The zinc finger protein ZPR1 is a potential modifier of spinal muscular atrophy

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Spinal muscular atrophy (SMA) is caused by mutation of the Survival Motor Neurons 1 (*SMN1*) gene and is characterized by degeneration of spinal motor neurons. The severity of SMA is primarily influenced by the copy number of the *SMN2* gene. Additional modifier genes that lie outside the SMA locus exist and one gene that could modify SMA is the Zinc Finger Protein (*ZPR1*) gene. To test the significance of ZPR1 down-regulation in SMA, we examined the effect of reduced ZPR1 expression in mice with mild and severe SMA. We report that the reduced ZPR1 expression causes increase in the loss of motor neurons, hypermyelination in phrenic nerves, increase in respiratory distress and disease severity and reduces the lifespan of SMA mice. The deficiency of SMN-containing sub-nuclear bodies correlates with the severity of SMA. ZPR1 is required for the accumulation of SMN in sub-nuclear bodies. Further, we report that ZPR1 overexpression increases levels of SMN and promotes accumulation of SMN in sub-nuclear bodies in SMA patient fibroblasts. ZPR1 stimulates neurite growth and rescues axonal growth defects in SMN-deficient spinal cord neurons from SMA mice. These data suggest that the severity of disease correlates negatively with ZPR1 levels and ZPR1 may be a protective modifier of SMA.

INTRODUCTION

Spinal muscular atrophy (SMA) is a neuromuscular disorder characterized by the loss of spinal motor neurons, muscle weakness, respiratory failure and death (1). Mutation of the *SMN1* gene and retention of the *SMN2* gene result in low levels of SMN protein and cause SMA (2,3). The *SMN1* and *SMN2* genes functionally differ by a single nucleotide that alters splicing and results in majority of the transcript from *SMN2* lacking exon 7 (4,5). The truncated SMNΔ7 protein does not efficiently oligomerize with itself or full-length SMN (6) and its ability to interact with other proteins is reduced, including ZPR1 (7). The reduced ability of SMNΔ7 to form protein complexes and defects in nuclear accumulation (8) result in an unstable protein with rapid turnover (9)

and cause defect in the snRNP assembly, a major cause of defective pre-mRNA splicing in SMA (10,11).

The defect in the accumulation of SMN in sub-nuclear bodies, including gems and Cajal bodies, is one of the hallmarks of SMA pathogenesis (3,7,12). ZPR1 interacts with SMN and is required for the accumulation of SMN in sub-nuclear bodies (13). The interaction of ZPR1 with SMN is disrupted in cells derived from SMA patients (7). The function of ZPR1 is unclear. However, ZPR1 may contribute to the function of SMN that requires SMN localization to sub-nuclear bodies. ZPR1 deficiency causes defects in pre-mRNA splicing (7), similar to defects caused by SMN deficiency (14). ZPR1 is a part of cytoplasmic pre-import snRNP complexes containing snurportin 1 and importin β (15). The loss of cytoplasmic pool of snRNPs coupled with the loss of SMN-containing

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nuclear bodies caused by ZPR1 deficiency (13) suggests that ZPR1 may be involved in the nucleocytoplasmic trafficking of SMN-containing snRNPs and contribute to the snRNP biogenesis, a major target of SMN activity (16). Mutation of the *Zpr1* gene causes embryonic lethality in mice (13). Reduced *Zpr1* gene dosage in mice results in progressive loss of spinal motor neurons (17) and a phenotype similar to mice with reduced *Smn* gene dosage that have mild SMA (18). Notably, SMA patients express low levels of ZPR1 (19). Because the reduced expression of ZPR1 results in the loss of spinal motor neurons (17), it is possible that the downregulation of ZPR1 observed in SMA patients might contribute to the severity of SMA.

The severity of SMA phenotypes correlates with *SMN2* copy number and SMN protein levels (3,20). However, there are SMA families with siblings that have lost *SMN1*, have identical *SMN2* copy number and inherited a haploidentical region of chromosome 5 but have discordant phenotypes (21,22). These findings indicate that genes outside of the 5q region can modify the SMA phenotype. Recently, the Plastin 3 (*PLS3*) gene was identified as a protective modifier of SMA and was upregulated in unaffected *SMN1*-deleted females with identical number of *SMN2* copies (23). In this study, we investigated how alteration in the expression of *Zpr1* gene modifies the SMA phenotype in mice. Reducing the level of ZPR1 in both $Smn^{-/+}$ mice and in SMA mice [$Smn^{-/-}$; $SMN2^{+/+}$; $SMN\Delta7^{+/+}$] resulted in the increase of severity and decrease in the lifespan of SMA mice. The decrease in ZPR1 levels results in acute hypermyelination and degeneration of axons in phrenic nerves that may contribute to the respiratory distress and the severity of SMA. The increase in ZPR1 expression in SMA patient cells results in higher SMN levels and accumulation of SMN in the nucleus. ZPR1 overexpression in SMN-deficient spinal cord neurons from SMA mice rescues the defective axonal length phenotype. We suggest that change in ZPR1 levels can modulate the severity of the SMA phenotype.

RESULTS

The effect of reduced *Zpr1* gene dosage on levels of SMN

It is reported that SMA patients express low levels of ZPR1 (19). However, why SMA patients express low levels of ZPR1 and what is the significance of reduced ZPR1 in SMA is unclear. To address these questions and to understand the contribution of low levels of ZPR1 in the severity and pathogenesis of SMA, we examined the effect of reduced *Zpr1* gene dosage on the levels of SMN and the severity of disease in mice with SMA. To test whether there is a genetic interaction between ZPR1 and SMN and the change in ZPR1 expression can influence *in vivo* SMN levels, we examined the effect of reduced *Zpr1* gene dosages on the levels of SMN protein in the spinal cords of wild-type ($Smn^{+/+}$) and heterozygous (het) mice ($Smn^{-/+}$). Mutation of one allele of $Zpr1^{-/+}$ results in lower levels of ZPR1 ($49 \pm 3.3\%$, mean $\pm$ SD; $n = 3$) and SMN ($73 \pm 5.2\%$, $n = 3$), and mutation of one allele of $Smn^{-/+}$ results in lower levels of SMN ($56 \pm 4.9\%$, $n = 3$) and ZPR1 ($79 \pm 4.6\%$, $n = 3$) compared with wild-type mice. Interestingly, mutation of one allele of both

Smn and *Zpr1* genes in double-het [$Smn^{-/+}$; $Zpr1^{-/+}$] mice caused profound decrease and reduced levels of both SMN ($22 \pm 4.8\%$, $n = 3$) and ZPR1 ($28 \pm 4.7\%$, $n = 3$) (Fig. 1A, bar graph). The one-way analysis of variance (ANOVA) indicates that the decrease in ZPR1 levels in $Smn^{-/+}$ mice ($P = 0.002$) and the decrease in SMN levels in $Zpr1^{-/+}$ mice ($P = 0.003$) are significant. The comparison of $Smn^{-/+}$ mice with double-het [$Smn^{-/+}$; $Zpr1^{-/+}$] mice shows the decrease in SMN ($P = 0.008$) and ZPR1 ($P = 0.001$) is significant, also the comparison of $Zpr1^{-/+}$ mice with double-het [$Smn^{-/+}$; $Zpr1^{-/+}$] mice shows the decrease in SMN ($P = 0.001$) and ZPR1 ($P = 0.005$) is significant. Together, these data show that the *in vivo* changes in expression of either ZPR1 or SMN result in the alteration of levels of both proteins and indicate genetic interaction between SMN and ZPR1.

To test whether low levels of SMN affect the levels of ZPR1 in humans, we examined the expression of SMN and ZPR1 in fibroblasts derived from normal ($SMN1^{+/+}$), carriers of disease ($SMN1^{-/+}$) and SMA patients ($SMN1^{-/-}$). The marked decrease in ZPR1 levels in three unrelated SMA type-I patients is consistent with the previous finding that SMA patients express low levels of ZPR1 (19). Notably, the decrease in SMN expression in carriers of SMA disease ($SMN1^{-/+}$) also resulted in low levels of ZPR1 (Fig. 1B). However, carriers of SMA (GM03814 and GM03815) expressed different levels of SMN. This may be due to a different copy number of the *SMN2* gene in two carriers (7). The comparison of levels of ZPR1 and SMN in normal, carriers of disease and SMA patients indicates that the change in levels of SMN alters the levels of ZPR1 in humans (Fig. 1B, bar graph). Since the interaction of SMN and ZPR1 is disrupted in SMA patients (7) and SMN is stabilized by formation of protein complexes (9), the data from double-het [$Smn^{-/+}$; $Zpr1^{-/+}$] mice and SMA patient cells indicate that the decrease in SMN–ZPR1 complexes *in vivo* may contribute to the rapid degradation and further reduced levels of ZPR1 and SMN proteins.

The effect of reduced *Zpr1* gene dosage on the severity of disease in mice with mild SMA

Reduced ZPR1 expression causes neuron degeneration and results in progressive loss of spinal motor neurons in mice (17). It is possible that the reduced ZPR1 expression observed in SMA patients may contribute to the severity of disease because the primary defect in SMA is the loss of spinal motor neurons. To test this, we examined the effect of reduced *Zpr1* gene dosage in $Smn^{-/+}$ mice with mild form of disease similar to SMA type III (18). Reduced dosages of $Zpr1^{-/+}$ and $Smn^{-/+}$ gene cause occasional seizures and gait abnormalities with increased paw abduction compared with wild-type mice. The defects in gait abnormalities, including paw abduction, were increased in double-het male and female mice with mild SMA compared with wild-type mice and heterozygous mice (Fig. 1C).

To determine whether reduced expression of ZPR1 affects neuron degeneration in $Smn^{-/+}$ mice (mild SMA), we examined the effect of ZPR1 deficiency on the brain and spinal cord. Major morphologic differences between the brains of 12-month-old wild-type and $Zpr1^{-/+}$, $Smn^{-/+}$ and [$Smn^{-/+}$;
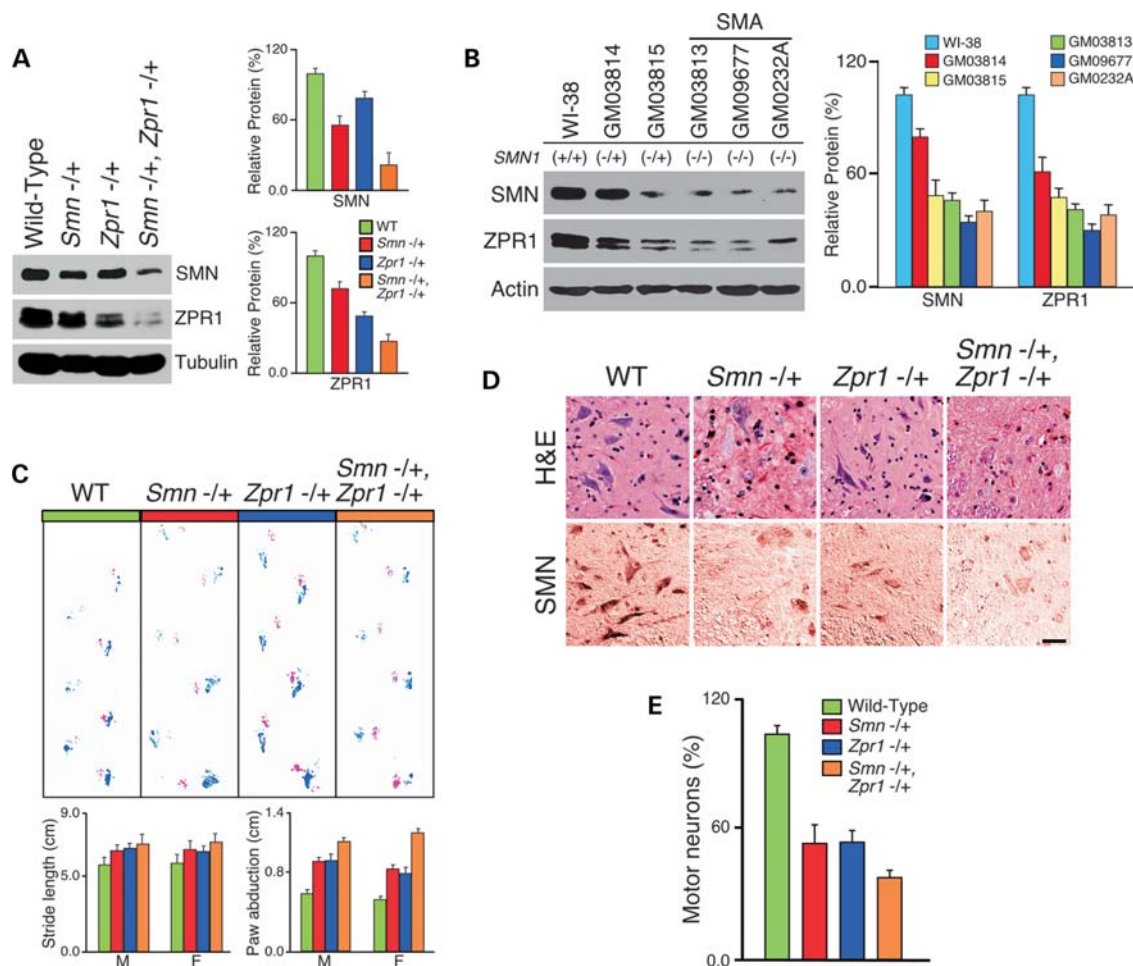
Figure 1. Reduced expression of ZPR1 causes increase in the loss of spinal motor neurons and the severity of disease in mice with mild SMA. (**A**) Reduced dosage of *Zpr1* or *Smn* genes results in decreased levels of SMN and ZPR1 proteins in the spinal cords. The amounts of ZPR1, SMN and tubulin in spinal cords from 12-month-old mice were examined by immunoblot analysis. The relative change in amounts of proteins was calculated using the amount of each protein normalized to tubulin in wild-type (WT) as reference point (100%). (**B**) SMA disease carriers and patients (SMA type-I) express low levels of ZPR1. The amounts of SMN, ZPR1 and actin were examined by immunoblot analysis of cell lysates prepared from fibroblasts of non-SMA human ($SMN1^{+/+}$) (WI-38), carriers of SMA ($SMN1^{-/+}$) mother (GM03814) and father (GM03815) of SMA child (GM03813) and SMA patients ($SMN1^{-/-}$) [GM03813, GM09677 and GM0232A]. The relative amounts of SMN and ZPR1 proteins normalized to β-actin are presented as (mean ± SD; $n = 3$). (**C**) ZPR1 deficiency increases defects in the gait of mice with mild SMA ($Smn^{-/+}$). Footprint patterns of 12-month-old WT, $Smn^{-/+}$, $Zpr1^{-/+}$ and [$Smn^{-/+}$; $Zpr1^{-/+}$] littermates (red, fore-paws; blue, hind-paws) were examined. Quantitative analysis of stride length and paw abduction for males (M) and females (F) was performed three times (five mice/group) and results are presented as (mean ± SD). (**D**) Histochemical staining with H&E and immunohistochemical staining with SMN antibody of the lumbar region of spinal cords from 12-month-old mice (scale bar, 50 μm). (**E**) Motor neurons were counted in sections of the lumbar (L1–L5) region of spinal cords (mean ± SD; five mice/group) from 12-month-old mice. The loss of motor neurons was calculated using the number of motor neurons in wild-type mice as the reference point (100%).

$Zpr1^{-/+}$] mice were not detected. The analysis of spinal cord sections stained with hematoxylin and eosin (H&E) and antibodies against SMN indicated the loss of spinal motor neurons. To estimate the effect of ZPR1 deficiency on the loss of motor neurons, we examined the number of motor neurons in the anterior horns of spinal cords. Reduced gene dosage of either *Smn* (539 ± 40, mean ± SD; $n = 5$) or *Zpr1* (548 ± 24.9, $n = 5$) results in ~45% decrease in the number of motor neurons compared with WT (1047 ± 20.5, $n = 5$). This is consistent with previous findings that reduced expression of either SMN (18) or ZPR1 (17) causes degeneration of spinal motor neurons. Mutations of one allele of both the *Smn* and *Zpr1* genes in double-het mice resulted in the increased loss (~64%) of spinal motor neurons (412 ± 38.5, $n = 5$)

(36 ± 3.2%) compared with wild-type mice (1047 ± 20.5, $n = 5$) (100%) (Fig. 1D and E). ZPR1 deficiency further increased (24 ± 3%, $n = 5$) the loss of motor neurons in $Smn^{-/+}$ mice. The increase in the loss of motor neurons caused by ZPR1 deficiency in mild SMA mice is significant ($P = 0.001$) as indicated by ANOVA. These data suggest that reduced expression of ZPR1 increases degeneration of the spinal motor neuron in mice with mild SMA.

The loss of spinal cord motor neurons can be associated with defects in the peripheral nervous system (PNS) of patients with motor neuron diseases (MNDs), including SMA (24,25). We therefore examined the effect of ZPR1 deficiency on the PNS by examining the sciatic and phrenic nerves. Sciatic nerves from 12-month-old wild-type and
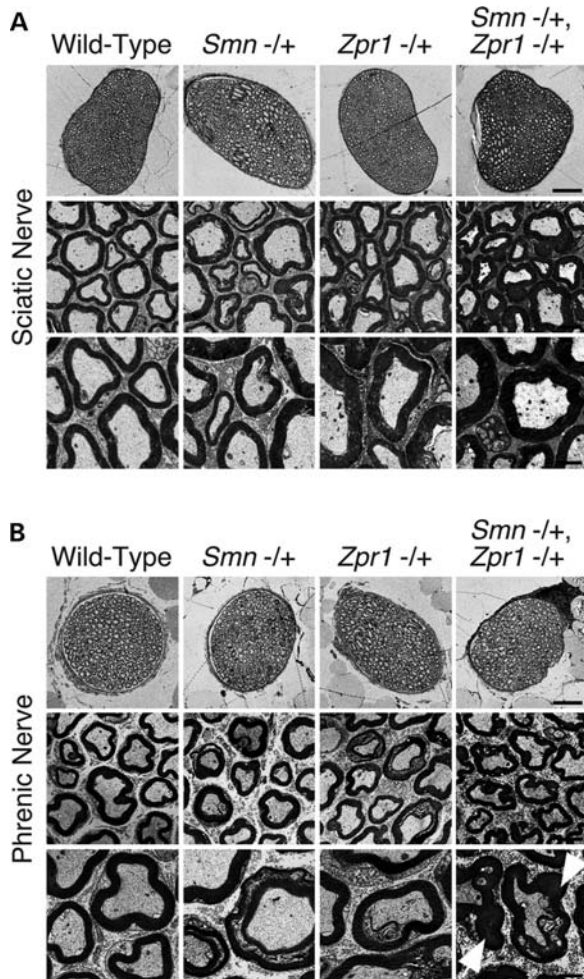
Figure 2. Low levels of ZPR1 cause myelin defects in the PNS of mice with mild SMA. (**A**) Transverse ultra-thin sections of sciatic nerves of 12-month-old wild-type, $Smn^{-/+}$, $Zpr1^{-/+}$ and [$Smn^{-/+}$; $Zpr1^{-/+}$] mice were examined by TEM. Scale bars are 60 μm (upper), 4 μm (middle) and 2 μm (lower) panels. (**B**) Transverse ultra-thin sections of phrenic nerves of 12-month-old mice were examined by TEM. Arrows indicate hypermyelination and myelin folding (tomaculi). Scale bars are 60 μm (upper), 4 μm (middle) and 2 μm (lower) panels.

$Zpr1^{-/+}$, $Smn^{-/+}$ and [$Smn^{-/+}$; $Zpr1^{-/+}$] mice did not show any striking differences when examined by light microscopy. Analysis by electron microscopy indicated some myelin degeneration in sciatic nerves from $Zpr1^{-/+}$, $Smn^{-/+}$ and [$Smn^{-/+}$; $Zpr1^{-/+}$] mice (Fig. 2A). In contrast, striking differences between phrenic nerves of $Zpr1^{-/+}$, $Smn^{-/+}$ and [$Smn^{-/+}$; $Zpr1^{-/+}$] mice were detected. Many axons with hypermyelination and invagination (folding) were present in double-het [$Smn^{-/+}$; $Zpr1^{-/+}$] mice compared with $Zpr1^{-/+}$ and $Smn^{-/+}$ mice (Fig. 2B) and increased myelin and axon degeneration was also observed in [$Smn^{-/+}$; $Zpr1^{-/+}$] mice (Fig. 2B). Together, these data show that reduced expression of ZPR1 causes axonopathy in the phrenic nerve of mice with mild SMA. These findings suggest that in addition to the defects caused by decreased levels of SMN, such as the loss of spinal motor neurons, reduced ZPR1 expression causes specific myelination defects in the phrenic nerve that may contribute to the respiratory distress and the severity of SMA disease.

However, whether the myelination defect in the phrenic nerve is because of further reduced levels of SMN or specifically caused by low levels of ZPR1 is unclear. It is possible that ZPR1 contributes to tissue-specific myelination process independent of functions of ZPR1/SMN complexes.

ZPR1 deficiency contributes to the severity and decreases the lifespan of mice with severe SMA

To test the significance of the downregulation of ZPR1 observed in patients with severe forms of SMA (Fig. 1B), we examined the effect of reduced *Zpr1* gene dosage in SMA mice [$Smn^{-/-}$; $SMN2^{+/+}$; $SMN\Delta7^{+/+}$] with severe phenotype (26). The general morphological examination of littermates non-SMA (WT), SMA and SMA mice with *Zpr1* mutation (SZ) [$Smn^{-/-}$; $SMN2^{+/+}$; $SMN\Delta7^{+/+}$; $Zpr1^{-/+}$] shows smaller body size with less fur for SZ compared with SMA mice (Fig. 3A). Mutation of one allele of *Zpr1* in SZ mice resulted in a profound decrease of ZPR1 levels (22 ± 3.5%, mean ± SD; n = 3) and caused additional ∼13% decrease in SMN and resulted in lower (13 ± 4.2%, n = 3) levels of SMN in SZ mice (Fig. 3B). These data indicate that the reduced expression of ZPR1 results in further decrease in the levels of SMN in mice with severe SMA. The tail hanging analysis to examine weakness of limbs shows clasping of both front and hind limbs in SZ compared with SMA mice (Fig. 3C). Clasping of all limbs indicates increased muscle weakness (27). The analysis of the growth of littermates shows that differences in size and weight between SMA and SZ mice gradually increase throughout the lifespan (Fig. 3D). The severe weakness was noted in SZ mice compared with SMA mice at early stages (age 5 days) of life. The SZ mice show considerable difficulty in righting compared with SMA mice when placed on their backs and were unable to stand on paws or to walk. Progressive weakness in SZ mice resulted in a marked reduction of limb movement activity and increased lethargy at the age of 10 days (Supplementary Material, Movie S1). The Kaplan–Meier survival analysis shows that reduced expression of ZPR1 decreases the lifespan of SMA mice with *Zpr1* mutation (SZ). The maximum survival of 20 days (SMA) was reduced to 13 days in SZ mice. The average survival (11.26 ± 0.7 days, mean ± SD; n = 35) of SMA mice was reduced to the average survival (6.03 ± 0.5 days, mean ± SD; n = 30) of SZ mice (Fig. 3E). The marked reduction in the average survival (46%) and maximum survival (35%) of SMA mice with *Zpr1* mutation (SZ) is highly significant (P = 0.000) and indicates that the increase in the severity of disease caused by ZPR1 deficiency may be a major cause of significantly reduced lifespan of SMA mice.

Reduced ZPR1 expression increases the loss of spinal motor neurons in mice with SMA

The low levels of ZPR1 result in progressive loss of spinal motor neurons in mice (17). It is possible that the significant reduction in the average and maximum survival of mice caused by low levels of ZPR1 in SMA mice might be due to the increased loss of motor neurons that could contribute to the severity of disease. To determine whether reduced lifespan
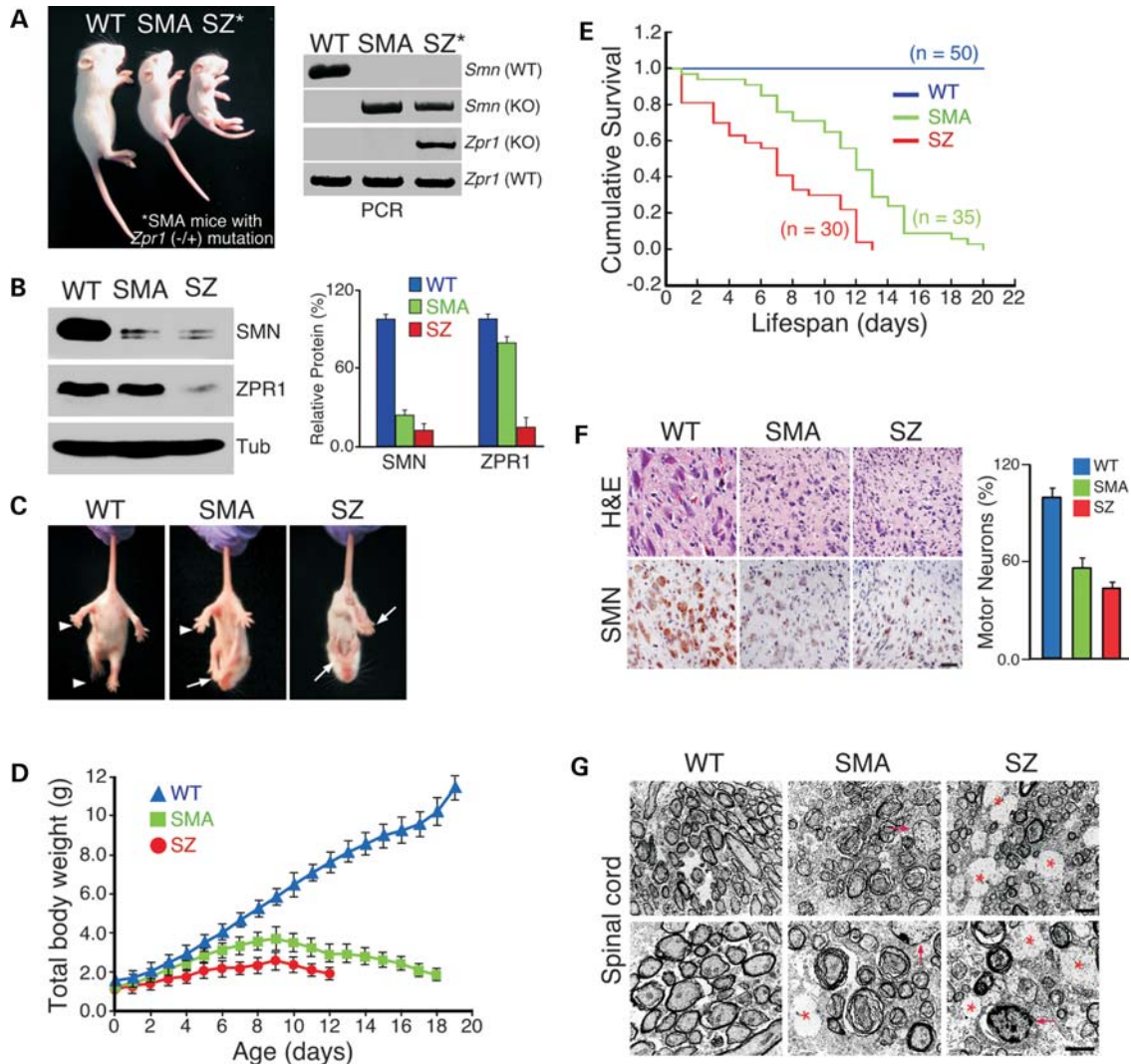
Figure 3. Reduced *Zpr1* gene dosage increases the severity of disease and decreases the lifespan of mice with severe SMA. (**A**) ZPR1 deficiency increases weakness and severity in SMA mice. A photograph of 10-day-old littermates, non-SMA (WT) [*Smn*$^{+/+}$; *SMN2*$^{+/+}$; *SMNΔ7*$^{+/+}$; *Zpr1*$^{+/+}$], SMA [*Smn*$^{-/-}$; *SMN2*$^{+/+}$; *SMNΔ7*$^{+/+}$; *Zpr1*$^{+/+}$] and SMA mouse with *Zpr1* mutation (SZ) [*Smn*$^{-/-}$; *SMN2*$^{+/+}$; *SMNΔ7*$^{+/+}$; *Zpr1*$^{-/+}$]. Genotypes were examined by PCR. The Quick-Time movie (Supplementary Material) of 10-day-old littermates with WT, SMA and SZ genotypes shows motor activity. (**B**) Reduced *Zpr1* gene dosage causes decreased expression of ZPR1 and SMN in the spinal cord of SMA mice. The amounts of ZPR1, SMN and tubulin in tissue were examined by immunoblot analysis. The change in amounts of proteins was calculated by normalizing to the levels of tubulin in wild-type as the reference point (100%). (**C**) Reduced expression of ZPR1 increases the severity of disease in mice with SMA. Tail hanging of WT, SMA and SZ mice shows opening (arrowheads) and clasping (arrows) of front and hind limbs, respectively. (**D**) ZPR1 deficiency causes defects in the growth of mice with SMA. Analysis of growth as total body weights (g) with age (days) in WT (blue triangles), SMA (green squares) and SZ (red circles) mice is shown as growth curves. The results are presented as (mean ± SD, 10 mice/group). (**E**) Reduced expression of ZPR1 results in the decrease of lifespan in mice with SMA. Kaplan–Meier survival analysis of WT (blue, triangles), SMA mice (green, squares) and SMA mice with *Zpr1* mutation (SZ) (red, circles). (**F**) ZPR1 deficiency causes increase in the loss of spinal cord motor neurons. Histochemical staining with H&E and immunohistochemical staining with SMN antibody of the spinal cords sections from 10-day-old WT, SMA and SZ littermates (scale bar, 50 μm). Bar graph shows the loss of motor neurons in the thoracic region (T9–T12) of spinal cords from SMA and SZ mice compared with WT mice as the reference point (100%) (mean ± SD; five mice/group). (**G**) The ultra-thin sections of the spinal cords of WT, SMA and SZ littermates were examined by TEM. The arrows indicate heterochromatin condensation in the nucleus. Asterisks indicate void spaces. Scale bar is 2.0 μm.

of SMA mice is because of increased neurodegeneration, we examined spinal cords from SMA and SZ mice. Histochemical analysis of spinal cord sections stained with H&E and anti-SMN antibodies indicated increased degeneration of spinal motor neurons in SZ mice compared with SMA mice (Fig. 3F). The examination of the spinal cord from SMA mice by transmission electron microscopy (TEM) shows decompression of myelin associated with axonal swelling

and myelin degeneration. In contrast, the spinal cord from SZ mice shows hypomyelination, and axons are compact and smaller in diameter compared with SMA mice (Fig. 3G). In addition, SZ mice show condensation of heterochromatin in the nucleus, indicating the beginning of cell death. These data indicate that ZPR1 deficiency may accelerate the loss of spinal motor neurons in mice with SMA. To examine whether ZPR1 deficiency increased total loss of

motor neurons, we counted neurons in the anterior horns (Supplementary Material, Fig. S3) of the spinal cord thoracic region (T9–T12) from 10-day-old non-SMA (WT), SMA mice (SMA) and SMA mice with $Zpr1^{-/+}$ mutation (SZ) littermates. The total numbers of neurons in 20 sections (every fifth section) of non-SMA mice were 723 ± 35 (mean $\pm$ SD; $n = 6$); SMA mice, 402 ± 12 ($n = 6$); and SZ mice, 321 ± 14 ($n = 6$). The comparison of the number of motor neurons relative to non-SMA (wild-type) mice shows the loss of $44 \pm 3.05\%$ ($n = 6$) in SMA mice and $55.61 \pm 4.5\%$ ($n = 6$) in SZ mice (Fig. 3F, bar graph). The ANOVA of these data shows that ZPR1 deficiency increases ($\sim$12%) the loss of motor neurons in SZ mice, which is very significant ($P = 0.002$). This finding suggests that reduced expression of ZPR1 may increase the severity of disease in SMA mice by increasing the loss of spinal cord motor neurons.

ZPR1 deficiency causes hypermyelination in phrenic nerves of mice with SMA

It is established that the respiratory distress contributes to the severity of SMA and leads to the death of SMA patients (24). However, the defects that cause respiratory failure in SMA patients are unclear. The defects in the PNS, such as hypermyelination coupled with axon shrinking, are found in patients with MNDs, including hereditary neuropathies (28). Since the phrenic nerve regulates respiration, it represents a relevant tissue that might be affected in SMA. The increased myelination in phrenic nerves caused by ZPR1 deficiency was noted in [$Smn^{-/+}$; $Zpr1^{-/+}$] mice (Fig. 2). To determine whether low level of ZPR1 causes axonopathy in the PNS of SMA mice, we examined phrenic and sciatic nerves of SMA mice with $Zpr1$ mutation (SZ) and compared with SMA mice. The differences in the axonal and myelin degeneration between sciatic nerves of SMA and SZ mice were not detected (Fig. 4A). In contrast, phrenic nerves from SMA and SZ mice show striking differences. Reduced expression of ZPR1 resulted in hypermyelination (tomaculi), marked invagination and acute shrinking/degeneration of axons in phrenic nerves of SZ mice (Fig. 4B). The marked decrease in SMN levels in severe SMA [$Smn^{-/-}$; $SMN2^{+/+}$; $SMN\Delta7^{+/+}$] mice compared with mild SMA ($Smn^{-/+}$) mice did not cause hypermyelination in the phrenic nerve. In contrast, the decrease in ZPR1 levels resulted in increased myelination and acute hypermyelination in phrenic nerves of [$Smn^{-/+}$; $Zpr1^{-/+}$] (Fig. 2B) and SZ mice (Fig. 4B), respectively. To test whether the spinal cord neurons (motor and sensory) that are present in the cervical region and form phrenic nerve are affected by ZPR1 deficiency, we counted the number of axons in phrenic nerves. The number of axons in phrenic nerves shows similar loss of axons in both SMA (244.66 ± 3.29, $n = 3$) and SZ (241.63 ± 1.69, $n = 3$) mice compared with wild-type (normal) (263.33 ± 1.24, $n = 3$) mice. However, comparison of the number of axons did not show any significant difference between SMA (244.66 ± 3.29) and SZ (241.33 ± 1.69) mice. These data suggest that the number of neurons in the cervical region of the spinal cord that contribute to the formation of phrenic nerves in SMA and SZ mice is not further reduced by ZPR1 deficiency. Together, these data provide support for the hypothesis that the hypermyelination
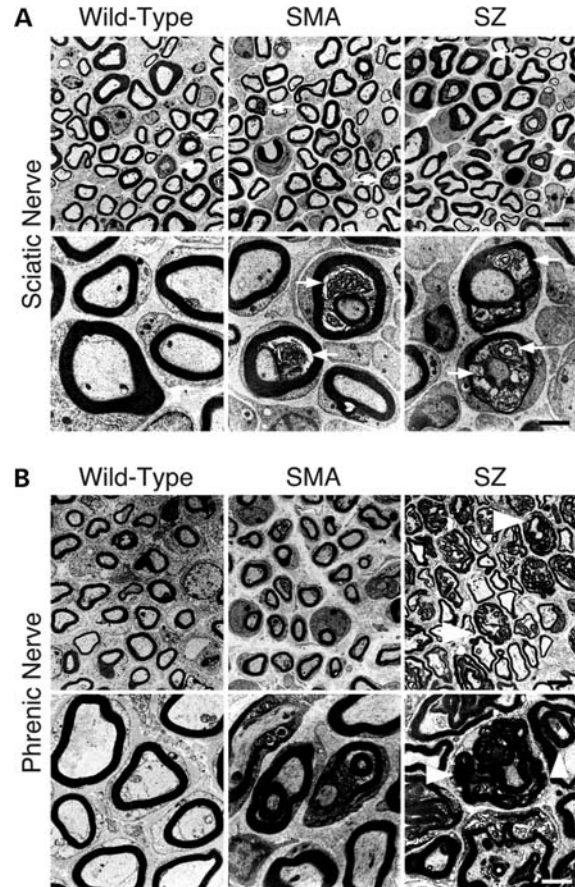
Figure 4. ZPR1 deficiency causes axonal degeneration and hypermyelination in the phrenic nerve of SMA mice. (**A**) Transverse ultra-thin sections of sciatic nerves of wild-type (WT), mice with SMA (SMA) and SMA mice with $Zpr1^{-/+}$ mutation (SZ) littermates were examined by TEM. The arrows indicate myelin degeneration. Scale bar is 2 μm (upper), 1 μm (lower). (**B**) Transverse ultra-thin sections of phrenic nerves of WT, SMA and SZ littermates were examined by TEM. Arrowheads indicate hypermyelination and myelin folding (tomaculi). Scale bar is 2 μm (upper), 1 μm (lower).

defect in the phrenic nerve may be caused by reduced ZPR1 expression. The axonal shrinking and hypermyelination defects observed in phrenic nerves of SZ mice are known to affect the electrical conductivity of the nerve that causes defects in diaphragm movement and contribute to the respiratory distress (28,29). Together, these findings suggest that ZPR1 deficiency may contribute to the severity of disease by increasing the respiratory distress in SMA.

ZPR1 promotes nuclear accumulation and increases SMN levels in SMA patient cells

The deficiency of SMN-containing gems and Cajal bodies in the nucleus might be one of the major causes of defects in functions of SMN in the nucleus, including aberrant pre-mRNA splicing (11). ZPR1 is required for the accumulation of SMN in sub-nuclear bodies, and low levels of ZPR1 result in decreased accumulation of SMN in the nucleus (30). To test whether the increase in ZPR1 levels will affect nuclear accumulation of SMN, we examined the effect of
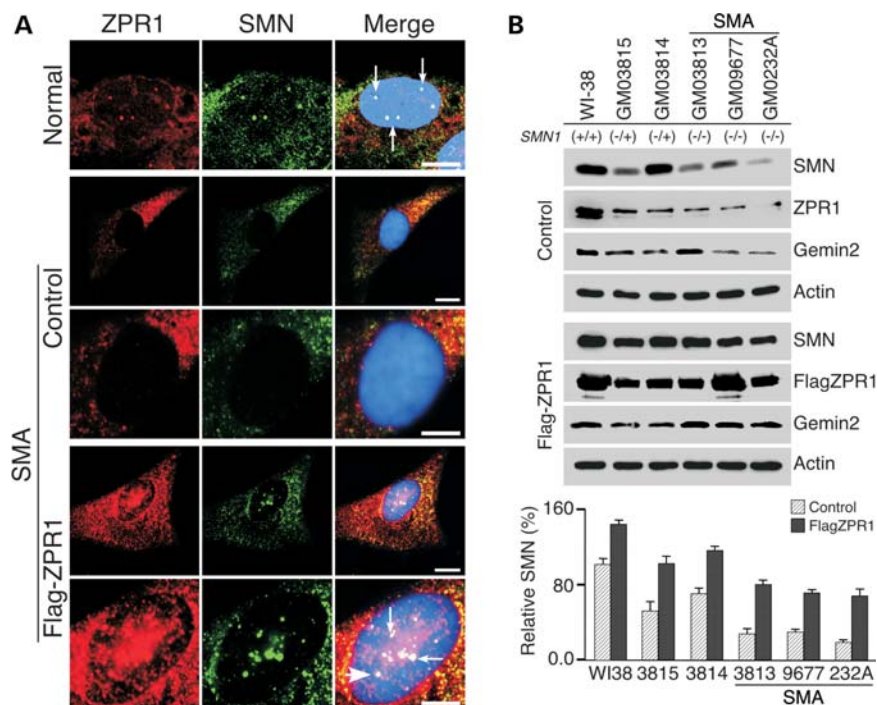
Figure 5. ZPR1 promotes nuclear accumulation of SMN and increases levels of SMN in SMA patient cells. (**A**) The fibroblasts from non-SMA human (WI-38) (normal) and SMA type-I patient (GM03813) were cultured, mock-transfected (control) and transfected with pcDNA3/FlagZPR1 (Flag-ZPR1), processed and examined by immunofluorescence analysis. The sub-cellular distribution of ZPR1, SMN and FlagZPR1 was examined using monoclonal antibodies to ZPR1, SMN and FLAG (M2) for the detection of recombinant Flag-ZPR1. The colocalization (yellow) and accumulation of ZPR1 (red) and SMN (green) in the nucleus is indicated by arrows. DNA was stained with DAPI (blue). Scale bar is 10 μm. (**B**) The fibroblasts from normal (WI-38), SMA carriers (GM03814 and GM03815) and SMA patients (GM03813, GM09677 and GM0232A) were infected with empty Ad5CMV (control) and with recombinant adenovirus (Ad5CMV) expressing FlagZPR1 (Flag-ZPR1). The amounts of ZPR1, Flag-ZPR1, SMN, Gemin2 and β-actin in cell lysates were examined by immunoblot analysis. The change in the amounts of proteins (mean ± SD; $n = 3$) was calculated using the amount of each protein normalized to actin in WI-38 as the reference point (100%).

ZPR1 overexpression in fibroblasts from SMA patients. In the non-SMA (normal) fibroblast, both SMN and ZPR1 colocalize in sub-nuclear bodies (Fig. 5A). In contrast, both ZPR1 and SMN did not colocalize into sub-nuclear bodies in SMA patient cells (control) (Fig. 5A). Notably, transient expression of ZPR1 results in the accumulation of SMN in sub-nuclear bodies in SMA patient cells (FlagZPR1) (Fig. 5A). Further we show that newly formed sub-nuclear bodies in SMA patient cells also contain Gemin2 that binds SMN (Supplementary Material, Fig. S1A). The FlagZPR1 was also able to accumulate in the Cajal bodies stained with p80 coilin (Supplementary Material, Fig. S1B). These results suggest that the increase in ZPR1 expression may partially correct the defect in nuclear accumulation of SMN and help restore the normal functions that require SMN localization to sub-nuclear bodies, including Cajal bodies. The increase in the number of SMN-containing nuclear bodies in SMA patient cells suggests that ZPR1 overexpression may help reduce the severity of disease because the severity of SMA correlates negatively with the number of SMN-containing nuclear bodies (3).

The reduced ZPR1 expression results in lower levels of SMN in mice. Similarly, the decrease in SMN results in low levels of ZPR1 in mice (Fig. 1). Since ZPR1 and SMN form complexes, it is possible that the increase in ZPR1 may influence levels of SMN. To test whether ZPR1 can result in higher levels of SMN, we examined the effect of ZPR1 overexpression on SMN levels in SMA patient cells. Overexpression of

ZPR1 in non-SMA (WI-38) and carriers of SMA (GM03814 and GM03815) resulted in 1.0−1.5-fold increase in SMN levels (Fig. 5B). Notably, ZPR1 overexpression resulted in a marked increase in SMN levels (2−3-fold) in SMN-deficient fibroblasts from SMA patients (Fig. 5B, bar graph). To determine whether the increase in SMN levels by ZPR1 in patient cells is because of the increase in the expression of mRNA, we examined levels of transcripts of SMN and SMNΔ7 using RT–PCR. The change in the levels of transcripts of SMN and SMNΔ7 was not detected (Supplementary Material, Fig. S2). Therefore, the increase in SMN levels by ZPR1 in normal and SMA patient cells suggests that ZPR1 may stabilize the turnover and increase levels of SMN. The stabilization of SMN complexes by ZPR1 is supported by the increase in another protein of SMN complexes, Gemin2 (Fig. 5B). Importantly, the increase in levels of SMN by ZPR1 overexpression in fibroblasts from carriers and SMA patients suggests that ZPR1 may function in the context of SMN deficiency. Since the severity of SMA correlates negatively with SMN levels, these data suggest that upregulation of SMN levels by ZPR1 overexpression may help reduce the severity of disease and modify SMA phenotype.

ZPR1 stimulates axonogenesis in motor neuron-like cells

The axonal growth defects, including pathfinding and inability to innervate caused by low levels of SMN, may contribute to
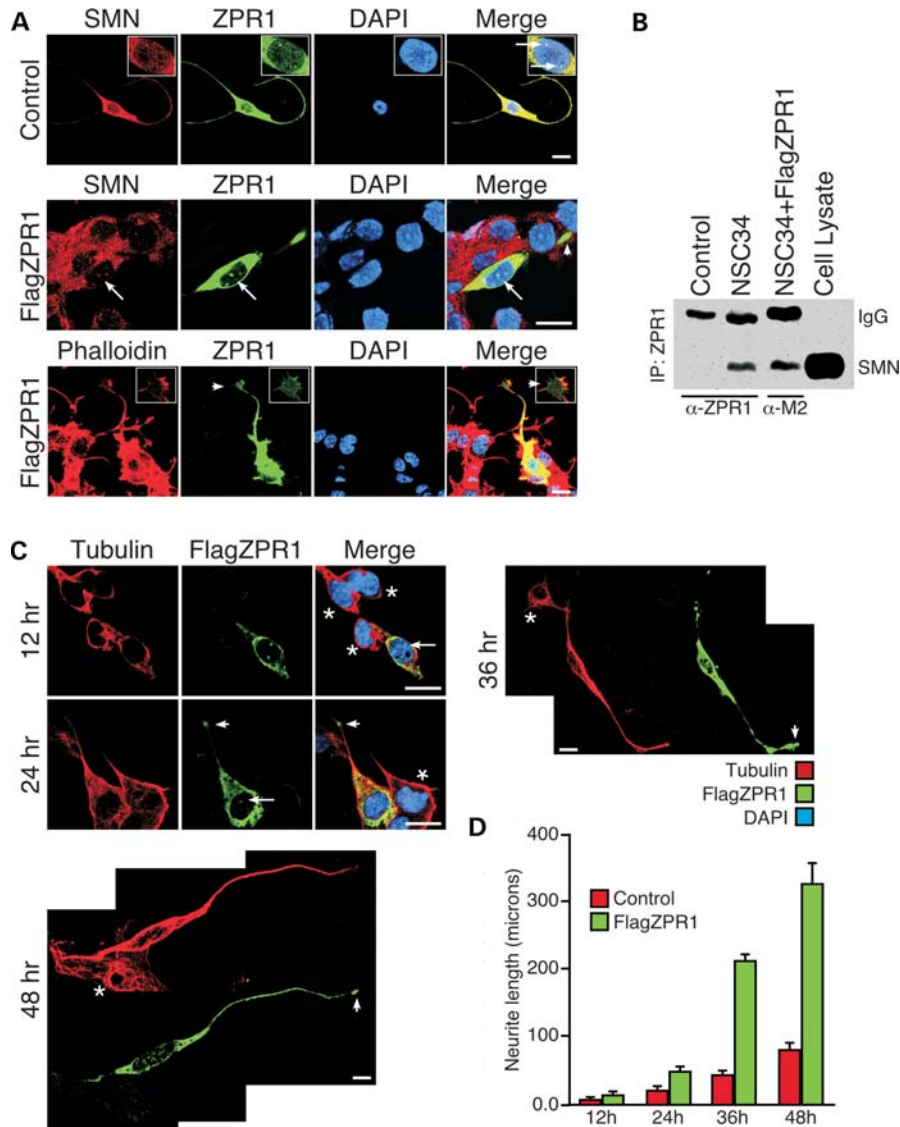
Figure 6. ZPR1 stimulates neuron differentiation and axonal growth in motor neuron-like cells. (**A**) Colocalization of endogenous and overexpressed ZPR1 with SMN in motor neuron-like (NSC-34) cells. Undifferentiated NSC-34 cells were mock-transfected or transfected with pCDNA3/FlagZPR1. After 24 h of transfection, cells were differentiated into neurons. Cells were harvested at 24 h after differentiation, fixed and stained with antibodies to SMN (red) and ZPR1-FITC (green) (control), SMN (red) and FLAG (M2)-FITC (green) (center panel), phalloidin (red) and FLAG epitope (M2) (green) (bottom panel). Arrows indicate sub-nuclear bodies. Arrowheads show growth cones. Nuclei were stained with DAPI (blue). Scale bar is 20 μm. (**B**) Interaction of ZPR1 with SMN in NSC-34 cells. Endogenous and overexpressed ZPR1 proteins were immunoprecipitated with antibodies to ZPR1 and FLAG M2, respectively. Immune complexes were separated by SDS−PAGE and SMN was detected by immunoblot analysis. (**C**) ZPR1 stimulates axon growth and differentiation in cultured motor neuron-like cells. NSC-34 cells transfected with FlagZPR1 were differentiated into neurons after 24 h. Cells were harvested at 12, 24, 36 and 48 h post-differentiation, examined by immunofluorescence using antibodies to FLAG (M2) (green) and neuron-specific class-III β-tubulin (red). DNA was stained with DAPI (blue). Arrows indicate nuclear bodies. Arrowheads indicate growth cones. Asterisks indicate untransfected cells. Scale bar is 20 μm. (**D**) The bar graph shows the average neurite length (mean ± SD, $n = 50$) in untransfected (control) and transfected (FlagZPR1) neurons.

SMA pathogenesis (31). To gain insight into the function of ZPR1 in growth and differentiation of neurons, we examined the effect of ZPR1 overexpression in a neuroblastoma fused with spinal cord (NSC-34) hybrid cells that can be induced to differentiate into motor neuron-like cells (13,32). Both SMN and ZPR1 present in the soma and axons and colocalize in the cytoplasm and in the sub-nuclear bodies of differentiated NSC-34 cells (Fig. 6A, top panel). The recombinant FlagZPR1 colocalizes with SMN in the soma, sub-nuclear bodies (Fig. 6A, middle panel) and in the growth cones

stained with phalloidin (Fig. 6A, bottom panel). We tested the formation of ZPR1−SMN complexes in NSC-34 cells, using co-immunoprecipitation followed by immunoblot analysis. Both endogenous ZPR1 and recombinant FlagZPR1 formed complexes with SMN in NSC-34 cells (Fig 6B). These data indicate that FlagZPR1 can be a part of the endogenous SMN complexes and may contribute to the function of SMN. Further, we examined the effect of FlagZPR1 on the axonal growth and differentiation of NSC-34 cells. Overexpression of ZPR1 stimulated differentiation and axonal
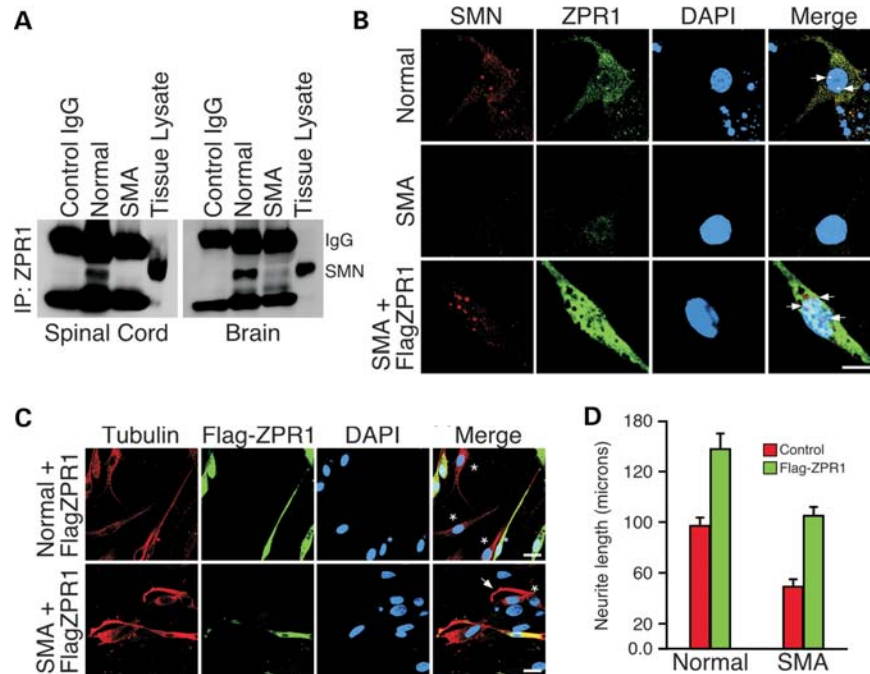
Figure 7. ZPR1 promotes accumulation of SMN in the nucleus and rescues axon length phenotype in SMN-deficient neurons derived from SMA mice. (**A**) Interaction of ZPR1 with SMN is disrupted in SMA mice. ZPR1 was immunoprecipitated with antibodies to ZPR1 from the brain and spinal cords of normal and SMA mice. Immune complexes were separated by SDS–PAGE, and SMN was detected by immunoblot analysis. (**B**) Colocalization of endogenous and overexpressed ZPR1 with SMN in spinal cord neurons from non-SMA mice (normal) and SMA mice (SMA) expressing recombinant ZPR1 (SMA+ FlagZPR1). Spinal cord explant neuronal cultures were infected with adenovirus, empty virus (Ad5CMV) and with recombinant ZPR1 (Ad5CMV-FlagZPR1), harvested after 48 h of infection and stained with antibodies to SMN (red), ZPR1 (green) and FLAG (M2) to detect recombinant Flag-ZPR1 (green). Nuclei were stained with DAPI (blue). Arrows indicate colocalization of ZPR1 and SMN in sub-nuclear bodies. Scale bar is 20 μm. (**C**) ZPR1 stimulates axonogenesis and rescues the axon length in SMN-deficient spinal motor neurons from SMA mice. Spinal cords neurons from 7-day-old non-SMA (normal) and SMA mice were infected with Ad5CMV-FlagZPR1, fixed after 48 h post-infection and stained with antibodies to tubulin (red) and FLAG (M2) to detect recombinant FlagZPR1 (green). Nuclei were stained with DAPI (blue). Asterisks indicate neurons that do not express FlagZPR1. Neurons that express FlagZPR1 are stained in green. Arrows indicate bending and retraction of SMN-deficient spinal cord neurons. Scale bar is 20 μm. (**D**) The bar graph shows average neurite length [mean ± SEM, $n = 30$ (cells), 3 mice/group] in untransfected (control) and transfected (FlagZPR1) spinal cord neurons from non-SMA (normal) and SMA mice (SMA).

growth, including formation of growth cones in motor neuron-like cells within 24 h of ZPR1 expression (Fig. 6C, panel-24 h). The average 3–4-fold increase in neurite length (FlagZPR1/Con) [mean ± SEM (μm); $n = 10$] within 24 h (44.76 ± 1.18/18.67 ± 1.03), 36 h (211.41 ± 4.40/41.11 ± 1.06) and 48 h (326.98 ± 13.68/77.02 ± 5.33) induced by ZPR1 in motor neurons was highly significant ($P = 0.000$) (Fig. 6D). These data suggest that ZPR1 can induce neuron differentiation and stimulate axonal growth in motor neuron-like cells.

ZPR1 rescues axonal growth defects in SMN-deficient spinal cord neurons from SMA mice

The interaction of ZPR1 with SMN is disrupted in SMA patient cells (7). We show that the interaction of ZPR1 with SMN is also disrupted in mice with SMA as indicated by IP of ZPR1 from the brain and spinal cord tissues of normal and SMA mice (Fig. 7A). To address one of the important questions—whether ZPR1 can also promote nuclear accumulation of SMN in spinal cord neurons from SMA mice, we expressed FlagZPR1 in cultured spinal cord neurons from 7-day-old SMA mice, using Ad5CMV-FlagZPR1. The expression of FlagZPR1 promoted nuclear accumulation of SMN in spinal cord neurons from SMA

mice (Fig. 7B) and this is consistent with the data from SMA patient cells (Fig. 5A). Further we tested whether ZPR1 can stimulate neurite growth in cultured primary mouse spinal cord neurons. The control experiments were performed using spinal cord cultured neurons from normal (non-SMA) mice and infection with Ad5CMV-GFP and Ad5CMV-FlagZPR1-GFP. The overexpression of GFP did not induce or stimulate neurite growth (96.4 ± 5.6, $n = 30$) [mean ± SEM (μm)] (GFP) compared with neurons without GFP (96.6 ± 5.2, $n = 30$) (Con). Notably, overexpression ZPR1-GFP resulted in the stimulation of neurite growth in primary spinal cord neurons, similar to FlagZPR1 (Supplementary Material, Fig. S4 and Fig. 7D). In the control experiments (normal), FlagZPR1 resulted in the stimulation of neurite growth in spinal cord neurons from normal (non-SMA) mice (Fig. 7C). The significant increase in neurite length ($P = 0.000$) from 96.40 ± 5.6 ($n = 30$) (control) to 158.21 ± 11.79 ($n = 30$) (Con + FlagZPR1) with three mice/group supports the idea that ZPR1 can stimulate axonogenesis in spinal cord neurons (Fig. 7D). To test whether ZPR1 may be a protective modifier of SMA and can rescue axonal growth defects in SMN-deficient neurons, we examined the effect of ZPR1 in spinal cord neurons from SMA mice. The growth of SMN-deficient motor neurons (48.74 ± 3.4, $n = 30$) was severely impaired

compared with neurons from normal (non-SMA) mice and consistent with previous findings (Fig. 7C) (23,33). The defects in the axonal growth of SMN-deficient neurons were rescued by ZPR1 overexpression, and the neurite length of SMA neurons with FlagZPR1 (106.98 ± 3.6; $n = 30$) was comparable with wild-type neurons (96.40 ± 5.6; $n = 30$) (Fig. 7C). The one-way ANOVA shows that the rescue of axonal growth in SMA neurons within 48 h of increase in ZPR1 levels is highly significant ($P = 0.000$). These data suggest that ZPR1 may function in SMN-deficient neurons and has the potential to rescue axonal growth defects associated with SMA pathogenesis.

DISCUSSION

SMA is one of the leading genetic causes of infant death, with a carrier frequency of 1 in 35–39 (20). Currently, there is no treatment available to cure or reduce the burden of the severity of SMA mainly because of limited knowledge of modifier genes and the biochemical and molecular mechanisms associated with the pathogenesis of SMA (34). It has been established that the severity of SMA is primarily influenced by the copy number of the *SMN2* gene and correlates inversely with the levels of SMN protein (3,20). However, genes outside the 5q SMA locus, including *PLS3* (Chr Xq23), can modify the SMA phenotype because individuals with homozygous *SMN1* deletion and identical *SMN2* copy numbers show discordant phenotypes compared with the siblings (21–23). Since the SMA phenotype is primarily modified by levels of SMN, the identification of genes and proteins that upregulate levels of SMN will provide insight into the molecular details of SMN biogenesis and open new possibilities for therapeutic interventions of SMA. In this study, we show that the alteration in ZPR1 expression changes levels of SMN, and ZPR1 has a potential to modify the SMA phenotype.

The genetic interaction between ZPR1 and SMN

The *ZPR1* gene is located on Chr 11q23.2 in humans and expressed ubiquitously. Genetic studies have established that the *Zpr1* gene is essential for embryonic viability in mice (13). Reduced *Zpr1* gene dosage in mice causes progressive loss of spinal motor neurons and results in a phenotype similar to mice with mild SMA (17,18). The expression of ZPR1 is downregulated in SMA patients. However, the mechanism of ZPR1 downregulation and contribution of ZPR1 deficiency to SMA pathogenesis is unclear. We show that the reduced expression of ZPR1 downregulates the levels of SMN in $Zpr1^{-/+}$ mice. The reduced expression of SMN resulted in low levels of ZPR1 in $Smn^{-/+}$ mice. The marked reduction in the levels of both ZPR1 and SMN in double-het [$Smn^{-/+}$; $Zpr1^{-/+}$] mice and the downregulation of ZPR1 in carriers and SMA patients with SMN deficiency indicate that the levels of ZPR1 and SMN are interdependent. These findings suggest that alteration in levels of either SMN or ZPR1 can influence *in vivo* levels of each protein and indicate genetic interaction between ZPR1 and SMN that could modify SMA phenotype.

Reduced *Zpr1* gene dosage increases the severity of disease in SMA mice

We show that unrelated SMA patients express low levels of ZPR1, which is consistent with previous findings (19). However, the significance of downregulation of ZPR1 in SMA is unclear and whether low levels of ZPR1 contribute to the pathogenesis of SMA remains to be established. To address one of the important questions—whether downregulation of ZPR1 contributes to the severity of SMA, we show that the reduced *Zpr1* gene dosage in mice with mild SMA results in ambulatory defects, including increased paw abduction, that are associated with mild SMA phenotype. The increased loss of spinal motor neurons caused by ZPR1 deficiency may contribute to muscle weakness and abnormal gait in mild SMA [$Smn^{-/+}$; $Zpr1^{-/+}$] mice. Further, we show that the reduced ZPR1 expression in mice with severe SMA [$Smn^{-/-}$; $SMN2^{+/+}$; $SMN\Delta7^{+/+}$; $Zpr1^{-/+}$] (SZ) results in a marked reduction of motor activity, retarded growth and shortened lifespan compared with SMA [$Smn^{-/-}$; $SMN2^{+/+}$; $SMN\Delta7^{+/+}$] (SMA) mice. The modification of phenotype that includes increase in limb weakness (clasping), marked decrease in motor activity and retarded growth in SZ mice compared with SMA mice indicates that ZPR1 deficiency may increase severity of SMA. The increase in severity caused by ZPR1 deficiency in SMA mice correlates with additional loss (∼10%) of spinal motor neurons. These findings suggest that reduced expression of ZPR1 may contribute to the severity of SMA by increasing the loss of spinal motor neurons. However, a small increase in the loss of motor neurons alone might not account for the marked reduction (46%) in the survival of SZ mice. It is possible that ZPR1 deficiency causes other defects, such as axonopathy in the PNS that might also contribute to the severity and reduce the lifespan of SMA mice.

ZPR1 deficiency contributes to the respiratory distress in SMA mice

It is established that the respiratory failure is the cause of death in SMA patients (1). SMA type-I and some type-II patients require ventilator for life support because of acute respiratory distress. However, the molecular details of pathogenesis that leads to respiratory failure and infant death in SMA are unclear. The respiratory distress is caused by defects in intercostal muscles, diaphragm and phrenic nerves. SMN deficiency results in the weakness of intercostal muscles in mice that may contribute to the respiratory distress (26,35). The comparison of intercostal muscles did not show any differences between SMA and SZ mice (data not shown). However, contribution of diaphragm dysfunction caused by defects in the phrenic nerve to the respiratory distress in SMA is unclear. We show that the reduced ZPR1 expression selectively causes hypermyelination in phrenic nerves of SMA mice with ZPR1 deficiency (SZ). The phrenic nerve contains motor, sensory and sympathetic nerve fibers and is the only motor supply to the diaphragm that regulates respiration. The comparison of the number of axons in phrenic nerves did not show any significant difference in SMA and SZ mice and suggested that the neurons in the cervical region of the spinal cord that form the phrenic nerve are not affected by ZPR1

deficiency. However, hypermyelination in the phrenic nerve caused by ZPR1 deficiency might be a major cause of the respiratory distress and shortening of the lifespan of SZ mice because hypermyelination causes defects in the nerve conduction velocity of motor and sensory nerve fibers that contribute to diaphragm dysfunction (36). Therefore, it is possible that hypermyelination in the phrenic nerve caused by ZPR1 deficiency may result in increased respiratory distress and contribute to the severity of SMA. These findings suggest that the marked reductions in the average survival (46%) and the maximum survival (35%) of SZ mice compared with SMA mice may be a consequence of increased respiratory distress caused by ZPR1 deficiency.

The comparison of the phenotype of phrenic nerves of mice with mild SMA [$Smn^{-/+}$] and severe SMA [$Smn^{-/-}$; $SMN2^{+/+}$; $SMN\Delta7^{+/+}$] did not show any change in levels of myelination (Figs 2 and 4). These data suggest that the marked decrease in SMN levels from 66 to 26% did not affect levels of myelination. In contrast, decrease in ZPR1 levels caused by reduced *Zpr1* gene dosage resulted in hypermyelination in both mice with mild SMA ($Smn^{-/+}$; $Zpr1^{-/+}$) and severe SMA [$Smn^{-/-}$; $SMN2^{+/+}$; $SMN\Delta7^{+/+}$; $Zpr1^{-/+}$] (SZ). These data suggest that low levels of ZPR1 result in hypermyelination of axons in the PNS. This is consistent with the finding that ZPR1 deficiency causes hypermyelination in the femoral nerve of 6-week-old mice (17). The mechanism of hypermyelination caused by ZPR1 deficiency in the phrenic nerve is unclear. The precise signaling between axonal ligands and glial cell receptors is critical to determine the accurate amount of myelination required for normal nerve function (36). The profound hypermyelination observed in phrenic nerves of SZ mice may be a result of altered axon/glial cell signaling. Axonal ligand, neuregulin 1 (Nrg1) and epidermal growth factor (EGF) receptor tyrosine kinases (ErbB2 or HER2)-mediated signaling regulates myelin sheath thickness in the PNS (37). Hypermyelination is caused by increased Nrg1/ErbB2 signaling (37). ZPR1 binds to the cytoplasmic signaling domain of tryrosine kinase receptors, including EGF receptor, and controls downstream signaling (38). It is possible that the low levels of ZPR1 may result in increased ErbB2 receptor signaling and contribute to hypermyelination observed in phrenic nerves of SZ mice. However, further studies are required to delineate the mechanism of ZPR1-mediated myelination in the PNS. Nevertheless, the observation that the ZPR1 deficiency causes hypermyelination in phrenic nerves of mice with mild and severe SMA provides strong support for the hypothesis that the phrenic nerve dysfunction may contribute to the respiratory distress in SMA. Since the levels of ZPR1 are downregulated in SMA patients, our data indicate that low levels of ZPR1 may contribute to the respiratory distress and SMA pathogenesis. Together, these findings suggest that the phrenic nerve may be a potential target tissue for developing therapeutic strategies to reduce the burden of the respiratory distress in SMA.

ZPR1 increases SMN levels and its nuclear accumulation in SMA patient cells

The defect in the nuclear accumulation of SMN$\Delta7$ and low levels of SMN and SMN-containing bodies in the nucleus

are major causes of biochemical defects in SMA. Importantly, the severity of disease negatively correlates with the amount of nuclear SMN and the number of SMN-containing nuclear bodies (3,8). ZPR1 binds to SMN and is required for the accumulation of SMN in sub-nuclear bodies but the interaction of ZPR1 with SMN is disrupted in SMA (7). The function of nuclear bodies is unclear. However, the role of sub-nuclear bodies has been implicated in several aspects of RNA biogenesis, including maturation and sorting of spliceosomal snRNPs (39,40). The nuclear SMN plays a role in the splicing; therefore, the deficiency of SMN in the nucleus may be a major cause of defects in pre-mRNA splicing in SMA mice (11). The function of ZPR1 in the nucleus is unclear. However, ZPR1 may contribute to the function of SMN that requires its accumulation in sub-nuclear bodies (13). Immunodepletion of ZPR1 from nuclear extract causes defects in pre-mRNA splicing, similar to defects caused by SMN deficiency (7,14). We show that ZPR1 overexpression promotes the accumulation of SMN in the nucleus and results in the increase of the number of SMN-containing sub-nuclear bodies in SMA patient cells. These data suggest that the increase in ZPR1 levels may partially correct defects in nuclear accumulation of SMN and result in gain of function in the nucleus. Because the severity of SMA correlates negatively with the number of SMN-containing nuclear bodies, our data suggest that the increase in nuclear accumulation of SMN by ZPR1 may partially restore biochemical function of SMN in the nucleus and help reduce the severity of SMA.

It is established that the levels of SMN primarily influence the severity of SMA (3,20). We show that ZPR1 overexpression increases levels of SMN in SMA patient cells. The mechanism of increase in SMN levels by ZPR1 is unclear. Because ZPR1 and SMN form complexes, it is possible that the increased formation of ZPR1–SMN complexes and nuclear accumulation of SMN may reduce degradation and enhance half-life of SMN protein but remains to be tested. This is supported by the data that ZPR1 did not change the levels of transcription of SMN and SMN$\Delta7$ mRNA. It is reported that the SMN is stabilized by increased formation of SMN–protein complexes upon treatment with proteosome/protease inhibitors in SMA patient cells (9). The stabilization of SMN–ZPR1 complexes is further supported by the finding that the increase in SMN levels using antisense oligonucleotides results in higher levels of ZPR1 in SMA patient cells that express low levels of ZPR1 (41). However, the molecular mechanisms involved in the increase of SMN levels by ZPR1 remain to be investigated. Nevertheless, positive effects of the increase in SMN levels on the rescue of SMA phenotype have been established (42,43). Therefore, the current findings that ZPR1 overexpression increases the levels of SMN in SMA patient cells suggest that the increase in ZPR1 levels may help reduce severity of SMA.

ZPR1 rescues axonal growth in spinal cord neurons from SMA mice

SMA is characterized by the degeneration of spinal motor neurons caused by low levels of SMN. The axonal defects, including slow neurite outgrowth and reduced synapse that result in abnormalities in the formation of neuromuscular

junctions, are major contributors to motor neuron degeneration in SMA (31,35,44). A protective modifier of SMA should have functions in the growth and differentiation of neurons and exhibit potential to rescue defects in the growth of SMN-deficient neurons similar to one of the first identified SMA modifier genes *PLS3* (23). We show that the increase in ZPR1 expression stimulates differentiation, formation of growth cones and axonal growth in cultured motor neuron-like cells (NSC-34). These data suggests that ZPR1 may play an important role in the growth and differentiation of neurons. This is supported by findings that ZPR1 deficiency causes neuron degeneration and progressive loss of spinal motor neurons in mice (17). Further, we show that ZPR1 overexpression in SMN-deficient spinal motor neurons derived from SMA mice stimulates neurite outgrowth and rescues axonal growth phenotype that is similar to rescue by PLS3 protein, a protective modifier of SMA (23). Therefore, ZPR1 may represent a positive modifier of SMA.

In conclusion, ZPR1 deficiency causes phrenic nerve dysfunction that contributes to the respiratory distress and increases the severity of SMA in mice. Because SMA patients express low levels of ZPR1, our data suggest the phrenic nerve as a potential therapeutic target tissue to reduce the burden of the respiratory distress in SMA. The ZPR1 overexpression elevates SMN levels, corrects the defect in nuclear accumulation of SMN in SMA patient cells and rescues the axonal growth phenotype in SMN-deficient neurons from SMA mice, which suggests that ZPR1 may be a protective modifier of SMA. The identification of ZPR1 as a protective modifier will open new avenues for the development of novel therapies for the treatment of SMA.

MATERIALS AND METHODS

Mice

The $Zpr1^{-/+}$ (C57BL/6J) (13) mice were bred with $Smn^{-/+}$ mice (18) to generate double-het mice. The SMA model mice, Smn $(-/-)$ mouse rescued with human transgenic $SMN2$ [$Smn^{-/+}$; $SMN2^{+/+}$; $SMN\Delta7^{+/+}$] (26), were bred with $Zpr1^{-/+}$ (FVB) to generate SMA mice with $Zpr1$ mutation. All mice were genotyped by PCR using tail DNA. Gait was examined by applying non-toxic paint to the hind (blue) and fore (red) feet of the mice. Footprint patterns were analyzed for two parameters: stride length and paw abduction. Mean values were measured from three tests/mouse and six mice/group (17). All mice were housed in a facility accredited by the American Association for Laboratory Animal Care. All studies were approved by the IACUC and TTUHSC.

Spinal cord, sciatic and phrenic nerve morphology

The spinal cord tissue was fixed in 4% paraformaldehyde (24 h) prior to processing and embedding in paraffin wax. Paraffin sections were cut at 7 μm and stained with H&E and SMN antibody (clone 8, Transduction Laboratories). Motor neurons were counted in anterior horns of the spinal cord as indicated by a dotted-line boundary in stained spinal cord sections (Supplementary Material, Fig. S3) in every fifth section (20 sections/per region) of the lumbar (L1–L5) and thoracic

(T9–T12) regions of the spinal cord and tallied after correcting for double counts of split nucleoli (six mice/group) as described earlier (17,26,45). The morphologic identity of motor neurons was established using staining with antibodies to ChAT. Immunohistochemical staining was performed using monoclonal antibodies to SMN and polyclonal antibody to ChAT (Abcam) using standard methods (17).

Transmission electron microscopy

Tissues for TEM were washed with 0.5 M Na cacodylate–HCl buffer (pH 7.0) and fixed with 5 ml of 1.25% glutaraldehyde for 30 min at 30°C and overnight at 4°C with 5 ml of 2.5% glutaraldehyde in cacodylate buffer (13). The tissue was post-fixed (1 h) in 1% osmium tetraoxide (w/v) in 0.1 M phosphate buffer (pH 7.2). The fixed nerves were embedded in epoxy resin. Semi-thin sections for light microscopy were stained with toludine blue. Ultra-thin sections for transmission microscopy were mounted on copper support grids in serial order, contrasted with lead citrate and uranyl acetate, and examined by transmission electron microscope (17).

Mammalian cells and mouse spinal cord neuron culture

Fibroblasts from non-SMA human (WI-38), SMA type-I patients (GM03813, GM09677, GM0232A) and clinically unaffected parents of GM03813 (GM03814 and GM03815) were cultured and transfected with pCDNA3/FlagZPR1 (1 μg) using Lipofectamine 2000 (7) or infected with recombinant adenovirus Ad5CMVempty, Ad5CMV-GFP, Ad5CMV-FlagZPR1 expressing Flag (M2)-tagged ZPR1 and Ad5CMV FlagZPR1-GFP (Gene Transfer Vector Core, University of Iowa). Mouse spinal cord neurons were cultured using spinal cords from 7-day-old non-SMA (normal) [$Smn^{+/+}$; $SMN2^{+/+}$; $SMN\Delta7^{+/+}$] and SMA [$Smn^{-/-}$; $SMN2^{+/+}$; $SMN\Delta7^{+/+}$] littermates. Spinal cord explants (1.0 mm thick) were cultured *in vitro* for 5 days in eight-well chambers coated with poly-D-lysine/laminin in neurobasal medium with B-27, glutamine and penicillin/streptomycin. Primary neurons were infected with Ad5CMV-GFP, Ad5CMV-FlagZPR1 and Ad5CMV-FlagZPR1-GFP and harvested after 48 h. NSC-34 cells were transfected with pCDNA3/FlagZPR1. NSC-34 cells were incubated in differentiating medium [DMEM/F12 (1:1) with 1% FBS] after 12 h of transfection. NSC-34 cells were harvested at 12, 24, 36 and 48 h post-differentiation for immunofluorescence analysis (17).

Immunofluorescence analysis

Cells cultured on glass cover slips were fixed with methanol and acetone (13). Primary spinal cord neurons were fixed with 4% paraformaldehyde. The identity and morphology of the spinal cord neurons and glial cells were established by staining with specific markers, including ChAT (Abcam), tubulin (Covance) and GFAP and MAP2 (Cell Signaling). The recombinant FlagZPR1 was detected by monoclonal antibody to FLAG (M2) (Sigma). Growth cones of neurons were stained with phalloidin coupled with Alexa 546 (Molecular Probes). Double labeling (Flag-ZPR1/tubulin) was performed by sequential incubations (1 h) with (i) anti-β-tubulin III

(neuron specific), (clone TUJ1) and Alexa 546-conjugated anti-rabbit IgG secondary antibody and (ii) anti-FLAG (M2) antibody and FITC-conjugated anti-mouse IgG secondary antibody (7). Double labeling (ZPR1or FlagZPR1/SMN) was carried out using anti-SMN, Alexa 488-conjugated anti-mouse IgG secondary antibody and then with Alexa 546-conjugated anti-ZPR1 (clone LG1) or CY3-conjugated anti-FLAG (M2) antibody (Sigma) (7). Spinal cord neurons were double-labeled (ChAT and FlagZPR1; ChAT and tubulin; FlagZPR1 and tubulin) to identify the population of ChAT-positive neurons for quantitative analysis of neurite length. The triple labeling of primary spinal cord neurons infected with Ad5CMV-GFP or Ad5CMV-FlagZPR1-GFP was carried out sequentially using rabbit polyclonal antibody to ChAT and anti-rabbit IgG Alexa 546 and mouse monoclonal antibody to tubulin followed by anti-mouse IgG Alexa 633. The GFP and FlagZPR1-GFP were detected with 488 nm excitation laser. The cover slips were mounted on slides, using Vecta-shield with DAPI (Vector Lab), to stain DNA, and immuno-fluorescence was examined using confocal microscopy (Leica SP5-AOBS equipped with 405 nm UV laser).

Immunoprecipitation and immunoblot analysis

Cultured cells and mouse tissues (brain and spinal cord) were dissected and proteins were extracted using Triton lysis buffer (7). ZPR1 was immunoprecipitated using monoclonal anti-bodies against ZPR1 (clone C61). Proteins were separated by SDS–PAGE and electrotransferred to PVDF membrane (Millipore). Immunoblot analysis was performed using anti-bodies against ZPR1 (C61), SMN (Clone 8, Transduction lab), α-tubulin (Sigma) and β-actin (Sigma). HRP-conjugated donkey anti-mouse IgG (1:5000) secondary antibodies were used for detection by chemiluminescence and autoradiography. Quantitation of immunoblots was performed using UVP BioImager. The relative amounts of proteins (mean ± SD) normalized to either β-actin or tubulin are presented as bar graphs.

Statistical analysis

The quantitative data are presented as mean ± SD. Neurite length data are presented as mean ± SEM. Statistical significance (*P*-values) of data was calculated by ANOVA analysis using the online software available at http://www.danielsoper.com/statcalc/calc43.aspx. *P*-value ≤0.05 was considered significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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