An X-linked *PLXNB3* mutation identified in patients with congenital heart disease with neurodevelopmental disabilities

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Background: Congenital heart disease (CHD) is the most common birth defect and is often accompanied by neurodevelopmental disabilities (NDD) which increase the associated mortality. Plexin families are known to play a key role in the development of heart and the occurrence of neurodevelopmental anomalies. However, there has been no report of *PLXNB3* mutation in isolated CHD or CHD with concomitant NDD.

Methods: We performed whole-exome sequencing (WES) on a proband with CHD with neurodevelopmental anomalies and his family members. Targeted sequencing, conservation analysis, AlphaFold, and PyRosetta were performed to identify more pathogenic mutations of *PLXNB3*. Scratch wound assay, Ki-67 assessment by flow cytometry, and gene expression analysis of heart development related pathway by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) were conducted after 24 h transfection in AC16 and HEK293T to investigate the effect of the target mutation.

Results: We identified a pathogenic mutation in the X-linked *PLXNB3* gene (c.A4319T p.E1440V). In addition, we found 4 other pathogenic mutations in a cohort of 75 patients with sporadic CHD with NDD. AlphaFold and PyRosetta predicted that these 4 mutations could cause dramatic changes of the PLXNB3 protein structure (root-mean-square deviation score >10 Å). Further functional analysis revealed that this p.E1440V variant inhibits cell migration and proliferation, and affects the activity of key factors in the Notch signaling pathway, myocardial contraction pathway, and neurodevelopmental pathways.

Conclusions: These findings suggest that *PLXNB3* and the p.E1440V variant may be related to the pathogenesis of CHD associated with NDD.

Keywords: Congenital heart disease (CHD); neurodevelopmental disability (NDD); PLXNB3; PLXNB3 mutations

Introduction

Congenital heart disease (CHD), one of the most common birth defects, refers to an abnormality of the heart or large blood vessels identified at birth (1). During the period from 1970 to 2017, the global prevalence of CHD gradually increased. The estimated incidence of CHD in Asia is 9.342/1,000 (2), and according to a prospective study, the estimated overall prevalence of CHD in China is 8.98/1,000 (3). CHD is often associated with other neurodevelopmental disabilities (NDD) and genetic or syndromic conditions (4,5): 10% of children with CHD and 50% of those with severe CHD also have NDD (6). Epidemiological studies suggest that environmental or genetic factors are involved in the causation of approximately 20–30% of CHD cases, while other unexplained CHD with NDD cases are believed to have a multifactorial causation (7). In recent years, an increasing number of studies have identified single-gene variants that are closely related to CHD. These single-gene variants mainly pertain to transcription factors, cell signaling and adhesion proteins which are closely related to the development of the heart (8,9).

PLXNB3, a member of the highly conserved plexin family, is the receptor for the axon guiding molecule Sema5A (10). PLXNB3 is a large transmembrane protein; its extracellular domain mainly includes Sema, 3 plexin-semaphorin-integrin (PSI) domains, and 4 plexin, and transcription factor (IPT) repeats, while the intracellular domain is mainly composed of a GTPase-activating protein (GAP) domain and Rho GTPase-binding domain (RBD) insert (11). Plexin families are involved in many aspects of heart development, such as myocardial compaction and trabeculation, neural crest and cardiac outflow tract development, epicardial development, pulmonary vein patterning, autonomic innervation of the heart, and vascular patterning (12). Gene mutations or abnormalities in the Sema/plexin signaling pathway can cause a variety of cardiovascular diseases. For example, in one study, Sema3E/plexinD1 pathway was shown to affect ventricular development by regulating Notch signaling pathway and extracellular cell matrix (ECM) dynamics; mice with abnormal Sema3E/plexinD1 pathway showed left ventricular noncompaction (LVNC) (13). Recent study has reported that the R1229C mutation of PLXND1 is pathogenic in persistent truncus arteriosus (14).

No report yet exists concerning the *PLXNB3* mutation in isolated CHD or CHD with NDD. In this study, we performed whole-exome sequencing (WES) on a patient with CHD complicated with neurodevelopmental anomalies and the patient's family members in order to identify a new pathogenic *PLXNB3* mutation which may play an important role in CHD with NDD. We present the following article in accordance with the MDAR reporting checklist (available at https://tp.amegroups.com/article/view/10.21037/tp-22-556/rc).

Highlight box

Key findings
- These findings suggest that PLXNB3 and the p.E1440V variant may be related to the pathogenesis of CHD associated with NDD.

What is known and what is new?
- Plexin families are involved in many aspects of heart development and the occurrence of neurodevelopmental anomalies, and gene mutations or abnormalities in the Sema/plexin signaling pathway can cause a variety of cardiovascular diseases.
- In this study, we first identified PLXNB3 as a pathogenic gene in patients with CHD with NDD, our research revealed that p.E1440V variant affected the cardiac muscle contraction and neurodevelopmental pathway, which is involved in the pathogenesis of CHD with NDD.

What is the implication, and what should change now?
- We consider PLXNB3 to be a possible gene marker for diagnosis of CHD with NDD. However, this hypothesis should be verified by more samples from CHD with NDD patients carried PLXNB3 mutations.

Methods

Patient recruitment

All children who presented with CHD-associated NDD at the Children's Hospital of Fudan University (CHFU) were enrolled in the study from May 2015 to December 2020. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of Children's Hospital of Fudan University (code No. 2016121) and written informed consent was obtained from the parents or legal guardians of the children.

WES and Sanger sequencing

Using the QIAamp DNA Blood Mini Kit (Qiagen), genomic DNA for WES and targeted sequencing was extracted from the peripheral blood samples of both

parents and individuals. Blood samples were extracted for whole WES by an Illumina Hiseq X Ten platform. The raw data obtained were compared with the reference genome (GRCh37/hg19), and the following 3 parts of information analysis process were completed: sequencing data quality assessment, variant detection, and variant screening and prediction of association with CHD. For the filtered sequencing results, the core family line was used as a whole for genetic analysis, and the 3 aspects of chromosomal dominant inheritance, invisible inheritance, and compound heterozygosity were screened to identify the possible disease-causing variants, with Sanger sequencing being later applied to verify and minimize the false positives. The Sanger sequencing primers were synthesized by Generay Biotech as follows: PLXNB3, forward, 5'-CTCATCCACACCCTGGAGGAGC-3' and reverse, 5'-CTGCGTAGCATGAGCTTGGGG-3'.

De novo homology modeling by AlphaFold and PyRosetta

We firstly used the SWISS-MODEL and CABS-fold servers to model the structure of *PLXNB3*; however, the obtained models were not acceptable because of the low homology with the template proteins. Subsequently, AlphaFold, the deep learning algorithm developed by DeepMind, was used to predict the model of *PLXNB3* in order to obtain more stable wild type (WT) structures. We then performed molecular dynamics (MD) simulations using PyRosetta, the script-based interface for implementing MD simulations algorithm, to obtain the protein structure of the mutant. In addition, Pymol, the molecular graphics system, as used to align and calculate the root-mean-square deviation (RMSD) score between the WT and mutant protein structure.

Cell culture and cell passage

The HEK293T and AC16 cell lines were grown in high-glucose Dulbecco's Modified Eagle Medium (DMEM; Gibco, Thermo Fisher Scientific) with 10% fetal bovine serum (FBS; Gibco), and 1X penicillin-streptomycin (Gibco) at 37 ℃ in a 5% CO_2 atmosphere. The *PLXNB3* ORF from the complement DNA (cDNA) library was subcloned into the pcDNA3.1 vector containing the Flag-tag produced the WT *PLXNB3* construct. PLXNB3-E1440V was created using the KOD-Plus Mutagenesis Kit (Toyobo). Following the manufacturer's instructions, Lipofectamine 3000 (Invitrogen, Thermo Fisher Scientific) was used to transfect

plasmids into cells in Corning culture dishes at a density of 50–70%. Given on the consistent protein expression at 24 and 48 h collected after transfection, the transfected cells were used for further investigation after 24 hours.

Western blotting

The cells were placed on ice after 24-hour transfection. Total proteins were extracted using RIPA buffer and a stop protease inhibitor cocktail (Thermo Fisher Scientific). A bicinchoninic acid (BCA) protein assay kit (Takara Bio) was used to measure the protein concentrations in accordance with the manufacturer's instructions. Subsequently, polyacrylamide gel was used to isolate an equal number of proteins and transfer them to polyvinylidene difluoride membranes (MilliporeSigma). The membranes were incubated overnight with primary antibodies at 4 ℃ and incubated with secondary antibodies for 2 hours at room temperature after being blocked in 5% skimmed milk for 90 minutes. Antibody against Flag, antibody against GAPDH, and antibody against rabbit were obtained from Cell Signaling Technology.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

HEK293T is a good cell model for exploring mechanisms of gene functions. To investigate the effect of mutations on PLXNB3 function, HEK293T cell RNA was extracted using TRIzol reagent (Thermo Fisher Scientific) and was reverse-transcribed into cDNA using a PrimeScript RT reagent Kit (Takara Bio). cDNA was them amplified by RT-qPCR using the TB Green Premix Ex Taq (Takara Bio). Human plexin-B3, GAPDH, and other signaling pathway–related primers are listed in Table S1.

Scratch wound assay

AC16 cells were plated into 6-well culture dishes. After attainment of 80% confluence, transient transfection was completed with the Flag, WT, and mutant plasmids according to the Lipo3000 (Thermo Fisher Scientific) manufacturer's protocol. After AC16 cells had attained confluent growth, a wound was produced by using 20-µL pipette tip. Cells were washed twice using phosphate-buffered saline (PBS) to remove floating cells and debris. The wound area was then monitored and measured at 0 and

24 hours.

Flow cytometry for assessment of cell proliferation

Cells were acquired using the Cytofix/CytopermTM Fixation/Permeabilization Solution Kit (BD Biosciences), and then the percentage of Ki67-positive cells was determined. Anti-Ki67 antibody was added (1:500), and the group of tubes without antibody was used as the control. Antibody against Ki67 and antibody against rabbit immunoglobin G (IgG); H+L; Phycoerythrin (PE) were obtained from Abcam.

RNA sequencing

The overexpression efficiency of HEK293T cells transfected with Flag, WT, and mutant plasmids was found to be consistent. Subsequently, transcriptome sequencing and data analysis were performed by Novogene.

Statistical analysis

All data were processed as mean ± standard deviation (SD) values from at least 3 independent experiments. Student *t*-test and 2-tailed Fisher exact test were used for all analyses. $P<0.05$ was considered indicative of statistical significance. Figures were created using GraphPad Prism 9.0 (GraphPad Software).

Results

Identification of the PLXNB3 mutation in patients

In a clinic in the Yunnan province of China, we found a proband aged 29 years who had CHD with neuromuscular malformations (*Figure 1A*). At the age of 6 years, he gradually developed limb weakness, and by the age of 26 years, he was completely unable to walk. In the past 5 years, he had recurrent exacerbations of congestive heart failure and heart enlargement. A 10-year-old child in the family pedigree had similar clinical manifestations. By tracking the medical history, we identified 3 suspected patients in this pedigree who died at the age of 30 years.

We performed WES to identify the missense mutation in the X-linked *PLXNB3* gene (c.A4319T p.E1440V), which was confirmed by Sanger sequencing (*Figure 1B*, Figure S1). The mutation site was highly conserved among human, mouse, zebrafish, and other species (*Figure 1C*), which

indicated that this missense variant was important for the function of the protein. We used AlphaFold and PyRosetta for *de novo* homology modeling of *PLXNB3* structure to compare the 3-dimensional structure of the WT PLXNB3 protein and the mutant p.E1440V protein. We found that the p.E1440V variant would cause structural change in amino acid, and the RMSD score was 26.859 Å (*Figure 1D*). The p.E1440V variant was predicted to be possibly pathogenic by Combined Annotation Dependent Depletion (CADD), Polyphen2 algorithm, and Sortig Intolerant From Tolerant (SIFT) program (*Table 1*).

We then recruited 285 patients with sporadic CHD who had concomitant NDD for targeted sequencing. As shown in *Table 2*, we observed 4 missense mutations which qualified the filtering criteria [Minor Allele Frequency (MAF) <0.01% and CADD >20]. Among these 285 patients, 4 were diagnosed as CHD with NDD (*Table 3*). In addition, 4 patients were hemizygote boys. The mutation sites were mainly concentrated on the non-Sema domain (*Figure 1E*). These variants were verified using Sanger sequencing (*Figure 1F*). To further explore whether these mutations alter the structure of PLXNB3, we used AlphaFold and PyRosetta to model protein structures *de novo*. All the mutations were found to cause dramatic changes in protein structures (RMSD >10 Å; Figure S2).

The above findings suggested that the X-linked *PLXNB3* gene and the p.E1440V variant may play an important role in the occurrence of CHD with NDD.

The influence of the PLXNB3 mutation in migration and proliferation of AC16 cells

In order to observe whether the *PLXNB3* mutation affects messenger RNA (mRNA) expression, we performed RT-qPCR by overexpressing the Flag, WT, and mutant plasmids in the AC16 cells. As shown in *Figure 2A*, the identified possible pathogenic variant was found to have no effect on mRNA expression. We also used western blotting to assess the influence of mutation on protein expression. As shown in *Figure 2B*, there was no significant difference in protein expression between the WT and the mutant groups.

It was reported that *PLXNB3* is linked to cell migration (15). To verify whether the p.E1440V mutant affects cell migration, we used scratch wound assay on AC16 cells. In the AC16 cells transfected with WT plasmid, the migration ability was lower than that of the Flag plasmid, but in the AC16 cells transfected with the p.E1440V mutation plasmid, the migration ability was higher than
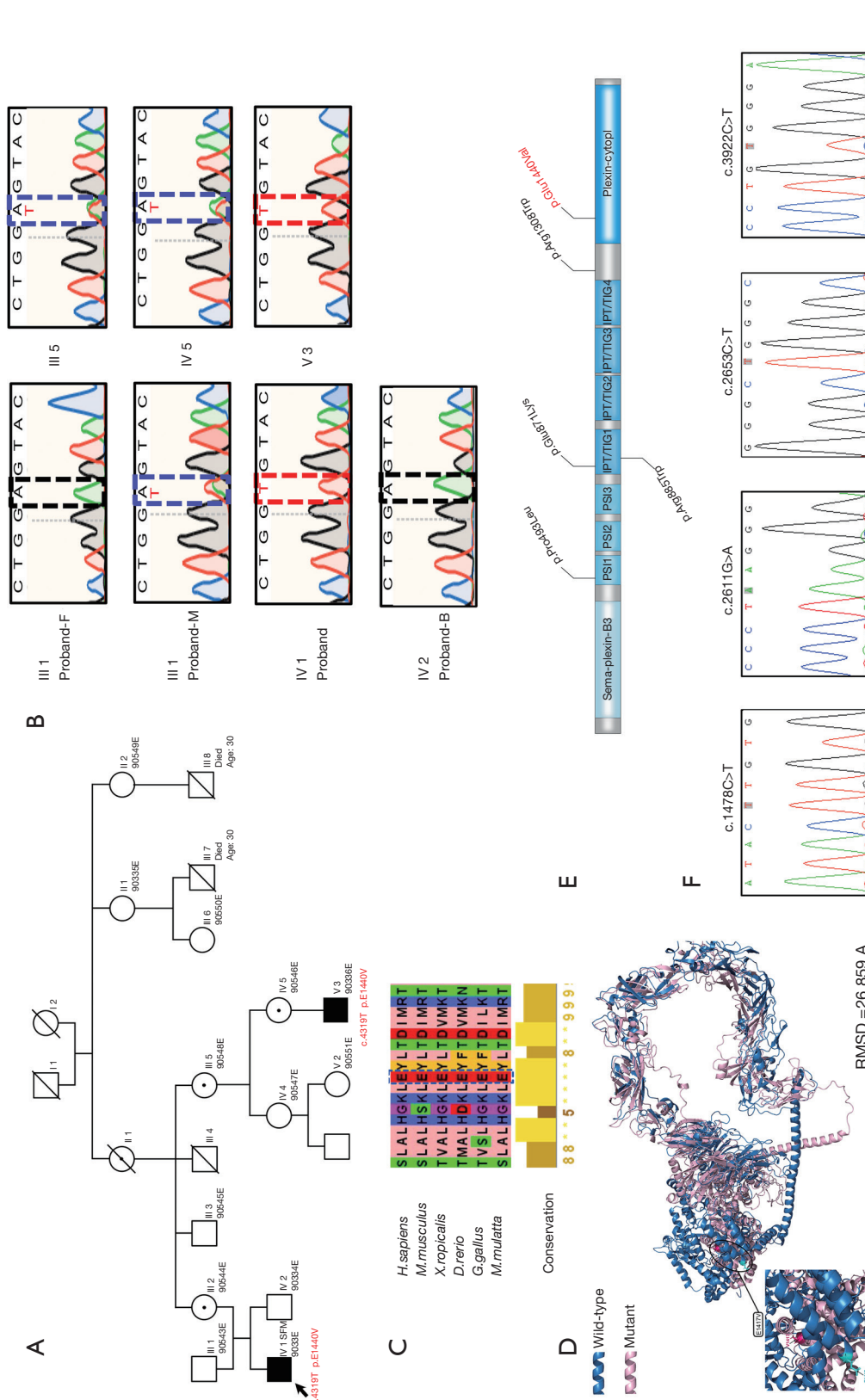
Figure 1 Genetic and phenotype analysis of the *PLXNB3* gene associated with CHD with NDD. (A) Pedigree of the Yunnan family with CHD and neuromuscular malformations. A square or circle with an origin represents a carrier, a square or circle with a diagonal line represents a deceased member, an arrow indicates the proband. (B) Sanger sequencing confirmation for a *PLXNB3* mutation in the family. (C) The p.E1140V mutant identified in the pedigree is highly conserved among the 6 species. (D) The PLXNB3 wild type and mutant protein structure predicted by AlphaFold and PyRosetta. (E) The position of all mutations in the protein domain. (F) Sanger sequencing confirmation for *PLXNB3* mutations in 4 patients with sporadic CHD with NDD. Proband-F, proband's father; Proband-M, proband's mother; Proband-B, proband's brother; RMSD, root-mean-square deviation. CHD, congenital heart disease; NDD, neurodevelopmental disability.

Table 1 Genetic findings in the Yunnan family with the *PLXNB3* variants

Gene	Variant type	Variant	Chromosomal position (GRCh37/hg19)	rs ID	CADD (v1.6)	Polyphen2	SIFT	gnomAD	
								Total	East Asian
PLXNB3	Missense	c.A4319T, p.E1440V	chrX:153040774	NA	28.5	D	T	Absent	Absent

NA, not available; CADD, Combined Annotation Dependent Depletion; SIFT, Sortig Intolerant From Tolerant; T, tolerated; gnomAD, The Genome Aggregation Database.

Table 2 Identified mutations in *PLXNB3*

Patient ID	Sex	Exon	Chromosomal position (GRCh37/hg19)	Variant	Zygosity	Impact	gnomAD		CADD (v1.6)
							East Asian	Total	
NO_2727	M	7	chrX:153034630	c.1478C>T, p.P493L	Hemizygote	Missense variant	–	–	20.5
NO_1097	M	16	chrX:153037343	c.2611G>A, p.E871K	Hemizygote	Missense variant	–	–	23.3
NO_1116	M	16	chrX:153037385	c.2653C>T, p.R885W	Hemizygote	Missense variant	0	6.40E-05	25.5
NO_3077	M	24	chrX:153040183	c.3922C>T, p.R1308W	Hemizygote	Missense variant	–	–	33

gnomAD, The Genome Aggregation Database; CADD, Combined Annotation Dependent Depletion.

Table 3 Clinical characteristics of the patients with CHD and *PLXNB3* mutations

Patient ID	Sex	Age (days)	Phenotype	
			Congenital heart defects	Neurodevelopmental disabilities
NO_2727	M	239	PDA	Brain atrophy
NO_1097	M	1666	TGA	Epilepsy
NO_1116	M	25	VSD, ASD	Epilepsy, Hypospadias
NO_3077	M	186	TGA, PDA	Brain dysplasia

CHD, Congenital Heart Defect; PDA, Patent Ductus Arteriosus; TGA, Transposition of Great Arteries; VSD, Ventricular Septal Defect; ASD, Atrial Septal Defect.

that of WT plasmid (*Figure 2C*). To assess the effect of this mutation on cell proliferation, we performed flow cytometry to detect Ki67 expression. As shown in *Figure 2D*, compared with that in the control group, the percentage of Ki67+ in the WT group was significantly decreased; however, in comparison to that of the WT group, proliferation of AC16 cells of the p.E1440V mutation group was significantly enhanced.

These results showed that this mutation may inhibit cell migration and cell proliferation.

The effect of the PLXNB3 mutation in Notch signaling pathway, cardiac muscle contraction pathway, and neurodevelopmental pathway

We subsequently performed RNA sequencing to detect the changes in the signaling pathway caused by the p.E1440V variant. In comparison to the WT group, there were 1764 differentially expressed genes ($|\log_2$ [fold change]$|>0$ and P value <0.05) in the mutant group, of which 826 were upregulated and 938 were downregulated (*Figure 3A*).
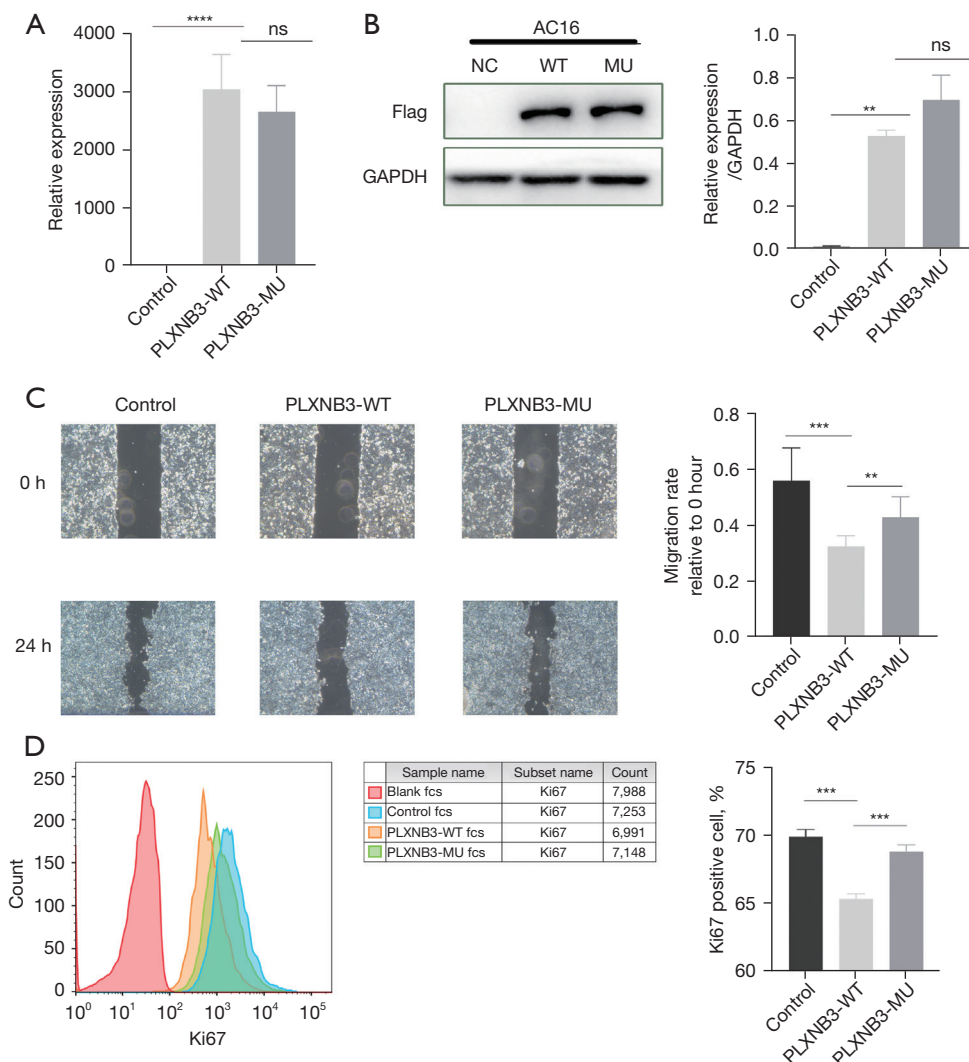
Figure 2 Functional changes caused by the *PLXNB3* E1140V mutation. (A) *PLXNB3* p.E1440V mutant has no effect on its own mRNA expression level. (B) There is no change in the protein expression level of the mutant. (C) Scratch wound assay (original magnification 40×) shows increased migration ability of AC16 cells overexpressing *PLXNB3* MU plasmids compared with AC16 cells overexpressing WT plasmids. (D) Results of flow cytometry showing enhanced Ki67 expression of AC16 cells overexpressing *PLXNB3* MU plasmids compared with AC16 cells overexpressing WT plasmids. **, P<0.01; ***, P<0.001; ****, P<0.0001; ns, no significance; NC, negative control; MU, mutant; WT, wild type; mRNA, messenger RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Differentially expressed genes were concentrated in signal pathways such as ribosome, Huntington disease, oxidative phosphorylation, Parkinson disease, Alzheimer disease, nonalcoholic fatty liver disease (NAFLD), and spliceosome and cardiac muscle contraction by KEGG enrichment analysis (*Figure 3B*). We also found changes in genes related to the Notch signaling pathway during differential gene analysis. Among these, the signaling pathways that were clearly related to heart development included Notch and

cardiac muscle contraction pathway. The pathways related to neurodevelopmental diseases included Huntington disease, Parkinson disease, and Alzheimer disease.

We further verified the effect of the p.E1440V variant on the above pathways by RT-qPCR. We tested some key genes of the Notch pathway (*Figure 3C*). Compared to *PLXNB3* in control group, the *PLXNB3* in WT group and mutant groups were overexpressed, and the transfection efficiency of the WT group and mutant group was
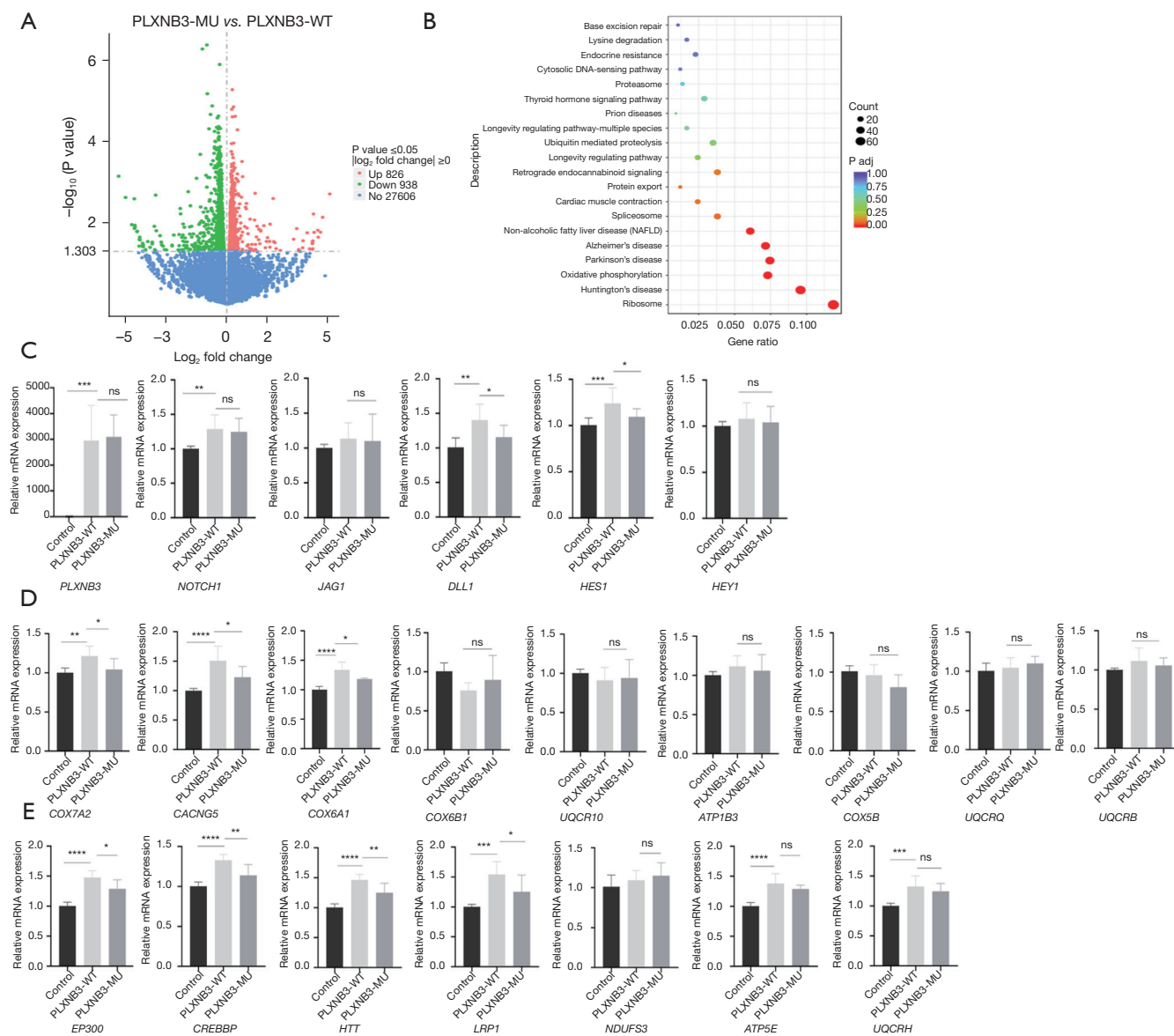
Figure 3 Impact of the *PLXNB3* p.E1440V mutant on its own expression and downstream signaling pathways. (A) Volcano plot showing genes differentially expressed between the MU group and WT group. There were 826 upregulated and 938 downregulated genes ($|\log_2$ fold change$|>0$ and P value <0.05) in the MU group. The abscissa is the $\log_2$ FoldChange value, the ordinate is the $-\log_{10}$ P value, and the blue dotted line represents the threshold line of the screening criteria for differential genes. (B) KEGG enrichment scatter plot shows the highly expressed pathways of the MU group compared with the WT group. The abscissa is the ratio of the number of differential genes to the total number of differential genes in KEGG pathway. The ordinate is KEGG pathway. (C) RT-qPCR analysis of Notch pathway genes using RNA isolated from the control, WT, and MU groups. (D) Expression levels of *COX7A2*, *CACNG5*, *COX6A1*, *COX6B1*, *UQCR10*, *ATP1B3*, *COX5B*, *UQCRQ*, and *UQCRB*, as measured by RT-qPCR using RNA isolated from the control, WT, and MU groups. (E) mRNA levels of *EP300*, *CREBBP*, *HTT*, *LRP1*, *NDUFS3*, *ATP5E*, and *UQCRH*, as observed by RT-qPCR using RNA isolated from the control, WT, and MU groups. Data are presented as mean ± standard error of the mean (SEM). *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001; ns, no significance; mRNA, messenger RNA; MU, mutant; WT, wild type; KEGG, Kyoto Encyclopedia of Genes and Genomes; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

consistent. In comparison to the control group, the other groups showed significant upregulation of *DLL1* and *HES1*. The mRNA levels of *DLL1* and *HES1* in the HEK293T cells transfected with the p.E1440V variant plasmid were lower than those of the WT plasmid. We did not find significant differences in *NOTCH1*, *JAG1*, and *HEY1* between the WT group and the p.E1440V variant group. We next examined some significantly different genes in the cardiac muscle contraction pathway (*Figure 3D*). We observed a remarkable increase of mRNA expression of only *COX7A2*, *CACNG5* and *COX6A1* in the WT group, and significantly lower mRNA expression of only *COX7A2*, *CACNG5* and *COX6A1* in the p.E1440V variant group compared to the WT group. To further evaluate whether this mutant influenced the activity of neurodevelopmental pathway, we selected several genes with obvious differences in RT-qPCR (*Figure 3E*). In the WT group, *CREBBP*, *LRP1*, *HTT*, *EP300* showed significant increase relative to the control group, but showed decrease relative to the mutant group. *UQCRH*, *NDUFS3*, and *ATP5E* showed no significant difference between HEK293T cells transfected with the WT plasmid and HEK293T cells transfected with the p.E1440V variant.

The above results indicated that the p.E1440V variant may downregulate the activity of Notch and cardiac muscle contraction pathway. Moreover, it affected the activity of neurodevelopmental-related factors.

Discussion

An accumulating body of evidence has indicated an important role of the plexin family in heart development, neurodevelopment, and cancer progression, but most reports have focused on PLXND1 (12). In this study, we not only identified the PLXNB3 p.E1440V variant in a pedigree with CHD and neurodevelopmental anomalies, but also identified 12 other variants in patients with sporadic CHD. These 13 identified mutations were all located on the non-Sema domain; therefore, we speculate that the non-Sema domain in the protein may be closely related to CHD. Among all the pathogenic mutations identified in patients with sporadic CHD, 4 patients had only CHD, and 7 patients had CHD with NDD. Patients with CHD who have NDD outnumbered those with CHD. Therefore, we speculate that the *PLXNB3* mutation may be more dominant in the category of CHD with NDD, which requires further investigations in the CHD population.

In this study, we found that the p.E1440V mutation

may play a key role in cardiogenesis and neurogenesis by regulating the Notch signaling pathway, myocardial contraction pathway, and neurodevelopmental pathway. Notch signaling plays an important role in cardiogenesis and disease models. Almost all cell types associated with the cardiovascular system are regulated directly or indirectly by Notch signaling (16). Aberrant Notch signaling always appears to cause disease phenotypes in each system examined (17). Effectors of the Notch signaling pathway (*DLL1*, *HES1*) are important for heart development. We found that these genes were reduced in HEK293T cells expressing mutant plasmids. *Hes1* is expressed in second heart field (SHF). Moreover, the lack of HES affects SHF and cardiac neural crest cell populations, resulting in defects in the proliferation of the SHF, decreased cardiac neural crest cells, and the inability to fully expand the outflow tract in the early mice embryo (18). In one study, the loss of function of *DLL1* in embryos was shown to cause asymmetric development of the heart, with the direction of embryonic turning being random (19). The downregulation of these effectors may lead to the interruption of signaling pathways essential for heart development, leading to heart defects. Hence, we consider *PLXNB3* to be a possible gene marker for diagnosis of CHD. However, this hypothesis should be further verified by larger sample of patients with CHD.

The *COX7A2* and *COX6A1* genes belong to a cytochrome oxidase family, which impairs the systolic and diastolic function of muscle (20). In a previous study, *COX7A1* knockout mice showed heart damage with impairment of systolic and diastolic function (21). We found that the mRNA expression levels of *COX7A2* and *COX6A1* in the mutant group were reduced, which is consistent with the muscle phenotypes of our patients.

Our data suggest a reduced expression level of *CREBBP*, *LRP1*, *HTT*, and *EP300* in the mutation group, indicating that the *PLXNB3* p.E1440V variant may cause a neurological disease phenotype. *LRP1* has been shown to play a key role in the generation of oligodendrocytes, while the lack of *LRP1* in the neural stem cells was shown to significantly reduce their ability to differentiate into oligodendrocytes (22). Other research reports that mice with the *LRP1* p. C4232R variant had a phenotype of a atrioventricular septal defect with a double-outlet right ventricle, and a similar phenotype can occur with the conditional deletion of *LRP1* in cardiac neural crest cells (23). The *HTT* gene is essential for early neural development. Decreased expression of HTT in neuroepithelial cells was found to increase neuronal cell

apoptosis, reduce proliferation, and impair migration ability (24). In patients with Huntington disease caused by a loss of *HTT* function, the metabolism of cardiomyocytes may be impaired, and neurological symptoms may be accompanied by cardiovascular pathology (25). Previous reports have pointed out that the loss of function of the *CREBBP* and *EP300* genes can lead to Rubinstein-Taybi syndrome (RSTS), which is a congenital disease characterized by intellectual disability, developmental delay, and various malformations (26,27). Deletion or decreased expression of these genes may be related to the occurrence of neurological diseases. Therefore, we speculate that the p.E1440V mutation may be related to the neurodevelopmental defects of the proband.

Normal growth of the embryonic heart is maintained by highly proliferating cardiac tissue cells and a precisely regulated cardiomyocyte proliferation cycle (28). Proliferation and migration of cardiomyocytes are necessary for ventricular trabeculation and cardiac outflow tracts (29). Several studies have shown that *PLXNB3* is closely related to cell migration and has a certain effect on cell proliferation. However, the contemporary studies on the effects of *PLXNB3* on cell migration and proliferation have been mainly conducted in the context of tumors. *PLXNB3* was shown to significantly inhibit glioma cell migration and cell invasion caused by Sema stimulation, but this effect almost disappeared after knockdown of *PLXNB3* (30). In the study by Sadanandam *et al.* (31), knockdown of *PLXNB3* in microvascular endothelial cells (HMEC-1) was found to inhibit cell proliferation stimulated by Sema5A. However, there are few reports on the role of *PLXNB3* mutations associated with CHD with neuromuscular malformations in cell migration and proliferation. Therefore, we further explored whether the p.E1440V variant affects cell migration and proliferation, and we found that the p.E1440V variant has an inhibitory effect on the migration and proliferation of AC16 cells compared with the WT. *HES1* is a transcriptional regulatory factor that plays an important role in cell proliferation, differentiation, and migration. Murata *et al.* (32) demonstrated that *HES1* may promote cell proliferation by altering *P27 KIP1* gene expression. Therefore, we speculate that the *PLXNB3* p.E1140V variant may affect cell proliferation and migration by reducing the expression of *HES1*.

Some limitations of our study should be acknowledged. First, the role of changes in cell proliferation and migration induced by *PLXNB3* p.E1440V is unclear in CHD with NDD. Second, we didn't detect the expression changes of

PLXNB3 in the patients with CHD with NDD because of difficulty of recalling patients. Therefore, we will try to collect more samples from new CHD with NDD patients carried *PLXNB3* mutations to detect the PLXNB3 expression and explore the pathogenic role of *PLXNB3* mutations in isolated CHD or CHD with NDD.

Conclusions

In this study, we identified a new pathogenic *PLXNB3* p.E1440V mutation in a family with CHD and NDD which may expand the spectrum of *PLXNB3* variants associated with CHD with NDD. Our study shows that the functional changes caused by *PLXNB3* pathogenic mutation may be a potential genetic mechanism of CHD with NDD.

Acknowledgments

Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://tp.amegroups.com/article/view/10.21037/tp-22-556/rc

Data Sharing Statement: Available at https://tp.amegroups.com/article/view/10.21037/tp-22-556/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tp.amegroups.com/article/view/10.21037/tp-22-556/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of Children's Hospital of Fudan University (code

No. 2016121) and written informed consent was obtained from the parents or legal guardians of the children.

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Table S1 Primer pairs for RT-qPCR analysis

Primer name	Sequences
PLXNB3-F	CGCTTCTCCGCACCTAATACC
PLXNB3-R	CAGGGCTGTCGATTACAGGG
GAPDH-F	GGAGCGAGATCCCTCCAAAAT
GAPDH-R	GGCTGTTGTCATACTTCTCATGG
Notch1-F	GAGGCGTGGCAGACTATGC
Notch1-R	CTTGTACTCCGTCAGCGTGA
Jag1-F	GTCCATGCAGAACGTGAACG
Jag1-R	GCGGGACTGATACTCCTTGA
DLL1-F	GATTCTCCTGATGACCTCGCA
DLL1-R	TCCGTAGTAGTGTTCGTCACA
HES1-F	TCAACACGACACCGGATAAAC
HES1-R	GCCGCGAGCTATCTTTCTTCA
HEY1-F	GTTCGGCTCTAGGTTCCATGT
HEY1-R	CGTCGGCGCTTCTCAATTATTC
UQCRB-F	GGTAAGCAGGCCGTTTCAG
UQCRB-R	AGGTCCAGTGCCCTCTTAATG
UQCRQ-F	CGCGAGTTTGGGAATCTGAC
UQCRQ-R	TAGTGAAGACGTGCGGATAGG
COX7A2-F	CTCGGAGGTAGTTCCGGTTC
COX7A2-R	TCTGCCCAATCTGACGAAGAG
UQCR10-F	ATCGTGGGCGTCATGTTCTTC
UQCR10-R	ATGTGGTCGTAGATAGCGTCC
COX6B1-F	CTACAAGACCGCCCCTTTTGA
COX6B1-R	GCAGAGGGACTGGTACACAC
COX5B-F	ATGGCTTCAAGGTTACTTCGC
COX5B-R	CCCTTTGGGGCCAGTACATT
ATP1B3-F	AACCCGACCACCGGAGAAT
ATP1B3-R	TGAGAGTCTGAAGCATAACCCA
CACNG5-F	GGCGTTGCTTCACCATAGAAT
CACNG5-R	GCTGAGCGGATCATCTTTAGAAC
CREBBP-F	CAACCCCAAAAGAGCCAAACT
CREBBP-R	CCTCGTAGAAGCTCCGACAGT
ATP5E-F	GGCTGGACTCAGCTACATCC
ATP5E-R	TTACGTTGCTGCCAGAAGTCT
EP300-F	AGCCAAGCGGCCTAAACTC
EP300-R	TCACCACCATTGGTTAGTCCC
HTT-F	AAACTTCTGGGCATCGCTATG
HTT-R	GTTGAGGCATTCGTCAGCCA
UQCRH-F	GAGGACGAGCAAAAGATGCTT
UQCRH-R	CGAGAGGAATCACGCTCATCA
LRP1-F	CTATCGACGCCCCTAAGACTT
LRP1-F	CATCGCTGGGCCTTACTCT
NDUFS3-F	ACTGTCAGACCACGGAATGAT
NDUFS3-R	GGGCAAGATTTCAGCCACATAC

RT-qPCR, reverse transcription-quantitative polymerase chain reaction.
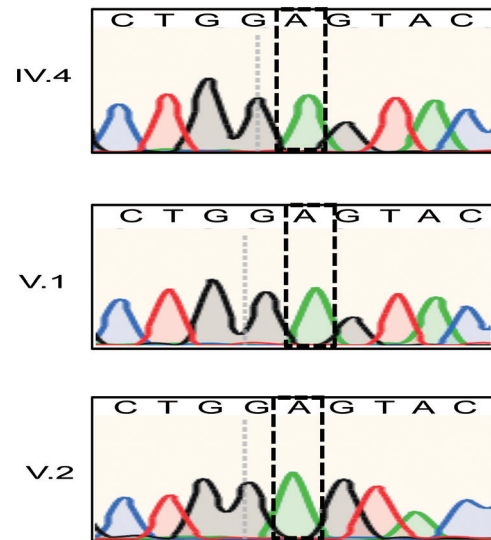

Figure S1 Sanger sequencing in the other members of the Yunnan family for confirmation of the c.A4319T mutation.

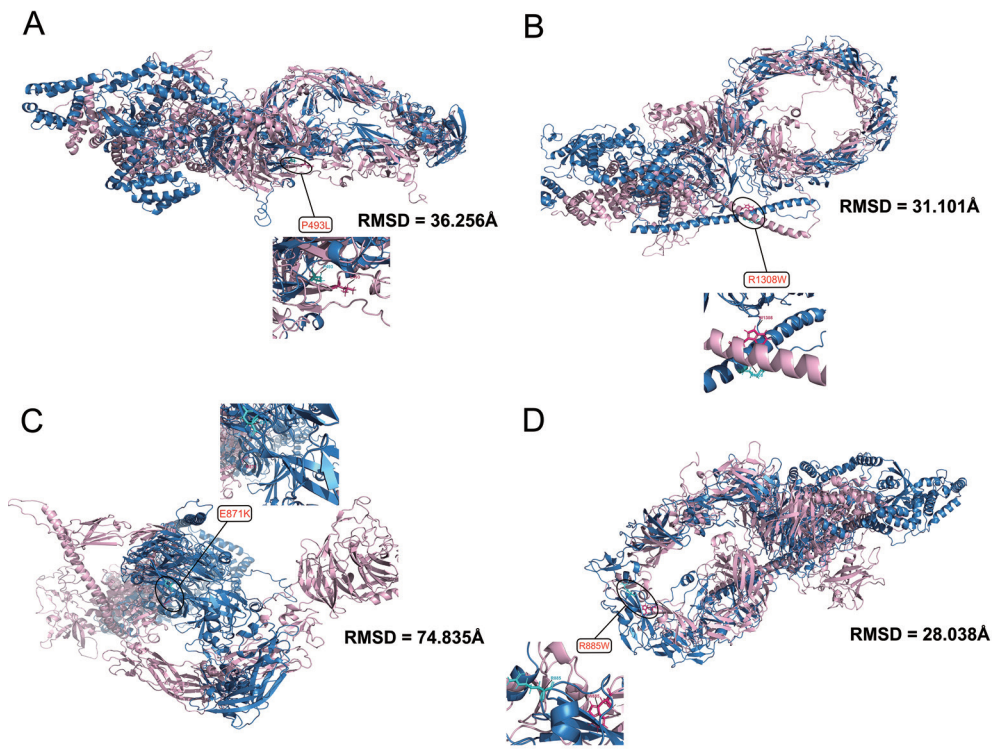**Figure S2** Structural changes in PLXNB3 protein caused by four mutations predicted by Alphafold and PyRosetta.