Brief Communications

# Substrate Availability of Mutant SPT Alters Neuronal Branching and Growth Cone Dynamics in Dorsal Root Ganglia

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**Serine palmitoyltransferase (SPT) is a key enzyme in the first step of sphingolipid biosynthesis. Mutations in the *SPTLC1* gene that encodes for SPT subunits cause hereditary sensory neuropathy type 1. However, little is understood about how mutant SPT regulates mechanisms of sensory neuron and axonal growth. Using transgenic mice overexpressing the C133W SPT mutant, we found that mutant dorsal root ganglia (DRG) during growth *in vitro* exhibit increased neurite length and branching, coinciding with elevated expression of actin-cross-linking proteins at the neuronal growth cone, namely phosphorylated Ezrin/Radixin/Moesin. In addition, inhibition of SPT was able to reverse the mutant phenotype. Because mutant SPT preferentially uses L-alanine over its canonical substrate L-serine, we also investigated the effects of substrate availability on DRG neurons. Supplementation with L-serine or removal of L-alanine independently restored normal growth patterns in mutant SPTLC1$^{C133W}$ DRG. Therefore, we report that substrate availability and selectivity of SPT influence the regulation of neurite growth in DRG neurons.**

*Key words:* **dorsal root ganglia; Ezrin/Radixin/Moesin; L-alanine; L-serine; neurite development; sensory neuropathy**

**Significance Statement**

**Hereditary sensory neuropathy type 1 is an autosomal-dominant disorder that leads to a sensory neuropathy due to mutations in the serine palmitoyltransferase (SPT) enzyme. We investigated how mutant SPT and substrate levels regulate neurite growth. Because SPT is an important enzyme in the synthesis of sphingolipids, our data are of broader significance to other peripheral and metabolic disorders.**

## Introduction

Hereditary sensory neuropathy type 1 (HSN-1) is a disorder of the peripheral nervous system (PNS), leading to progressive axonal degeneration and sensory loss accompanied by symptoms of lancinating/shooting pain. The *SPTLC1* gene, which encodes for subunits of the enzyme serine palmitoyltransferase (SPT), is mutated in HSN-1 (Bejaoui et al., 2001).

Normally, SPT initiates the *de novo* synthesis of sphingolipids, specifically the condensation of palmitoyl-CoA with L-serine. In HSN-1, however, mutant SPT loses enzymatic selectivity and incorporates L-alanine as an alternative substrate (Gable et al., 2010). The enzymatic promiscuity of mutant SPT is suggested to be the cause of pathology (Eichler et al., 2009; Penno et al., 2010). Despite the known deleterious impact of mutant SPT in HSN-1, how it regulates the mechanisms of axonal growth in sensory neurons remains poorly understood.

Here, we isolated dorsal root ganglia (DRG) neurons of transgenic SPTLC1$^{C133W}$ mice, which overexpress the C133W SPT mutant (McCampbell et al., 2005). Neurite growth *in vitro* was assessed by analyzing length, branching, and the expression of p-ERM actin cross-linking proteins at the neuronal growth cone. In neurons, p-ERM is localized at neurites and growth cones, links the cytoskeleton to plasma membrane proteins, and is important for growth and axon guidance via modulation of the actin cytoskeleton during normal and regenerative growth (Gonzalez-Agosti and Solomon, 1996; Haas et al., 2004; Khan et al., 2013). We confirmed the effects of mutant SPT using myriocin, a potent SPT inhibitor (Wadsworth et al., 2013). Further, we manipulated the availability of SPT substrates to determine how they influence SPTLC1$^{C133W}$ DRG growth. Because previ-

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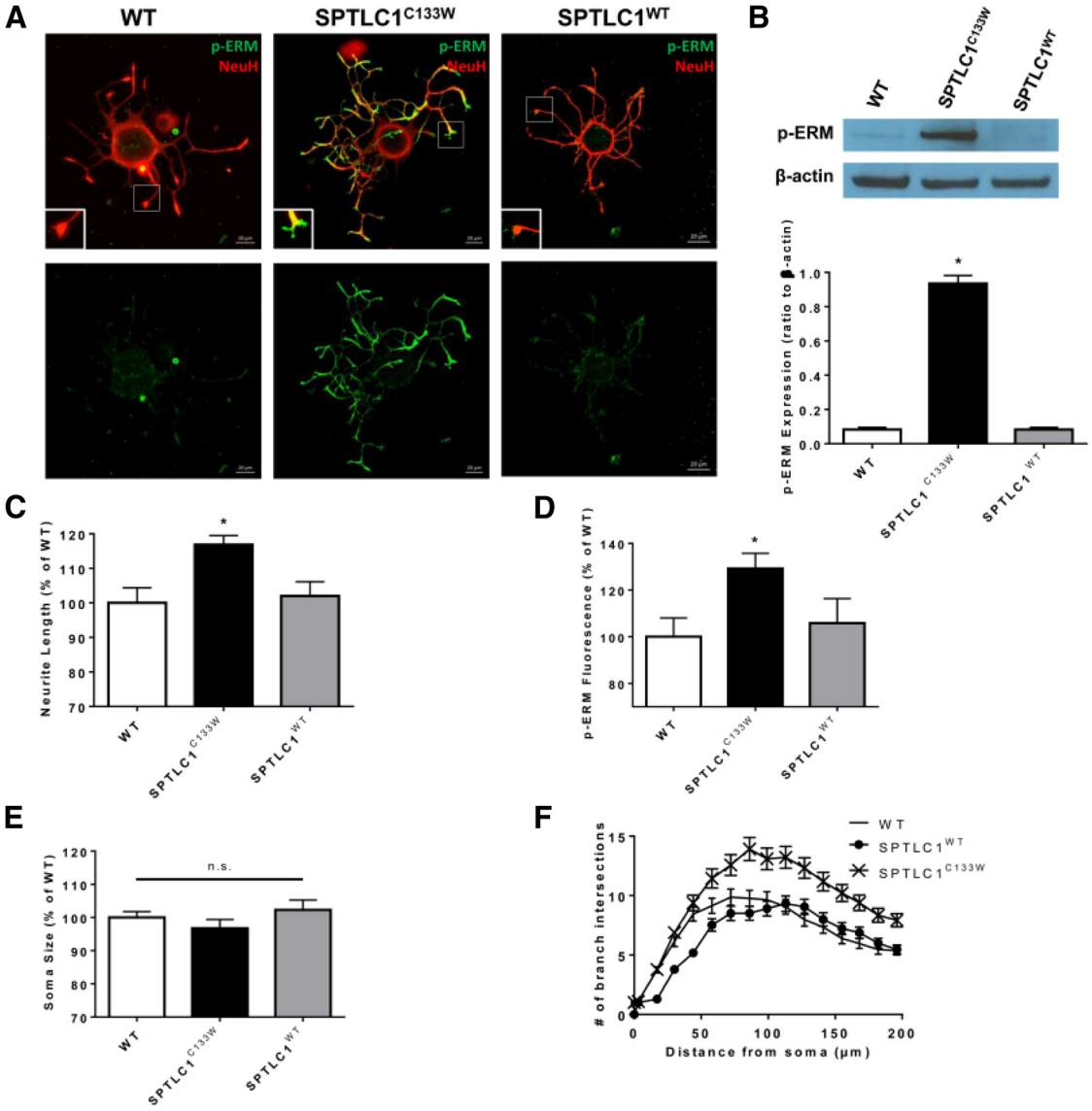


**Figure 1.** Characterization of the mutant SPTLC1 $^{C133W}$ DRG phenotype in *in vitro*. ***A***, DRG from WT, SPTLC1 $^{C133W}$, and SPTLC1 $^{WT}$ mice ($n = 3$) were stained with p-ERM and NeuH. Scale bar, 20 $\mu$m. ***B***, Western blot revealing an increase in p-ERM in SPTLC1 $^{C133W}$, but not in WT or SPTLC1 $^{WT}$, DRG. ***C***, SPTLC1 $^{C133W}$ DRG had significantly longer neurite length compared with WT and SPTLC1 $^{WT}$. ***D***, Expression of p-ERM at the neuronal growth cone is significantly elevated in SPTLC1 $^{C133W}$ DRG compared with WT and SPTLC1 $^{WT}$. ***E***, Soma size of DRG showed no significant difference across all groups. ***F***, Sholl analysis revealed increased neuronal branching in SPTLC1 $^{C133W}$ DRG compared with WT and SPTLC1 $^{WT}$.

ous *in vivo* studies found that varying L-serine or L-alanine levels can influence disease severity in HSN-1 (Garofalo et al., 2011), we examined their role in DRG growth *in vitro*.

## Materials and Methods

*Transgenic mice.* Generation of transgenic mice has been described previously (McCampbell et al., 2005). Overexpression of WT and C133W mutant copy of *SPTLC1* was driven by the chicken $\beta$-actin promoter. Mice were generated in the BL6/C57 background. SPTLC1 $^{C133W}$ mice were HSN-1 models and WT and SPTLC1 $^{WT}$ mice were controls.

*Neuronal culture.* Mice were anesthetized and killed at 6 months of age. Experiments were conducted with DRG from three male animals per genotype. DRG were extracted, digested with 0.05% trypsin (Life Technologies), and collagenase-IV/dispase (1 mg/ml and 0.25 mg/ml; Worthington Biochemical); resuspended in DMEM (Life Technologies) with 10% fetal bovine serum (Atlanta Biologicals) and DNase-I (Sigma-Aldrich); and triturated with heat-polished Pasteur pipettes. On chamber slides precoated with poly-D-lysine and laminin (Sigma-Aldrich), cells were plated in neurobasal or L-alanine-free medium (AFM) supplemented with 2% B27 (Life Technologies). Neurobasal medium is modified DMEM with optimized concentrations of components (Brewer et al., 1993). However, because neurobasal medium contains L-alanine, we developed AFM for use in our experiments. AFM was prepared using DMEM with optimized formulation of certain components (0.4 mM L-asparagine, 0.26 mM L-cysteine, 0.5 mM L-glutamate, 0.06 mM L-proline, $5 \times 10^{-6}$ mM vitamin B12, and 26.1 mM sodium bicarbonate).

*Amino acid supplementation.* After plating, cells were supplemented with L-serine or L-alanine at a final concentration of 10 mM (Sigma-Aldrich) and grown for 2 d *in vitro* (DIV).

*SPT inhibition.* After 1 DIV, cells were treated with myriocin (Santa Cruz Biotechnology) at a final concentration of 10 or 20 $\mu$M.

*Immunofluorescence.* Cells were fixed in 4% paraformaldehyde (Affymetrix). Primary antibodies were added in blocking solution containing 2% normal goat serum (Vector Labs) and 0.1% Triton X-100 (Sigma-Aldrich) overnight. Cells were washed and incubated with secondary antibodies for 1 h. Antibodies were against phosphorylated-ERM (rabbit anti-phospho-ezrin/radixin/moesin, 1:700; Cell Signaling Technology), neurofilament-heavy-chain (mouse-monoclonal SMI-32R,

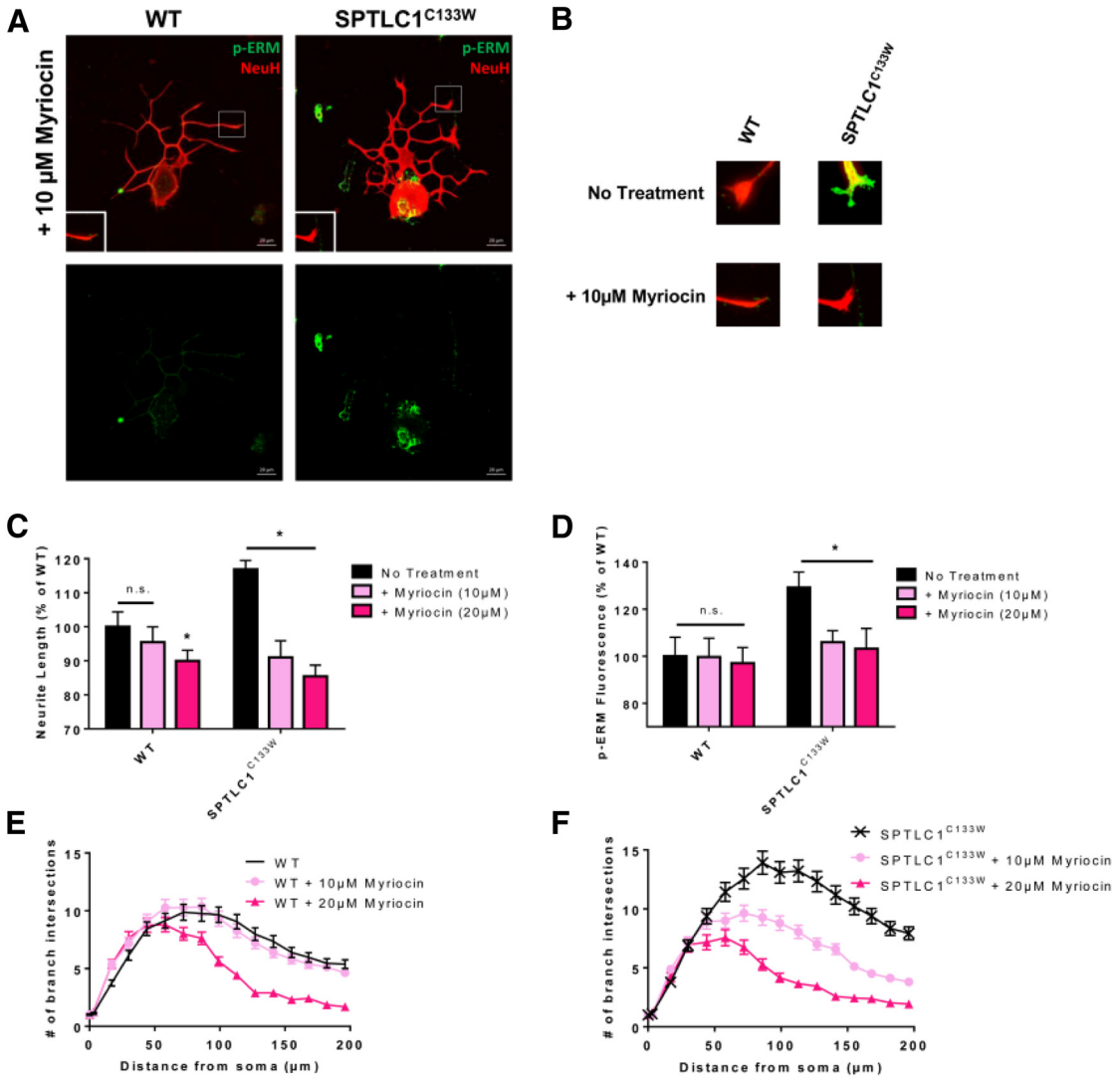


**Figure 2.** SPT inhibition via myriocin alleviates the mutant SPTLC1 $^{C133W}$ condition. ***A***, DRG from WT and SPTLC1 $^{C133W}$ mice treated with 10 $\mu$M myriocin ($n = 3$) were stained with p-ERM and NeuH. Scale bar, 20 $\mu$m. ***B***, Representative images of WT and SPTLC1 $^{C133W}$ DRG growth cones before and after treatment. ***C***, WT DRG exhibit significantly decreased neurite length when treated with 20 $\mu$M myriocin, but not at 10 $\mu$M. SPTLC1 $^{C133W}$ DRG show significantly decreased neurite length after 10 and 20 $\mu$M myriocin treatments. ***D***, There is no significant difference in p-ERM expression at the growth cones of WT DRG after treatment with 10 or 20 $\mu$M myriocin. In SPTLC1 $^{C133W}$ DRG, p-ERM expression is significantly reduced after 10 and 20 $\mu$M myriocin treatments. ***E***, Sholl analysis revealed no difference in branching of WT DRG after 10 $\mu$M myriocin, but a reduction at 20 $\mu$M. ***F***, Branching of SPTLC1 $^{C133W}$ DRG after 10 and 20 $\mu$M myriocin treatments is decreased in a dose-dependent manner.

1:700; Covance), goat anti-rabbit Alexa Fluor 488, and goat anti-mouse Alexa Fluor 555 (1:200; Life Technologies).

*Western blotting.* DRG were collected in RIPA buffer (Sigma-Aldrich) with Complete Protease Inhibitor Cocktail (Roche). Samples were separated on NuPAGE 4–12% Bis-tris gels (Invitrogen), transferred onto PVDF membranes, blocked with 5% milk in PBS containing 0.05% Tween 20, and probed with antibody against phosphorylated-ERM (1:1000) and $\beta$-actin (rabbit anti-$\beta$-actin, 1:5000; Santa Cruz Biotechnology). Membranes were developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) after incubation with HRP-conjugated secondary antibodies (Abcam).

*Microscopy and analysis.* Images were captured using Zeiss LSM510 confocal microscope and Zen 2009 software, keeping exposure/gain settings constant. Analyses were performed on ImageJ software by a blinded experimenter, with minimum 18 neurons analyzed per mouse. Using the NeuronJ plug-in, the longest neurite length of a DRG was measured, the average value calculated for the neurons of each mouse, and the mean neurite length derived from the three average values per genotype (subsequent mean values were determined similarly). Neurites were traced manually from the soma outward, excluding those $<10$ $\mu$m. For branching, the Sholl analysis plug-in was used to quantify branch intersections. Neurons with considerable overlap were excluded to avoid overestimation. The expression of p-ERM was measured as the mean fluorescence intensity (MFI) in neuronal growth cones. The MFI were background subtracted and averaged. Soma size was quantified by manually tracing the area around the neuron. All quantitative data were normalized to the WT control.

*Statistics.* Statistical analyses were performed on GraphPad Prism 6. Genotype comparisons among SPTLC1 $^{C133W}$, SPTLC1 $^{WT}$, and WT were analyzed via one-way ANOVA. Comparisons after treatment (myriocin, L-serine, L-alanine, and AFM) were analyzed via two-way ANOVA. Analyses were followed up by Bonferroni's *post hoc* tests. The criterion for significance was $^*p < 0.05$. Error bars are expressed as $\pm$ SEM.

## Results

### Mutant DRG exhibit increased neurite growth *in vitro*

After extraction from the spinal cord, DRG began to grow neuronal processes *in vitro*. Initial characterization revealed mor-

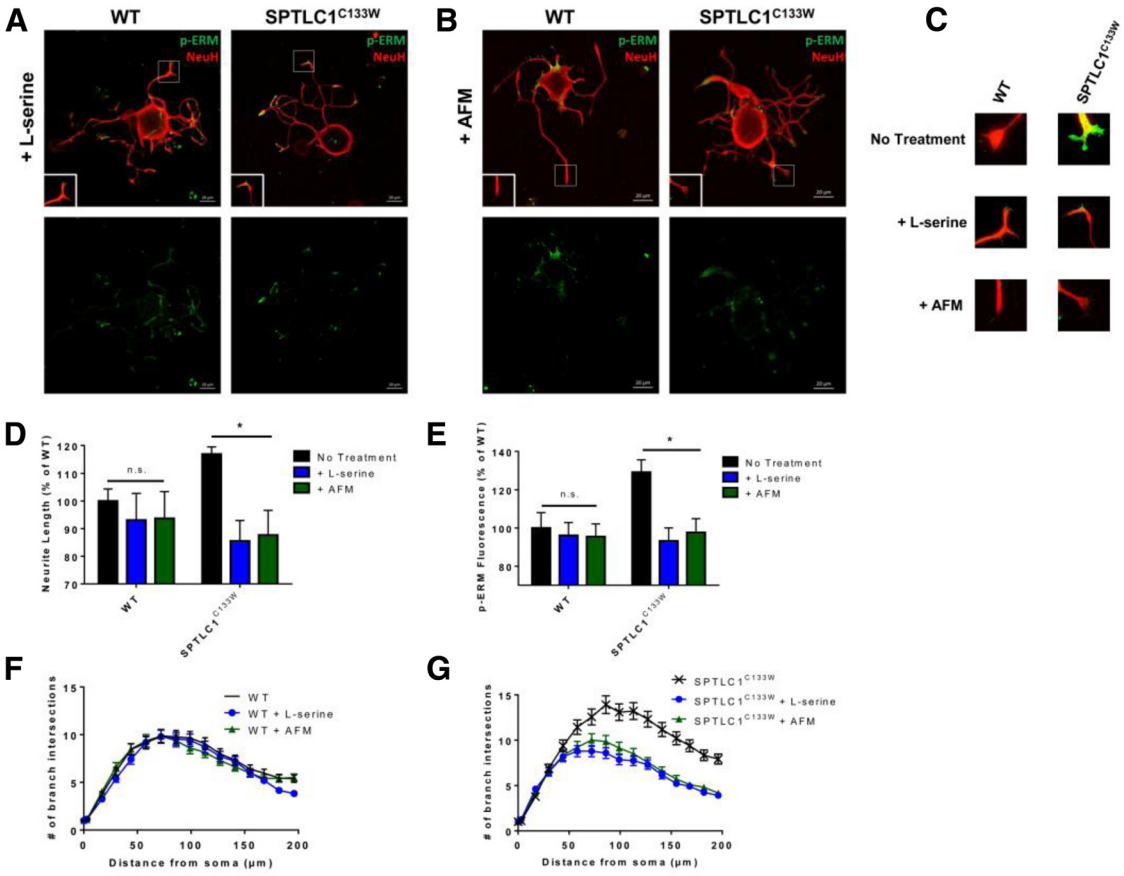


**Figure 3.** Supplementation with L-serine or removal of L-alanine rescues the mutant SPTLC1$^{C133W}$ condition. ***A***, ***B***, DRG from WT and SPTLC1$^{C133W}$ mice treated with 10 mM L-serine (***A***) or with AFM (***B***) ($n = 3$) were stained with p-ERM and NeuH. Scale bar, 20 $\mu$m. ***C***, Representative images of WT and SPTLC1$^{C133W}$ DRG growth cones before and after treatment. ***D***, SPTLC1$^{C133W}$ DRG treated with L-serine or AFM exhibit significantly decreased neurite length. WT DRG show no significant difference in neurite length after L-serine or AFM. ***E***, Expression of p-ERM is significantly decreased in SPTLC1$^{C133W}$ DRG growth cones after treatment with L-serine or AFM. There is no significant difference in p-ERM expression in WT DRG after L-serine or AFM. ***F***, Sholl analysis revealed no difference in branching of WT DRG after L-serine or AFM treatment. ***G***, Branching of SPTLC1$^{C133W}$ DRG after L-serine or AFM treatment is reduced to levels similar to WT DRG.

phology of mutant SPTLC1$^{C133W}$ DRG (Fig. 1*A*). To eliminate the possibility of the transgene overexpression having adverse effects, SPTLC1$^{WT}$ DRG were also used as controls. WT and SPTLC1$^{WT}$ DRG were not significantly different in any quantitative measures analyzed in this study. Compared with controls, SPTLC1$^{C133W}$ DRG exhibited a marked 29.2% increase ($^*p = 0.002$, $^*p = 0.004$) in p-ERM expression at neuronal growth cones, as detected by immunofluorescence and Western blotting (Fig. 1*B*,*D*). Mutant SPTLC1$^{C133W}$ DRG also exhibited a 16.9% increase ($^*p = 0.004$, $^*p = 0.011$) in neurite length and branching compared with controls (Fig. 1*C*,*F*), suggesting that the mutation is regulating axon elongation at neuronal growth cones. Quantification of soma size revealed no significant differences across all conditions (Fig. 1*E*) and we found no evidence of apoptosis (data not shown).

### SPT inhibition rescues the mutant phenotype

We confirmed whether the mutant SPTLC1$^{C133W}$ phenotype was linked to mutant enzymatic activity by using myriocin, a SPT inhibitor. WT and SPTLC1$^{C133W}$ DRG were treated with 10 or 20 $\mu$M myriocin (Fig. 2*A*). After treatment, we found that the expression of p-ERM at the neuronal growth cone was significantly decreased ($^*p = 0.003$, $^*p = 0.004$) in SPTLC1$^{C133W}$ DRG by 13.3% and 15.8% (Fig. 2*B*,*D*), which corresponded with 15.86% and 31.46% decreases in neurite length ($^*p = 0.003$, $^*p < 0.001$; Fig. 2*C*). However, myriocin had minimal effects on WT DRG, only reaching significance at the 20 $\mu$M concentration ($^*p = 0.021$), which decreased neurite length by 15.8% (Fig. 2*C*,*D*). In terms of branching, myriocin led to a dose-dependent decrease in SPTLC1$^{C133W}$ DRG, but only showed a noticeable effect at 20 $\mu$M in WT DRG (Fig 2*E*,*F*). Overall, the mutant SPTLC1$^{C133W}$ condition, but not the WT, responds strongly to SPT inhibition.

### Restorative effects of L-serine supplementation or L-alanine removal on mutant DRG

Supplementation with either L-serine or L-alanine has been shown to influence myelination, axon diameter, and sensory performance of HSN-1 mice (Garofalo et al., 2011). Specifically, excess L-serine ameliorates peripheral neuropathic symptoms, whereas excess L-alanine exacerbates those symptoms. Here, we found that the mutant condition was rescued when SPTLC1$^{C133W}$ DRG were treated with 10 mM L-serine (Fig. 3*A*). Not only did L-serine lead to a 31.36% decrease ($^*p = 0.001$) in neurite length and branching of mutant SPTLC1$^{C133W}$ DRG (Fig. 3*D*,*G*), it also reduced the expression of p-ERM by 36% ($^*p < 0.001$) to resemble the WT level (Fig. 3*A*,*C*). Conversely, L-serine had no significant effects on the WT neurite length or p-ERM expression ($p = 0.155$, $p = 0.351$; Fig. 3*A*,*D*–*F*).

Further, removal of L-alanine had similar restorative effects as L-serine supplementation on SPTLC1$^{C133W}$ DRG (Fig. 3*B*). When cultured in AFM, the mutant neurons exhibited a 29.2% decrease ($^*p = 0.012$) in neurite length and branching (Fig.

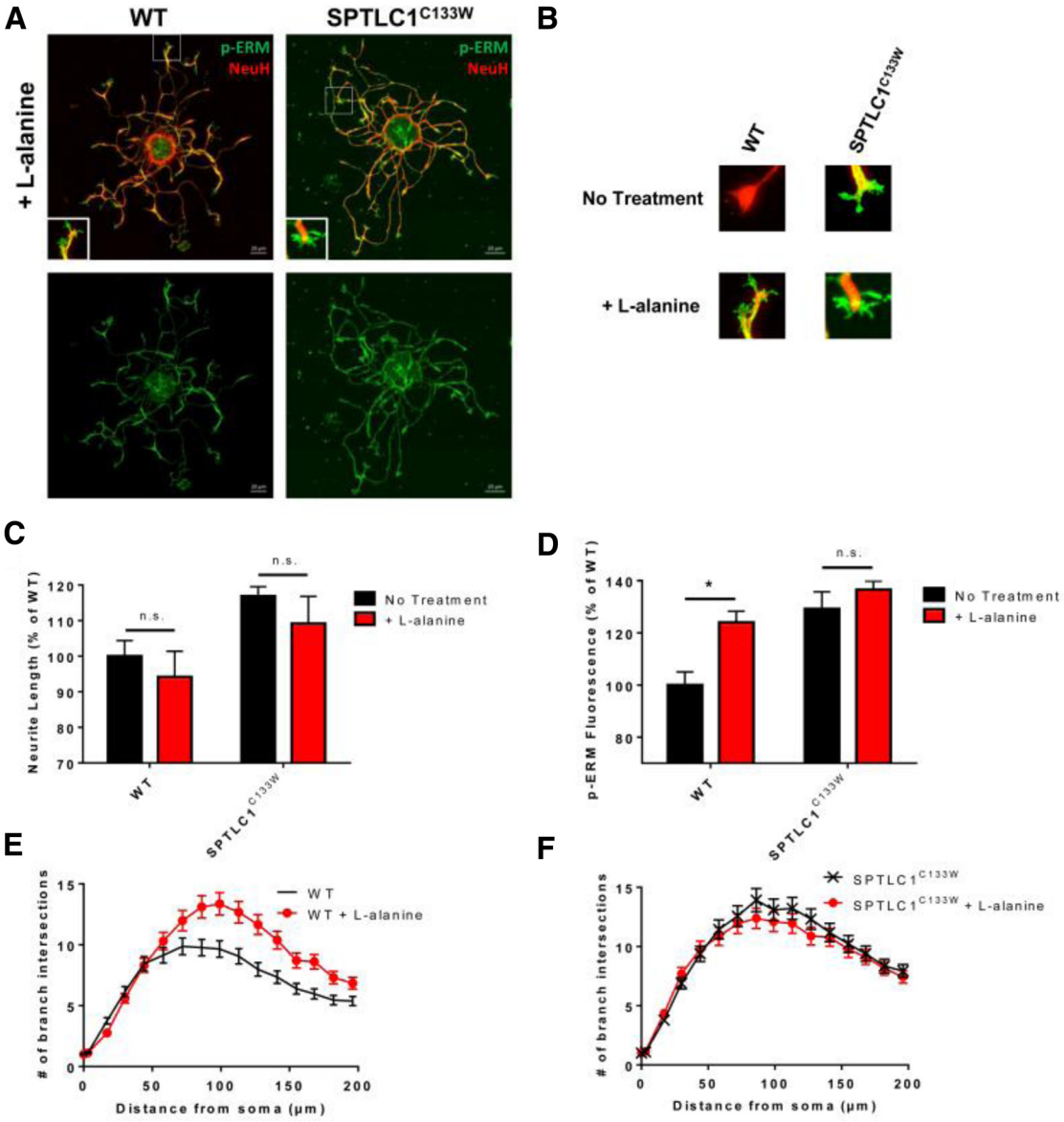


**Figure 4.** Supplementation with L-alanine increases WT DRG branching and p-ERM expression. ***A***, DRG from WT and SPTLC1 $^{C133W}$ mice treated with 10 mM L-alanine ($n = 3$) were stained with p-ERM and NeuH. Scale bar, 20 $\mu$m. ***B***, Representative images of WT and SPTLC1 $^{C133W}$ DRG growth cones before and after treatment. ***C***, Treatment with L-alanine has no significant effect on neurite length of WT or SPTLC1 $^{C133W}$ DRG. ***D***, Expression of p-ERM is significantly increased in WT DRG growth cones after L-alanine treatment. L-alanine treatment had no significant effect on p-ERM expression in SPTLC1 $^{C133W}$ DRG. ***E***, Sholl analysis revealed branching of WT DRG after L-alanine treatment is elevated to levels similar to mutant SPTLC1 $^{C133W}$ DRG. ***F***, Branching of SPTLC1 $^{C133W}$ DRG is not altered after L-alanine treatment.

3*D*,*G*) and a 31.58% reduction in p-ERM expression (*$p$ = 0.001; Fig. 3*C*,*E*). The absence of L-alanine had no significant effects on the WT condition ($p = 0.211$, $p = 0.304$; Fig. 3*B*,*D*–*F*).

### Excess L-alanine alters WT phenotype

Conversely, when treated with excess 10 mM L-alanine, mutant SPTLC1 $^{C133W}$ DRG showed no significant differences in neurite length ($p = 0.095$), branching, or p-ERM expression ($p = 0.131$; Fig. 4*A*,*C*,*D*,*F*). However, WT DRG treated with excess L-alanine in part resembled the mutant phenotype (Fig. 4*A*): branching was elevated and p-ERM expression was increased by 24% (*$p$ = 0.004) to resemble the mutant condition (Fig. 4*B*,*D*,*E*). Although we also expected an increase in WT neurite length, we found no significant difference ($p = 0.116$; Fig. 4*C*), suggesting that mechanisms of neuronal branching via growth cone proteins such as p-ERM is more susceptible to L-alanine availability than neurite outgrowth from the soma itself.

## Discussion

Mutations in genes encoding subunits of SPT are one of the most common causes of hereditary sensory neuropathies (Dawkins et al., 2001; Rotthier et al., 2011, 2012). Although the progressive axonopathy and accumulation of atypical sphingolipid metabolites in HSN-1 suggests putative neurotoxicity (Penno et al., 2010), it remains poorly understood how the altered activity of mutant SPT leads to selective damage of the PNS.

Our current *in vitro* study provides evidence of increased neuronal growth and branching after extraction of DRG from HSN-1 mice and its dependence upon SPT substrate availability. We observed corresponding increases of p-ERM expression in the

neuronal growth cones of SPTLC1$^{C133W}$ DRG, implicating these actin-cross-linking proteins as regulators of neurite growth. The increased neurite growth could be a result of elevated p-ERM in mutant DRG, which can aid axon elongation via attractive growth cone guidance or modulation of adhesion to the plasma membrane (Marsick et al., 2012). Further, the upregulation of growth cone proteins such as ERM has been linked to neuroregeneration after injury (Haas et al., 2004). However, the current *in vitro* model of neurite growth does not include a preconditioned *in vivo* injury, suggesting that the mutant morphology is indicative of compensatory rather than regenerative growth. Because progression of HSN-1 pathology is often exacerbated by peripheral injury in patients, a detailed investigation of the injury response in HSN-1 and how it affects the regulation of p-ERM and other relevant growth cone proteins may help to elucidate their role in the PNS.

Although it has been suggested that sphingolipid metabolites (Canals et al., 2010; Gandy et al., 2013) and various kinases (Ramesh 2004) can regulate p-ERM activation, how mutant SPT in HSN-1 leads to ERM phosphorylation is not known. We previously reported increased levels of TNF-$\alpha$ in SPTLC1$^{C133W}$ DRG and sciatic nerves (Eichler et al., 2009). This proinflammatory cytokine can act downstream via PKC or p38 MAPK to phosphorylate ERM proteins (Koss et al., 2006). Further, it is conceivable that canonical sphingolipid levels may be disrupted in HSN-1 due to altered activity of the mutant SPT enzyme. Although existing data on canonical sphingolipid levels in HSN-1 remain inconclusive (Dawkins et al., 2001; Dedov et al., 2004), the accumulation of neurotoxic deoxysphingolipids is well described (Eichler et al., 2009; Rotthier et al., 2011). Previous studies have reported that exogenous treatment of at least 1 $\mu$M deoxysphingolipids caused neurite loss and apoptosis (Cuadros et al., 2000; Penno et al., 2010). Endogenous levels are lower, however, ranging from 100 to 500 nM in plasma (Penno et al., 2010; Rotthier et al., 2011). We suggest that these metabolites may affect neuronal growth and viability indirectly via regulation of kinase activity (Hannun and Bell, 1987; Sánchez et al., 2008; Fyrst and Saba, 2010), alterations in sphingolipid metabolism (Dawkins et al., 2001; Zitomer et al., 2009), or disruption of cellular membranes (Jiménez-Rojo et al., 2014). A closer examination into how mutant SPT regulates these pathways and alters ERM activation will be important to help understand the molecular mechanisms of disease progression in HSN-1.

Because mutant SPT preferentially incorporates L-alanine over L-serine (Gable et al., 2010; Penno et al., 2010), we manipulated their availability *in vitro*. The beneficial effects of L-serine upon behavior and nerve pathology in HSN-1 mice have been described previously (Garofalo et al., 2011). We demonstrate rescue of the SPTLC1$^{C133W}$ condition, not only after L-serine supplementation, but also after removal of L-alanine, presumably due to restoration of canonical SPT activity. Despite its increased affinity for L-alanine, mutant SPT retains residual affinity for L-serine ($K_m$ = ~1.4 mM and $V_{max}$ = ~275 pmol/mg/min vs wild-type $K_m$ = ~0.75 mM and $V_{max}$ = ~1350 pmol/mg/min; Gable et al., 2010), with the result that mutant SPT can still incorporate L-serine, especially in the case of excess L-serine or lack of L-alanine.

Conversely, L-alanine supplementation elevated branching and p-ERM expression in WT DRG to resemble the mutant condition, which is not surprising because L-alanine exacerbates peripheral neuropathic symptoms in HSN-1 mice (Garofalo et al., 2011). However, because both L-serine and L-alanine are gluconeogenic amino acids involved in pathways regulating oxidative stress and neurotransmission (Rowsell et al., 1969; de Koning et al., 2003; Grosser et al., 2004; Fuchs et al., 2006), we cannot categorically rule out that the observed effects on DRG were due to their metabolic and neurotrophic properties.

Indeed, L-serine is crucial for lipid synthesis, neuronal survival, and function in the CNS (de Koning and Klomp, 2004; Hirabayashi and Furuya, 2008). In the absence of L-serine, neurons from the CNS do not grow as robustly as those treated with L-serine *in vitro*, because glia are the main suppliers of L-serine to neurons *in vivo* (Savoca et al., 1995; Furuya et al., 2000). After neuronal insult, the PNS receives glial support needed for regeneration (Scheib and Höke, 2013). This trophic activity is important for axons, especially those in the metabolically isolated PNS (Nave, 2010). Glia in the PNS tend to ensheath axons in a much lower ratio (1:1) than in the CNS (up to 60:1), emphasizing the importance of the neuron–glia interaction in peripheral nerves (Nave and Werner, 2014). Therefore, the distinct environment of the PNS may render peripheral axons susceptible to certain metabolic fluctuations. Our current *in vitro* data show contrasting effects of L-serine and L-alanine upon the mutant DRG phenotype, indicating a selective vulnerability to these amino acids, as suggested by studies of HSN-1 mice (Garofalo et al., 2011). Manipulating the L-serine/L-alanine ratio in HSN-1 may be of broader relevance to other peripheral nerve disorders and sphingolipidoses.

Here, we describe aberrant morphology of SPTLC1$^{C133W}$ DRG characterized by increased neurite growth, branching, and expression of p-ERM at neuronal growth cones. This phenotype indicates that neurite growth is being altered by the SPTLC1$^{C133W}$ mutation and is suggestive of compensatory growth *in vitro*. Because the eventual course of HSN-1 is distal axonal degeneration, it will be important to understand how the mutation *in vivo* leads to a length-dependent axonopathy. Further, because SPT inhibition and treatment with the canonical SPT substrate L-serine was able to rescue the mutant phenotype of elevated p-ERM, a detailed examination into how the mutation regulates the expression of important growth cone proteins will help ro identify therapeutic targets for HSN-1.

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