PiT2 regulates neuronal outgrowth through interaction with microtubule-associated protein 1B

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PiT2 is a member of the inorganic phosphate transporter family, and is extensively expressed in the nervous system. It was found that loop7 domain of PiT2 is not required for retroviral recognition and transport function. The exact functions of loop7 remain poorly understood. Here we show that loop7 of PiT2 is necessary for the transport of PiT2 protein to the cell surface. Further, loop7 is also related to the outgrowth of neurite in Neuro2A cells interacts with the light chain 1 of microtubule-associated protein 1B (MAP1B). PiT2 with mutated MAP1B binding sites affect neurite outgrowth whereas Pi transport function deficient mutants of PiT2 do not. We also show that Drosophila dPiT interacts with microtubule-associated protein Futsch, and dPiT is crucial for the normal development of neuromuscular junctions (NMJs). These results indicate that PiT2 might participate in the regulation of neuronal outgrowth by interacting with MAP1B and independently of its Pi transport function in the nervous system.

PiT2 is a member of the inorganic phosphate transporter family (Transport Classification Database Number 2.A.20), and is encoded by SLC20A21,2. SLC20A2 was cloned by Van Zeijl et al. in 1994 and was found to be comprised of 11 exons1. PiT2 possesses dual functions including as retroviral receptor GLVR2 or Ram-1 (Murine leukemia virus receptor)4–6 and sodium-dependent phosphate transporter7,8. As a member of the Na+-coupled mammalian type-III inorganic phosphate (Pi) transporters, PiT2 transports inorganic phosphate across the cell membrane against a chemical and electrical gradient2,9. In the mouse brain, PiT2 is mainly expressed in neurons, and is also known to be expressed in astrocytes and vascular endothelial cells10–12. Our recent study linked SLC20A2 to familial idiopathic basal ganglia calcification (IBGC). Loss-of-function mutations in SLC20A2 result in calcium phosphate deposition due to regional Pi accumulation in extracellular matrix of the brain13.

PiT2 consists of 652 amino acids14. According to bioinformatics predictions, cysteine scanning, epitope tagging and in vitro glycosylation studies, the topological model of PiT2 has 12 transmembrane domains (TMDs) with extracellular N- and C-terminal tails, 2 ProDom domains I11-L161 (N-PD1131) and V492-V640 (C-PD1131) located in the N-terminal and C-terminal of PiT2 respectively14,15. Corresponding to the protein functions of PiT2, loop regions in PD domain, such as 67–91, 107–141, 517–530 amino acid residues are required for amphotrophic murine leukemia virus (A-MuLV) binding16,17, and PD domains also play an important role in maintaining transport function18. In IBGC families, 23 missense variants have been found in SLC20A2, and these missense variants are mainly located in two PD domains of PiT219. The PiT2 also contains a 246-aa (about 38 percent amino acids of PiT2) large intracellular loop7 domain between N-PD1131 and C-PD113120. Böttger and Pedersen had reported that the PiT2 with deleted loop7 had normal retroviral recognition, and transport functions21. So far, there is no definite evidence that missense variants in loop7 affect the transport function of PiT2 which result in IBGC19. Therefore, it remains an intriguing question regarding the function of loop7 domain in the nervous system.

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To investigate possible functions of loop7 domain of PiT2 in the nervous system, we conducted immunofluorescence assays of Neuro2A cells transfected with PiT2 that lack loop7, and found that loop7 deletion affected the subcellular localization of PiT2 protein and neurite outgrowth in Neuro2A cells. To reveal the function of loop7 in PiT2, we performed yeast two-hybrid screening and identified microtubule-associated protein 1B (MAP1B) as a novel interactor of loop7 in PiT2. MAP1B is first synthesized and then cleaved to generate a heavy chain (HC) and a light chain (LC) 21. As a cytoskeletal protein that regulates actin and microtubule dynamics, MAP1B plays important roles in axonal elongation and regeneration, neuronal migration, axonal guidance, dendritic spine morphology, as well as expression, trafficking and activity of neurotransmitter receptors22,23. Differentiation assay showed that MAP1B binding site mutants of PiT2 decreased the length of neurites in Neuro2A cells. In Drosophila, CG42575 (encoding dPiT protein) is homologous to human SLC20A2, and there is only one representative of MAP1 family: the futsch gene24. Futsch protein is cleaved similarly to MAP1 proteins in vertebrates 25. Futsch is also implicated in neuronal development26,27. To dissect the neuronal function of loop7 domain in vivo, we generated transgenic lines that could be used to tissue-specifically overexpress dPiT with or without loop7. We performed co-immunoprecipitation and confirmed the interaction between Drosophila dPiT and Futsch. Immunochemical analyses showed that dPiT was crucial for the normal development of neuromuscular junctions (NMJs). This study reveals a novel function of PiT2 in neuronal outgrowth by interacting with MAP1B in vivo and in vitro.

Results

**The loop7 domain is essential for PiT2 localization and might impact neurite outgrowth in Neuro2A cells.** To get precise information about loop7 function in the nervous system, we first performed immunofluorescence assays of Neuro2A cells transfected with wild-type (PiT2-WT) or loop7 deletion mutant, in which residues 254–483 of PiT2 were deleted (PiT2-Δloop7). The PiT2-WT proteins were localized on plasma membranes in undifferentiated (Supplementary Fig. S1a) and differentiated Neuro2A cells (Fig. 1a), but most of the PiT2-Δloop7 proteins were found in the cytoplasm, and aggregated in a specific region of the cytoplasm (Fig. 1b). These findings indicated that loop7 might be necessary for trafficking of PiT2 protein to the cell surface. In differentiated Neuro2A cells transfected with PiT2-Δloop7, we observed that deletion of loop7 induced a decrease in neurite length compared with Neuro2A cells transfected with WT (Fig. 1a,b,f). To further explore the biological function of loop7 in Neuro2A cell differentiation, we performed neuritogenesis assay. Following induction of differentiation by retinoic acid (RA) treatment, lengthening of Neuro2A cell neurites were detected. Knockdown of PiT2 by shRNA-PiT2 significantly decreased the length of the longest neurites by about one half compared with negative control (Fig. 1c–e and Supplementary Fig. S2). These results indicate that PiT2 might participate in the growth and development of the nervous system.

**Figure 1.** The loop7 domain of PiT2 impacts neurite outgrowth in Neuro2A cells. (a,b) Differentiated Neuro2A cells stained with anti-HA/Alexa Flour488 (green) and DAPI (blue) with overexpression of HA-tagged wild type PiT2 (a) or HA-tagged PiT2-Δloop7 (b). Scale bar, 20 μm. (c) Neuro2A cells were transiently transfected with pSIH-PiT2 or pSIH-scramble. Cell lysates were immunoblotted with anti-PiT2 and anti-actin antibodies. Full length blots are shown in Supplementary Fig. S2. (d,e) Differentiated Neuro2A cells stained with DAPI (blue) with transfection of pSIH-scramble (d) or pSIH-PiT2 (e). Scale bar, 20 μm. (f) Quantification of neurite length of differentiated Neuro2A cells transfected with wild type PiT2 or PiT2-Δloop7 plasmids. (g) Average length of the longest neurite of Neuro2A cells transfected with scramble and shRNA-PiT2 were statistically analyzed. Error bars show the mean ± s.e.m. of 100 randomly selected cells from each group in three independent experiments. *** means P < 0.001.
Identification of MAP1B as a novel interaction partner of PiT2 by yeast two-hybrid screening. To search for interaction proteins involved in the subcellular localization or neurite outgrowth regulation of PiT2, yeast two-hybrid screening was performed. Residues 235–482 (loop7 domain) were used as bait (Fig. 2a), and was fused to the Gal4 DNA-binding domain. Through mating of the fetal brain cDNA library and Y187/pGBKT7-loop7 we succeeded in screening approximately 400,000 independent clones. After selection of fetal brain cDNA library, 183 positive yeast clones showing His-reporter and Ade-reporter gene activity were selected. Further high-stringency selection and sequencing of the AD plasmid inserts led to the identification of two independent clones containing the light chain 1 (LC1) of MAP1B (Fig. 2b). The interaction between PiT2-loop7 and LC1 in yeast was reconfirmed by co-transformation of LC1 of MAP1B and loop7 domain of PiT2 showed significant growth on SD/–Ade/–His/–Leu/–Trp selection agar plates compared with negative control. (d) Five C-terminal deletion mutants of loop7 were assayed for interaction with MAP1B using the yeast two-hybrid, only residues 235–402 interacted with LC1. (e) Loop7 truncation mutants were examined for interaction with MAP1B by the yeast two-hybrid assay, and only yeasts co-transformed with residues 356–402 and LC1 constructs showed growth on selection agar plates. (f) Alanine-scanning constructs designed to narrow the domains involved in interaction between residues 384 and 397. Mutations of residues 386–389 and 388–390 prevented the interaction between PiT2 and MAP1B. +, growth on stringent selection plates; –, no growth.

Figure 2. Yeast two-hybrid screen for the interacting protein of PiT2, and localization of MAP1B interaction site within loop7 of PiT2. (a) Schematic representation of PiT2, loop7 domain (residues 235–482, marked in red) was used as the bait for the yeast two-hybrid screen. (b) Schematic of the two yeast clones of MAP1B identified in the yeast two-hybrid screen. (c) Reconfirmation of the interaction between MAP1B and PiT2 in yeast. The transformants co-transformed with light chain of MAP1B and loop7 domain of PiT2 showed significant growth on SD/–Ade/–His/–Leu/–Trp selection agar plates compared with negative control. (d) Five C-terminal deletion mutants of loop7 were assayed for interaction with MAP1B using the yeast two-hybrid, only residues 235–402 interacted with LC1. (e) Loop7 truncation mutants were examined for interaction with MAP1B by the yeast two-hybrid assay, and only yeasts co-transformed with residues 356–402 and LC1 constructs showed growth on selection agar plates. (f) Alanine-scanning constructs designed to narrow the domains involved in interaction between residues 384 and 397. Mutations of residues 386–389 and 388–390 prevented the interaction between PiT2 and MAP1B. +, growth on stringent selection plates; –, no growth.

PiT2 interacts with MAP1B in vitro and in vivo. We substantiated the interaction between loop7 domain and LC1 by GST pulldown assay. The purified GST–PiT2-loop7 fusion protein, instead of GST alone, was able to pull down FLAG-LC1 fusion protein, indicating a direct association between loop7 and LC1 in vitro.
(Fig. 3a and Supplementary Fig. S3a). Then full-length PiT2 and LC1 fusion protein expressing vectors were co-transfected into Hela cells. Lysates from co-transfected cells were immunoprecipitated with GFP antibody. Western blotting showed that GFP antibody was capable of pulling down LC1 and PiT2 fusion protein complexes in Hela cells (Fig. 3bc and Supplementary Fig. S3b,c). We then carried out co-immunoprecipitation in mouse brain and Neuro2A cells lysates using LC1 antibody followed by Western blotting with PiT2 antibody, the results showed interaction between PiT2 and MAP1B (Fig. 3de and Supplementary Fig. S3d,e). After PiT2 knockdown, this interaction was weakened in Neuro2A cells (Supplementary Fig. S4). In vivo, no interaction was detected in the supernatant brain lysates of PiT2 knockout mice (Fig. 3d and Supplementary Fig. S3d).

MAP1B plays an important role in neurite extension during neuronal differentiation. We performed co-immunoprecipitation in DMSO- or RA-treated Neuro2A cells. Compared with undifferentiated Neuro2A cells, PiT2 proteins co-precipitating with LC1 were roughly doubled in the differentiated Neuro2A cells (Fig. 4a,b). This indicates that mutating the MAP1B binding sites in loop7 leads to reduced neurite length.

Mapping and verification of the MAP1B binding site on PiT2. To define the LC1 binding site in loop7 domain, we generated three constructs expressing truncated loop7 domain based on bioinformatics analysis (Supplementary Fig. S6a). Yeast two-hybrid analysis revealed that deletion of either residues 356–482 or 235–402 prevented binding with LC1, while mutants with deletion of residues 235–355 and 403–482 retained interaction with LC1 (Fig. 2e, Supplementary Fig. S6b). Therefore, we constructed a series of mutants with C-terminal of loop7 truncated and found that only mutants containing residues 235–402 could interact with LC1 (Fig. 2d, Supplementary Fig. S6c). To further localize the binding site of LC1 in PiT2, we generated six overlapping 4-residue alanine substitution mutations in the loop7 domain between residues 384 and 397. Among them, two alanine substitution mutants (residues 386–389 A and 388–390 A) failed to interact with LC1 (Fig. 2f, Supplementary Fig. S6c), which indicate that residues 386–390 (YTCYT) are necessary for interaction between PiT2 and MAP1B.

To further verify the interaction sites, we performed a co-immunoprecipitation experiment in Neuro2A cells. HA epitope tagged PiT2–386–390A mutants were expressed in Neuro2A cells. Western blotting showed that LC1 antibody was able to pull down WT protein, but not 386–390A or Δloop7 mutant proteins (Fig. 3f and Supplementary Fig. S3f).

Previous research results have demonstrated that LC1 is linked to the membrane localization of some ion channels and transmembrane receptor. However, by immunofluorescence assay, we found that WT and 386–390A mutant PiT2 proteins localized to plasma membranes, and there were no considerable differences in the fluorescence intensity between WT and 386–390A mutant (Supplementary Fig. S1a,b).

Mutating the MAP1B binding site in PiT2 affects neurite length. Next, we conducted immunofluorescence assay and found that overexpressing PiT2 (386–390A) significantly decreased the length of neurites in Neuro2A cells (Fig. 3c,g). This indicates that mutating the MAP1B binding sites in loop7 leads to reduced neurite length.

We also evaluated whether transport function of PiT2 underlies the regulation of neurite outgrowth, and performed neuritogenesis assays in Neuro2A cells transfected with three PiT2 mutants found in IBGC families (S601W, R254* and V507Efs*2). Sequence comparison showed that the loop7 domain of dPiT is 38% identical and 56% similar to human PiT2. We construct dPiT loss of function mutants dPiT2–386–390A and dPiT2–386–390A by the CRISPR/Cas9 technology (Supplementary Fig. S7). dPiT2–386–390A and dPiT2 are frame-shift mutants carrying one base pair deletion at 62th and 615th nucleotide of the dPiT gene, respectively. These mutants produced a truncated 43 and 191 amino acid peptides, respectively, and only 20 and 178 amino acids, respectively, in the C-terminal of this peptide are in common with WT dPiT protein. Drosophila dPiT which carries each of the mutation on one chromosome and the deficiency Df(3L)ED4470 or Df(3L)BSC817 that removes the entire dPiT gene on the other, were all embryonic lethal. Ubiquitous and neuronal overexpression of dPiT-GFP in dPiT loss of function mutant background by actin-Gal4 or elav-Gal4, respectively, rescued the lethality of loss of function. However, ubiquitous or neuronal overexpression of dPiT-Δloop7-GFP in dPiT loss of function mutant background by actin-Gal4 or elav-Gal4 could not rescue the embryonic lethality. These results suggest that dPiT is an essential gene for Drosophila development. The loop7 domain of dPiT is crucial for the function of dPiT.

Deletion of loop7 domain affects subcellular distribution of dPiT in Drosophila neurons. Since aforementioned in vitro study showed that the loop7 domain played a crucial role in the trafficking of PiT2, we then investigated the distribution of dPiTWT and dPiT-Δloop7 in the neuronal system in vivo. Both dPiT-GFP and dPiT-Δloop7-GFP, when driven by elav-Gal4 in the wild-type background, were abundantly...
expressed in the cell body of Drosophila brain or ventral ganglions. While dPiT-GFP could also be detected in the axon and the terminal of NMJ, there were little distribution of dPiT-Δloop7-GFP in the axon, and it was hardly detectable in the NMJ (Fig. 5a,b' and 5e,f'). dPiT-GFP was found to be located in all parts of the sensory

**Figure 3.** Interaction of PiT2 with MAP1B. (a) GST pulldown assays analyzing the interaction between PiT2-loop7 and LC1. Proteins pulled down were detected by using anti-flag antibodies. Full length blots are shown in Supplementary Fig. S3a. (b,c) Hela cells were co-transfected with PiT2 and LC1 expressing vectors. (b) Flag-tagged PiT2 constructs were co-transfected with a GFP-tagged LC1 construct in Hela cells, the GFP-tagged proteins were immunoprecipitated with control IgG or anti-GFP antibodies. Full length blots are shown in Supplementary Fig. S3b. (c) Hela cells were co-expressing GFP-tagged PiT2 and flag-tagged LC1, the cell lysates were immunoprecipitated with control IgG or anti-GFP antibodies. The precipitates were immunoblotted with antibodies indicated. Full length blots are shown in Supplementary Fig. S3c. (d) Interaction of PiT2 with MAP1B in wild type or slc20a2 knockout (KO) mice brains. Lysates of mouse brains were immunoprecipitated with LC1 antibody, the precipitates were immunoblotted with anti-PiT2 antibodies. Full length blots are shown in Supplementary Fig. S3d. (e) Interaction of PiT2 with MAP1B in Neuro2A cells. Lysates were immunoprecipitated with LC1 antibody, and then blotted with anti-LC1 or anti-PiT2 antibodies. Full length blots are shown in Supplementary Fig. S3e. (f) Neuro2A cells were transfected with HA-tagged PiT2-WT, PiT2–386–390A, and PiT2-Δloop7, and the cell lysates were immunoprecipitated with anti-LC1 antibodies. The precipitates were analyzed by immunoblot analysis using the antibodies indicated. Full length blots are shown in Supplementary Fig. S3f.
neuron system, including the cell body and dendritic branches. Nonetheless, dPiT-Δloop7-GFP mainly existed in the cell body and hardly observed in the dendritic branches of elav-Gal4/+;UAS-dPiT-Δloop7-GFP/+ flies (Fig. 5c,d'). These results indicate that loop7 domain affects subcellular localization of dPiT in neurons in vivo.

dPiT is required for normal the development of Drosophila NMJ synapses. To determine whether dPiT plays a role in synaptic development, we stained larval NMJs with anti-horseradish peroxidase (HRP) to label the neuronal membrane, and anti-cysteine string protein (CSP) to label synaptic vesicles. Knocking down dPiT in neurons or overexpressing dPiT-Δloop7-GFP was found to be associated with striking NMJ abnormalities as compared with controls or flies overexpressing of dPiT-GFP. The efficacy of the RNAi lines was validated

Figure 4. PiT2 with mutated MAP1B binding sites reduces neural outgrowth. (a) Co-immunoprecipitation of PiT2 with MAP1B in DMSO- or RA-treated Neuro2A cells. PiT2 interaction with LC1 of MAP1B is significantly enhanced in differentiated cells compared with undifferentiated cells. (b) Quantitative analysis (n = 3 independent experiments, * p < 0.05 by Student's t-test. Data are presented as mean ± s.e.m). Full length blots are shown in Supplementary Fig. S5a. (c–f) Differentiated Neuro2A cells stained with anti-HA/Alexa Flour488 (green) and DAPI (blue) with overexpression of HA-tagged PiT2–386–390A, PiT2-R254*, PiT2-S601W or PiT2-V507Es*2 fusion proteins. Scale bar, 20 μm. (g) Quantification of neurite length of differentiated Neuro2A cells transfected with the respective plasmids. Bars represent averages of 100 cells from each group in three independent experiments. The error bars represent the mean ± s.e.m. of 100 randomly selected cells from each group in three independent experiments. ns. means no significantly difference; *** means P < 0.001.
by Western blot analysis with dPiT antibody that we generated and reduction of dPiT levels can be observed (Supplementary Fig. S8e).

Comparing with the controls, NMJ length and bouton numbers were significantly decreased when dPiT is knocked down in neurons (Supplementary Fig. S8). The length of NMJ 4 from the abdominal segment A3 was significantly decreased from 110.3 ± 3.4 µm in elav-Gal4/+ (n = 37) to 98.3 ± 4.3 in elav-Gal4/+;UAS-dPiT RNAi/+ (n = 30, P < 0.05) (Fig. S8a,b,f). The average NMJ length in knockdown dPiT neurons of flies was 89% that of elav-Gal4/+ control. We quantified the total number of boutons of NMJ 4 from the abdominal segment A3 and found that knocking down dPiT in neurons significantly decreased bouton numbers. Total number of boutons in the control genotype elav-Gal4/+ was 22.4 ± 0.7 (n = 40) and 18.6 ± 1.2 in elav-Gal4/+;UAS-dPiT RNAi/+ (n = 37) (Supplementary Fig. S8b). When overexpressing dPiT in neurons with elav-Gal4, the NMJ length (114.8 ± 4.6, n = 20, P > 0.05) and bouton number (23.2 ± 0.6, n = 20, P > 0.05) were not significantly different from controls (Supplementary Fig. S8a).

Comparing with genetic control and neuronal overexpression of dPiT-GFP, NMJ length and bouton number were significantly decreased in neuronal overexpression of dPiT-Δloop7-GFP (Supplementary Fig. S8a,c,d,h,i).

**Figure 5.** The dPiT loop7 domain plays a crucial role in localization of dPiT. (a–f') The brains from wandering third instar larvae stained with anti-HRP (red), anti-GFP (green) and T3605 (blue) from flies with neuronal overexpression of dPiT-GFP (a,a') or dPiT-Δloop7-GFP (b,b'), driven by elav-Gal4 in wild type background. Scale bar, 10 µm. The dendrites from wandering third instar larvae segment 4 stained with anti-HRP (red), anti-GFP (green) of flies expressing dPiT-GFP (c,c') or dPiT-Δloop7-GFP (d,d') driven by elav-Gal4 in wild type background. Scale bar: 10 µm. Representative muscle 4 NMJs of the abdominal segment A3 are shown for dPiT-GFP (e,e'), dPiT-Δloop7-GFP (f,f') in wild type background. Scale bar, 5 µm. Arrows in a-b' indicate axons. Arrows and arrowheads in c-d' indicate dendrite branches.
The length of NMJ 4 from the abdominal segment A3 was significantly decreased from 116.9 ± 3.9 μm in *elav-Gal4/+* (n = 40, P < 0.001), 123.4 ± 4.7 μm in *UAS-dPiT-GFP/+* (n = 40, P < 0.001), 108.2 ± 6.0 μm in *UAS-dPiT-Δloop7-GFP/+* (n = 38, P < 0.05), 107.7 ± 4.5 μm in *elav-Gal4/+; UAS-dPiT-GFP/+* (n = 20, P < 0.01) to 86.3 ± 3.6 μm (n = 22) in *elav-Gal4/+; UAS-dPiT-Δloop7-GFP/+* flies. The average NMJ length in neuronal overexpression of *dPiT-Δloop7-GFP/+* flies was 74% of the *elav-Gal4/+* control (Supplementary Fig. S8h). We quantified the total number of boutons of NMJ 4 from the abdominal segment A3 and found that neuronal overexpression of *dPiT-Δloop7-GFP* significantly decreased bouton numbers. Total number of boutons in genetics control *elav-Gal4/+* (23.3 ± 1.0, n = 40, P < 0.001), *UAS-dPiT-GFP/+* (23.6 ± 0.9, n = 41, P < 0.001), *UAS-dPiT-Δloop7-GFP/+* (19.6 ± 1.0, n = 38, P < 0.01) decreased to 15.8 ± 0.6 (n = 22) in *elav-Gal4/+; UAS-dPiT-Δloop7-GFP/+* (Supplementary Fig. S8i). Meanwhile, there was significant difference between *elav-Gal4/+; UAS-dPiT-GFP/+* and *elav-Gal4/+; UAS-dPiT-Δloop7-GFP/+* (P < 0.001) in NMJ bouton number (Supplementary Fig. S8i).

**dPiT regulates NMJ development by interaction with Futsch.** Microtubule-associated protein, Futsch is specifically expressed in *Drosophila* nervous system, and colocalizes with microtubule cytoskeleton in the well-studied *Drosophila* larval NMJ[26,35]. To test whether dPiT interacts with Futsch in the central nervous system, we performed immunoprecipitation using *Drosophila* brain. Western blotting of the immunoprecipitates exhibited an interaction between dPiT and Futsch in the brain (Fig. 6a,b and Supplementary Fig. S9).

To investigate the localization pattern of dPiT in *futsch* mutant background, we constructed dPiT::GFP fly that expressed the reporter gene GFP under the *elav* control. While the futsch expression level was significantly decreased to 20% of wild type in *futsch N94*, the dPiT::GFP intensity was also decreased in axon tracts of ventral nerve cord compared with control, illustrating an effect of Futsch on subcellular localization of dPiT (Supplementary Fig. S10a-b). Meanwhile, comparing with the control, the average dPiT::GFP intensities (6.5 × 10^−4 ± 0.2 × 10^−4, n = 3), normalized to corresponding HRP staining of NMJ in control flies was also decreased (Supplementary Fig. S10c-d) to 3.9 × 10^−4 ± 13.5 × 10^−4 (n = 6, P < 0.05) in futsch^N94^ mutants (Supplementary Fig. S10e).

*futsch^N94^* mutant animals have a distinct phenotype with a reduction in bouton number and an enlargement in bouton size (Fig. 6f,i,34). The dPiT mutants show phenotypes in bouton number and bouton size similar to futsch mutants (Fig. 6d,e,i,j). Total number of boutons in wild type (24.5 ± 1.4, n = 18) decreased to 18.1 ± 0.7 (n = 26, P < 0.001) in *dPiT^15–1/+* and 16.2 ± 0.7 (n = 25, P < 0.001) in *dPiT^21–4/+* (Fig. 6c,d,e). The bouton size in wild type 6.7 ± 0.3 μm^2 (n = 18) increased to 8.1 ± 0.4 μm^2 (n = 26, P < 0.001) in *dPiT^15–1/+* and 8.5 ± 0.3 μm^2 (n = 25, P < 0.001) in *dPiT^21–4/+* (Fig. 6c,d,e). We tested for genetic interactions between dPiT and futsch using double mutants. Bouton number and size phenotypes in *dPiT* mutants on wild-type background is not significantly different from *dPiT* mutants on futsch^N94^ background, suggesting that dPiT and Fusch function in a common pathway to regulate bouton growth (Fig. 6). The bouton numbers of *dPiT^15–1/+* and *dPiT^21–4/+* mutants on futsch^N94^ background is 16.4 ± 1.0 (n = 26, P > 0.05) and 15.5 ± 1.5 (n = 25, P > 0.05), comparable with *dPiT* mutants on wild-type background (Fig. 6i). The bouton size of *dPiT^15–1^* and *dPiT^21–4^* mutants on futsch^N94^ background is 8.2 ± 0.4 μm^2 (n = 26, P > 0.05) and 8.4 ± 0.4 μm^2 (n = 26, P > 0.05) has no significantly difference with in *dPiT* mutants on wild-type background (Fig. 6i).

**Discussion**

Previous studies and bioinformatics prediction showed that PIT2 is a highly hydrophobic protein consisting of 12 transmembrane domains (TMDs) and a large central intracellular loop (loop7) whose function remains unknown[1,20]. In this study, we found that MAP1B was a new interacting protein of loop7 domain. The interaction between PIT2 and MAP1B was demonstrated by yeast two-hybrid, GST pulldown and co-immunoprecipitation analysis. We found that the interaction was enhanced during the differentiation of Neuro2A cells. Overexpression of PIT2 with mutated MAP1B binding site resulted in a significant decrease in the neurite length of Neuro2A cells compared with wild type. Overexpression of Pi transport function deficient mutants PIT2-S601W and PIT2-V507Efs*2 did not affect neurite outgrowth in Neuro2A cells. These results suggest that PIT2 modulates neurite outgrowth independently of its Pi transport function. In vivo studies showed that dPiT possessed similar functions in *Drosophila*. *Drosophila* dPiT interacts with Futsch, and dPiT is crucial for normal development of *Drosophila* NMJ synapses. Our data support the notion that loop7 domain of PIT2 is implicated in the growth and development of neurons by interacting with the adaptor protein MAP1B.

Most of the PIT2-Δloop7 proteins were localized to a specific region of cytoplasm (Supplementary Fig. S1c). Previous studies have reported that MAP1B can mediate microtubular trafficking of Nav1.6 and 5-HT6R to the cell surface[29,30]. On the other hand, MAP1B interacts with CaV2.2 and 5-HT3A to reduce their expression in the plasma membrane and promoting their desensitization[11,32]. In this study, we found that mutations in residues 386–390 (YTCTY) impeded the interaction between PIT2 and MAP1B but did not affect its localization (Supplementary Fig. S1b). In vivo studies also revealed that dPiT-Δloop7-GFP fusion proteins predominantly existed in the cell body but not in axons, the branches of dendrites or the terminal of motor neurons in the *elav-Gal4*-driven *UAS-dPiT-Δloop7-GFP* flies (Fig. 5a–f). Our results demonstrate that loop7 domain is required for membrane localization of PIT2 and interaction between PIT2 and MAP1B, but these two functions depend on different regions of loop7 domain.

MAP1B is highly expressed during early neuronal development[16,37]. MAP1B is principally expressed in neurons, oligodendrocytes, astrocytes, and their progenitor cells[38,39]. The expression pattern of MAP1B is similar to that of PIT2. MAP1B can regulate the dynamic balance between actin and microtubules, and it is essential for axonal growth, branching and nerve regeneration in developing nervous system[39–41]. In this study, we found that knockdown of PIT2 decreased the length of neurites in Neuro2A cells (Fig. 1d,e,g). The interaction

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**SCIENTIFIC REPORTS** | (2017) 7:17850 | DOI:10.1038/s41598-017-17953-3
between MAP1B and PiT2 was enhanced in differentiated Neuro2A cells (Fig. 4a,b) and abolishing the interaction decreased the length of neuritis (Fig. 4c,g). These findings suggested that the interaction between PiT2 and MAP1B might be involved in the differentiation of Neuro2A cells. Fly embryonic lethality of dPiT loss-of-function mutant illustrated that dPiT is an essential gene for fly development. We checked the NMJ phenotypes in dPiT loss-of-function mutant flies rescued with ubiquitous or neuronal expression of dPiT, respectively, and did not find any significant difference with wild type control in NMJ length and total bouton number (data not shown). This suggests that the crucial role dPiT plays development takes place in the neurons.

Immunoprecipitation assays showed that dPiT formed a complex with Futsch in Drosophila (Fig. 6a,b). Co-immunoprecipitation assays analyzing the interaction between dPiT and Futsch in wild type Drosophila. Lysates of Drosophila brains were immunoprecipitated with anti-Futsch or anti-dPiT antibody. The precipitates were immunoblotted with antibodies indicated. Full length blots are shown in Supplementary Fig. S9. (c–h) Confocal images of muscle 4 NMJ synapses of abdominal segment A3 double-labeled with anti-HRP (red) and anti-CSP (green). Representative NMJ synapses of different genotypes are shown: WT control (wild type; c), dPiT mutants dPiT21–4/+(d) and dPiT15–1/+(e), futschN94 (f), futschN94; dPiT21–4/+(g) and futschN94; dPiT15–1/+(h). Scale bar: 5 μm. Quantification of the total number of boutons (i) and bouton size (j) in different genotypes. Comparisons were made between each genotype and its corresponding control by one-way ANOVA unless indicated otherwise. * Means P < 0.05; ** means P < 0.01; *** means P < 0.001. Error bars indicate s.e.m.

Figure 6. dPiT interacts with Futsch and regulates synaptic growth in Drosophila. (a,b) Co-immunoprecipitation assays analyzing the interaction between dPiT and Futsch in wild type Drosophila. Lysates of Drosophila brains were immunoprecipitated with anti-Futsch or anti-dPiT antibody. The precipitates were immunoblotted with antibodies indicated. Full length blots are shown in Supplementary Fig. S9. (c–h) Confocal images of muscle 4 NMJ synapses of abdominal segment A3 double-labeled with anti-HRP (red) and anti-CSP (green). Representative NMJ synapses of different genotypes are shown: WT control (wild type; c), dPiT mutants dPiT21–4/+(d) and dPiT15–1/+(e), futschN94 (f), futschN94; dPiT21–4/+(g) and futschN94; dPiT15–1/+(h). Scale bar: 5 μm. Quantification of the total number of boutons (i) and bouton size (j) in different genotypes. Comparisons were made between each genotype and its corresponding control by one-way ANOVA unless indicated otherwise. * Means P < 0.05; ** means P < 0.01; *** means P < 0.001. Error bars indicate s.e.m.
Although Pi uptake of PiT2 in *Xenopus laevis* oocytes showed that loop7 domain is not required for Pi transport function\(^{15}\), there might be other regulatory mechanism for Pi uptake in the nervous system. PiT2 is a highly expressed inorganic phosphate transporter in the nervous system\(^{24–26}\). PiT2 could interact with actin and change their conformation to adapt to a changing ambient Pi concentration\(^{16–26}\). These results indicate that cytoskeletal proteins might play an important role in the regulation of PiT2 transport activity, and this might be related to the interaction between PiT2 and MAP1B in neuronal outgrowth regulation. In this study, we found that Pi transport function deficient mutant PiT2-S601W and PiT2-V507Efs*2 did not affect neurite outgrowth in Neuro2A cells (Fig. 4d,f,g). On the other hand, similar to PiT2-Δloop7, PiT2-R254* which also removes loop7 showed abnormal cytoplasmic localization and significantly decreased length of neurites in Neuro2A cells (Fig. 4e,g). These results show that PiT2 modulates neural outgrowth independently of its Pi transport function.

In summary, we identify a novel function of PiT2, which takes part in the growth and development of nerve cells. Furthermore, we find that PiT2 regulated the differentiation of nerve cells through interaction with MAP1B and independently of its Pi transport function. These findings might provide a novel mechanism that PiT2 regulates neural outgrowth, a process that might contribute to neuronal development.

**Methods**

**Yeast Two-hybrid Assay.** Yeast two-hybrid experiments were performed using the Matchmaker\textsuperscript{TM} Library Construction & Screening Kits (Clontech Laboratories, Inc., 630445). Briefly, the cDNA sequences encoding the human loop7 domain of PiT2 was amplified from KSM-hPiT2 vector\(^{13}\) and subcloned into the pGBK7T7 vector for use as “bait” in the yeast two-hybrid screen. A human fetal brain cDNA library as “prey” was purchased from Clontech (Clontech Laboratories, Inc., Mate & Plate\textsuperscript{TM} Library-Human Fetal Brain, 630469). The fetal brain cDNA library was screened by yeast mating, and then the mating mixture was spread onto complete medium lacking leucine, tryptophan, histidine and adenine (SD-Leu/Trp/His/Ade). In order to fully separate AD/library plasmid, candidate clones were restreaked on SD-Leu/Trp/His/Ade medium 2 times, and the β-galactosidase activity was monitored.

**Plasmid and Antibodies.** Human MAP1B-LC1 cDNA was subcloned into p3×flag-CMV-7.1 and pEGFP-N1 vector. The full-length of wild type human SLC20A2 cDNA (NM_006749) was amplified from KSM-hPiT2 construct and subcloned into pEGFP-N1 vector. Full-length of human SLC20A2 cDNA with HA epitope tag sequence was subcloned into pCDNA3.1 (−) vector, HA tag sequence was inserted into C terminus of PiT2 by PCR using two overlapped reverse primers. The pCDNA3.1-PiT2 construct was used as the parental plasmid to generate the deletion and alanine substitution mutant constructs via PCR mediated mutagenesis\(^{15,47}\). The directed tests of the interaction between LC1 and loop7 mutants were performed using LiAc-mediated yeast transformation. The primers are listed in Supplementary Table S1.

**For immunodetection,** the following antibodies were used at the following dilutions: mouse anti-glutathione S-transferase antibody (Abclonal, AE001; 1:3000 for WB), mouse anti-GFP antibody (Proteintech, 66002–1-Ig; 1:5000 for WB, 1:100 for IP), mouse anti-flag antibody (MBL, M185–3L; 1:5000 for WB, 1:100 for IP), mouse anti-HA antibody (sigma, clone A-7, H3663; 1:2000 for WB), mouse anti-PiT2 antibody (Santa Cruz Biotechnology, sc-101298; 1:200 for WB), rabbit anti-LC1 antibody antibody (Santa Cruz Biotechnology; 1:600 for WB, 1:500 for IP), mouse anti-cysteine string protein antibody [Developmental Studies Hybridoma Bank (DSHB) at the University of Iowa, AB 2307345; 1:500], mouse anti-Futsch antibody (DSHB, AB528403; 1:500) and Texas Red-conjugated goat anti-HRP antibody (1:100; Jackson Laboratory). A rabbit polyclonal anti-dPiT antiserum was raised against the synthetic peptide QSPKEEQKSKTNSIGTD (amino acids 382–398 of dPiT1) (Supplementary Fig. S7b).

**Cell culture and transfection.** Neuro-2A cells and HeLa cells were respectively cultured in Dulbecco’s modified Eagle medium (DMEM, Thermo Fisher scientific) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher scientific) at 37 °C and in 5% CO\(_2\). Transiently transfection of cells with plasmid DNA was performed using Lipofectamine\textsuperscript{®} 2000 Transfection Reagent (Thermo Fisher scientific) in Opti-MEM\textsuperscript{® I Reduced Serum Medium) by following the manufacturer’s instructions. For induction of differentiation, Neuro2A cells were transiently transfected as mentioned above. 24h after transfection, the medium was carefully replaced with an equal volume of DMEM with 1% fetal bovine serum and supplemented with 10μM Retinoic acid (RA) for another 48 h to induce neurite outgrowth.

**Mice.** Wild type C57BL/6NTac mice and Slc20a2 knockout mice C57BL/6NTac-Slc20a2\(^{29\text{m1a}(+)/(–)}\) (European Mouse Mutant Archive (EMMAW) Wtsi) were kindly provided by Prof. Xue Zhang (Chinese Academy of Medical Sciences & Peking Union Medical College). Mouse experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Tongji Medical College, Huazhong University of science and Technology (2015 IACUC number: 389). All experimental procedures were performed according to relevant guidelines and regulations set by the IACUC.
**Drosophila stocks and husbandry.** Flies were cultured on standard cornmeal medium at 25 °C unless otherwise specified. w^{118} is used as the wild-type control. Other stocks used included the ubiquitous actin-Gal4, muscle-specific C57-Gal4, pan-neuronal elav-Gal4, Df(3L)ED4470 and Df(3L)BSC817 which removes dPiT completely (Bloomington Drosophila Stock Center). dPiT RNAi (v49971) line was obtained from Vienna Drosophila RNAi Center.

**Generation of UAS transgenic flies.** For overexpression studies, a UAS-dPiT-GFP construct was made by fusing the GFP with the C terminal of dPiT (NM_140184.4). Then we transformed it into Drosophila with a UAS-dPiT-GFP fusion vector to generate transgenic flies. We also generated the UAS-dPiT-Δloop7-GFP vector. The insertion fragment was amplified from dPiT cDNA, connecting the sequence that encoding the 239 amino acids of N terminal to that of 183 amino acids of C terminal. Then we fused GFP with the C terminal of dPiT-Δloop7 fragment to generate the UAS-dPiT-Δloop7-GFP transgenic flies. The primers are listed in Supplementary Table S3.

**Mutagenesis of dPiT by CRISPR/Cas9 System.** Drosophila Cas9/sgRNA system was used to mutagenize dPiT<sup>49,50</sup>. Two U6b-sgRNA plasmids (sgRNA1: gatgcagagcgagttcgag and sgRNA2: gatgcagatccgcttcgag) were co-injected into nos-Cas9 transgenic blastoderm embryos to induce double-strand break at the first or the second exon of the dPiT (Supplementary Fig. S7a). We got two frame shift mutants, dPiT<sup>21-4</sup> and dPiT<sup>15-1</sup>, which was induced by sgRNA1 and the sgRNA2 respectively. dPiT<sup>21-4</sup> was the mutation with one bp deletion at the position of 62<sup>th</sup> in dPiT cDNA. dPiT<sup>15-1</sup> also deleted one bp at the position of 535<sup>th</sup> in dPiT cDNA (Supplementary Fig. S7b). The primers are listed in Supplementary Table S3.

**Generation of endogenous dPiT::GFP by CRISPR/Cas9 System.** We generated dPiT::GFP flies by CRISPR/Cas9 System<sup>49,50</sup>. We injected pCDE3-sgRNA-dPiT vector, which target a double-strand break (DSB) at the carboxyl terminal of dPiT, into syncytiat blastoderm-stage embryos<sup>49,50</sup>. The sgRNA sequence was ACATGGGTGGGGCCTAAAGATGG. We also injected a circular double-stranded plasmid containing the GFP-coding sequence flanked by 1.7- and 1.4-kb homology arms from the dPiT locus into vasa-cas9 pCDE3-sgRNA-dPiT embryos. The donor template is designed to produce an in-frame insertion of GFP within the dPiT coding region, leading to a dPiT::GFP protein. The flies that expressed endogenous dPiT::GFP were screened by the GFP signal.

**GST pulldown assay.** GST tagged PiT2-loop7 fusion proteins were expressed by addition of isopropyl-β-D-galactoside (IPTG, 0.1 mM, Sigma) in E.coli strain BL21 at 30 °C for 4 h. The HeLa cells were transfected with p3×flag-LC1 voter. Then glutathione-sepharose beads (Merck Millipore) were used to purify the GST fusion proteins according to the manufacturer’s protocol and subsequently incubated with the HeLa cDNA lysates at 4 °C over night. The pulldown proteins bound to the beads were detected by Western blotting.

**Immunoprecipitation.** Expression vectors were transfected into Neuro2A cells using Lipofectamine 2000 (Thermo Fisher scientific). After culturing for 36 h, cells were lysed using IP lysis buffer (Beyotime) supplemented with cock-tail protease inhibitors (Roche). To detect endogenous interaction, 150 Drosophila (half male and half female adult flies emerging from the pupal cases within a week) heads or one newborn mouse brain was lysed using IP lysis buffer supplied with the Complete Protease Inhibitor Cocktail (Roche). Cell or tissue lysates were collected, and then centrifuged at 12,000 rpm, 4 °C for 10 min. Supernatants were incubated with appropriate primary antibody overnight at 4 °C. Then protein A-agarose beads (Merck Millipore) were added and the samples for another 2 h. For immunoprecipitation of Drosophila, lysates were incubated with appropriate primary antibody and Dynabeads® Protein G (Thermo Fisher scientific). The beads were washed with IP lysis buffer three times. The immunoprecipitates were analyzed by Western blotting.

**Western Blotting.** Lysates or immunoprecipitates were prepared using SDS sample buffer. Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked for 2 h at room temperature (RT) with 5% skim milk, and incubated with the appropriate primary antibodies overnight at 4 °C. Next day the membranes were washed three times with TBST buffer (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH7.6), and incubated in HRP-conjugated secondary antibodies (1:20000, goat anti-rabbit or goat anti-mouse, Thermo Fisher scientific) for 2 h at RT. After rinsing three times, the proteins were detected by using SuperSignal ELISA Femto Maximum Sensitivity Substrate (Thermo Fisher scientific). The protein bands were quantitatively analyzed by employing the Image J software package (http://imagej.nih.gov/ij/).

**Immunofluorescence and Microscopic Analysis.** For RNA interference experiment, Neuro2A cells were transfected with pSH-H1-copGFP shRNA vectors, containing either a sequence targeted to PiT2 gene (shRNA-PiT2) or a non-silencing sequence (scramble). Transfected cells were fixed in 4% paraformaldehyde for 10 min and permeabilized with 0.5% Triton X-100 for 2 min at RT. After staining with DAPI (1:1,000, Sigma) for 5 min, cells were preserved at 4 °C. For exogenous expression experiment, transfected cells were fixed in 4% formaldehyde for 10 min at RT and permeabilized with 0.5% Triton X-100 for 2 min. Cells were incubated with the primary antibody overnight at 4 °C. The next day the cells were incubated with the secondary antibody (Alexa Fluor 488-conjugated donkey anti-mouse IgG secondary antibody and Alexa Fluor 594 F(ab’)2 fragment of goat anti-rabbit IgG(H+L) secondary antibody, 1:500, Thermo Fisher scientific) at 30 °C for 1 h. After staining with DAPI for 5 min, cells were preserved at 4 °C. For Immunohemochromatography analysis of NMJ, third instar larvae were dissected in HL3 with all internal organs removed, followed by fixation in the 4% paraformaldehyde for 40 min. Dissection and antibody staining of third instar larvae were performed as previously described<sup>51,52</sup>. Immunofluorescent imaging
was performed using an Olympus Fluoview 1000 Laser Scanning Confocal Microscope mounted on an Olympus IX-81 inverted microscope. Image analysis was carried out using FV1000 Viewer and the Image J software. The length of the longest neurite in each Neuro2A cell stained green or NMJ length of Drosophila was measured from the swell of HRP staining to the terminal using Image J software package. All branches were calculated. For bouton size analyses, ImageJ 3.0 (NIH) was used to define anti-HRP-stained individual boutons. The software output reports the area for each bouton automatically. At least 16 NMJ terminals of different genotypes were analyzed. For quantification of GFP intensities in NMJ, staining signals were digitalized automatically using ImageJ and normalized to the average intensities in the corresponding HRP staining. 100 cells from each group in three independent experiments were captured for neurite outgrowth assay of Neuro2A cells.

Statistical analysis. Experiments were repeated at least three times. Statistical analyses were performed with T-test for the comparison of two groups, and one-way ANOVA for the comparison of three or more groups. Quantitative data were presented as mean ± s.e.m., p values < 0.05 were considered to be statistically significant.

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Acknowledgements
We are indebted to Prof. Liming Chen for kindly providing Neuro2A cell lines and Prof. Xue Zhang for kindly providing slc20a2 knockout mice. This study was mainly supported by the National Natural Science Foundation of China grants 31230045, 31671301 (J.Y.L), 31371423 (P.Y) and 31501019 (C.W), and by the state Key Program of National Natural Science Foundation of China (Grant Reference Number 81130021) (B.S.T).

Author Contributions
J.-Y.L. conceived the study. J.-Y.L., S.J. and P.Y. designed the experiments and obtained financial support. J.-Y.L., S.J. and X.-X.M. analyzed data and wrote the manuscript. X.-X.M., H.S. and K.L. performed the yeast two-hybrid assays. R.C., X.M. and S.D. were responsible for molecular cloning. X.-X.M. and S.F. conducted the cell culture and transfection. X.X. maintained mice and collected tissue samples. X.-X.M. and C.W. carried out GST pulldown, immunoprecipitation and western blot. X.L. and L.Z. carried out transfection. X.-X.M. and C.W. carried out GST pulldown, immunoprecipitation and western blot. X.L. and L.Z. carried out in vivo Drosophila experiments. X.-X.M. and X.J.Z. participated in the suggestion and revision of the manuscript.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-017-17953-3.
Competing Interests: The authors declare that they have no competing interests.
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