Sh3tc2 deficiency affects Neuregulin-1/ErbB signaling

Running title: Role of Sh3tc2 in axonal size sensing

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Abstract

Mutations in *SH3TC2* trigger autosomal recessive demyelinating Charcot-Marie-Tooth type 4C (CMT4C) neuropathy. Sh3tc2 is specifically expressed in Schwann cells and is necessary for proper myelination of peripheral axons. In line with the early onset of neuropathy observed in patients with CMT4C, our analyses of the murine model of CMT4C revealed that the myelinating properties of Sh3tc2-deficient Schwann cells are affected at an early stage. This early phenotype is associated with changes in the canonical Nrg1/ErbB pathway involved in control of myelination. We demonstrate that Sh3tc2 interacts with ErbB2 and plays a role in the regulation of ErbB2 intracellular trafficking from the plasma membrane upon Nrg1 activation. Interestingly, both the loss of Sh3tc2 function in mice and the pathological mutations present in CMT4C patients affect ErbB2 internalization, potentially altering its downstream intracellular signaling pathways. Altogether, our results indicate that the molecular mechanism for the axonal size sensing is disturbed in Sh3tc2-deficient myelinating Schwann cells, thus providing a novel insight into the pathophysiology of CMT4C neuropathy.

Introduction

Charcot-Marie-Tooth disease type 4C (CMT4C) is an autosomal recessive (AR) childhood-onset demyelinating neuropathy caused by mutations in *SH3TC2* (Senderek et al., 2003). Individuals affected by CMT4C present distal muscle weakness and wasting, distal sensory deficits and scoliosis. Recent evaluation of the frequency of *SH3TC2* mutations in populations from different geographic origins ranked CMT4C subtype as one of the most common forms of AR-CMTs (Azzedine et al., 2006; Claramunt et al., 2007; Fischer et al., 2011; Gosselin et al., 2008; Lassuthova et al., 2011; Senderek et al., 2003).

While the implication of SH3TC2 in peripheral neural system function was clearly established through genetic studies and the generation of *Sh3tc2*$^{-/-}$ mice (Arnaud et al., 2009), its exact role in myelin biology remains to be determined. Detailed analysis of the *Sh3tc2*$^{-/-}$ animals demonstrated that Sh3tc2 is required for normal myelination in mouse peripheral nerve. Nerve conduction velocity (NCV) measurements, morphometric and transcriptional analyses indicated an early onset of the disease in *Sh3tc2*$^{-/-}$ mice. The neuropathy was then slowly progressive; adult mutant mice NCV values were comparable to the values recorded in CMT4C patients. The conduction defects in *Sh3tc2*$^{-/-}$ mice are primarily a consequence of a combination of hypomyelination and nodal defects, both also present in patients. However, contrary to the data from human biopsies, the unmyelinated axons and basal lamina did not present major changes in *Sh3tc2*$^{-/-}$ mice.

Recent data demonstrated that Sh3tc2 localizes at the plasma membrane and in endocytic vesicles (Arnaud et al., 2009; Lupo et al., 2009; Roberts et al., 2010). Sh3tc2 further interacts with the small GTPase Rab11, which regulates the recycling of internalized membranes and receptors back to the plasma membrane. Protein binding studies and transferrin receptor trafficking assays also revealed that Sh3tc2 and Rab11 affect the dynamics of transferrin endocytic recycling (Stendel et al., 2010).

Myelination of axons by glial cells critically depends on tight neuron-glia communication. In the peripheral nervous system (PNS), the matching of myelin sheath thickness to axonal caliber is mostly controlled by interactions between neuronal Neuregulin-1 type III (Nrg1) and ErbB2/ErbB3 receptor heterodimers expressed by Schwann cells (Bunge, 1993; Taveggia et al., 2005). Nrg1 binds to ErbB3 and promotes ErbB2-mediated phosphorylation of tyrosine residues in the cytoplasmic domain of both ErbB2 and ErbB3 receptors (Birchmeier and Nave, 2008). The quantity of Nrg1 expressed at the surface of an axon determines the number of layers a Schwann cell will wrap around it; thus, Schwann cells in contact with big axons, which present more Nrg1 at their surface, produce more myelin than Schwann cells in contact with small axons expressing lower amounts of Nrg1 (Michailov et al., 2004). Consequently, decreased expression of axonal Nrg1 or conditional knockout of ErbB2 in Schwann cells leads to hypomyelination (Garratt et al., 2000; Michailov et al., 2004). Activation of the ErbB receptors leads to intracellular signaling through multiple cascades, including PI3K/Akt, Erk1/2, JNK and FAK (Newbern and Birchmeier, 2010). Importantly, ErbB receptor internalization upon activation by Nrg1 is essential for proper downstream signaling (reviewed by (Seto et al., 2002)).

Based on its well-established role in PNS myelination, we investigated Nrg1/ErbB signaling in Sh3tc2-null sciatic nerves and in various cellular paradigms. Our analyses revealed that Sh3tc2 plays a role in the modulation of ErbB receptor internalization and activity in Schwann cells. Our data thus indicate that the hypomyelinating phenotype of CMT4C patients is, at least in part, the consequence of molecular defects in Nrg1/ErbB signaling.

Material and methods

Animals

We used a previously established breeding colony of *Sh3tc2* knockout mice (Arnaud et al., 2009). All animals were housed in a controlled environment with a 12 h light/12 h dark cycle, with free access to water and standard chow diet. Biological samples were collected from littermates and were genotyped as previously described (Arnaud et al., 2009). All experiments were performed in accordance with the legal requirements of the University of Lausanne and the Canton of Vaud.

Antibodies

Rabbit anti-Nrg1, anti-ErbB2, ErbB3, active-ErbB2 (p-ErbB2) and mouse anti-JNK were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti active-ErbB3 (p-ErbB3), anti-Akt, active-Akt (p-Akt), anti-Erk, mouse anti-active-Erk (p-Erk) and rabbit anti-pJNK (Thr183/Tyr 185) were from Cell Signaling (Beverly, MA). Rabbit anti-HA was from Sigma (St. Louis, MO) and goat anti-Flag was from Novus Biological (Littleton, CO). Mouse anti-EEA1 was from BD Biosciences. Secondary antibodies: HRP-conjugated goat anti-mouse IgG, goat anti-rabbit IgG were obtained from Dako, while Alexa Fluor 488- or 546- or 633-conjugated goat anti mouse, donkey anti-goat and goat anti-rabbit IgG were from Molecular Probes (Invitrogen).

Plasmids

The human SH3TC2::HA, the constructs Δmyr, ΔTPR, ΔSH3, ΔSH3ΔTPR, and the R529Q construct carrying CMT4C pathological mutation were previously described (Lupo et al., 2009). Ct-2880 construct was generated by subcloning the amplified sequence of interest by PCR into the pcDNA3-HA vector (a gift from Dr. D. Barettino). The Flag-ERBB2 and Flag-

ERBB3 constructs were obtained by subcloning the full-length coding sequences of both human ERBB2 and ERBB3 into the pFLAG-CMV6 vector. The pFLAG-CMV6 empty vector (Sigma) was a gift from Dr. Pascual Sanz. The ERBB2 and the ERBB3 cDNAs were amplified by PCR from the pME18S::ERBB2 (kindly provided by Pr. Tadashi Yamamoto, Tokyo) and from pCMV-SPORT6::ERBB3 (from Open Biosystem (IMAGE ID: 6147464)) respectively. For each construct, a pair of forward and reverse primers was designed, containing adaptors with restriction sites that allowed in-frame cloning in the vector of interest. N881S CMT4C mutant construct was generated by PCR-based site-directed mutagenesis with specific primers containing the nucleotide changes and according to the instruction manual from the QuikChange TM Site-Directed Mutagenesis kit (Stratagene). The sequences of all the cDNA constructs were confirmed by sequencing on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems).

Real-time PCR

Quantitative PCR was performed as previously described (Arnaud et al., 2009). Quantitation was performed using a standard curve established from a serial dilution of a mix of the samples. Results were normalized using ubiquitin.

Nrg1/ErbB pathway analysis by Western Blotting

Sciatic nerves from post-natal ages of 2, 5 and 15 days were homogenized in lysis buffer containing 25 mM Tris-HCl, pH 7.5, 95 mM NaCl, 10 mM EDTA, 2% SDS, 1 mM NaF, 1 mM Na_3VO_4, and protease inhibitors (Complete®, Roche). All lysates were resolved on SDS/PAGE and analyzed by Western blotting. For anti-phospho antibodies, the blocking reagent was 5% BSA instead of non-fat-milk in T-TBS. For quantitative analysis, captured bands were analyzed with the Image J software (National Institutes of Health, Bethesda, MD).

Co-immunoprecipitation assays

HeLa or COS7 cells were transiently transfected with FuGENE^{TM}6 (Roche), harvested after 24 h, washed with PBS and lysed in co-immunoprecipitation lysis buffer (50 mM HEPES-KOH pH 7.2, 150 mM NaCl, 1 mM $MgCl_2$, and 1% Triton X-100) containing protease inhibitors. The lysates were centrifuged at 16000 g for 20 min at 4°C. For Western blots, aliquots of 50 µg of total proteins were saved from the supernatant to analyze the total cell fraction. For the immunoprecipitation, equal amounts of total protein from the supernatant were incubated for 4 h with anti-Flag (M2) or anti-HA antibodies (1:200). Then, 50 µL of pre-washed protein G sepharose beads (Protein G Sepharose 4 Fast Flow, GE HealthCare) were added. The immunoprecipitation was carried out on a rotating wheel for 16 h at 4ºC. The precipitates were washed three times with cold co-immunoprecipitation washing buffer (20 mM HEPES-KOH, pH 7.2, 150 mM NaCl, 1 mM $MgCl_2$, and 0.2% Triton X100) and boiled in SDS sample buffer to elute protein complexes. Samples were processed using standard SDS-PAGE and Western blotting procedures.

Myelin wrap analysis

Sciatic nerves were carefully dissected and post fixed by immersion in the fixative solution for 2 h at 4°C, washed in 0.1 M cacodylate buffer and osmicated for 4 h in 1% OsO_4 (Fluka). Nerves were rinsed in water, dehydrated, and embedded in Epon 812 substitute (Fluka). Ultra-thin sections were subsequently cut, collected on Formvar-Carbon coated single slot grids, and stained with uranyl acetate and lead citrate. Preparations were analyzed under the Philips CM 100 electron microscope. Large views of sciatic nerve ultrathin sections were acquired at 2200 and 4400-fold magnifications and the details of the myelin sheath were obtained at 96000-fold magnification to allow visualization of the myelin layers that were subsequently

counted manually and plotted as a function of axon diameter measured with Image J software on 4400x pictures.

Subcellular co-localization studies

To study the subcellular localization of SH3TC2 and internalized ERBB2, live HeLa cells or primary Rat Schwann cells transfected with pcDNA3-SH3TC2-HA and Flag-ERBB2 were pretreated with or without 5 µM PAO for 30 min and incubated at 4ºC for 30 min in DMEM containing goat anti-Flag to block endocytosis and to label the Flag-ERBB2 receptor on the cellular surface. Cells were then washed twice with cold PBS and incubated for the indicated times (0, 5, 15 and 30 min) with 10 nM Neuregulin (R&D Systems) to allow the internalization of Flag-ERBB2. Cells were fixed with 4% paraformaldehyde in PBS for 15 min and permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature. Cells were then incubated with Alexa Fluor 488 donkey anti-goat IgG for 1 h at room temperature in BSA 3% / PBS to detect goat anti-Flag. Subsequently, cells were incubated with primary antibodies rabbit anti-HA and mouse anti-EEA1. Finally, to detect both primary antibodies, the cells were incubated with the appropriate secondary fluorescent antibodies (Alexa 546- or 633-conjugated goat anti-rabbit or goat anti-mouse)) for 1 h at room temperature inBSA 3% / PBS. The samples were mounted with Fluoromount-G (Southern Biotech, Birmingham, AL) and were visualized using a Leica DMI-6000 microscope and SP8 Confocal System.

ERBB2 internalization assay

Mouse primary Schwann cells were derived from P2-P4 sciatic nerves of WT or *Sh3tc2*$^{-/-}$ pups as described (Honkanen et al., 2007). Isolated Schwann cells were cultivated in DMEM containing 10% horse serum, 2 ng/mL Neuregulin and 0.5 µM Forskolin. HeLa cells were grown in DMEM containing 10% FBS, and supplemented with 2 mM glutamine, 100 IU/ml

penicillin and 100 µg/ml streptomycin (Invitrogen). HeLa cells were processed for internalization assay 24 h after transfection with SH3TC2-HA. Untransfected HeLa cells were used as a control. To carry out the internalization assays, cells were biotinylated at 4ºC for 30 min with 0.5 mg/mL sulfo-NHS-SS-biotin (Pierce) in PBS. Cells were gently washed three times with cold PBS and then incubated for the indicated times at 37ºC with Neuregulin (10 nM) to activate internalization. Cleavage of the remaining surface biotin was performed with the glutathione cleavage buffer (50 mM glutathione, 75 mM NaCl, 10 mM EDTA, 1% BSA, pH 8.6). Finally, cells were washed twice with cold PBS and scraped into the modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 5 mM DTT, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxicholate supplemented with 1 mM Na_3VO_4, 1 mM NaF, and protease inhibitors (Complete®, Roche)). Cell lysates were centrifuged at 11000 g and incubated with the magnetic streptavidin beads (Pierce) over-night at 4ºC. An aliquot of cell lysate was saved for each sample to analyze the total ERBB2 protein. The beads containing the biotinylated surface proteins were washed, collected and diluted with 2X SDS-sample buffer. After boiling the beads at 96-100ºC the biotinylated surface proteins were analyzed by Western blot.

Results

Early onset Schwann cell myelination defect in *Sh3tc2*$^{-/-}$ mice

In a previous study (Arnaud et al., 2009), we showed that hypomyelination of peripheral nerve fibers in *Sh3tc2*$^{-/-}$ mice was detectable by elevated g-ratio at postnatal day 23 (P23) and onwards. Since we detected murine *Sh3tc2* mRNA early in developing postnatal sciatic nerves, doubling between P0 and P10 (**Supplemental Figure 1**), we decided to expand our analyses of myelination by electron microscopy (EM) in nerves from P0 to adult stages (**Figure 1**). As expected, in the one-year old (P365) control nerves the number of myelin wraps was proportional to axonal diameter. This number, as well as the scaling factor (represented by the slope of the regression trend line in figure 1), were severely reduced in *Sh3tc2*$^{-/-}$ nerves. A similar phenotype was detectable at P10. The hypomyelination in *Sh3tc2*$^{-/-}$ nerves, as detected by overall reduction in number of myelin wraps around axons of all sizes, was already detectable at P5. At this stage we also noticed a higher number of individual fibers already sorted out by Schwann cells but not yet myelinated in the *Sh3tc2*$^{-/-}$ nerves. However, the analysis of P0 sections showed no difference between axonal sorting in *Sh3tc2*$^{-/-}$ and control fibers. The latter suggests that hypomyelination of *Sh3tc2*$^{-/-}$ axons is not due to a defect or delay in axonal colonization and sorting by Schwann cells but rather a consequence of disturbed interactions and/or communication between axons and Schwann cells at the onset of myelination.

Neuregulin-1/ErbB signaling is affected in Sh3tc2-deficient peripheral nerves

In the peripheral nervous system (PNS), Nrg1/ErbB signaling is one of the main pathways that triggers myelination. Intriguingly, *Krox20-Cre/ErbB2*$^{flox/flox}$ peripheral nerves (Garratt et al., 2000) present a hypomyelinated phenotype similar to that of *Sh3tc2*$^{-/-}$ sciatic nerves. To investigate the mechanisms contributing to early myelination impairment in the

absence of Sh3tc2, we analyzed Nrg1/ErbB signaling at the early stage of myelination (P2 to P15) in the sciatic nerves of *Sh3tc2*$^{-/-}$ mice. No change in the amount of the Nrg1 ligand or ErbB2 and 3 receptors was detected at any of the stages tested (**Figure 2A**). However, Sh3tc2-deficient sciatic nerves expressed significantly increased levels of the activated (phosphorylated) forms of the two co-receptors ErbB2 and ErbB3. This increase was detectable at P2 and P5 but disappeared at P15. In order to evaluate the potential cellular consequences of the observed changes in the amount of phosphorylated ErbB2 and ErbB3 receptors, we analyzed the activation of some of their known signaling cascades. No hyperactivation of the downstream targets was observed in the *Sh3tc2*$^{-/-}$ samples. However, we detected a downregulation of phosphorylated 46 kDa c-Jun N-terminal kinase1 (JNK1) (**Figure 2B**).

SH3TC2 interacts and co-localizes with ERBB2

The changes in ErbB signaling in *Sh3tc2*$^{-/-}$ sciatic nerves, together with the capacity of ErbB receptors to interact with SH2 and/or SH3 domain containing proteins (Goodearl et al., 2001; Waterman and Yarden, 2001), suggested that Sh3tc2 and ErbB2- and/or ErbB3 might interact. To test for this, we performed co-immunoprecipitations in HeLa cells co-expressing HA-tagged SH3TC2 and either Flag-tagged ERBB2 or ERBB3. In these experiments, SH3TC2 specifically co-immunoprecipitated with ERBB2 but not with ERBB3 (**Figure 3A**). A reciprocal assay confirmed that ERBB2 co-immunoprecipitate with SH3TC2 (**Figure 3B**). We then assessed the topology of this interaction using various deletion mutants of SH3TC2 (**Figure 3C**). Remarkably, C-terminal truncated forms of SH3TC2 (i.e. ΔTPR or ΔSH3ΔTPR) showed a stronger interaction with ERBB2 in comparison with the wild-type form. In contrast, the C-terminal construct (Ct-2880) lost its ability to interact with ERBB2, while the

Δmyr construct showed a mild interaction. These results demonstrate that the NH_2-terminus of SH3TC2 is required for optimal interaction with ERBB2.

We previously showed that Sh3tc2 localized at the plasma membrane and in intracellular endocytosis vesicles such as endosomes or recycling compartments (Arnaud et al., 2009; Lupo et al., 2009). We therefore assessed subcellular localization of SH3TC2 with respect to ERBB2 by immunofluorescence in HeLa cells co-expressing SH3TC2-HA and Flag-ERBB2. These experiments demonstrated that both wild-type proteins co-localized at the plasma membrane and in intracytoplasmic vesicles (**Figure 4A**). Interestingly, after the treatment with phenylarsine oxide (PAO), a pharmacological inhibitor of endocytosis previously shown to lead to a block of EGF receptor-ligand and ErbB receptor internalization (Bild et al., 2002; Yang et al., 2005) ERBB2 together with SH3TC2 accumulated at the plasma membrane (**Figure 4A**). The retention of both proteins at the plasma membrane induced by PAO treatment was maintained even after the stimulation of ERBB receptor internalization with Nrg1.

To further study protein co-localization upon endocytosis stimulation, we performed an *in vitro* antibody feeding assay. When endocytosis was blocked at 4°C (t=0), wild-type SH3TC2 and ERBB2 co-localized at the plasma membrane. After Nrg1 induction and temperature shift to 37ºC, both proteins were concomitantly internalized from the plasma membrane to endocytic vesicles (t=5, 15, 30 min) (**Figure 4B**). Importantly, we have observed a similar co-internalization of SH3TC2 and ERBB2 in endocytic vesicles (labeled by EEA1, a marker of early endosomes) in rat primary Schwann cells (**Figure 5**). Taken together, these data suggest that SH3TC2 may play a role in ERBB2/3 receptor internalization and/or intracellular trafficking.

Sh3tc2 is involved in ErbB2 receptor internalization in Schwann cells

We next analyzed the effect of Sh3tc2 on the internalization of ErbB2 receptor. HeLa cells expressing endogenous ERBB2 were transfected with SH3TC2-HA and subsequently used for internalization assays. Overexpression of SH3TC2 induced an increase in the amount of internalized ERBB2 (**Figure 6A**). Importantly, two SH3TC2 mutants (R529Q and N881S) failed to increase ERBB2 internalization (**Figure 6A**). ~~These data indicate that SH3TC2 participates in the regulation of the ERBB2 trafficking from the cell surface~~. Hela cells is an heterologous system which was used because of the presence of endogenous ErbB2. Our results show that Sh3tc2 as an effect on the regulation of ERBB2 trafficking from the cell surface. However this system has limitation as the stability of total Erbb2 seems to be affected by the introduction of SH3TC2. We therefore validate the relevance of our results in the context of glial cells.~~To confirm the relevance of these observations specifically in the context of glial cells,~~ W~~w~~e analyzed the role of Sh3tc2 in ErbB2 receptor internalization in Sh3tc2-deficient Schwann cells. Primary neonatal Schwann cells were prepared from the sciatic nerves of *Sh3tc2*$^{-/-}$ mice and control littermates and used for ErbB2 internalization assays (**Figure 6B)**. We observed that the amount of internalized ErbB2 was reduced in Sh3tc2-deficient Schwann cells, underlying the role of Sh3tc2 in ErbB2 internalization.

Discussion

In this manuscript we present evidence for a role of Sh3tc2 in the regulation of Nrg1/ErbB signaling during early postnatal development of PNS. This role is compatible with the early onset neuropathy present in CMT4C patients. Upon axonal Nrg1 type III binding, ErbB2/3 receptors initiate activation of the canonical signaling pathways contributing to induction of myelination. Regulation of the amplitude and kinetics of signal transduction by ErbB receptors involves a complex succession of interactions with several proteins. This process has been studied in particular for ErbB1 (Sorkin and Goh, 2008; Waterman and Yarden, 2001). However, only limited information is available concerning the mechanism of regulation of activated ErbB2 and ErbB3 receptors in Schwann cells. Previous analyses showed that internalization and sorting of activated ErbB receptors require direct or indirect interactions with diverse proteins, in particular adaptor molecules containing SH2 and SH3 domains (Goodearl et al., 2001; Waterman and Yarden, 2001). We showed that SH3TC2, a predicted scaffold protein, interacts with ERBB2 but not with ERBB3. This interaction is independent of the typical SH3 and TPR binding domains, but involves the N-terminal region. Moreover, we showed that upon Nrg1 activation SH3TC2 follows the same cellular compartmentalization as ERBB2 from plasma membrane to endocytosis vesicles and is also retained at the plasma membrane when ERBB2 internalization is blocked with PAO. Therefore, we propose that SH3TC2 is an adaptor protein that interacts with ERBB2/3 after its activation at the plasma membrane.

Previous studies reported that Nrg1 increased the internalization of ErbB2 and ErbB3 receptors, a process necessary for the activation of their downstream effectors (Liu et al., 2007; Yang et al., 2005). The recruitment of adaptor proteins, hetero-dimerization and phosphorylation of both ErbB2 and ErbB3 are necessary for receptor internalization and activation of signaling cascades, which can occur from the plasma membrane as well as from

endosomes (Di Guglielmo et al., 1994; von Zastrow and Sorkin, 2007). Our *in vitro* internalization assay in HeLa cells as well as in primary Schwann cells show that SH3TC2 behaves as a regulator of ERBB2 receptor internalization after Nrg1 activation. Internalization of receptors upon activation is considered not only to attenuate signaling by targeting the receptors to degradation compartments, but also to compartmentalize the activated receptor in order to promote specific signaling. Our results suggest that Sh3tc2 is an adaptor protein participating in such compartmentalization of ErbB2 upon its activation in Schwann cells. Pathogenic or null SH3TC2 mutations impair its capacity to regulate ErbB trafficking in Schwann cells, thereby resulting in transient accumulation of activated ErbB (P-erbB).

We did not observe an over-activation of the ErbB downstream targets probably because activated ErbBs accumulate in the wrong compartment in absence of Sh3tc2. However, although Akt and Erk activation is not changing at the analyzed time-points (probably due to a secondary compensatory effect via other pathways), we observed that JNK activation is reduced in sciatic nerve extracts from *Sh3tc2*$^{-/-}$ animals at early stages. Together, these data suggest that SH3TC2 may regulate ERBB2 endocytosis cycle from the plasma membrane, thereby promoting ERBBs access to specific targets that are preferentially activated in particular endocytosis compartments. As previously described for JNK3 (McDonald et al., 2000), it is possible that JNK is also activated in specific intracellular Schwann cell compartments. In Schwann cells, JNK activation is at least in part mediated by ErbB signaling (Newbern and Birchmeier, 2010) therefore the fact that ErbB internalization is affected in *Sh3tc2*$^{-/-}$ Schwann cells could explain the reduced amount of p-JNK observed in the Sh3tc2-deficient sciatic nerve. So far, Jnk has mostly been described as important for proliferation (Parkinson et al., 2004) and migration (Miyamoto et al., 2012) of Schwann cells. Jnk is downregulated by Krox20 at the onset of myelination, however its active form is still present at P2 and P5 (Figure 2B). JNK is able to activate Paxillin inducing migration of the

Schwann cells (Myamoto et al., 2012) and importantly this protein is retrieved together with Focal adhesion kinase and beta-1 integrin in filopodia and basal lamina of Schwann cells, suggesting a role in myelination (chen et al., 2000). Therefore if JNK would play also a role also at this stage, the downregulation of p-JNK observed in Sh3tc2 KO may explain the defect in the onset of myelination. Analysis of Paxillin and beta-1 integrin might be informative. The proposed role of Sh3tc2 as an adaptor protein localized at the plasma membrane, which follows and guides the route of activated receptors via endocytosis to target their downstream effectors, suggests that activity of other surface receptors (e.g. beta-1 integrin or Gpr126) may be affected in CMT4C neuropathy.

In summary, we conclude that Sh3tc2 localization at the plasma membrane together with ErbB receptors is pivotal to promote proper myelination. Indeed, the alteration or loss of function of Sh3tc2 in CMT4C may affect ErbB2 internalization and potentially lead to changes in its subsequent sub-compartmentalization that contribute to a defect in its downstream signaling. Therefore, the hypomyelination present in CMT4C patients may be, at least in part, the consequence of disregulated Nrg1/ErbB signaling.

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Figure legends

Figure 1. The onset of hypomyelinating phenotype in *Sh3tc2*$^{-/-}$ mice. (A) Electron microscopy images of ultrathin cross-sections of sciatic nerve from control (*Sh3tc2*$^{+/+}$) and *Sh3tc2*$^{-/-}$ animals at P365, P10, P5 and P0. **(B)** The number of myelin wraps (# wraps) is represented as a function of the diameter of the corresponding axon. This quantification was not performed at P0 since many axons are still unmyelinated at this stage. Each dot represents one measured axon, Sh3tc2-deficient axons are depicted in grey and controls in black. Sciatic nerves from 3 mice for each genotype were analyzed at P0, P5 and P10; nerves from 2 mice for each genotype were analyzed at P365. The following number of fibers was characterized at P365, P10 and P5: 31, 50 and 72 in Sh3tc2$^{+/+}$ samples and 37, 69 and 84 in Sh3tc2$^{-/-}$ samples. The slope of the linear regression trend line (plain line) illustrates the scaling between the number of wraps and the axonal diameter for each genotype. While detectable in control nerves at P365 and P10, the scaling is not yet established at P5 due to the immaturity of PNS at this stage.

Figure 2. Nrg1/ErbB signaling in Sh3tc2-deficient sciatic nerves. (A) Activation of ErbB2 and ErbB3 receptors was quantified using relative amounts of phosphorylated forms (p-) versus total amount of each receptor at postnatal days P2, P5 and P15 (-/-: *Sh3tc2*$^{-/-}$ mice, +/+: control *Sh3tc2*$^{+/+}$ littermates, n=2-4). Representative results of 4 independent experiments are shown. The histograms at the bottom represent quantification of the assays (n=4, **p<0.01). Grey bars: controls, black bars: *Sh3tc2*$^{-/-}$. **(B)** Downstream activation of ErbB2/3 receptor targets was analyzed by Western blotting. Representative results of 3 independent experiments are shown. The histograms represent quantification of the assays (n=3, *p<0.05, ***p<0.001). Tubulin and Gapdh were used as controls of equal loading.

Figure 3. SH3TC2 interacts with ERBB2. (A, B) Proteins precipitated either with anti-Flag (IP α-Flag) or anti-HA (IP α-HA) antibodies were analyzed using anti-Flag (M2) to detect Flag-tagged ERBB2 or ERBB3 (IB: Flag) or anti-HA to detect HA-tagged SH3TC2 (IB: HA). pFlag and pCMV-HA were used as controls. TCL: total cell lysate. **(C)** SH3TC2 interacts with ERBB2 via its N-terminal region. The immunoprecipitation was performed using anti-Flag antibody (IP α-Flag). Precipitated proteins were immunoblotted with anti-Flag to detect Flag-ERBB2 (IB: Flag) or anti-HA to detect SH3TC2-HA constructs (IB: HA). Immunoprecipitation of pFLAG was used as a control. SH3TC2-HA: tagged full-length form of SH3TC2; Δmyr, ΔSH3, Ct-2880, ΔTPR, and ΔSH3ΔTPR: truncated forms of SH3TC2; Flag-ERBB2 or B3: tagged full-length form of ERBB.

Figure 4. SH3TC2 co-localizes with ERBB2 in HeLa cells. (A) Both SH3TC2 and ERBB2 were retained at the plasma membrane after 30 minutes of 5 µM PAO treatment. SH3TC2-HA is in green and Flag-ERBB2 is in red. Merged images are shown in the right column. **(B)** SH3TC2-HA and ERBB2 followed the same compartmentalization from the plasma membrane during internalization. T0, 5, 15 and 30 min: time after induction of internalization with 10 nM Nrg1 at 37°C.

Figure 5. Internalization of SH3TC2 and ERBB2 in primary rat Schwann cells.

SH3TC2-HA and ERBB2 co-internalize in endocytic vesicles. T0 (A), 5(B), 15(C) and 30 min (D): time after induction of internalization with 10 nM Nrg1 at 37°C. SH3TC2-HA is in green, Flag-ERBB2 is in red and EEA1 is in blue. Co-localization of the three proteins is showed in merged images as white color and a higher magnification is shown.

Figure 6. SH3TC2 is a positive regulator of ERBB2 internalization. (A) Internalization of endogenous ERBB2 in HeLa cells transfected (+SH3TC2) or not (control) with wild-type SH2TC2 or with SH3TC2 carrying pathological CMT4C mutations (R529Q and N881S). Representative results of 3 independent experiments are shown (**p<0.01). **(B)** Internalization of ErbB2 in control (WT) or *Sh3tc2*$^{-/-}$ (KO) primary mouse Schwann cells (n=5, *p<0.05). Quantification shown in histograms represents the ratio of the amount of immunoprecipitate ErbB2 over total input ErbB2. Longer exposure could result in 2 bands corresponding to ErbB2. The 2 bands are quantified and the same area square is for quantification of the total and internalized ErbB2

Supplemental Figure 1. Expression profile of Sh3tc2 during development of the sciatic nerve. Sh3tc2 mRNA was measured by qPCR in sciatic nerves of wild-type mice at embryonic stage E17, and postnatal stages P0 to P56. Results were normalized using ubiquitin transcript. The data represent the mean ± S. D. of triplicate measurements.