The rare mutation in the endosome-associated recycling protein gene VPS50 is associated with human neural tube defects

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Abstract

Background: Tight control of endosome trafficking is essential for the generation of a normally patterned embryo. Recent studies have found that VPS50 is a key ingredient in EARP which is required for recycling of internalized TfRs to the cell surface and dense-core vesicle maturation. However, the role of VPS50 in embryogenesis and human physiology are poorly understood.

Results: We identified a rare missense heterozygous VPS50 mutation (p. Gly169Val) in NTDs by high-throughput sequencing. In vitro functional analysis demonstrated that the p. Gly169Val was a loss-of-function mutation, delaying transferrin recycling and altering its interaction with VPS53. Using WISH during zebrafish embryogenesis, we demonstrated that vps50 gene was expressed throughout the early embryo, especially in the head. Abnormal body axis phenotypes were observed in those vps50 knock-down zebrafishes. Further rescue study in zebrafish suggested that the mutation displayed loss-of-function effects comparing with wild-type VPS50.

Conclusions: These findings thus demonstrated that the functional mutations in VPS50 might contribute to neurodevelopmental disorder and highlighted the critical importance of VPS50 function in cellular and organismal physiology.

Keywords: VPS50, EARP, WISH, Zebrafish, NTDs

Background

Endosomes, mediating transport of intracellular and extracellular cargos, are important functional elements in the organization of intracellular membrane dynamics [1]. In addition to the house-keeping functions common to all eukaryotic cells, endosomal transport appears to be of particular importance in polarized cell types such as the extension of axons and dendrites in neuronal cells [2]. For simplicity’s sake, endosomes’ components are frequently dismantled into discrete units such as early (sorting) endosomes, late endosomes (multivesicular bodies), recycling endosomes and lysosomes [3–5]. Once in endosomes, the fates of internalized proteins diverge. Some proteins are transported to lysosomes, other internalized proteins are delivered to the trans-Golgi network or back to the plasma membrane by recycling endosomes.

In general, recycling endosomes are found associated with unique sets of proteins [6]. Recent studies show that EARP is involved in recycling cargos from endosomes back to plasma membrane. It shares three identical proteins with GARP: Vps51, Vps52 and Vps53 [7], but uses VPS50 instead of VPS54 as the fourth subunit [8, 9]. This change determines differential localization of EARP to recycling endosomes and GARP to the Golgi complex. VPS50 is mainly associated with the cytosolic face of endosomes marked by the small GTPase Rab4, and promotes the recycling of TfRs from endosomes to the plasma membrane. The functions of EARP in TfRs transport are essential for the viability of mammalian organisms, as demonstrated by the embryonic lethality of mice with homozygous null mutations in this pathway,
showing abnormalities in the nervous system such as kinked neural tubes [10–12]. Recent genome-wide linkage analysis also identified mutations in VPS53 and VPS51 genes encoding different subunits of EARPS in patients with complex neurological diseases [13, 14]. These results raise the possibility that rare mutations in VPS50 may be associated with human NTDs.

**Material and methods**

**Study subjects and capture DNA sequencing**

100 patients with NTDs and 171 healthy controls were all ethnically Han Chinese (Table 1). All the cases were collected in Shanxi Province. The unrelated healthy controls \( (n = 171,17.9 \pm 1.3 \text{ years}) \) were collected from new recruits in Shandong and Henan province of China. Each sample was collected with the approval of the local ethics committee and institutional review board of Fudan University. Written consent was obtained from the patients’ parents. Whole genome sequencing was conducted using Illumina HiSeq X ten at WuXi AppTec. Genotyping was performed according to the Infinium HD protocol from Illumina. All controls were confirmed by Sanger sequencing as previously described [15]. Partial alignment of Vps50 amino acid sequences between human and other vertebrates was aligned using the Clustalx program. The effect of missense variants were predicted according to SIFT [16] (Sorting Intolerant from Tolerant; http://sift.jcvi.org/) and PolyPhen-2 [17] (Polymorphism Phenotyping version 2.1.0; http://genetics.bwh.harvard.edu/pph/) programs.

**Plasmid constructs and in vitro transcription of amplified cDNA**

Human VPS50 cDNA (NM_017667) was cloned into pEnter-Flag/His vector with puromycin screening marker. The plasmid constructs were verified by Sanger sequencing. The VPS50 vectors were linearized with Xhol restriction enzyme (NEB, USA) and transcribed with the T7 mMESSAGE mMACHINE kit (Ambion, USA). The reaction was carried out at 37°C for 1 h, followed by the addition of DNase I and incubation for 15 min. Ammonium acetate was added, and RNA was isolated by phenol/chloroform extraction and isopropanol precipitation. After centrifugation, the RNA pellet was resuspended in RNase-free water, and purity was determined by UV spectrophotometry and electrophoresis.

**Generation of VPS50 knockout HeLa cell lines**

For VPS50 KO Hela cell lines, the gRNA was designed and cloned in an U6 targeting vector [18] and the single clones were established by dilution cloning. Knockout efficiencies were confirmed by Western Blotting and Immunofluorescence. gRNA sequence used was:

VPS50 exon1 5′-CAAATCTCTCATGACCCGAC-3′

**Western blot**

Cells were lysed in RIPA buffer (Beyotime, China). The lysates were denatured at 100°C for 5 min and then cooled down on ice. Then lysates were loaded on sodium dodecyl sulfatepolyacrylamide gel (SDS-PAGE) (10%) and electrotransferred onto polyvinylidene difluoride (PVDF) membrane. After blocking with 5% nonfat milk in TBST (Tris-buffered saline, 0.1% Tween 20) for 2 h at room temperature, PVDF membranes were blotted with primary antibody at 4°C for 12 h, then incubated with HRP-labeled secondary antibody (CST, USA) at room temperature for 2 h. The bands were visualized using Tanon 5200 (Tanon, China). Primary antibodies are as follows: mouse monoclonal antibody to VPS50 (Abnova, China). Mouse monoclonal antibody to beta-actin (CST, USA).

**Co-immunoprecipitation**

HEK293T cells were transfected with 4μg wild-type or the VPS50 mutation and 4μg VPS53-HA vector per well using Lipofectamine2000 reagent (Life Technologies, USA) by manufacturer’s instructions with a 0.5:1 ratio of reagent to DNA. Cells were allowed to incubate with transfection media for 48 h then were washed with PBS and harvested in cold RIPA lysis buffer (Beyotime, China) containing protease inhibitors (Roche, Germany). Cells were lysed by rocking at 4°C for 20 min and then Immunoprecipitation with anti-HA mouse polyclonal agarose beads (Abmart, China).

**Immunofluorescence and transferrin chase**

Immunofluorescence was performed as described previously [19] on a Zeiss LSM700 microscope (Carl Zeiss, Germany).
Germany). Transferrin chase was carried out using a modification of a previously described protocol [8].

Whole mount in situ hybridization
A 626 bp cDNA fragment of vps50 was sub cloned into pGEM-T-easy vector (Promega, USA), which was conservative across species, with the primers as the following: 5′-GGCAGCCAAAAGCCATAGA-3′ (forward); 5′-TGCA GACCAGCGCAAGACA-3′ (reverse). Spe I and Nco I restriction enzyme (NEB, USA) were chosen to linearize the probe plasmid respectively. The Digoxigenin-labeled sense and antisense probes were synthesized by Sp6 and T7 mMESSAGE mMACHINE kit (Ambion, USA). The wild type (WT) AB zebrafish embryos were collected and fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) in the 4 °C for overnight. Then the embryos were dehydrated with gradient Methanol and PBST (Tween-20, 1%) mixture and stored in 100% Methanol in −20 °C. Whole mount in situ hybridization was subsequently followed the protocol described by Rodney M. Dale’s study [20].

Zebrafish embryo microinjections
The WT AB zebrafish were maintained and bred under standard conditions and embryo microinjection was performed following the standard protocol [21]. Morpholino oligos (MOs, Gene Tools) acted as a means to inhibit gene function in embryos [22]. We injected vps50 MOs, directed against the ATG of the vps50 gene, with a different concentration gradient, respectively, into zebrafish embryos during the one to two cell stages. To validate MOs knockdown efficiencies and verify the conservation of VPS50 function, we injected human mRNA of VPS50 with a different concentration gradient in pairwise combinations with MOs, respectively, into zebrafish embryos during the one to two cell stages. Post injection (48 h), embryos were observed by microscopy, phenotypes were scored, and images were taken using a QImaging microscope system. Zebrafish vps50 MOs and standard control MOs were purchased from Gene Tools and the sequences were:

- vps50 MOs: 5′–TTCTGCATCGAACAGTCGAGG
- Control MOs: 5′–CCTTTACCTCAGTTACAATTT

ExAc database (Table 2). By alignment of VPS50 ortholog protein sequences, we found that the p. Gly169 residue was highly conserved between species (Fig. 1b). Sanger sequencing is the gold standard for confirmation of minor variants detected by NGS (Fig. 1a). A more detailed sequencing results of VPS50 can be found in Additional file 1.

The p. Gly169Val mutation delayed transferrin recycling
To investigate the in vitro activity of the p. Gly169Val mutation, we generated VPS50 knockout Hela Cell Lines. Knockout efficiency was examined by Western Blot and Immunofluorescence (Fig. 2a and b). Then VPS50-KO Hela cells were transfected using Lipofectamine 3000 with various VPS50 plasmid constructs. After 24 h, puromycin was added into the DMEM for 24 h to filter untransfected cells (Additional file 2: Figure S1). Then cells were subjected to transferrin chase. As previously described [8], the intracellular Alexa Fluor 568 Conjugated transferrin was lost more rapidly in Hela cells than in VPS50-KO cells and the p. Gly169Val mutation followed the same pattern of delaying transferrin recycling as the VPS50-KO cells (Fig. 2c and d). These experiments thus demonstrated that the p. Gly169Val mutation partially disrupted the function of VPS50, delaying transferrin recycling.

The p. Gly169Val mutation disrupted the interaction of VPS50 with VPS53
Since EARP consists of ANG2, VPS52, VPS53 and VPS50, then we constructed the other three parts of EARP plasmids and tested their physical interaction by using Co-immunoprecipitation (Co-IP). We found that VPS50 could interact with VPS53 in vitro. Then, we co-transfected the various VPS50 plasmid constructs in

| Nucleotide change | Amino acid change | Case no. | Control no. | SIFT | PolyPhen2 | Sex | Age (weeks) | NTD type | MAF in cohort | MAF in CHB |
|-------------------|-------------------|---------|-------------|------|------------|-----|-------------|----------|--------------|-------------|
| c.G506T           | p. Gly169Val      | 1       | 0           | 0.04 | 1          | F   | 12          | anencephalus | 0.001845     | not reported |
| c.A1104T          | p. Glu368Asp      | 1       | 1           | 0.25 | 0.003      | F   | 24          | anencephalus | 0.00369      | 0.0049      |

For nucleotide numbering, +1 corresponds to the A of ATG of mRNA sequence 2. NM_017667.3
Reference protein sequence NP_060137.2
The threshold for intolerance is 0.05 and ranges from 0 to 1. The amino-acid substitution was predicted to be damaging if the score was <=0.05 and tolerated if the score was >0.05
The score is from 0 to 1; the amino-acid substitution was appraised qualitatively as benign or damaging based on pairs of false positive rate thresholds and optimized separately for each model.
pairwise combinations with the HA-tagged VPS53 cDNA into HEK293T cells. Immunoprecipitation with antibodies to HA and western blotting with antibodies to Flag. The results of Co-IP showed that the p. Gly169-Val mutation destroyed the interaction of VPS53 to a certain extent (Fig. 3).

Spatial expression of gene vps50 during zebrafish early development

Since our research group was interested in utilizing the zebrafish model to investigate the function of conserved genes during development, we obtained 11 identified or predicted chordate Vps50 proteins spanning from the zebrafish to Homo sapiens to generate a phylogenic tree [15, 23]. By protein sequence alignment, a phylogenetic tree demonstrated that the Vps50 proteins seemed to follow the predicted vertebrate phylogenetic order with higher amino acid conservation being observed within a subclass or clade of animals (Fig. 4a). Interestingly, even zebrafish and humans shared common ancestors 435 million years ago, the Vps50 amino acid identity was quite conserved between zebrafish and human (~ 84.6%) (Additional file 3: Figure S2).

The high conservation of Vps50 proteins between zebrafish and human suggested that zebrafish vps50 was a good candidate to study aspects of VPS50 function during embryogenesis. To map the expression of zebrafish vps50 during embryogenesis, we designed a specific sense and antisense riboprobe to determine its spatiotemporal expression by utilizing WISH. We chose the following zebrafish early stages individually: 0.5 dpf, 1 dpf, 2 dpf, and 3 dpf. The early stages tested with the antisense probe demonstrated the presence of vps50 mRNA, while the sense probe assayed at the same stages showed no staining (Fig. 4b).

All stages tested with the antisense probe demonstrated the presence of vps50 mRNA. By 0.5 dpf, embryonal transcripts of vps50 were expressed globally. By 1 dpf, we started to see a tissue specific expression of vps50. vps50 gene was expressed around the yolk sac, especially in the height of the head and a small amount of expression in the tail. By 2–3 dpf, the expression pattern of vps50 became restricted in the head and heart. Our experimental results revealed the time and spatial expression pattern of vps50 in the early development of zebrafish.

vps50 affecting the development of zebrafish embryos

The results of WISH showed that vps50 was widely expressed during embryogenesis. To explore the effects of vps50 deficiency on development of zebrafish embryos, we designed antisense MOs (vps50-MOs), directed against the ATG of vps50 gene. Injection of vps50-MOs, and not control MOs, into embryos resulted in different populations of abnormal embryos after 48 h post fertilization. These injected zebrafish embryos were clustered into four categories according to the severity of their morphology (grade 1: WT like; grade 2: mild, curving axis compared with WT embryos; grade 3: moderate, up to 1/3 shortened axis; grade 4: severe, the body axis just extend out of the range of the yolk ball, Fig. 5a). As the concentration of the vps50-MOs increased, the abnormal rate increased. When injecting with 6 ng vps50-MOs, 58.3% of the injected fish showed abnormal phenotype (Fig. 5b). To validate
vps50-MOs knockdown efficiencies and verify the conserved function of VPS50, we carried out rescue experiments. Dosage-dependent rescued of vps50 knock-down zebrafish embryo with human VPS50 was shown in Fig. 5b. With the dosage of 400 pg VPS50 mRNA, most of the mild and moderate phenotypes were rescued (Fig. 5b). To further clarify the effect of the VPS50 mutation, we injected 6 ng vps50-MOs with the VPS50 variant mRNA. Compared to WT VPS50, the p. Gly169Val mutation displayed the loss-of-function effects.

**Discussion**
The effect of EARP-disrupting mutations on development extends to a broad range of cellular functions. It is known that in yeast cells, the loss of other components of EARP complex causes identical phenotypes with reduced growth rates [24–26]. Recent studies also found that EARP complex could function in the maturation of dense-core vesicles and vps50 mutations could alter neuropeptide and dense-core vesicle protein levels at synapses; Knockdown of VPS50 expression in Hela Cells disrupted the balance.
of TfRs on the cytoplasmic membrane. Interestingly, \(TfR^{-/-}\) embryos were abnormal and died before embryonic day (E) 12.5 showing kinked neural tubes [10]. Pathogenic mutations in the subunits of EARP have been found to cause the neurological deficits in the patients due to partial loss of EARP [14]. These results raise the possibility that the rare mutations in \(VPS50\) may be associated with human NTDs, although \(VPS50\) has never been reported to be associated with any human disease.

By high-throughput sequencing, we identified a rare case-specific mutation (p. Gly169Val) in \(VPS50\) gene in a Chinese NTDs cohort. The p. Gly169Val mutation was located in the highly conserved residues in vertebrates, suggesting its critical function. In vitro results showed that the p. Gly169Val was a loss-of-function mutation, delaying transferrin recycling and disrupting its interaction with \(VPS53\).

The high conservation of Vps50 proteins between zebrafish and human suggested that zebrafish \(vps50\) was a good candidate to study functions of \(VPS50\) in early embryo development. Using WISH, we firstly identified that \(vps50\) was highly expressed in early development of zebrafish and the expression pattern of \(vps50\) changed with different developmental stages. Embryonal transcripts of \(vps50\) were expressed globally at 0.5 dpf. After 1 dpf, the

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**Fig. 3** Co-immunoprecipitation illustrated the effect of novel mutations on the physical interaction of \(VPS50\) with \(VPS53\). HEK293t cells were co-transfected into 4\(\mu\)g \(VPS50\) plasmid constructs in pairwise combinations with 4\(\mu\)g \(VPS53\) cDNA. HA immunoprecipitation, blotting with anti-Flag confirmed physical interaction between WT \(VPS50\) and \(VPS53\). This interaction was decreased in the p. Gly169Val mutation

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**Fig. 4** a A protein phylogenetic tree was constructed of obtained sequences. Zebrafish and other chordate Vps50 protein sequence were identified from the Uniprot database. Accession numbers: Human: Q96JG6; Rat: F1L5G8; Mouse: Q8C71I; Zebrafish: F1RA04; Chicken: Q52VK9; Chimpanzee: H2QUX7; Bovine: F1ML08; Western clawed frog: A9UM56; Japanese pufferfish: H2UPA7. b Expression patterns of zebrafish \(vps50\) during embryogenesis. Expression of \(vps50\) gene was detected around the yolk sac at 1 dpf. Once the embryo reached the hatching period (2–3 dpf), \(vps50\) expression was seen in the head and heart (Right Figure). Representative images of \(n = 3\) replicates where with each replicate having at least 15 embryos. Control images of sense strand probed on early embryos (Left Figure)
expression pattern of the vps50 gene became more concentrated and specific. By 2 dpf and 3 dpf, the late pharyngula stage, expression of the zebrafish vps50 gene was localized in the head and heart of the embryo. These results were consistent with the previous studies that many genes in the recycling endosome were found to be expressed throughout the early embryo [27, 28]. Since vps50 was highly expressed in zebrafish embryonic development, vps50 knockdown by MOs was carried out. Knockdown of vps50 during embryo development of zebrafish resulted in high ratio of abnormal body axis. These defects could be rescued significantly by injection of exogenous human VPS50. Knockdown and rescue experiments suggested that the presence of Vps50 in the early embryo might be indispensable during vertebrate development. Further rescue study in zebrafish suggested that p. Gly169Val displayed loss-of-function effects compared with wild-type VPS50.

**Conclusion**
This is the first report describing the association of VPS50 gene mutations with human NTDs. In vivo functional analysis demonstrated the expression pattern of vps50 during embryogenesis and found that it played an important role in the early development of zebrafish. Further understanding the roles of VPS50 in the regulations of post-endocytic transport pathways and development are critical and necessary.

**Additional files**

*Additional file 1:* List of VPS50 mutations. (XLSX 68 kb)
*Additional file 2:* Figure S1. The immunofluorescence showed that after using puromycin to filter untransfected cells, transfection efficiency was quite high. (DOCX 2395 kb)
*Additional file 3:* Figure S2. Sequence alignment of different vertebrate Vps50. (DOCX 1448 kb)
Abbreviations
EARP: Endosome-associated recycling protein; GARP: Golgi-associated retrograde protein; MOs: Morpholino oligos; NTDs: Neural Tube Defects; TRFs: Transferrin receptors; WSH: Whole mount in situ hybridization

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Availability of data and materials
Please contact the corresponding author for data requests.

Authors’ contributions
ZS, YZ and HW performed lab working. ZS and XH sorted out the experimental data and wrote the paper. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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