N-WASp is required for Schwann cell cytoskeletal dynamics, normal myelin gene expression and peripheral nerve myelination

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SUMMARY
Schwann cells elaborate myelin sheaths around axons by spirally wrapping and compacting their plasma membranes. Although actin remodeling plays a crucial role in this process, the effectors that modulate the Schwann cell cytoskeleton are poorly defined. Here, we show that the actin cytoskeletal regulator, neural Wiskott-Aldrich syndrome protein (N-WASp), is upregulated in myelinating Schwann cells coincident with myelin elaboration. When N-WASp is conditionally deleted in Schwann cells at the onset of myelination, the cells continue to ensheath axons but fail to extend processes circumferentially to elaborate myelin. Myelin-related gene expression is also severely reduced in the N-WASp-deficient cells and in vitro process and lamellipodia formation are disrupted. Although affected mice demonstrate obvious motor deficits these do not appear to progress, the mutant animals achieving normal body weights and living to advanced age. Our observations demonstrate that N-WASp plays an essential role in Schwann cell maturation and myelin formation.

KEY WORDS: Myelination, Schwann cells, N-WASp (Wasl), Mouse

INTRODUCTION
Axons ensheathed with myelin propagate action potentials in a saltatory manner resulting in significantly accelerated conduction velocity and reduced energy requirements. In the peripheral nervous system (PNS), myelin is elaborated by Schwann cells. Their precursors emerge from the neural crest early in development and distribute amongst elongating axons, where they continue to proliferate as well as extend and retract radially oriented processes between axons, ultimately ensheathing all large caliber axons in a process referred to as radial sorting (Webster et al., 1973; Grove et al., 2006). As development proceeds they form discrete Schwann cell families that encircle bundles of axons, where they continue to proliferate as well as extend and retract radially oriented processes between axons, ultimately ensheathing all large caliber axons in a process referred to as radial sorting (Webster et al., 1973; Grove et al., 2007; Woodhoo et al., 2009) (for a review, see Woodhoo and Sommer, 2008).

To initiate axon ensheathment, a Schwann cell develops a longitudinal groove that deepens as it extends processes circumferentially to fully enwrap the axon (Webster, 1971; Webster et al., 1973; Webster, 1984). Through mechanisms that remain poorly understood (Bunge et al., 1989), the Schwann cell membrane spirally wraps around the axon, following which, interlamellar Schwann cell cytoplasm is extruded and the apposing membrane layers are stabilized by myelin-associated proteins (Wood et al., 1990). In addition to their seemingly lamellipodia-like mesaxons, Schwann cells generate microvilli at the electrophysiologically specialized nodes of Ranvier that are located between successive myelin sheaths (Gatto et al., 2003).

The migratory, membrane-extending and microvilli-generating capacities of Schwann cells suggest an integral role for cytoskeletal remodeling in Schwann cell maturation. This possibility is supported by the growth cone-like properties displayed by the terminal processes of migrating Schwann cell precursors in vivo (Wanner et al., 2006) and by both the structure and composition of the mesaxons that are elaborated by myelinating Schwann cells in vitro (Bacon et al., 2007). Also, in Schwann cell-neuron co-cultures, myelin formation is attenuated when actin polymerization is disrupted by cytochalasin D (Fernandez-Valle et al., 1997). Similarly, in mice, myelin formation is attenuated when the Cdc42 or Rac1 GTPase cytoskeletal regulators are deleted (Nodari et al., 2007; Benninger et al., 2007). Additionally, Rho GTPase and its kinase, ROCK, have been implicated in Schwann cell- and/or oligodendrocyte-mediated myelination (Melendez-Vasquez et al., 2004) (for a review, see Feltrin et al., 2008).

Although a link between cytoskeletal dynamics and myelination is widely appreciated, the specific effectors that modulate cytoskeletal reorganization in developing and myelinating Schwann cells are not well defined. However, one likely effector is the neuronal Wiskott-Aldrich syndrome protein [N-WASp; also known

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as Wiskott-Aldrich syndrome-like (Wasl) – Mouse Genome Informatics], a member of the WASp family of cytoskeletal regulators. Like other family members, N-WASp links extracellular stimuli and actin polymerization (Wegner et al., 2008) via the binding of its verprolin homology/connecting/acid region domain to the Arp2/3 complex. In cultured Schwann cells, N-WASp localizes to the leading edge of extending processes where its activity depends upon interaction with Cdc42, a protein with essential roles in Schwann cell development (Benninger et al., 2007; Bacon et al., 2007). Further, the myelinating capacity of cultured rat Schwann cells is impaired by an inhibitor of N-WASp activity (Bacon et al., 2007), as is oligodendrocyte-mediated myelination in the central nervous system (CNS) when another WASp family member, Wave1, is deleted in vivo (Kim et al., 2006). Additionally, process remodelling in mature oligodendrocytes involves a complex including FAK, Fyn, the intracellular domain of Dcc and N-WASp (Rajasekharan et al., 2009).

Here, to investigate the in vivo role of N-WASp during myelination, we used conditional gene targeting to generate mice in which N-WASp (Wasl) is deleted in Schwann cells as they initiate myelin elaboration. N-WASp-deficient Schwann cells achieve a normal 1:1 relationship with large caliber axons, albeit on a delayed schedule. However, their capacity to elaborate myelin sheaths appears to be arrested completely and their expression of myelin-related genes is severely reduced. Additionally, the production of lamellipodia-like processes in vitro is markedly impaired. Although these mutant mice manifest motor deficits characterized by tremor and mild ataxia, they are viable into advanced age. These observations demonstrate that N-WASp is required for normal Schwann cell maturation and that it plays an essential role in myelin formation, potentially by regulating the cytoskeletal remodelling that is required for the spiral extension of Schwann cell processes around axons.

**MATERIALS AND METHODS**

**Conditional knockout and transgenic mice**

N-WASp floxed (N-WASpΔ/Δ), mPoTOTA-Cre (P0-Cre) and Z/EGER reporter mice have been reported by Lommel et al. (Lommel et al., 2001), Feltri et al. (Feltri et al., 1999a; Feltri et al., 1999b) and Novak et al. (Novak et al., 2000), respectively. C57BL/6 mice heterozygous for the N-WASp floxed allele were crossed with C57BL/6/P0-Cre mice to derive N-WASpΔ/Δ; P0-Cre. These were crossed with N-WASpΔ/Δ; P0-Cre mice to generate N-WASpΔ/Δ; P0-Cre (N-WASpΔ/Δ mutant) mice. Z/EGER homozygous mice were mated to P0-Cre mice to derive Z/EGER/P0-Cre mice for assessing P0-Cre activity. Genotypes were determined by PCR analysis of tail and/or sciatic nerve genomic DNA using primers that yield amplicons of 200 bp (wild-type), 306 bp (floxed) or 408 bp (recombined) from the N-WASp allele.

**Immunofluorescence analyses and primary cultures**

Sciatic nerve sections were fixed in 4% paraformaldehyde (PFA), blocked with 1 hour for 10% goat serum/0.1% Triton X-100 in PBS and incubated overnight at 4°C with primary antibodies. After washing in PBS, tissue sections were incubated for 1 hour with secondary antibody. Alternatively, Schwann cells were isolated from P4 sciatic nerves following nerve digestion with 1 mg/ml collagenase/dispase and 2.5% trypsin and Thy1.2 antibody/complement-mediated fibroblast lysis (Honkanen et al., 2007) and maintained in culture in DMEM supplemented with 10% fetal calf serum (FCS)/antibiotics. Dorsal root ganglia (DRG) were dissected as previously described from E13.5 wild-type mice (Päivälahtinen et al., 2008) and cultured for 2 days in neural basal medium containing 10% FCS followed by supplementation with 10^{-5} M uridine/5'-fluoro-2'-deoxyuridine (Sigma). Schwann cells and DRG were then co-cultured in myelination-promoting media containing 50 μg/ml ascorbic acid (Eldridge et al., 1987). For staining, Schwann cells or Schwann cell/DRG co-cultures were fixed in 4% PFA for 10 minutes at room temperature, the cells then permeabilized with 0.2% Triton X-100 for 5 minutes at room temperature and incubated with primary antibody in blocking buffer (1% BSA in PBS) overnight at 4°C, followed by incubation with secondary antibodies in blocking buffer for 1 hour at room temperature. Staining was visualized using an Olympus Fluoview 1000 inverted confocal microscope equipped with fluorescence optics and Olympus Fluoview FV1000 software.

**Immunoblotting analysis**

Sciatic nerves were homogenized with a chilled mortar and pestle in RIPA buffer containing protease inhibitors (Roche). The lysate was suspended in loading buffer, electrophoresed through a 10% polyacrylamide gel and transferred to nitrocellulose. After overnight blocking, as previously described (Badour et al., 2004), membranes were incubated for 1 hour with primary antibody followed by incubation for 1 hour with a 1/3000 dilution of horseradish peroxidase-conjugated secondary antibody (Bio-Rad), then ECL substrate (Amersham Biosciences) for 1 minute, followed by a 1- to 10-minute exposure to X-ray film (Eastman Kodak).

**Morphological analysis**

Sciatic nerves or spinal roots were immersion-fixed in either 4% PFA or by trans-cardiac perfusion with 2.5% glutaraldehyde/0.5% PFA in 0.1 M phosphate buffer saline (pH 7.4) and subjected to alcohol dehydration and Epon embedding. Alternatively, specimens were fixed for 2 hours in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, dehydrated and embedded in Spurr’s resin (Electron Microscopy Sciences). Semi-thin sections were stained with Toluidine Blue or Methylyene Blue and examined by light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate and examined by electron microscopy (JEOL JEM2010 electron microscope).

**Proliferation and TUNEL assays**

Mice were injected intraperitoneally with BrdU (300 mg/kg body weight) and sacrificed 24 hours later. The labeled sciatic nerves were then fixed with 4% PFA, embedded in paraffin and 5 μm sections stained with BrdU antibody. Apoptotic cell death was measured in transverse sciatic nerve sections by TUNEL labeling using calf thymus TdT, biotin-labeled UTP and horseradish peroxidase-streptavidin (Boehringer). Counterstaining was performed with Haematoxylin. Sections were dehydrated and coverslipped and the percentage of TUNEL-positive cells in a sample of 500 was determined.

**Gene expression assays**

Total RNA (200 ng) prepared from sciatic nerve samples using the Qiagen RNasy Kit was reverse transcribed with Superscript II (Invitrogen) according to the manufacturer’s protocol. SYBR Green-based qRT-PCR was performed using the ABI 7900HT system (Applied Biosystems). Primers were: Mag forward 5’- GTGGAGCTGAGTGTCATGTATG-3’ and 3’- AAATCAGCTACATTAGATATC-3’; 5’-ACTCTGTGCTGATCAGGCGGG-3’ and 5’-TGACAGAGAGAATGAATG-3’.
N-WASp is expressed in many regions of the developing and mature brain and also in cultured neurons, oligodendrocytes and Schwann cells (Tsuchiya et al., 2006; Bacon et al., 2007). To determine when N-WASp might participate in Schwann cell development in vivo, we first evaluated the developmental programming of N-WASp accumulation in the PNS of postnatal mice. N-WASp was readily detectable by immunocytochemistry in sciatic nerves at postnatal day (P) 28, a time when myelin sheaths are approaching a mature state. As demonstrated by its co-localization with S100B, the N-WASp signal was localized largely to a cytoplasmic domain adjacent to the external Schwann cell membrane. Intensely labeled profiles frequently contained Schwann cell nuclei with the N-WASp signal extending from the perinuclear cytoplasm to define much, but not all, of the fiber circumference. Similarly intense N-WASp labeling was not observed in either the Schwann cell cytoplasm adjacent to the axon nor in nuclei (Fig. 1A).

To determine when N-WASp expression initiates, we investigated sciatic nerve samples obtained from mice at earlier stages of postnatal development (Bray et al., 1977). As early as P1, coinciding with the initiation of PNS myelin elaboration, N-WASp was readily detectable by immunofluorescence imaging (Fig. 1B). A broad ring of N-WASp staining partially overlapping that of myelin basic protein (Mbp) and extending to the cell periphery was the most characteristic labeling pattern observed at this age (see Fig. S1 in the supplementary material). Staining intensity appeared...
to increase to peak levels near P5 and moderated by P13, at which
time a distribution pattern characterized by thinner and incomplete
peripheral rings, typical of more mature fibers, was common (Fig.
1B). Quantitative real-time PCR (qRT-PCR) assays revealed that
N-WASp and Mbp mRNA accumulated on similar programs and
indicated that N-WASp expression was coincident with, or
preceded, the initiation of myelination (Fig. 1C).

**Mice with N-WASp-deficient Schwann cells**

To explore the role of N-WASp in Schwann cell-mediated
myelination, we derived mice in which N-WASp was specifically
inactivated in premylinating Schwann cells. Mice homozygous for
an N-WASp floxed allele (N-WASp<sup>fl<sup>ex</sup>fl<sup>ex</sup>) were bred with N-WASp<sup>cre<sup>cre</sup> mice carrying a P0-Cre transgene (Feltri et al., 1999a; Lommel et
al., 2001). As the P0 (Mpz – Genome Mouse Informatics) sequence
drives expression in Schwann cells during the perinatal period
(Feltri et al., 1999a; Feltri et al., 1999b), we expected accumulated
Cre to delete floxed N-WASp at the beginning of PNS myelination.
In sciatic nerve samples, both a Cre-sensitive GFP reporter and Cre
expression followed the expected developmental program, both
being detectable at P1, increasing through P5 and stabilizing
thereafter (see Fig. S2 in the supplementary material). Evaluation
of Cre expression revealed ~65% of all DAPI-labeled nuclei to be
Cre-positive at P1, increasing to 99% by P28, thus suggesting that
most Schwann cells express effective levels of Cre.

Crosses involving floxed N-WASp and P0-Cre alleles resulted in
the predicted Mendelian ratios of wild-type, floxed, heterozygous
and homozygous mutant (N-WASp<sup>fl<sup>ex</sup>fl<sup>ex</sup>/P0-Cre, referred to as
N-WASp<sup>−/−</sup> offspring. The deleted N-WASp allele was detected only in Schwann cells from mice bearing the P0-
Cre transgene and the floxed N-WASp allele (see Fig. S3A in the
supplementary material). Consistent with widespread
inactivation of the N-WASp gene in Schwann cells, N-WASp
protein levels were profoundly reduced in mutant sciatic nerves
(see Fig. S3B in the supplementary material). Moreover, in
immunolabeled sciatic nerve samples, the perineurium labeled
intensely, whereas N-WASp was undetectable in all but rare
Schwann cells (see Fig. S3C in the supplementary material).

**Motor impairment in N-WASp<sup>−/−</sup> mice**

N-WASp<sup>−/−</sup> mice appeared normal at birth but, by P14, showed
locomotor anomalies including a wide-based ataxic gait with
extraneous steps and impaired rotarod endurance (Fig. 1D,E and
and see Movies 1 and 2 in the supplementary material). Mutants also
manifested an abnormally wide hindlimb stance while sitting and
low frequency (10-20 Hz) tremors (Fig. 1D,F). Interestingly, these
mice achieved normal body weights and their behavioral
phenotypes progressed minimally over an 8-month follow-up
period.

**N-WASp-null Schwann cells fail to elaborate myelin**

As shown in Fig. 2, all large caliber (>2 μm) axons coursing
through mutant sciatic nerves (Fig. 2A,B) and ventral roots (Fig.
2C) were ensheathed. However, most fibers were amylinated
(>99% in sciatic nerve; see Table S1 in the supplementary material)
and, as seen in many demyelinating or amylinating conditions,
axons without normal myelin sheaths failed to undergo normal
radial growth (see Table S1 in the supplementary material).
Exceptional myelinated fibers were encountered in the mutants
(<1%; see Table S1 in the supplementary material) and, consistent
with the enhanced radial growth of myelinated axons, such fibers
appeared as prominent components in nerve transverse sections
(Fig. 2C). Such myelin sheaths appeared qualitatively normal and
revealed normal interlamellar spacing (13.17±0.48, versus 13.41±0.46 nm in controls) suggesting that these rare Schwann
cells might have escaped N-WASp inactivation. Nonetheless,
relative to the calibers of their associated axones, these sheaths were of
modestly reduced thickness, achieving g-ratios of 0.79±0.06, versus
0.67±0.06 in controls (mean of 31 myelinated fibers).

Finally, regional heterogeneity may exist in the frequency at which
myelin sheaths arise in this model as they were encountered in
most spinal roots but were virtually absent in sciatic nerves (Fig.
2A,B). Interestingly, enlarged blood vessels were also prominent
in mutant spinal roots (Fig. 2C).

At birth, many axons in normal sciatic nerves remain closely
packed into bundles surrounded by Schwann cell families. Through
a complex program involving process extension/retraction and cell
division, Schwann cells segregate such bundles to arrive at a 1:1
relationship with large caliber axons. This radial sorting was
successfully achieved in sciatic nerves of mature N-WASp<sup>−/−</sup>
animals. However, in mutant nerves, axon bundles were still
prominent in the sciatic nerves of P5 mice, whereas in control mice
at this age radial sorting was largely complete (Fig. 2A,B; see Fig.
S4A and Table S1 in the supplementary material). Thus, the mutant
Schwann cells remained capable of completing the radial sorting
process, albeit over an extended period.

Mutant fibers that failed to elaborate myelin sheaths demonstrated numerous ultrastructural features shared with
promyelinating fibers in normal perinatal mice (Webster, 1971).
Processes enwrapping axons often comprised inner- and outer-facing Schwann cell membrane with little cytoplasm between.
Where their processes confronted each other, no evidence for
continued circumferential movements was apparent. Instead, such
mesaxon contacts remained in apposition (Fig. 2E,F) or developed
multiple short interdigitations (Fig. 2G). Also common were one
or more Schwann cell processes extending away from the axon into
interfiber space (Fig. 2E,F). All outward-oriented Schwann cell
membrane was associated with a continuous and non-redundant
basal lamina and collagen fibrils were deposited densely between
the amylinated fibers. In Remak fibers, where Schwann cells
ensheath multiple small diameter C-fibers, but elaborate no myelin,
 qualitative anomalies were not obvious (see Fig. S4B and Table S1
in the supplementary material) and neither the number nor caliber
of the ensheathed axons was remarkable (see Table S1 in the
supplementary material).

With the exception of Schwann cells, N-WASp was expressed
widely in mutant mice bearing both the P0-Cre transgene and
floxed N-WASp alleles (data not shown). Therefore, the
amyelination observed in the PNS of N-WASp<sup>−/−</sup> mutants was likely
to be the result of a Schwann-cell-autonomous defect. To test this
conclusively, we used mixed cultures in which mutant or wild-type
Schwann cells were assessed for their capacity to myelinate axons
extending from wild-type dorsal root ganglia neurons. Consistent
with a Schwann-cell-autonomous defect, myelination, as assessed
by Mb immunoreactivity, was detected only in cultures containing
wild-type Schwann cells (Fig. 2H).

**N-WASp deficiency alters Schwann cell numbers and proliferation**

We next compared Schwann cell numbers (Brown and Asbury,
1981), proliferation and apoptosis in mutant versus control-type
nerves. Cell numbers were equivalent at P1 (Fig. 3A,B), but by P7
and at all later ages examined the mutant nerves contained
significantly more cells and these demonstrated greater proliferation as revealed by Ki67 labeling and bromodeoxyuridine (BrdU) incorporation (Fig. 3B,C). By contrast, TUNEL assays revealed no differences in the percentages of TUNEL-positive cells in mutant compared with control (6.58±0.54% versus 6.64±0.83%, \( P = 0.81 \)) nerves (data not shown). As the extent of caspase 3 staining was also similar in mutant and control samples (data not shown), the enhanced Schwann cell proliferation observed in mutant nerves does not appear to be in response to increased Schwann cell death. Rather, their increased proliferation is consistent with the limited capacity of non-myelin-forming, as compared with myelin-forming, Schwann cells to extend for long distances along their associated axons. Thus, relatively more non-myelin-forming Schwann cells are required to ensheathe a comparable length of axon.

**N-WASp modulates myelin-related gene expression**

Genes encoding a group of myelin proteins are coordinately upregulated as Schwann cells initiate myelination. To determine whether the lack of N-WASp modulates this expression program, levels of three such myelin proteins were assessed in P5 sciatic nerve samples. In the hypercellular mutant samples, both immunoblotting and qRT-PCR analyses revealed reduced accumulation of Mag and P0 (Fig. 4A,B), whereas only trace amounts of transcript and negligible protein were apparent for the later-accumulating Mbp (Fig. 4A-C). Notably, these findings cannot be explained by a generalized transcription defect, as expression of Oct6 (Pou3f1 – Mouse Genome Informatics), Krox20 (Egr2 – Mouse Genome Informatics) and Sox10, which are all promyelinating transcription factors with key roles in Schwann cell differentiation and myelin gene activation (Svaren and Meijer, 2008; Ghislain and Charnay, 2006), was increased in the mutant compared with control samples (Fig. 4D).

**N-WASp modulates lamellipodia formation**

Cultured N-WASp-deficient Schwann cells were assessed for their capacity to form the actin-based processes that are thought necessary for spiral membrane wrapping and myelin formation (Bunge et al., 1989; Feltiri et al., 2002). After 16 hours in culture, similar numbers of processes extended from the main cell axis of both mutant and wild-type cells, but those extending from the mutant cells were only ~60% the normal length (Fig. 4E). Although phallolidin staining revealed that an equivalent number of lamellipodia formed at cell termini, only wild-type cells elaborated well-defined, F-actin-rich leading edges (Fig. 4F,G). Additionally, the number of processes extending from the periphery of the cell body was reduced in \( N-WASp^{-/-} \) cells.
DISCUSSION

Our study of Schwann cell maturation in the context of N-WASP deficiency identifies crucial roles for N-WASP in the elaboration of PNS myelin. The amyelination phenotype manifested by the mutant Schwann cells studied here is likely to be attributable, at least in part, to loss of N-WASP actin polymerization activity. The cytoskeleton supporting the growth cones elaborated by migrating and elongating Schwann cells is normally enriched in actin, and perturbation of the actin cytoskeleton is associated with defective Schwann cell morphology and myelin production (Fernandez-Valle et al., 1997; Melendez-Vasquez et al., 2004; Nodari et al., 2007; Benninger et al., 2007) (for a review, see Feltri et al., 2008). Thus, N-WASP deficiency might result in an impaired cytoskeletal capacity to evoke the inner Schwann cell mesaxon movement that drives the elaboration of spirally wrapped membrane needed for myelin sheath production. This possibility is supported by the absence of mesaxon-mediated plasma membrane circumnavigation following axon ensheathment in the N-WASP-deficient cells in vivo and also by the multiple cytoskeletal anomalies manifested by the N-WASP-deficient Schwann cells cultured from mutant nerves. Thus, in contrast to the actin-rich growth cones observed in both axial and radial lamellipodia of cultured wild-type Schwann cells, the axial processes elaborated by cultured N-WASP-deficient cells were reduced in length and lacked defined F-actin-rich leading edges. Numbers of radial lamellipodia also were decreased dramatically in the mutant cells. These findings are consistent with the aberrant process extension observed in cultured Schwann cells and oligodendrocytes treated with an N-WASP inhibitor (Bacon et al., 2007) and suggest that the crucial role for N-WASP in Schwann cell-mediated myelination reflects, at least partially, the regulation of actin rearrangements that are integral to the formation and function of Schwann cell lamellipodia.

In addition to actin regulatory roles for N-WASP at the Schwann cell membrane, the localization of N-WASP to multiple cellular compartments, including the perinuclear cytoplasm external to the myelin sheath, suggests that the influence of N-WASP on myelination might be realized through multiple effects on Schwann cell biology. When co-cultured with neurons, for example, Schwann cells normally undergo long extensions along the axon of contact prior to initiating the circumferential mesaxonal movement that drives deposition of spirally wrapped plasma membrane (Bunge et al., 1989). By contrast, as is consistent with the extensive Schwann cell hypercellularity observed in mutant nerves, longitudinal extension of N-WASP-deficient Schwann cells is markedly foreshortened. The consequent failure of pre-myelinating Schwann cells to achieve significantly extensive apposition with
the axon membrane may limit the signal exchanges necessary to support transition to the myelination program (Lemke and Chao, 1988). Such signals might involve, for example, induction of other cytoskeletal regulatory pathways or effectors key to the myelination process, such as Rho kinase (ROCK). Notably, ROCK deficiency leads to aberrant Schwann cell branching and elaboration of myelin with multiple shortened internodes, which is suggestive of a failure to form functionally cohesive mesaxons (Melendez-Vasquez, 2004). Similarly, the association of integrin α1, Rac1 or Cdc42 deficiency with defects not only in myelination, but also in Schwann cell proliferation and axonal sorting (Benninger et al., 2007; Nodari et al., 2007), implies that complex signaling interactions that enable Schwann cells to regulate their developmental relationship with axons are integrally involved in the myelination program. Rho family GTPases are also known to play key roles in F-actin-mediated Schwann cell membrane exocytosis processes (Ory and Gasman, 2010) and N-WASp effects on myelination may thus also reflect associations with the effectors that modulate the membrane synthesis and assembly needed for mesaxons to drive productive circumferential travel. Improved understanding of the basis for N-WASp effects on myelination will therefore require definition of the extent to which N-WASp interactions with these and other effector pathways influences Schwann cell maturation.

Of note, the in vivo effects of Cre-mediated N-WASp deletion were not restricted to myelin elaboration, but also included a transient delay in the earlier developmental events associated with axon radial sorting. Thus, the P0-regulated Cre transgene used in...
deriving the mutant Schwann cells investigated here must be expressed at levels sufficient to cause deletion of floxed N-WASp alleles prior to the initiation of myelination, when genes such as P0 are upregulated. Although the sorting anomaly implies a role for N-WASp in early Schwann cell maturation events, the eventual realization of the mature relationship between each Schwann cell and a single axon demonstrates that Schwann cells deficient in N-WASp do successfully complete radial sorting. Schwann cell mitosis is also not adversely affected by N-WASp deficiency, as intense Schwann cell proliferation accompanies the normal sorting process. In further support of this conclusion, the size of the Schwann cell population in the myelinated nerves of mature mutant mice was markedly increased.

N-WASp deficiency in Schwann cells was also associated with diminished expression of multiple myelin-associated genes, including Mag, P0 and Mbp. This finding was, however, not indicative of a global transcription deficit as expression of the promyelinating transcription factor Oct6, Krox20 and Sox10 appeared normal or increased. Thus, failure of the N-WASp-deficient Schwann cells to realize a normal myelin gene expression program could reflect their apparent developmental immaturity (Fernandez-Valle et al., 1997). However, evidence is growing for direct roles of nuclear actin and its modulating partners in transcriptional regulation (for a review, see Gieni and Hendzel, 2009); both N-WASp and related WASp molecules are now directly implicated in transcriptional activities relevant to their cellular functions. WASp, for example, has recently been shown to directly regulate epigenetic changes crucial to the transcriptional induction of the TBX21 gene required for the differentiation of T helper cells (Taylor et al., 2010). Similarly, N-WASp has been identified within a transcription complex that is recruited in response to retinoic acid to the HoxB2 gene enhancer, and the actin polymerization evoked by N-WASp is required for the induction of these genes (Ferrai et al., 2009). The extent to which transcriptional regulatory roles for N-WASp might contribute to the impaired expression of myelin genes in N-WASp-deficient Schwann cells requires further investigation.

Interestingly, the multiple Schwann cell deficits associated with N-WASp deficiency appear to be tolerated relatively well by the mutant mice, in which neither the amylolysed PNS phenotype nor the motor abnormalities significantly evolved with age. Such stability is remarkable as it contrasts with the severe axon pathology and progressive neuropathies associated with many myelin gene mutants (for a review, see Nave, 2010). The stability of this phenotype provides a unique opportunity to investigate the regulation of peripheral nerve fiber maturation in the amylolysed context and also renders these mutant mice of particular value in delineating the molecular pathways that connect N-WASp and/or N-WASp-modulated cytoskeletal remodeling to myelination and the control of myelin gene expression.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org//lookup/suppl/doi:10.1242/dev.058677/-/DC1

References


Supplemental Fig 1

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<tr>
<th>DAPI</th>
<th>N-WASp</th>
<th>MBP</th>
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10 μm

2 μm

PI
Table S1. Morphometric analyses of Schwann cells and axons in electron microscopy images from N-WASp<sup>−/−</sup> and control sciatic nerves

<table>
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<tr>
<th>Measures</th>
<th>Control</th>
<th>N-WASp&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tr>
<td><strong>Large caliber axons</strong></td>
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<td>Axon diameter (µm)</td>
<td>5.49±1.16 (n=55)</td>
<td>3.65±0.71 (n=55)</td>
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<td>Axon density (1000/mm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>23.30±0.50 (n=530)</td>
<td>18.20±0.49 (n=526)</td>
<td>0.09</td>
</tr>
<tr>
<td>Axons/Schwann cell</td>
<td>1±0 (n=1250)</td>
<td>1±0 (n=1250)</td>
<td>–</td>
</tr>
<tr>
<td>Myelinated axons (%)</td>
<td>100 (n=850)</td>
<td>0.67±0.29 (n=850)</td>
<td>–</td>
</tr>
<tr>
<td>G-ratio</td>
<td>0.67±0.06 (n=30)</td>
<td>0.89±0.04 (n=30)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Axons with escaped Schwann cell processes (%)</td>
<td>0 (n=150)</td>
<td>28.68±7.02 (n=150)</td>
<td>–</td>
</tr>
<tr>
<td>Unsorted bundles/1000 µm&lt;sup&gt;2&lt;/sup&gt; (*)</td>
<td>0 (n=8 fields)</td>
<td>1.19±0.22 (n=8 fields)</td>
<td>–</td>
</tr>
<tr>
<td><strong>Axons in Remak bundles</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Axon diameter (µm)</td>
<td>0.73±0.15 (n=67)</td>
<td>0.79±0.19 (n=58)</td>
<td>0.03</td>
</tr>
<tr>
<td>Axon density (1000/mm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>71.60±5.21 (n=600)</td>
<td>87.30±4.68 (n=600)</td>
<td>0.64</td>
</tr>
<tr>
<td>Axons/Schwann cell</td>
<td>10.03±2.81 (n=36)</td>
<td>9.13±3.53 (n=45)</td>
<td>0.34</td>
</tr>
</tbody>
</table>

*Measurements for large caliber (>2 µm) axons and small diameter Remak bundle axons were derived from sciatic nerve cross-sections obtained at P28-P34, with the exception of the quantification of unsorted bundles (*) which was performed on P5 samples. Values shown represent the mean ± s.e.m. with P-values determined where appropriate by Student’s t-test.