database of Genomic Variants (DGV), only one *PARD3* deletion (nsv524046, chr10:34459350-34941134,) was identified from 2026 Western controls (Shaikh, et al., 2009). However, nsv524046 was detected by low-resolution SNP chip (Illumina HumanHap550 Genotyping BeadChip v3) and not validated by PCR, so the accuracy remains uncertain. Although Fisher's exact test did not show *PARD3* deletion to be significantly associated with NTDs (1/138 in NTDs vs. 0/274 in controls, $p>0.05$), the low prevalence in this study population and DGV database (1/412 and 1/2026, frequency<0.5%) demonstrates that it is a rare event.

This singleton deletion crosses an exon for a *PARD3* isoform and several anonymous mRNAs (Supp. Fig S1A). The ENCODE dataset revealed evidence from a polyA RNA-Seq technique that the sequences within this segment are transcribed and poly-adenylated in some normal cell lines. We compared *PARD3* levels in the deletion carrier with that in 12 age-matched controls (Supp. Fig S1B). The NTD case had a significantly lower *PARD3* expression index than controls in both kidney (0.50 vs. 0.72, $p<0.01$ for 180KDa) and heart tissues (0.52 vs. 0.73, $p<0.05$ for 180KDa, Supp. Table S4), suggesting a negative impact of the intragenic deletion on production of *PARD3*. We then sequenced the non-deleted allele in this subject, but did not identify any rare deleterious SNV as a second hit.

Rare deleterious NTD-specific variants enriched in the aPKC-binding region of *PARD3*

Previous genotyping of 53 tag *PARD3* SNPs had confirmed that subjects in this cohort represent the same Chinese ethnic group (Gao, et al., 2012). After removing any questionable sequencing data and common variants, a total of 60 rare *PARD3* variants including synonymous, missense and splicing variants remained for comparison (Supp. Table S5). The proportion of NTD subjects with rare *PARD3* variants was significantly higher than the matched controls (28/138 vs. 32/274, $p<0.05$; Table 1). Furthermore, NTD subjects harbored significantly more deleterious variants than the matched controls (11/138 vs. 7/274, $p<0.05$; Table 2, Supp. Fig S2, Supp. Table S6), suggesting that rare deleterious *PARD3* variants confer an increased risk for cranial NTD (OR=3.3). These rare deleterious variants were all present as heterozygotes. Among them, four variants were novel and nine variants were reported in the public databases with or without an rs number; three variants were recurrent in NTD cases (p. G1249S, p.P913Q and p.T861S; Table 2). None of the control-specific deleterious variants was recurrent. Importantly, seven NTD-specific deleterious variants localized to key binding regions of *PARD3*, with six variants in the aPKC-binding region (protein 712-936, Fig 1), two of which were recurrent (p.P913Q and p.T861S). None of the control-specific variants localized to the aPKC-binding region (6/138 vs. 0/274, $p<0.01$). All NTD-specific variants have also been submitted to ClinVar (<http://www.ncbi.nlm.nih.gov/clinvar/>, SCV000299306, SCV000299312). We also reviewed all *PARD3* SNVs from the ExAC database, and only the missense and loss-of function variants

in the aPKC-binding region that occurred at <0.5% frequency in the 4325 East Asian individuals were extracted for comparison. Although we didn't predict a deleterious effect for those ExAC variants as well as for our NTD-specific variants (which increased the potential for false deleterious variants within the ExAC database), the Fisher's exact test revealed that cranial NTD cases had significantly more rare aPKC-binding variants than the East Asian cohort (6/138 vs. 49/4325, $p<0.01$). The genotyping data from 800 normal children confirmed that four NTD-specific variants (p.P913Q, p.D783G, p.R349H, c.583-3T>C, Table 2,) were very rare (0 alleles in 2424 normal Chinese chromosomes) in the Chinese population. Two of these were in the aPKC-binding region (p.P913Q, p.D783G) and one was recurrent (p.P913Q). Considering only very rare aPKC-binding variants still yielded a significant different enrichment between NTD case and controls (3/138 vs. 0/274, $p<0.05$). Thus, multiple genetic analyses supported rare deleterious *PARD3* variants in the aPKC region as risk factor for cranial NTDs.

Among the cranial NTD cases carrying very rare deleterious variants, 60% (3/5) presented with craniorachischisis (Supp. Table S7). The Sanger sequences and species conservation of very rare NTD-specific variants of *PARD3* are shown in Fig 1 and Supp. Fig S3. Maternal inheritance was excluded for variants P913Q, c.583-3T>C, and R349H using DNA from the mother's blood, but paternal samples were not available to test whether the variants are *de novo* or inherited. For the D783G variant, the parental sample confirmed maternal inheritance. The NTD Epidemiological Register Database indicated that this mother suffers from neurobehavioral delays with communication difficulties. No rare copy number variant (>30kb) was detected from their whole genomic array data. *In silico* programs predicted that D783G and P913Q alterations result in loss of aPKC binding activity. Consequently, these two NTD-specific variants were chosen to perform downstream functional analyses.

Decreased binding to aPKC of *PARD3* variants in HEK293T cells

Given that the *PARD3*/aPKC interaction is essential for *PARD3* function during the establishment of apical tight junctions and subsequent polarity of neuroepithelial cells (Horikoshi, et al., 2009; McCaffrey and Macara, 2009a), we examined the binding affinity of *PARD3* containing the deleterious variants (D783G and P913Q) to aPKC using an *in vitro* HEK293T transient transfection system (Pk-myc-*PARD3*). In these experiments, both mutant *PARD3* variants (D783G and P913Q) showed reduced co-precipitation with endogenous aPKC (Fig 2A, 2D) compared to wildtype *PARD3* (wt-*PARD3*), suggesting a lower affinity for the aPKC of the *PARD3* variants. When exogenous aPKC was overexpressed (flag-aPKC, Fig. 2B, 2D), reduced co-precipitation was again seen for the D783G variant, but not for P913Q, consistent with the greater disruption of aPKC binding by D783G variant with endogenous aPKC observed in the previous set of experiments (Fig. 2A).

Phosphorylation status of *PARD3* variants in HEK293T cells

aPKC is a serine/threonine protein kinase which binds *PARD3* via its kinase domain. Once bound, *PARD3* acts as a substrate for aPKC phosphorylation and stimulates multiple downstream pathways including: NUMB, CRUMBS, PTEN and *PARD6* (Goldstein and Macara, 2007). There are 129 predicted phosphorylation sites in *PARD3* (Henrique and

Schweisguth, 2003; Nagai-Tamai, et al., 2002). The D783G and P913Q variants are close to S781, a known phosphorylation site. Using the *in vitro* HEK293T transient transfection assay, we observed that P913Q variants showed increased phosphorylation compared with wt-PARD3 (Fig 2C,2E) while the D783G variant did not show a significant difference in phosphorylation.

PARD3 variants disrupted ZO-2 colocalization and tight junction formation in MDCK cells

The tight junction is an epithelium-specific junction structure that is important for maintaining apical cell polarity, and functions as the “fence” separating apical from basal domains (Denker and Nigam, 1998; Suzuki, et al., 2001). The co-localization of PARD3/aPKC with other molecules is essential for tight junction formation (Horikoshi, et al., 2009; Suzuki, et al., 2001). MDCK cells have been recognized as a model for examining tight junction function. We analyzed the co-localization of wt/mutant exogenous PARD3 with a classic tight junction protein, zonula occludens-2. A higher level of PARD3 expression was observed in MDCK cell transfected with exogenous *PARD3* (exo-PARD3 cells) than null-transfected MDCK cell (endo-PARD3 cells) (Supp. Fig S4). The exogenous wt-PARD3 co-localized with ZO-2 at the tight junction region, and the morphology of the exo-PARD3 cells was the same as that of the endo-PARD3 cells with integrated tight junction formation. For mutant-PARD3, the morphology of mutant exo-PARD3 cells changed (clumped and crowded) with obscure or negative ZO-2 staining. Tight junctions between cells disappeared (Fig 3A). These results suggested that *D783G* and *P913Q* variants disrupt tight junction formation in epithelial cells.

It is well known that tight junction formation is calcium-dependent. When cells are incubated in media with low calcium (LC) concentration, the monolayers of MDCK cells lack cell-cell contact and intercellular junctions. However, providing normal calcium concentrations after low calcium incubation triggers a series of molecular events to restore tight junction formation (Denker and Nigam, 1998). This is referred to as a low calcium switch experiment, and it offers a model system to study how the related tight junction proteins are synthesized and co-localize to the intercellular region (Cereijido, et al., 1998).

We further investigated whether the synthesis or co-localization of tight junction protein was influenced by PARD3 variants (D783G and P913Q) using low calcium switch assay. First, we sought to determine the suitable time point to compare the synthesis and co-localization of endogenous ZO-2 during a pilot experiment. We performed ZO-2 staining in the MDCK cells at different time lapse (0.5h, 2h and 8h intervals) after standard incubation (Supp. Fig S5) and finally chose 30 minutes as the appropriate time point because both positive ZO2 and PARD3 expression can be observed at this point before tight junction formation is complete. In wt-*PARD3* cells, there was significant co-localization of ZO-2 with exogenous PARD3. The integrated tight junction formed normally between the exo-PARD3 cells and the endo-PARD3 cells. However, in mutant-PARD3 cells, the endogenous ZO-2 expressed normally, but didn't co-localize with exogenous PARD3 (Fig 3B). These results suggested that the two *PARD3* variants (D783G and P913Q) disrupt tight junction formation by affecting the co-localization of ZO-2 to the intercellular region.

Altered cell polarity distribution in human neural progenitor cells after *PARD3* knockdown

Because two aPKC-binding variants showed disruption of tight junction formation, we transfected siRNA into human neural progenitor cells (NPCs) to develop an *in vitro* cellular model with *PARD3* knockdown, and then examine the phenotypic characteristics of the NPCs. The *PARD3*/*PARD6* complex was reported to display apical polarity in human neural progenitors, which is different from what was seen in MDCK cells (Khazaei and Puschel, 2009; Wolf, et al., 2008). We confirmed that *PARD3* was apically localized on NPCs undergoing mirror-symmetry divisions (white arrow in Supp. Fig S6).

After transfection with the siRNA universal negative control, the co-localization of *PARD3* and *PARD6* was seen clearly on the apical aspect of NPCs with single long axon-like synapse, and the mirror-symmetry distribution between two daughter NPCs maintained (Fig 4A). *PARD3* knockdown resulted into reduced *PARD3* expression, loss of mirror-symmetry distribution between two daughter NPCs and the development of multiple short processes (Fig 4B), suggesting that the cell polarity characteristics and differentiation state of the NPCs altered after *PARD3* knockdown.

Short abnormal tail bud phenotype, abnormal epithelial polarization process of neural tube in chick embryo after *cPard3* knockdown

The chick is an excellent model with which to study the neurulation process, possessing several advantages over mammalian model systems including: low cost, easy manipulation of the embryo *in vitro* that recapitulate normal *in ovo* development, the zipper-like NTC from multiple sites which is similar to that in human embryos, and a short period of embryogenesis (Eom, et al., 2012).

Using chick embryos we explored whether the aforementioned cellular polarization abnormalities are potentially responsible for abnormal NTC in neuroepithelial tissue. We performed *in ovo* shRNA-induced lentiviral infection to produce *cPard3* knockdowns in chick embryos, and then analyzed the neural tube phenotypes and the morphology of neuroepithelial tissue. Briefly, the lentiviral strain with high efficiency and low lethality was chosen by infecting chick embryo neuroepithelial cells. Higher GFP expression and survival were observed for the LV3 and LV4 strains than the LV1 strain (Supp. Fig S7) and RT-PCR validated approximately 75% *cPard3* knockdown (Supp. Fig S8). After *in ovo* microinjection, mosaic GFP expression was constant from HH18 to HH38 stage in brain tissues (Supp. Fig S9), demonstrating a continuous *cPard3* knockdown in the treated chick embryos.

Within the *cPard3* knockdown chick embryos, we observed two kinds of abnormal developmental phenotypes: small forebrains and small tails (Fig 5). Occasionally, the knockdown chicks presented with edematous torsos. Significantly more small tail bud types of embryos existed in *cPard3* knockdown chicks than in control chicks (11/20 vs. 0/9, $p < 0.05$, Table 3). The prevalence of small forebrain phenotypes in the *cPard3* knockdown embryos did not achieve statistical significance (5/20 vs. 0/9, $p > 0.05$). However, no classic cranial NTD phenotype (craniorachischisis, anencephaly) were observed in *cPard3* knockdown chick embryos.

We analyzed the localization and integrity of *cPard3* and the epithelial polarization process of the chick spinal neural tube (Fig 6A). *cPard3* expression was continuous and integrated on the apical surface of the epithelia (Fig 6A–10) that predominantly converged and aggregated on the ventral midline region (Fig 6A–1,5 and 9) where the median hinge point (MHP) emerged during NTC. This implies that normal epithelial polarization phenomena occurred predominantly within the ventral MHP site of the neural tube. With the *cPard3* knockdown, *cPard3* expression was reduced and discrete on the apical aspect of the epithelia. More mesenchymal-like cells (Fig 6A–3,7 and 11) rather than the neuroepithelial cells aggregated in the ventral region. The inner lumen of the neural tube was also more coarse and broad compared with the control embryo (Fig 6A–10, 12). Multiple ectopic hinge-like invaginations were observed in *cPard3* knockdown chick embryos (Fig 6A–6,8). Taken together, these observations suggest that the process of epithelial polarization was compromised. The process of epithelial polarization in the caudal neural tube was also analyzed (Fig 6B). It revealed a coarse neural lumen, abnormal epithelial polarization process, and multiple ectopic hinge-like invaginations in the *cPard3* knockdown chick embryos.

Discussion

The contribution of known polarity genes to human cranial and spinal NTDs

The establishment and maintenance of the PCP and ABP pathways is a crucial step for the morphogenesis of the neuroepithelium (McCaffrey and Macara, 2009b). Rare mutations in PCP pathway genes, including: *VANGL1*, *VANGL2*, *CELSR1*, *FZD6*, *PRICKLE1*, *FUZZY* and *SCRIB* have been recognized as risk factors for human NTDs (Allache, et al., 2012; Bosoi, et al., 2011; De Marco, et al., 2012; Kibar, et al., 2009; Kibar, et al., 2011; Kibar, et al., 2007; Lei, et al., 2010; Robinson, et al., 2012; Seo, et al., 2011). The overall incidence of mutations in PCP genes detected in human NTD samples is between 0.75%–16.7%, due to complex phenotypic heterogeneity amongst the types of NTDs and ethnic differences amongst the study populations.

Recently, the *Pard3* complex was found to have an independent role in neurulation separate from the PCP pathway. The zebrafish NTC model revealed that *Pard3* is critical in orchestrating the midline crossing during neurulation, affecting mirror-symmetric polarized cell division (Geldmacher-Voss, et al., 2003; Sottocornola, et al., 2010). Cross-talk between the PCP and *Pard3* complex has also been observed (Cha, et al., 2011; Courbard, et al., 2009; Wolf, et al., 2008; Zhang, et al., 2007). For example, the core PCP protein Dishevelled, is regulated by aPKC kinase activity to control axon differentiation (Zhang, et al., 2007); Wnt interacts with phosphorylation of aPKC (Wolf, et al., 2008; Zhang, et al., 2007). The ABP protein Scribble and the PCP protein Vangl interact during anterior-posterior axon guidance (Courbard, et al., 2009). A study in *Xenopus* oocytes found that Vangl2 also interacts with aPKC directly (Cha, et al., 2011). Collectively, these data suggest the importance of the *PARD3* complex in the process of human neurulation. However, the possible contribution of rare mutation in the *PARD3* complex to human NTDs has not yet been studied.

Our association study supported that rare deleterious variants of *PARD3*, particularly those variants in the aPKC-binding region, are significant risk factors for human cranial NTDs. The prevalence of very rare deleterious variants in the aPKC-binding region of *PARD3* was 2% (3/138) in a Chinese cranial NTD cohort. To our knowledge, this is first study to identify rare deleterious variants of *PARD3* in human cranial NTDs, which further supports the concept that human NTDs are a collection of disorders with high genetic heterogeneity, and both PCP and ABP genomic variants contribute to their occurrence, albeit at a low individual prevalence. These results serve to enhance our understanding of pathogenesis of NTDs in humans, highlighting the importance of the ABP genes to their etiology.

The mechanisms involved in human cranial NTDs is thought to differ from spinal NTDs (Copp and Greene, 2010). Although the differential role of the polarity genes in human cranial and spinal NTDs has not yet been fully resolved, the differential SNV yield of polarity genes from different NTD subgroups implies that rare mutations in polarity genes is most often associated with cranial rather than spinal NTDs. For example, the yield of *CELSR1* mutations in Western spinal NTDs cases was only 3% (Lei, et al., 2014). Conversely, 16.7% of *CELSR1* pathogenic mutations were reported in Caucasian fetuses with craniorachischisis (Robinson, et al., 2012), which is significantly higher than for spinal NTDs (6/36 vs. 13/462, $p < 0.01$) (Allache, et al., 2012; Juriloff and Harris, 2012). For the Chinese NTD cohort, *VANGL2* mutations were identified in 2.9% (3/105) of cranial NTDs; however, no mutations were identified in spinal NTD cases (Lei, et al., 2010). The skewed yield supported the specificity of the risk from variants in human PCP genes with respect to cranial NTDs. In our study, three of five cases (60%, Supp. Table S6) carrying rare deleterious *PARD3* variants presented craniorachischisis, in which the lower spine region is equally involved as the cranial region, so the contribution of rare *PARD3* variants to spinal NTDs cannot be excluded. In the future, the samples with isolated cranial/spinal NTDs should be studied to compare the pathogenesis of rare *PARD3* variant in different types of NTDs.

***PARD3* loss-of-function variants in aPKC binding region alter tight junction function**

It has been previously demonstrated that mutant *PARD3* lacking the aPKC binding region (residues 827–856) is unable to associate with aPKC and influences cell-cell contact in MDCK cells (Hirose, et al., 2002). Site-directed mutagenesis and *in vitro* expression studies demonstrated that a *PARD3* mutation at Ser827 (S827A) formed a stable complex with aPKC, which suppressed tight junction formation, whereas the mutant (S827E) can't bind to aPKC (Nagai-Tamai, et al., 2002). The effect of *Pard3* point mutations on the localization of tight junction proteins has been previously studied. The mutant *Pard3* was unable to localize to the tight junction regions in MDCK cells, resulting in abnormal/multiple cysts or duct formation (Hirose, et al., 2002; McCaffrey and Macara, 2009a).

Based on the molecular and cellular evidence from an *in vitro* over-expression system, we confirmed that two deleterious *PARD3* variants in the aPKC binding region resulted in defective tight junction formation during epithelium morphogenesis. Surprisingly, a differential contribution was observed between D873G and P913Q variant. The D783G brought down less aPKC than did the P913Q, and but didn't produce significant altered

phosphorylation levels as did the P913Q, although it is physically close to Ser827. We speculate that either there are other regulators such as small GTPases involved in Pard3 phosphorylation (Joberty, et al., 2000), or there is a feedback phenomenon in order to compensate for the reduced aPKC binding of mutant PARD3 (McCaffrey and Macara, 2009b).

Suppression of *PARD3* expression disrupts the polarity distribution of human NPCs

Neural progenitor cells can differentiate *in vitro* into mature neurons, providing a suitable cell model to study the function of individual genes during neural development. In neural tube formation, polarized cell division (referred to here as C-division) is an important step that deposits one daughter cell on either side of the midline. Using a GFP-tag to follow tissue dynamics and cell polarization during zebrafish neural tube development, Tawk and co-workers (Tawk, et al., 2007) found that the C-division is a mirror-symmetric division in which Pard3 is expressed only on the apical pole of neural progenitor cells. Time-lapse photography revealed Pard3–GFP is expressed and localized consistently at the apical poles of the two daughter cells throughout division. After injection with a morpholino (MO) and a mutant Pard3–GFP which lacks the aPKC binding domain, Pard3–GFP was expressed widely in the cell membrane rather than being restricted to apical poles. We confirmed the apical polarity of PARD3 complex in normal human NPCs. However, *PARD3* knockdown disrupted this apical polarity of PARD3 complex and mirror-symmetry distribution of daughter NPCs. This further established the role of PARD3 in maintaining polar characteristics of human NPCs.

***PARD3* loss-of-function results in abnormal process of epithelial polarization and ectopic hinge-like invaginations**

It is well established that NTC is a complex epithelium remodeling process. During this process, dynamic epithelial polarization is observed, including apical constriction/basal expansion and nuclear migration and asymmetric epithelial distribution (Doudney and Stanier, 2005; Shimokita and Takahashi, 2011). Specifically, the polarization process at some special site of the epithelium including ventral midline/median hinge point (MHP) and dorsolateral hinge points (DLHPs) are critical steps to facilitate NTC (Copp and Greene, 2010). A recent study using a chick embryo model demonstrated that cPard3 was recruited to the apical region of the MHP to induce apical constriction by apical tight junction formation (Eom, et al., 2012). This study suggests that *Pard3* is a key mediator/molecule to promote MHP formation by apical tight junction formation and constriction, promoting the process of epithelial polarization. In this chick model with abnormal NTC, *cPard3* expression was reduced on the apical region of the MHP region, while ectopic hinge-like points appeared (Eom, et al., 2012).

On the spinal neural tube of *cPard3* knockdown chick embryos, we observed a reduction in the epithelial polarization of the ventral MHP region due to an insufficiency of cPard3. The multiple ectopic hinge points instead of the specific MHP points emerged on the coarse and broad neural tube lumen, which are similar to those observed in chick embryos with NTDs. These phenomena supported our hypothesis that *Pard3* insufficiency in the developing chick disrupts the apical tight junction formation/constriction of the neuroepithelium on the MHP

region, and affects the normal epithelial polarization process, ultimately contributing to abnormal NTC.

In view of the human genetic evidence and the *in vitro* cellular/chick transfection system, we firmly believe that rare deleterious variants of *PARD3* in the aPKC-binding region exert a loss-of function effect on NTC, adversely affecting the binding activity and co-localization with other polarity components. This results in disruption of apical tight junction formation and the neuroepithelium polarization process, and contributes to abnormal NTC.

Malformed tails were previously reported in mouse/chick PCP mutants as well as in a mouse model of NTDs. Knockout *Vangl2* mutant mice presented with craniorachischisis, while heterozygous *Vangl2* mutants mice are normal except for a looped tail and/or the occasional spina bifida (Copp, et al., 1994). The *dact*^{-/-} (a regulator of Vangl) mouse has a shorter tail than does the wildtype (Yang and Cheyette, 2013). In an retinoic acid treated mouse model, defects of the tail bud were also observed (Griffith and Wiley, 1991). In the *cPard3* knockdown chick embryos, although no cranial NTD phenotype was observed, a significantly increased prevalence of a smaller tail bud than found in the controls suggests that *cPard3* and some PCP molecules may be involved in the same neurulation developmental pathway to control NTC.

Considering the absence of an NTD phenotype in *cPard3* knockdown chick embryos, we believe that the complex genetic inheritance of NTDs provides the most plausible explanation. Most mutant mice with NTDs, particularly mutant mice with a cranial NTD phenotype, harbor digenic mutations on other modifier/effector/interacting genes in addition to PCP genes (reviewed by (Juriloff and Harris, 2012)). For human NTDs, although we recognize the rare PCP variants as genetic risk factors for NTDs, we cannot exclude other loci as co-contributors. In this study, the D783G variant detected in the cranial NTD cases is very rare and NTD-specific, but was maternally inherited. The parental validation supports the concept that human NTDs are a collection of highly heterogeneous genetic disorders, suggesting that other modifier variants in addition to *PARD3* contribute to cranial NTD occurrence. In the future, next generation sequencing of all target genes will help us to reveal the complete burden of genetic variants and their co-interactions contributing to NTD formation. In addition, the timing of the microinjections could represent another possible explanation. In the chick, primary neurulation occurs between HH stages 7–13. We microinjected *cPard3* shRNA lentivirus at the HH12–14 stage. Generally it will take 48h for shRNA lentivirus to exert its knockdown effect (validated by real-time PCR, Supp. Fig S8). The short overlap between these two temporal windows may not be sufficient for the shRNA to affect primary neurulation. Another possibility is that the knockdown efficiency in chick embryo was insufficient because the knockdown efficiency in cultured chick neuroepithelial cells might not be as same as that in chick embryos following *in ovo* microinjections.

In summary, this study explored a potential pathogenicity of rare deleterious *PARD3* genomic variants on risk for human cranial NTDs. By *in vitro* functional analysis, we demonstrated that deleterious *PARD3* variants in the aPKC binding region showed altered *PARD3*-aPKC interaction and tight junction formation. The abnormal phenotypes from human NPCs and chick embryos after *cPard3* knockdown suggest that the disrupted apical

tight junction formation and compromised polarization process of the neuroepithelium is a potential mechanism of NTDs. These results enhance our understanding of the etiology of human cranial NTDs, highlighting the importance of ABP genes in the development of these malformations, and also supports the concept that human NTDs are a collection of disorders with a high degree of genetic heterogeneity, and both PCP and ABP genomic variants contribute to their occurrence, albeit at a low individual prevalence (2%). In the future, the co-contribution of ABP and PCP genes should be studied in detail to assess the genetic heterogeneity and the phenotypic complexity of human NTDs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Allache R, De Marco P, Merello E, Capra V, Kibar Z. Role of the planar cell polarity gene CELSR1 in neural tube defects and caudal agenesis. *Birth Defects Res A Clin Mol Teratol.* 2012; 94(3):176–81. [PubMed: 22371354]
- Bosoi CM, Capra V, Allache R, Trinh VQ, De Marco P, Merello E, Drapeau P, Bassuk AG, Kibar Z. Identification and characterization of novel rare mutations in the planar cell polarity gene PRICKLE1 in human neural tube defects. *Hum Mutat.* 2011; 32(12):1371–5. [PubMed: 21901791]
- Cereijido M, Valdes J, Shoshani L, Contreras RG. Role of tight junctions in establishing and maintaining cell polarity. *Annu Rev Physiol.* 1998; 60:161–77. [PubMed: 9558459]
- Cha SW, Tadjuidje E, Wylie C, Heasman J. The roles of maternal Vangl2 and aPKC in *Xenopus* oocyte and embryo patterning. *Development.* 2011; 138(18):3989–4000. [PubMed: 21813572]
- Chen X, Guo J, Lei Y, Zou J, Lu X, Bao Y, Wu L, Wu J, Zheng X, Shen Y, et al. Global DNA hypomethylation is associated with NTD-affected pregnancy: A case-control study. *Birth Defects Res A Clin Mol Teratol.* 2010; 88(7):575–81. [PubMed: 20641100]
- Chen X, Shen Y, Gao Y, Zhao H, Sheng X, Zou J, Lip V, Xie H, Guo J, Shao H, et al. Detection of copy number variants reveals association of cilia genes with neural tube defects. *PLoS One.* 2013; 8(1):e54492. [PubMed: 23349908]
- Copp AJ, Checiu I, Henson JN. Developmental basis of severe neural tube defects in the loop-tail (Lp) mutant mouse: use of microsatellite DNA markers to identify embryonic genotype. *Dev Biol.* 1994; 165(1):20–9. [PubMed: 8088438]
- Copp AJ, Greene ND. Genetics and development of neural tube defects. *J Pathol.* 2010; 220(2):217–30. [PubMed: 19918803]
- Courbard JR, Djiane A, Wu J, Mlodzik M. The apical/basal-polarity determinant Scribble cooperates with the PCP core factor Stbm/Vang and functions as one of its effectors. *Dev Biol.* 2009; 333(1):67–77. [PubMed: 19563796]
- De Marco P, Merello E, Rossi A, Piatelli G, Cama A, Kibar Z, Capra V. FZD6 is a novel gene for human neural tube defects. *Hum Mutat.* 2012; 33(2):384–90. [PubMed: 22045688]

- Denker BM, Nigam SK. Molecular structure and assembly of the tight junction. *Am J Physiol.* 1998; 274(1 Pt 2):F1–9. [PubMed: 9458817]
- Doudney K, Stanier P. Epithelial cell polarity genes are required for neural tube closure. *Am J Med Genet C Semin Med Genet.* 2005; 135C(1):42–7. [PubMed: 15800847]
- Eom DS, Amarnath S, Fogel JL, Agarwala S. Bone morphogenetic proteins regulate hinge point formation during neural tube closure by dynamic modulation of apicobasal polarity. *Birth Defects Res A Clin Mol Teratol.* 2012; 94(10):804–16. [PubMed: 22865775]
- Fan S, Hurd TW, Liu CJ, Straight SW, Weimbs T, Hurd EA, Domino SE, Margolis B. Polarity proteins control ciliogenesis via kinesin motor interactions. *Curr Biol.* 2004; 14(16):1451–61. [PubMed: 15324661]
- Feuchtbaum LB, Currier RJ, Riggle S, Roberson M, Lorey FW, Cunningham GC. Neural tube defect prevalence in California (1990–1994): eliciting patterns by type of defect and maternal race/ethnicity. *Genet Test.* 1999; 3(3):265–72. [PubMed: 10495925]
- Gao Y, Chen X, Shangguan S, Bao Y, Lu X, Zou J, Guo J, Dai Y, Zhang T. Association Study of PARD3 Gene Polymorphisms With Neural Tube Defects in a Chinese Han Population. *Reprod Sci.* 2012; 19(7):764–71. [PubMed: 22447895]
- Geldmacher-Voss B, Reugels AM, Pauls S, Campos-Ortega JA. A 90-degree rotation of the mitotic spindle changes the orientation of mitoses of zebrafish neuroepithelial cells. *Development.* 2003; 130(16):3767–80. [PubMed: 12835393]
- Goldstein B, Macara IG. The PAR proteins: fundamental players in animal cell polarization. *Dev Cell.* 2007; 13(5):609–22. [PubMed: 17981131]
- Griffith CM, Wiley MJ. Effects of retinoic acid on chick tail bud development. *Teratology.* 1991; 43(3):217–24. [PubMed: 2014484]
- Hamburger V, Hamilton HL. A series of normal stages in the development of the chick embryo. 1951. *Dev Dyn.* 1992; 195(4):231–72. [PubMed: 1304821]
- Henrique D, Schweisguth F. Cell polarity: the ups and downs of the Par6/aPKC complex. *Curr Opin Genet Dev.* 2003; 13(4):341–50. [PubMed: 12888006]
- Hirose T, Izumi Y, Nagashima Y, Tamai-Nagai Y, Kurihara H, Sakai T, Suzuki Y, Yamanaka T, Suzuki A, Mizuno K, et al. Involvement of ASIP/PAR-3 in the promotion of epithelial tight junction formation. *J Cell Sci.* 2002; 115(Pt 12):2485–95. [PubMed: 12045219]
- Hong E, Jayachandran P, Brewster R. The polarity protein Pard3 is required for centrosome positioning during neurulation. *Dev Biol.* 2010; 341(2):335–45. [PubMed: 20138861]
- Horikoshi Y, Suzuki A, Yamanaka T, Sasaki K, Mizuno K, Sawada H, Yonemura S, Ohno S. Interaction between PAR-3 and the aPKC-PAR-6 complex is indispensable for apical domain development of epithelial cells. *J Cell Sci.* 2009; 122(Pt 10):1595–606. [PubMed: 19401335]
- Joberty G, Petersen C, Gao L, Macara IG. The cell-polarity protein Par6 links Par3 and atypical protein kinase C to Cdc42. *Nat Cell Biol.* 2000; 2(8):531–9. [PubMed: 10934474]
- Juriloff DM, Harris MJ. A consideration of the evidence that genetic defects in planar cell polarity contribute to the etiology of human neural tube defects. *Birth Defects Res A Clin Mol Teratol.* 2012; 94(10):824–40. [PubMed: 23024041]
- Khazaei MR, Puschel AW. Phosphorylation of the par polarity complex protein Par3 at serine 962 is mediated by aurora a and regulates its function in neuronal polarity. *J Biol Chem.* 2009; 284(48):33571–9. [PubMed: 19812038]
- Kibar Z, Bosoi CM, Kooistra M, Salem S, Finnell RH, De Marco P, Merello E, Bassuk AG, Capra V, Gros P. Novel mutations in VANGL1 in neural tube defects. *Hum Mutat.* 2009; 30(7):E706–15. [PubMed: 19319979]
- Kibar Z, Salem S, Bosoi CM, Pauwels E, De Marco P, Merello E, Bassuk AG, Capra V, Gros P. Contribution of VANGL2 mutations to isolated neural tube defects. *Clin Genet.* 2011; 80(1):76–82. [PubMed: 20738329]
- Kibar Z, Torban E, McDearmid JR, Reynolds A, Berghout J, Mathieu M, Kirillova I, De Marco P, Merello E, Hayes JM, et al. Mutations in VANGL1 associated with neural-tube defects. *N Engl J Med.* 2007; 356(14):1432–7. [PubMed: 17409324]

- Kibar Z, Vogan KJ, Groulx N, Justice MJ, Underhill DA, Gros P. Ltap, a mammalian homolog of *Drosophila Strabismus*/Van Gogh, is altered in the mouse neural tube mutant Loop-tail. *Nat Genet.* 2001; 28(3):251–5. [PubMed: 11431695]
- Lary JM, Edmonds LD. Prevalence of spina bifida at birth—United States, 1983–1990: a comparison of two surveillance systems. *MMWR CDC Surveill Summ.* 1996; 45(2):15–26. [PubMed: 8602137]
- Lei Y, Zhu H, Duhon C, Yang W, Ross ME, Shaw GM, Finnell RH. Mutations in planar cell polarity gene *SCRIB* are associated with spina bifida. *PLoS One.* 2013; 8(7):e69262. [PubMed: 23922697]
- Lei Y, Zhu H, Yang W, Ross ME, Shaw GM, Finnell RH. Identification of Novel *CELSR1* Mutations in Spina Bifida. *PLoS One.* 2014; 9(3):e92207. [PubMed: 24632739]
- Lei YP, Zhang T, Li H, Wu BL, Jin L, Wang HY. *VANGL2* mutations in human cranial neural-tube defects. *N Engl J Med.* 2010; 362(23):2232–5. [PubMed: 20558380]
- Li Z, Ren A, Zhang L, Ye R, Li S, Zheng J, Hong S, Wang T. Extremely high prevalence of neural tube defects in a 4-county area in Shanxi Province, China. *Birth Defects Res A Clin Mol Teratol.* 2006; 76(4):237–40. [PubMed: 16575897]
- Martin-Belmonte F, Mostov K. Regulation of cell polarity during epithelial morphogenesis. *Curr Opin Cell Biol.* 2008; 20(2):227–34. [PubMed: 18282696]
- McCaffrey LM, Macara IG. The Par3/aPKC interaction is essential for end bud remodeling and progenitor differentiation during mammary gland morphogenesis. *Genes Dev.* 2009a; 23(12):1450–60. [PubMed: 19528321]
- McCaffrey LM, Macara IG. Widely conserved signaling pathways in the establishment of cell polarity. *Cold Spring Harb Perspect Biol.* 2009b; 1(2):a001370. [PubMed: 20066082]
- Mitchell LE. Epidemiology of neural tube defects. *Am J Med Genet C Semin Med Genet.* 2005; 135C(1):88–94. [PubMed: 15800877]
- Nagai-Tamai Y, Mizuno K, Hirose T, Suzuki A, Ohno S. Regulated protein-protein interaction between aPKC and PAR-3 plays an essential role in the polarization of epithelial cells. *Genes Cells.* 2002; 7(11):1161–71. [PubMed: 12390250]
- Rajasekaran SA, Palmer LG, Moon SY, Peralta Soler A, Apodaca GL, Harper JF, Zheng Y, Rajasekaran AK. Na,K-ATPase activity is required for formation of tight junctions, desmosomes, and induction of polarity in epithelial cells. *Mol Biol Cell.* 2001; 12(12):3717–32. [PubMed: 11739775]
- Robinson A, Escuin S, Doudney K, Vekemans M, Stevenson RE, Greene ND, Copp AJ, Stanier P. Mutations in the planar cell polarity genes *CELSR1* and *SCRIB* are associated with the severe neural tube defect craniorachischisis. *Hum Mutat.* 2012; 33(2):440–7. [PubMed: 22095531]
- Rothenberg SM, Mohapatra G, Rivera MN, Winokur D, Greninger P, Nitta M, Sadow PM, Sooriyakumar G, Brannigan BW, Ulman MJ, et al. A genome-wide screen for microdeletions reveals disruption of polarity complex genes in diverse human cancers. *Cancer Res.* 2010; 70(6):2158–64. [PubMed: 20215515]
- Seo JH, Zilber Y, Babayeva S, Liu J, Kyriakopoulos P, De Marco P, Merello E, Capra V, Gros P, Torban E. Mutations in the planar cell polarity gene, *Fuzzy*, are associated with neural tube defects in humans. *Hum Mol Genet.* 2011; 20(22):4324–33. [PubMed: 21840926]
- Shaikh TH, Gai X, Perin JC, Glessner JT, Xie H, Murphy K, O'Hara R, Casalunovo T, Conlin LK, D'Arcy M, et al. High-resolution mapping and analysis of copy number variations in the human genome: a data resource for clinical and research applications. *Genome Res.* 2009; 19(9):1682–90. [PubMed: 19592680]
- Sheridan SD, Theriault KM, Reis SA, Zhou F, Madison JM, Daheron L, Loring JF, Haggarty SJ. Epigenetic characterization of the *FMR1* gene and aberrant neurodevelopment in human induced pluripotent stem cell models of fragile X syndrome. *PLoS One.* 2011; 6(10):e26203. [PubMed: 22022567]
- Shi Y, Ding Y, Lei YP, Yang XY, Xie GM, Wen J, Cai CQ, Li H, Chen Y, Zhang T, et al. Identification of novel rare mutations of *DACT1* in human neural tube defects. *Hum Mutat.* 2012; 33(10):1450–5. [PubMed: 22610794]
- Shimokita E, Takahashi Y. Secondary neurulation: Fate-mapping and gene manipulation of the neural tube in tail bud. *Dev Growth Differ.* 2011; 53(3):401–10. [PubMed: 21492152]

- Sottocornola R, Royer C, Vives V, Tordella L, Zhong S, Wang Y, Ratnayaka I, Shipman M, Cheung A, Gaston-Massuet C, et al. ASPP2 binds Par-3 and controls the polarity and proliferation of neural progenitors during CNS development. *Dev Cell*. 2010; 19(1):126–37. [PubMed: 20619750]
- Suzuki A, Yamanaka T, Hirose T, Manabe N, Mizuno K, Shimizu M, Akimoto K, Izumi Y, Ohnishi T, Ohno S. Atypical protein kinase C is involved in the evolutionarily conserved par protein complex and plays a critical role in establishing epithelia-specific junctional structures. *J Cell Biol*. 2001; 152(6):1183–96. [PubMed: 11257119]
- Tawk M, Araya C, Lyons DA, Reugels AM, Girdler GC, Bayley PR, Hyde DR, Tada M, Clarke JD. A mirror-symmetric cell division that orchestrates neuroepithelial morphogenesis. *Nature*. 2007; 446(7137):797–800. [PubMed: 17392791]
- Torban E, Wang HJ, Groulx N, Gros P. Independent mutations in mouse Vangl2 that cause neural tube defects in looptail mice impair interaction with members of the Dishevelled family. *J Biol Chem*. 2004; 279(50):52703–13. [PubMed: 15456783]
- Ueno N, Greene ND. Planar cell polarity genes and neural tube closure. *Birth Defects Res C Embryo Today*. 2003; 69(4):318–24. [PubMed: 14745972]
- Wallingford JB, Rowning BA, Vogeli KM, Rothbacher U, Fraser SE, Harland RM. Dishevelled controls cell polarity during *Xenopus* gastrulation. *Nature*. 2000; 405(6782):81–5. [PubMed: 10811222]
- Wolf AM, Lyuksyutova AI, Fenstermaker AG, Shafer B, Lo CG, Zou Y. Phosphatidylinositol-3-kinase-atypical protein kinase C signaling is required for Wnt attraction and anterior-posterior axon guidance. *J Neurosci*. 2008; 28(13):3456–67. [PubMed: 18367611]
- Wolff T, Rubin GM. Strabismus, a novel gene that regulates tissue polarity and cell fate decisions in *Drosophila*. *Development*. 1998; 125(6):1149–59. [PubMed: 9463361]
- Xiao KZ. Epidemiology of neural tube defects in China. *Zhonghua Yi Xue Za Zhi*. 1989; 69(4):189–91. 14. [PubMed: 2673491]
- Yang X, Cheyette BN. SEC14 and spectrin domains 1 (Sestd1) and Dapper antagonist of catenin 1 (Dact1) scaffold proteins cooperatively regulate the Van Gogh-like 2 (Vangl2) four-pass transmembrane protein and planar cell polarity (PCP) pathway during embryonic development in mice. *J Biol Chem*. 2013; 288(28):20111–20. [PubMed: 23696638]
- Zhang X, Xu Y, Liu D, Geng J, Chen S, Jiang Z, Fu Q, Sun K. A modified multiplex ligation-dependent probe amplification method for the detection of 22q11.2 copy number variations in patients with congenital heart disease. *BMC Genomics*. 2015; 16:364. [PubMed: 25952753]
- Zhang X, Zhu J, Yang GY, Wang QJ, Qian L, Chen YM, Chen F, Tao Y, Hu HS, Wang T, et al. Dishevelled promotes axon differentiation by regulating atypical protein kinase C. *Nat Cell Biol*. 2007; 9(7):743–54. [PubMed: 17558396]
- Zhang Z, Sun P, Yu F, Yan L, Yuan F, Zhang W, Wang T, Wan Z, Shao Q, Li Z. Transgenic quail production by microinjection of lentiviral vector into the early embryo blood vessels. *PLoS One*. 2012; 7(12):e50817. [PubMed: 23251391]

Fig 1. DNA sequencing and locations of the rare deleterious variants of *PARD3*

(A) The sequence chromatographs of four rare, NTD-specific variants of *PARD3*. The top is the sequence from an NTD case, and the bottom is reference sequence from GenBank. Two independent PCR experiments and bidirectional sequencing were performed for each variant. (B) Schematic diagram of *PARD3* shows the differential locations of rare deleterious variants of *PARD3* detected in the NTD cases (blue) and the controls (red). The protein structure and domain of *PARD3* was described based on information from The Universal Protein Resource (UniProt, <http://www.uniprot.org/uniprot/Q8TEW0>). Six NTD-specific variants are in the aPKC-binding region.

Fig 2. Altered binding to aPKC and phosphorylation status of *PARD3* variants (D783G and P913Q)

(A–B) Human HEK293T cells transfected with wt/mutant myc-*PARD3* vectors (A) or co-transfected wt/mutant myc-*PARD3* and flag-aPKC vectors (B) were lysed for IP and immunoblotting experiment to test binding activity of wt/mutant *PARD3* to endogenous (A) or exogenous aPKC (B). Human HEK293T cells without transfection or transfected flag-aPKC alone were used as the negative controls. The total *PARD3* levels extracted from cell lysates are shown on the bottom. The HEK293T cells with transfected flag-aPKC alone showed a weak and non-specific band for anti-myc due to protein G immunoprecipitation. (C) Human HEK293T cells transfected with wt/mutant myc-*PARD3* vectors were lysed, pulled down by anti-myc to test *PARD3* phosphorylation levels using anti-phos.G140. The myc-*PARD3* levels extracted from cell lysates are shown on the bottom. (D) Quantification analysis for aPKC binding activity of wt/mutant *PARD3*. The relative binding to aPKC of wt/mutant *PARD3* was normalized by total myc-*PARD3* levels pulled down by anti-myc. (E) Quantification analysis for *PARD3* phosphorylation levels induced by aPKC. The relative phosphorylation status of wt/mutant *PARD3* was normalized by total myc-*PARD3* levels pulled down by anti-myc. Each histogram value represents the average of three independent experiments; bars represent the SD. The differences with $p < 0.05$ (two-sided) were considered statistically significant.

Fig 3. Colocalization of PARD3 and ZO-2 in MDCK cells transfected with wt/mutant *PARD3*.

(A) 46h after transfection, MDCK cells were fixed and stained. In MDCK cells expressing wt-*PARD3* vectors, the exo-PARD3 co-localized with the endogenous ZO-2. In order to form integrated tight junctions between cells, the ZO-2 protein is exported from the exo-PARD3 cell to touch the endo-PARD3 cells and a clear intact tight junction was formed between them (thin white arrow). In MDCK cells expressing mutant-*PARD3* vectors (D783G and P913Q), the mutant exo-PARD3 cells detached from adjacent endo-PARD3 cells, and the ZO-2 protein staining was obscure despite high levels of exogenous PARD3 expression (broad white arrows). The ZO-2 staining was inversely associated with the mutant PARD3 expression. Scale bars: 10um in D783G, 25um in WT and P913Q. (B) 36h after transfection, MDCK cells were cultured overnight in low Ca²⁺ medium, and then transferred to normal calcium medium for another 1/2h culture to initiate tight junction formation. In MDCK cells expressing wt-*PARD3*, although ZO-2 was discontinuous at the tight junction region due to short recovery time, it clearly co-localized with myc-PARD3. Two tight junction proteins emitted radically from the exo-PARD3 cell to touch the endo-PARD3 cells in order to form integrated tight junctions (long white arrow). In MDCK cells expressing mutant *PARD3* (D783G and P913Q), the ZO-2 didn't co-localize with exo-PARD3 (short white arrows). Scale bars: 10um in WT and P913Q, 25um in D783G.

Fig 4. Loss of mirror-symmetric distribution and apical polarity in human NPCs with *PARD3* siRNA-induced knockdown

48h after *PARD3* siRNA transient transfection, NPCs were fixed and stained. (A) In progenitor with control siRNA, PARD3 and PARD6 co-localize to the apical poles of NPC, the mirror-symmetry distribution between two daughter NPCs is maintained (white arrow). Most NPCs maintained a single and long axon-like synapse to the adjacent cell (red arrow). (B) In progenitor with *PARD3* siRNA, PARD6 localized along the entire membrane or cytoskeleton, instead of on the apical poles of NPC (low white arrow). In addition, the mirror-symmetry distribution between two daughter NPCs disappeared (top white arrow), and some knockdown NPCs harbored multiple minor processes (red arrow). Blue: DAPI; Red: PARD3; Green: PARD6.

Fig 5. Abnormal developmental phenotypes in *cPard3* knockdown chick embryos

A shows chick embryos at HH35 stage, and B shows chick embryos at HH37 stage. Control embryos were microinjected by GFP-tag lentiviral supernatant. Compared with age-matched controls, *cPard3*-KD chick presented with small forebrain (broad white arrow) and small or absent tail (thin white arrows).

Fig 6. The epithelial polarization process and the hinge point of neural tube disappeared in *cPard3* knockdown chick embryo

Transverse sections at the rostrocaudal axis were obtained from the posterior spinal cord (A) and tail (B) of chick embryos (HH34-35). The epithelial structure of the neural tube was stained. Green: Pard3, Blue: DAPI. (A) The square regions of panels 1, 3, 5 and 7 were captured and magnified on panels 5, 7, 9, 11. In the normal chick embryos, The Pard3 expressed on the apical location of epithelia, and was restricted to the MHP region (red arrows in panel 10). The epithelia predominantly aggregate on the ventral midline region (columnar cell, white arrows in panels 1, 5 and 9) rather than the dorsal midline region. The

inner lumen appears smooth, with the hinge point on the ventral tip (yellow arrowheads in panel 6, 10). With *cPard3* knockdown, the undifferentiated mesenchymal cells rather than epithelia cells aggregated evenly on the ventral and dorsal midline point (round cells, white arrows in panels 3, 7 and 11). The inner lumen of the neural tube was coarser, and the hinge point on the ventral tip was broader (yellow arrowheads in panel 8, 12). Multiple ectopic hinge-like invaginations presented in *cPard3* knockdown chick (yellow arrows in panel 8, 12) compared the control chick. (B) For the chicks injected with control shRNA, the smooth and wedge-like neural tube was formed with epithelial cells distributing neatly on three hinge points (white arrows). In the *cPard3* knockdown chicks, the undifferentiated mesenchymal cells formed the broader and coarse tube with multiple ectopic hinge-like invaginations (white arrows).

Table 1

The genomic variant burden of *PARD3* in the cranial NTD cases and the matched controls

Single nucleotide variants of <i>PARD3</i> ^a	138 cranial NTDs	274 matched controls	<i>p</i> value ^b
Rare whole genomic variant burden of <i>PARD3</i>	28	32	0.02
Rare missense and splicing variant	17	21	0.14
Missense variant	15	20	
Splicing variant	2	1	
Rare deleterious variant	11	7	0.018
Missense variant	9	6	
Splicing variant	2	1	
Rare deleterious variant in the binding region ^c	7	0	0.000
Missense variant	7	0	
Splicing variant	0	0	
Rare deleterious variant in the aPKC-binding region ^d	6	0	0.001
Missense variant	6	0	
Splicing variant	0	0	

^a*PARD3*:NM_019619.3.

^bThe proportion of cranial NTD cases with rare variants was compared with that of the matched controls, Fisher's exact test, two-tailed.

^cIncluding the variant in the PDZ region, aPKC-binding region.

^dThe region between protein 716 to 937.

Table 2

The frequencies of the rare deleterious variants of *PARD3* in the cranial NTD cases, the matched controls, the normal children cohort

Rare deleterious variants of <i>PARD3</i> ^a	Novel or reported (rs ID)	Cranial NTDs (138)	Controls (274)	Children cohort (800)
Chr10:34400183, G>A; c.3985C>T, p.R1329W	rs565005004	0	1	NA
Chr10:34400423, C>T; c.3745G>A, p.G1249S ^c	Reported	2	0	1
Chr10:34408604, C>A; c.3614G>T, p.R1205L	rs61735569	0	1	NA
Chr10:34558757, A>G; c.3256T>C, p.F1086L	Novel	a	1	NA
Chr10:34558808, C>G; c.3205G>C, p.E1069Q	rs3758458	0	1	NA
Chr10:34606028, G>A; c.3074+7C>T ^c	Novel	1	1	NA
<i>Chr10:34620149, G>T; c.2738C>A, p.P913Q^{b, c}</i>	<i>Novel</i>	<i>2</i>	<i>0</i>	<i>0</i>
<i>Chr10:34625160, T>A; c.2581A>T, p.T861S^{b, c}</i>	<i>Reported</i>	<i>3</i>	<i>0</i>	<i>4</i>
<i>Chr10:34630624, T>C; c.2348A>G, p.D783G^b</i>	<i>Novel</i>	<i>1</i>	<i>0</i>	<i>0</i>
Chr10:34671498, C>T; c.1369G>A, p.G457S	rs374261501	0	1	NA
Chr10:34671821, C>T; c.1046G>A, p.R349H	rs199923448	1	0	0
Chr10:34690842, C>T; c.718G>A, p.E240K	rs201544508	0	1	NA
Chr10:34739379, A>G; c.583-3T>C ^b	rs557643577	1	0	0
<i>Total</i>		<i>11</i>	<i>7</i>	<i>NA</i>

^a*PARD3*:NM_019619.3; all variants are heterozygous.

^bVariant in the aPKC-binding region.

^cRecurrent variant in this study; NA: not available.

Table 3

Abnormal developmental phenotypes in chick after *cPard3* RNAi

RNAi Strand	Successful Injections ^a	Embryo Survival to HH34-38 (%)	Embryo with GFP expression (%)	Small forebrain		Small tail	
				N	P value ^b	N	P value ^b
LV3 group	18	14(78%)	9(50%)	2	0.23	5	0.01
LV4 group	18	13(72%)	11(61%)	3	0.1	6	0.01
GFP control	15	12(80%)	9(60%)	0		0	
Non-injected control	12	11(92%)	0	0		0	

^aThe egg number with successful microinjection at HH 12-14stage.

^bTwo-tailed Fisher's exact test, compared with GFP control.