Knockdown of Pnpla6 protein results in motor neuron defects in zebrafish

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SUMMARY
Mutations in patatin-like phospholipase domain containing 6 (PNPLA6), also known as neuropathy target esterase (NTE) or SPG39, cause hereditary spastic paraplegia (HSP). Although studies on animal models, including mice and Drosophila, have extended our understanding of PNPLA6, its roles in neural development and in HSP are not clearly understood. Here, we describe the generation of a vertebrate model of PNPLA6 insufficiency using morpholino oligonucleotide knockdown in zebrafish (Danio rerio). Pnpla6 knockdown resulted in developmental abnormalities and motor neuron defects, including axon truncation and branching. The phenotypes in pnpla6 knockdown morphants were rescued by the introduction of wild-type, but not mutant, human PNPLA6 mRNA. Our results also revealed the involvement of BMP signaling in pnpla6 knockdown phenotypes. Taken together, these results demonstrate an important role of PNPLA6 in motor neuron development and implicate overexpression of BMP signaling as a possible mechanism underlying the developmental defects in pnpla6 morphants.

INTRODUCTION
Motor neuron diseases (MNDs) are an etiologically heterogeneous group of disorders that are characterized by muscle weakness and/or spastic paralysis, which results from the degeneration of motor neurons. Hereditary spastic paraplegia (HSP) is the second-most common group of MNDs, whose main feature is progressive spasticity in the lower limbs. The identification of numerous spastic paraplegia (SPG) loci that are associated with different forms of HSP underscored a varied etiology and pathology in HSP. Currently, more than 40 SPG loci and 20 causative genes have been identified, which provides important insights into pathogenesis leading to HSP (Dion et al., 2009). In 2008, a relatively rare type of HSP with marked distal wasting in all four limbs was reported and the gene responsible for it was named SPG39 (Rainier et al., 2008; Rainier et al., 2011). SPG39 protein, i.e. patatin-like phospholipase domain containing 6 (PNPLA6), is a protein that had been studied for many years in neurotoxicology of organophosphate poisoning. But, until now, little is known about its exact role in HSP pathogenesis.

The protein product of PNPLA6 is neuropathy target esterase (NTE), a specific serine esterase that was originally identified as the target of organophosphorus esters that cause a delayed neuropathy in human and other vertebrates (Pope et al., 1993; Wilson et al., 1990). This organophosphate-induced delayed neuropathy (OPIDN) syndrome is characterized by paralysis of the lower limbs and degeneration of long axons in the spinal cord and peripheral nerves. Although much research has shown that functional loss of NTE is an initiating event in OPIDN (Glynn, 2005; Glynn, 2006), the exact role of NTE is not well established. The PNPLA6 mutations in HSP occur in the key catalytic domain of NTE and abolish NTE activity. The fact that OPIDN and the PNPLA6-related HSP exhibit similar symptoms also suggests a possible link between NTE function and motor neuron development.

PNPLA6, a conservative protein found in many species ranging from yeast to mammals (Chang et al., 2008; Kaur et al., 2006; Kretzschmar et al., 1997; Zaccheo et al., 2004), is abundantly expressed in the central nervous system and peripheral nerves (Johnson, 1982; Lush et al., 1998). PNPLA6 is anchored to the cytoplasmic side of endoplasmic reticulum (ER) and displays potent phospholipase B activity that is responsible for converting phosphatidylcholine to glycerophosphocholine in mammalian cells (Zaccheo et al., 2004). Although its tissue distribution and catalytic function are well established, how it functions in the nervous system has remained elusive. Drosophila was the first in vivo model established for PNPLA6 study. When swiss cheese (sws), the ortholog of PNPLA6 in Drosophila, was mutated, the activity of NTE was totally abolished. The sws mutant Drosophila exhibited neural degeneration including abnormal, multilayered wrappings around glia and neuron death. However, this phenotype can be rescued by mouse Nte, suggesting that PNPLA6 contributes to neural homeostasis (Kretzschmar et al., 1997; Mühlig-Versen et al., 2005). Complete inactivation of Nte gene results in embryonic lethality in mouse, whereas Nte⁰/⁻ mice do not exhibit apparent defects in the nervous system (Winrow et al., 2003). In a neuron-specific Nte knockout mouse strain, the absence of Nte results in disruption of ER, abnormal reticular aggregation and vacuolization of nerve cell bodies (Akassoglou et al., 2004). Furthermore, Read et al. observed distal degeneration of the longest spinal axons in nestin-cre:NTefl/fl mice that closely resembled the axonal lesion in HSP (Read et al., 2009). These data indicate that PNPLA6 plays a key role in normal neural development and axon maintenance.

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**TRANSLATIONAL IMPACT**

**Clinical issue**
Hereditary spastic paraplegias (HSPs) are a clinically and genetically heterogeneous group of conditions that are characterized by progressive weakness and spasticity in the lower limbs. They are caused by axonal degeneration, which might involve mechanisms including impaired cellular membrane trafficking and mitochondrial dysfunction. More than 40 loci (designated SPG1 through SPG48, in order of discovery) and 20 genes have been identified for different HSPs. Discovering how mutations in these genes cause cellular and molecular defects affecting axons will enhance our understanding of the etiology of HSPs and contribute to the development of effective treatments.

**Results**
In this paper, the authors investigated the involvement of a previously identified HSP gene, patatin-like phospholipase domain containing 6 (*PNPLA6*), in the pathology of HSP using a zebrafish model. They created and characterized zebrafish with reduced expression of the orthologous *pnpla6* gene by using morpholino oligonucleotides targeting the *pnpla6* mRNA translational start site. Compared with control zebrafish, the authors found that morpholino-injected zebrafish had fewer motor neurons and abnormal axons. Reduced *pnpla6* expression severely impaired axon development and resulted in increased axonal truncation and branching. Reduced *pnpla6* expression also caused other phenotypes such as curly tail, aberrant eyes and reducedotic vesicle size. Finally, *pnpla6* morphants exhibited over-activation of BMP signaling that could be attenuated with the BMP pathway inhibitor dorsomorphin.

**Implications and future directions**
These results reveal crucial functions for *pnpla6* in zebrafish motor neuron development, at least in part through its ability to inhibit the BMP signaling pathway. Thus, it is possible that, in humans, *PNPLA6* mutations might lead to overactive BMP signaling, causing impaired maintenance of long axons and the axonal transport machinery; this would explain the degeneration of distal axons observed in HSP patients. Future studies will aim to identify compounds that modulate PNPLA6 esterase activity as a potential therapeutic strategy for HSP.

Zebrafish are becoming more popular as a model organism because the large and transparent embryos can be observed and manipulated with relative ease. Its genomic organization and embryonic development have been well characterized (Dodd et al., 2000). The relatively simple nervous system, gene knockdown technology with morpholinos (MOs), and the availability of many cell-type-specific antibodies provide additional advantages for study of genes related to neural function in zebrafish (Kabashi et al., 2011). We studied the function of Pnpla6 protein in zebrafish neural development and gained some insight into the role of PNPLA6 in HSP pathogenesis.

**RESULTS**

**Knockdown of *pnpla6* expression leads to morphological changes in zebrafish embryos**
Zebrafish *pnpla6* gene (Gene ID: 560986) consists of 35 exons that span over 50 kb. The transcript of 4144 bp (XM_001921173.2) is predicted to encode for a protein of 1348 amino acids (XP_001921208.1). Based on the predicted zebrafish *pnpla6* mRNA sequence, we designed five pairs of primers for PCR amplification and obtained the complete *pnpla6* coding sequence of zebrafish. The zebrafish *pnpla6* coding sequence we obtained contains 4026 bp and encodes 1342 amino acids. Zebrafish Pnpla6 protein shares 73% identity with human PNPLA6 and is highly conserved compared with Pnpla6 protein in other species including *Drosophila* and mouse. The catalytic domain of zebrafish Pnpla6 protein is similar to that in other vertebrate species, consisting of the serine hydrolase signature motif GXXSG (Glynn, 2006).

An antisense morpholino oligonucleotide targeting the *pnpla6* mRNA translation start site was used to decrease the overall expression of Pnpla6 protein. Before analyzing the phenotypes of *pnpla6* knockdown zebrafish, we first tested the effects of different concentrations of *pnpla6* MOs on zebrafish development. The morphants that stayed alive were scored at different time points for each MO concentration injected. The survival rate of embryos decreased with increasing dose of MO injected (data not shown). The most prominent phenotype in *pnpla6* MO morphants is curly tail (Fig. 1A,B). The proportion of morphants with curly tail increased with increasing *pnpla6* MO concentration (Fig. 1C). This dose-dependent phenotypic severity indicates the specificity of *pnpla6* knockdown. To avoid the toxic effects caused by high doses of MOs, 0.15 pmol of *pnpla6* MO was chosen for subsequent experiments.

Injection of *pnpla6* MO caused dramatic changes in the gross morphological appearance of *pnpla6* morphants from 6 to 72 hours post-fertilization (hpf). Because the tail malformation in *pnpla6* morphants is probably caused by anterior-posterior (AP) pattern defect, early AP pattern formation was examined. Before somite furrowing, no significant difference was observed between *pnpla6* morphants and controls (injected with control MO). At the four-somite stage, the AP axis was shorter in *pnpla6* morphants, or with a longer gap between head and tail (Fig. 1C,D). At the six-somite stage, the tails in *pnpla6* morphants still appeared shorter than in control animals (Fig. 1E,F). A summary of the straight-line distances between head and tail of embryos measured at the four-somite stage is shown in Fig. 1P. Aberrant eye development (Fig. 1G,H) and reducedotic vesicle size (Fig. 1I,J) were also apparent in *pnpla6* morphants. Compared with control animals, *pnpla6* morphants have smaller eyes and some morphants had unclosed eye fissures. Other abnormalities, such as midbrain–hindbrain boundary (MHB) defect (Fig. 1K,L) and swelling of the pericardium (Fig. 1M,N), reduced heart rate and reduced blood flow (data not shown) were also observed in some *pnpla6* morphants.

The *pnpla6* morphant phenotypes can be rescued by wild-type human *PNPLA6* mRNA, but not by mutant *PNPLA6* mRNA
To further test the specificity of *pnpla6* MO knockdown, rescue experiments with human *PNPLA6* mRNA were performed. When human wild-type *PNPLA6* mRNA was co-injected with *pnpla6* MO, the *pnpla6* morphant phenotypes were rescued. Most embryos receiving co-injection demonstrated normal tail formation (Fig. 2B,C,H). Only 14.2% of embryos co-injected with wild-type mRNA showed curly tails, a significant decrease from that in the *pnpla6* MO morphants (53.7%).

To clarify whether the phenotypes of *pnpla6* knockdown were a result of lack of catalytic activity conferred by the serine-centered domain (Fig. 2A), we prepared three mutant human mRNAs. The three key amino acids at the catalytic domain were mutated to alanine. None of the three mutant RNAs (D960A mRNA, S966A mRNA and D1086A mRNA) was able to rescue the abnormal phenotypes of the *pnpla6* morphants (Fig. 2E–H). The results
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**Fig. 1. Knockdown of *pnpla6* leads to distinct morphological change.**
(A) 36-hpf embryo injected with control MO. (B) 36-hpf *pnpla6* morphant displays a curly tail. (C-F) Distinct shorter axis is seen in zebrafish embryos injected with *pnpla6* MO: four-somite control MO embryo (C), four-somite *pnpla6* MO morphant (D), six-somite control MO embryo (E) and six-somite *pnpla6* MO morphant (F). (G,H) Eye development in control (G) and morphant (H) embryo with unclosed eye fissures (black arrow). (I,J) Otic vesicle development in control (I) and morphant (J) embryos. (K,L) Analysis of 25-hpf MHB defects, with the arrow denoting the location of the MHB: lateral view of MHB in control (K) and *pnpla6* MO (L) embryos. (M) 72-hpf embryo injected with control MO. (N) 72-hpf *pnpla6* morphant displays a swelling of the pericardium. (O) The dose-dependent effect on curly tails; *P*<0.01 compared with control MO group. (P) The average distance between head and tail (straight-line length between two arrowheads) of early-stage (four-somite period) zebrafish embryos; *P*<0.01 compared with control MO group. Scale bars: 200 μm (A-F); 50 μm (G,H); 100 μm (I,J); 50 μm (K,L); 200 μm (M,N).

Obtained with the rescue experiments confirmed that the phenotypes in the *pnpla6* knockdown morphants were due to reduced esterase activity.

**Pnpla6 is required for primary motor neurons and axon development**

It has been reported that *PNPLA6* mutation contributes to motor neuron disease in humans (Rainier et al., 2008). Another study showed that *PNPLA6* might play a crucial role in maintenance of axons in sensory and motor spinal tracts on adult axons (Read et al., 2009). To determine whether reduced *pnpla6* expression could also affect the growth of spinal motor neurons in zebrafish, we examined the motor neurons and their axon outgrowth. The spinal cord motor neurons were immunostained with antibody 39.4D5, which recognizes the islet-1 transcription factor in the nuclei of motor neurons and Rohon-Beard sensory neurons. Immunostaining of islet-1 revealed that *pnpla6* morphants displayed fewer motor neurons than control embryos (Fig. 3A,B). Quantification of motor neurons at 30 hpf showed that the number of islet-1-positive spinal motor neurons was significantly lower than in controls (Fig. 3I).

To observe the effect of *pnpla6* knockdown on axon outgrowth, *pnpla6* MO-injected embryos were fixed and whole-mount antibody staining was performed using the Znp-1 antibody (Melançon et al., 1997), which stains caudal primary motor axons and middle primary motor axons. The *pnpla6* MO morphants showed aberrant axon branching (Fig. 3D). The axons in *pnpla6* morphants also appeared to be truncated, failing to join the ventral muscles (Fig. 3D). At 36 hpf, the incidence of axon branching and truncation (10 axons at segments 11-20 per side or 20 axons per embryo) was also increased over that in controls. Although 69.2% of axons observed exhibited the phenotype of truncation in *pnpla6* morphants, only 31.3% exhibited the phenotype in control embryos (Fig. 3J). When *pnpla6* MO morphants were co-injected with wild-type human *PNPLA6* mRNA, a significant drop in the number of aberrant motor axons was observed (Fig. 3E,J). However, these axon defects in *pnpla6* morphants could not be rescued by co-injection of mutant mRNA (Fig. 3F,J).

Increase in apoptosis could contribute to the impairment in primary motor neuron development in *pnpla6* morphants. We therefore evaluated the level of apoptosis in *pnpla6* morphants. Using Acridine Orange staining, cell death and apoptosis were analyzed in *pnpla6* morphant embryos. We found that there were more apoptotic cells in *pnpla6* morphants than in control animals, especially in the central nervous system (data not shown). Because morpholino itself can lead to cell death through the p53 pathway, we used p53 knockdown co-injection for rescue. Knockdown of p53 can rescue cell death in control but not in *pnpla6* MO-injected animals. Consistent with our observations using Acridine Orange for apoptosis analysis, TUNEL assay showed that more apoptotic cells were detected in morphants (Fig. 3G,H).

Because most of the *pnpla6* morphants demonstrated curved tails, we determined whether the axon defect was due to the deformed body. First of all, we assessed the correlation between curly tail and axon defect in *pnpla6* morphants. Although the morphants with severely impaired axons had severely curly tails, 15% of morphants with curly tails did not show axon defects. On the other hand, 65% of morphants without curly tails exhibited axon defects. Next, we scored the number of the motor axon defects located in the curved region (segments 13-20) and non-curved region (segments 5-12) in *pnpla6* morphant embryos at 36 hpf. There was no significant difference in the percentage of sides with
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at least one of the axon defects between the curved and non-curved regions (truncation: \( 56.5\pm4.4\% \) and \( 50.7\pm2.2\% \), respectively; branching: \( 46.6\pm2.4\% \) and \( 49.0\pm4.2\% \), respectively; for all experiments, \( n=98 \)). To further test whether the axon defects were secondary to the curved body axis of \( \text{ppla6} \) morphants, we performed spinal neuron cultures. Cells were prepared from \( \text{ppla6} \) morphant and control embryos. After the primary cells were cultured for 8 hours in vitro, the lengths and branches of motor neurons were determined using Znp-1 immunofluorescence staining. As shown in Fig. 4, motor neurons of \( \text{ppla6} \) morphants exhibited axons that were much shorter and more branched than in controls. These results are consistent with defects seen in the entire embryos and argue against deformed body being the cause of the impaired motor neuron development.

To examine whether \( \text{ppla6} \) functions in a cell-autonomous fashion, we established chimeric zebrafish embryos. Donor embryos were injected with rhodamine-lysine-dextran (RLDx), along with control MO or \( \text{ppla6} \) MO, at the one-cell stage. At 4-6 hpf, a small group of cells at the neuroectoderm were transplanted into the tail-bud region of wild-type recipients. At 36 hpf, the recipients were fixed and immunostained with Znp-1 antibody. As shown in Fig. 5, most motor neurons developed from \( \text{ppla6} \) MO cells in the chimeric embryos exhibited axon defects, resembling the motor neuron phenotype of \( \text{ppla6} \) morphants.

Knockdown of \( \text{ppla6} \) has no obvious effect on interneurons and sensory neurons in zebrafish embryos

Because our results demonstrated a role for \( \text{ppla6} \) in motor neurons, we wondered whether \( \text{ppla6} \) function is also crucial to other types of neurons. Hindbrain Mauthner neurons are the most important interneurons in zebrafish. At 34 hpf, embryos were immunolabeled with 3A10 antibody, which specifically labels the commissural axon of the Mauthner neuron (McWhorter et al., 2003). Compared with controls, \( \text{ppla6} \) MO morphants did not exhibit a significant difference in the formation and arrangement of Mauthner neuron axons. Moreover, \( \text{ppla6} \) knockdown was not observed to affect the projection from interneurons (Fig. 6A,B).

To determine the effect of \( \text{ppla6} \) knockdown on sensory neurons, acetylated tubulin monoclonal antibody staining was employed to analyze sensory neuron development. As shown in...
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Fig. 6C,D, the Rohon-Beard, lateral line sensory neurons and axons from them appeared normal in *pnpla6* morphants. We next used neuronal antibodies HuC/D to analyze *pnpla6* morphants and control embryos, and found that neither the number nor the arrangement of mature neurons was significantly affected by the knockdown of *pnpla6* (Fig. 6E-H).

**The BMP pathway is affected by *pnpla6* MO treatment**

It has recently been reported that bone morphogenetic protein (BMP), one of the major secreted morphogens, plays a key role in HSP pathogenesis. Several HSP proteins, including NIPA1, spastin, spartin and atlastin, are all confirmed to be inhibitors of BMP signaling (Fassier et al., 2010; Tsang et al., 2009). Taking into consideration their similarity to Pnpla6 protein in cellular location and in phenotypes caused by reduced gene expression in zebrafish, we next investigated whether the BMP pathway is involved in the abnormal neural development in *pnpla6* morphants. As shown in Fig. 7A, there was a striking increase in the level of phosphorylated Smad1/5/8 in *pnpla6* morphants compared with control embryos at 24 hpf. Furthermore, the expression of phosphorylated Smad1/5/8 appeared to be reduced to the normal level in embryos rescued with wild-type human *PNPLA6* mRNA. Consistent with the results of the rescue experiments mentioned above, mutant RNA failed to attenuate the expression of phosphorylated Smad1/5/8 in *pnpla6* morphants.

To determine whether the developmental defects observed in *pnpla6* morphants were caused by the upregulation of BMP signaling, dorsomorphin (DM), a small molecule that blocks the kinase activity of BMP receptors (Yu et al., 2008), was used to test possible involvement of BMP signaling in zebrafish *pnpla6* morphants. The *pnpla6* morphants and control embryos were each divided into two groups that were treated with DM or dimethyl sulfoxide (DMSO) at the ten-somite stage, and the typical phenotype (curly tail) was examined at 36 hpf. The phenotypes of *pnpla6* morphants, including curly tail, could be rescued by treatment with DM, but not by DMSO (Fig. 7B,C).

**DISCUSSION**

Here, we describe a vertebrate model of *pnpla6* insufficiency. Decreased *pnpla6* activity in embryonic zebrafish resulted in developmental abnormalities including curly tail, short AP axis and small eyes. Knockdown of *pnpla6* also results in motor neuron loss and axon growth defects including truncation and branching. The following lines of evidence suggest that loss of *pnpla6* is directly responsible for these phenotypes: first, a mismatch control MO does not cause the same phenotypes; and second, co-injection of...
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Because Pnpla6 is essential for embryonic development and Pnpla6 knockout mice died around embryonic day 9 (Moser et al., 2004; Winrow et al., 2003), it is difficult to study its function in early development using mice. Employing zebrafish as a model system, our in vivo studies revealed several new functions for \textit{pnpla6} in motor neuron development, AP axis development and eye development. With regard to the role of \textit{pnpla6} in motor neuron development, \textit{pnpla6} morphants showed prominent motor neuron loss and defects of motor neuron axon outgrowth, including abnormal branching and truncation. Using a neuron-specific NTE knockout mouse model, Akassoglou et al. showed that loss of NTE results in neuron defects in hippocampus, thalamus and cerebellum (Akassoglou et al., 2004). Recently, the distal degeneration of the spinal axons was observed in 3-week-old NTE-deficient mice (Read et al., 2009). Furthermore, progressive degeneration involved both motor and sensory spinal tracts, although the incidence of lesions in sensory tracts was much lower than that in motor tracts. In contrast to these results, no obvious defects of interneurons and sensory neurons were found in \textit{pnpla6} morphant zebrafish. In our zebrafish model, morpholino antisense oligonucleotides were used to knockdown NTE, and the effect of morpholino can last only about 5-7 days. The effect caused by morpholino knockdown should be much less than that in knockout mice, in which sustained NTE deficiency over many months can result in much severer phenotypes. Furthermore, the axon lesion in Pnpla6 conditional knockout mice, including Wallerian-like morphology, closely resembled that in HSP. It was proposed that PNPLA6 deficiency affects membrane trafficking, which eventually results in distal axon lesion and consequent motor defects in HSP patients (Read et al., 2009). In our study, zebrafish \textit{pnpla6} MO morphants were found to exhibit more truncation and branching in motor neuron axons. Therefore, it is reasonable to assume that \textit{pnpla6} plays a key role in axon development and maintenance and, when deficient, leads to the axon pathology of HSP.

The most distinct morphological phenotype in \textit{pnpla6} MO morphants is the curly tails, resembling the phenotype observed in zebrafish with morpholino knockdown of KIAA0196. KIAA0196, also named SPG8, is the eighth HSP locus whose mutation leads...
wild-type human PNPLA6 mRNA. By contrast, co-injection of pnpla6 MO with each of the three mutated PNPLA6 mRNAs failed to rescue the phenotypes of pnpla6 MO morphants, confirming the key role of the catalytic domain for its physiological function. In Drosophila, Ser985 of sws, which aligns precisely with Ser966 of vertebrate NTE, has been shown to be the active residue. The observation that sws with a point mutation in Ser985 failed to functionally replace wild-type sws further confirmed the importance of the active site (Mühlig-Versen et al., 2005). Each of two point mutations (M1012V, R890H) in PNPLA6 can lead to a motor neuron disease in humans (Rainier et al., 2008). A recent report revealed that introduction of these mutations can also change the NTE catalytic activity (Hein et al., 2010). Both mutations are located in the conserved region of the serine-centered catalytic domain. These findings are consistent with our data demonstrating a role for NTE catalytic activity in vertebrate development and motor neuron maintenance.

Despite the genetic heterogeneity of HSP, the similar clinical phenotypes shared by different SPGs suggests a common biochemical pathway. Spichthyin, the Drosophila ortholog of SPG6, is the first HSP protein reported to function as a BMP inhibitor (Wang et al., 2007). The elevated levels of phosphorylated smad and BMP receptors in spichthyin mutant animals suggests that spichthyin antagonizes BMP signaling by regulating its receptor traffic. It has also been demonstrated in human cells that NIPA1 inhibits BMP signaling by promoting endocytosis and lysosomal degradation of BMP receptors. SPG4 (spartin) and SPG20 (spartin) protein are also demonstrated to be BMP inhibitors (Tsang et al., 2009). Another study showed that SPG3A protein, also known as atlastin, can inhibit BMP signaling in zebrafish, and that the atlastin morphant phenotype can be rescued by BMP repression (Fassier et al., 2010). A recent study confirmed that two HSP-associated proteins, ATLASTIN-1 and NIPA1, are direct binding partners and proposed that these two proteins are members of a common biochemical pathway for maintenance of motor neuron axons (Botzolakis et al., 2011). Our present study also demonstrates the link between pnpla6 and the BMP pathway. In pnpla6 morphants, phosphorylated smad was expressed at abnormally high levels, hinting that pnpla6 is an inhibitor of BMP signaling. This notion was substantiated by the result that the BMP inhibitor DM could rescue the abnormal phenotype. It was also reported that deletion of PNPLA6 results in disruption of ER and aberrant membrane trafficking. Although the exact mechanism is still unknown, results obtained on BMP signaling strongly suggested that PNPLA6 might affect the trafficking of BMP-related protein which, when impaired, could lead to motor neuron defects. Furthermore, previous study has suggested that knockdown of atlastin leads to abnormal architecture of motor neurons with excessive branching. In the current study, we have shown that knockdown of pnpla6 also leads to abnormal architecture of motor neurons with excessive truncation, although branching is also observed. Interestingly, both atl1 and pnpla6 genes affect BMP signaling. We believe that both Atlastin and Pnpla6 might be involved in motor axon development. However, they might play different functional roles in this process. It is possible that BMP signaling is the common downstream pathway.

In summary, we have described a zebrafish model of neural dysfunction as a result of the knockdown of the pnpla6 gene, which...
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is responsible for HSP in humans. This zebrafish model offers the opportunity to study the irregularities in BMP signaling or in other pathways during the pathogenesis of HSP.

**MATERIALS AND METHODS**

**Animal husbandry and treatment**

Wild-type TU-strain zebrafish (\textit{Danio rerio}) were maintained at 28.5°C under standard laboratory conditions (Westerfield, 1995). Embryos were obtained by natural cross and were staged by hours or days after fertilization at 28.5°C using morphological criteria (Kimmel et al., 1995).

Dorsomorphin (DM; EMD Biosciences/Calbiochem), a BMP type I receptor antagonist, was used to downregulate the BMP pathway. For the BMP inhibition experiment, \textit{pnpla6} morphant and control embryos at 10 hpf were dechorionated and each divided into two groups, which were treated with 6 μmol/l DM or DMSO and the phenotype of different groups scored at 36 hpf (Yu et al., 2008).

**Construction of vectors**

D16 vector was kindly provided by Yong Li (Leicester University, UK) and total cDNA of human \textit{PNPLA6} was cloned into pcDNA3.1 vector. Point mutations were introduced to \textit{PNPLA6} cDNA at positions corresponding to amino acids 960, 966 and 1086, respectively.

Based on the predicted zebrafish \textit{pnpla6} sequence, five pairs of primers were designed for the amplification of zebrafish \textit{pnpla6}. Total cDNA from 72 hpf zebrafish embryos was used as template and five overlapping fragments were amplified. Using appropriate endonucleases distributed in the five fragments, the complete \textit{pnpla6} coding region was obtained. Sequencing of the complete sequence further verified its \textit{pnpla6} identity.

**Microinjection of embryos**

Injections were performed on wild-type zebrafish embryos using a PV830 Pneumatic PicoPump (WPI) under a Nikon SMZ 645 stereomicroscope. Antisense MOs were purchased from Gene Tools (Philomath, OR). One translation blocking morpholino against the ATG-containing sequence (+1 to +25) was designed (5'-CTGTGTCGATGTGCTCCTCCCAT-3') and named \textit{pnpla6} MO. The control MO oligo (5'-CTcTGTgCGATG-TcCCTCCTCgAT-3') had five mismatched bases (shown in lowercase) compared with \textit{pnpla6} MO. The MOs were resuspended in RNase-free water with Phenol Red and injected into the yolk of one- to two-cell stage embryos. Different doses (0.15 pmol or 0.3 pmol) of MO were injected. Human wild-type and mutant \textit{PNPLA6} mRNA were in vitro transcribed from linearized pCS2+ constructs using SP6 Message Machine Kit (Ambion). For the rescue experiments, injections were performed as mentioned above, with 0.15 pmol MO plus 60 pg human \textit{PNPLA6} mRNA.

**Analysis of embryos**

Embryos were examined with an Olympus SZX16 dissecting microscope, and images were captured using a DP-71 camera and Olympus DP2-BSW controller program. Figures were generated using Adobe Illustrator CS4 or Adobe Photoshop CS2. For the analysis of distance between the head and tail, embryos were captured and measured using the DP2-BSW controller program.

**Whole-mount immunohistochemistry**

Immunohistochemistry was performed using standard procedures for zebrafish (Beattie, 2000). The following monoclonal antibodies (mAb) were used: mAb Znp-1 (1:2000; Developmental Studies Hybridoma Bank, DSHB, Iowa City, IA), mAb acetylated tubulin and HuC/D (1:200; Santa Cruz Biotechnology), 39.4D5 (1:200; DSHB), 3A10 (1:200; DSHB). Primary antibody binding was visualized using a 3,3'-diaminobenzidine tetrahydrochloride substrate kit (Zhongshan Goldenbridge Biotechnology, Beijing, China).

**Chimeric embryos**

To generate chimeric embryos, cell transplantations were performed as previously described with modifications (Ho and Kane, 1990; van Eeden et al., 1996). Donor embryos for the
transplants were injected with rhodamine-lysine-dextran (RLDx, 25 mg/ml), along with control MO or npplatl MO, at the one-cell stage. These embryos were allowed to develop to 4-6 hpf. Several cells near the animal pole of donor embryos were transplanted into the midline of the neural plate adjacent to the tail bud of the recipients. After immunostaining with mAb Znp-1, images were acquired with a confocal laser scanning microscope (Zeiss LSM 780) or with a fluorescent microscope.

**Primary neuron culture**

The 18-somite stage embryos were used for primary neuron culture. Briefly, the spinal cords were isolated by removing the yolk bag, head and tail. They were then treated with Custom ATV solution (0.6 mM EDTA, 5.5 mM glucose, 5.4 mM KCl, 136.8 mM NaCl, 5.5 mM Na2CO3 and 0.05% w/v trypsin). The fully dissociated spinal cords were plated onto a cover glass in a Petri dish. Eight hours after plating at room temperature, cells were fixed with 4% paraformaldehyde for immunofluorescence analysis. The mAb Znp-1 (1:100; DSHB) was used for analysis of motor neuron growth and the lengths of axons were measured.

**Detection of cell death**

Apoptotic and necrotic cells were detected by incubation of 30-hpf embryos in Acidine Orange solution (5 µg/ml) (Furutani-Seiki et al., 1996). Cells loaded with the dye were visualized using a tetramethylrhodamine isothiocyanate filter on the Olympus BX51 fluorescent microscope. For TUNEL assay, embryos were fixed with 4% paraformaldehyde overnight, rehydrated with decreasing concentrations of methanol, washed with PBST (PBS containing 0.1% Tween 20), treated with proteinase K (10 mg/ml) at 37°C for 30 minutes, refixed with 4% paraformaldehyde, washed in PBST, and lastly incubated with 0.1 M citrate solution (0.1% citrate in PBST). TUNEL staining was performed with the One Step TUNEL Apoptosis Assay Kit (Beyotime, Jiangsu, China).

**Protein blot analysis**

Embryos collected at suitable developmental stages were dechorionated, deyolked and lysed in RIPA lysis buffer supplemented with 1 mM PMSF, 1 mM NaF and 1 mM sodium vanadate (Na3VO4). Equal amounts of protein (40 µg) of the embryos were electrophoresed into 10% SDS-PAGE gel and transferred to nitrocellulose membranes. Membranes were blocked with PBS buffer containing 5% non-fat dry milk and 0.01% Tween 20 for at least 1 hour at room temperature, followed by overnight incubation at 4°C with primary antibodies against phosphorylated Smad1/5/8 (1:400; Cell Signaling), Smad1 (1:500; Abcam), BMPRI (1:300; Santa Cruz Biotechnology) or actin (1:5000; Sigma). After washing, the membranes were incubated with horseradish peroxidase-labeled anti-rabbit (Sigma), anti-mouse (Sigma) secondary antibodies for 1 hour at 20-23°C. Chemiluminescence detection was performed by ECL kit (Thermo).

**Statistical analysis**

Statistical significance between experimental groups was determined by one-way analysis of variance (ANOVA), and data from two groups was compared by Student’s t-test. A difference between means was considered significant at P<0.05.

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**COMPETING INTERESTS**

The authors declare that they do not have any competing or financial interests.

**AUTHOR CONTRIBUTIONS**

Y.G. and M.W. conceived and designed the experiments. Y.S. and M.W. analyzed the data. Y.G., M.W. and C.S. prepared and edited the manuscript prior to submission.

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