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Prenylation-Defective Human Connexin32 Mutants Are Normally Localized and Function Equivalently to Wild-Type Connexin32 in Myelinating Schwann Cells

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Mutations in *GJB1*, the gene encoding the gap junction protein connexin32 (Cx32), cause the X-linked form of Charcot-Marie-Tooth disease, an inherited demyelinating neuropathy. The C terminus of human Cx32 contains a putative prenylation motif that is conserved in Cx32 orthologs. Using [^{3}H]mevalonolactone ([^{3}H]MVA) incorporation, we demonstrated that wild-type human connexin32 can be prenylated in COS7 cells, in contrast to disease-associated mutations that are predicted to disrupt the prenylation motif. We generated transgenic mice that express these mutants in myelinating Schwann cells. Male mice expressing a transgene were crossed with female *Gjb1*-null mice; the male offspring were all *Gjb1*-null, and one-half were transgene positive; in these mice, all Cx32 was derived from expression of the transgene. The mutant human protein was properly localized in myelinating Schwann cells in multiple transgenic lines and did not alter the localization of other components of paranodes and incisures. Finally, both the C280G and the S281x mutants appeared to "rescue" the phenotype of *Gjb1*-null mice, because transgene-positive male mice had significantly fewer abnormally myelinated axons than did their transgene-negative male littermates. These results indicate that Cx32 is prenylated, but that prenylation is not required for proper trafficking of Cx32 and perhaps not even for certain aspects of its function, in myelinating Schwann cells.

Key words: **X-linked Charcot-Marie-Tooth disease; CMT; neuropathy; myelin; Cx32; gap junction**

Introduction

Inherited demyelinating neuropathies are a clinically and genetically heterogeneous diseases that include the dominantly inherited, demyelinating forms of Charcot-Marie-Tooth disease (CMT1), as well as milder and more severe neuropathies (Lupski and Garcia, 2001; Wrabetz et al., 2004; Shy et al., 2005). Mutations in *GJB1*, the gene encoding the gap junction protein connexin32 (Cx32), cause the X-linked form of CMT1 (CMT1X). Gap junctions are channels that allow the diffusion of ions and small molecules (up to ~1000 Da) between closely apposed cells and have diverse functions, including the propagation of electrical signals, metabolic cooperation, growth control, and cellular differentiation (Bruzzone et al., 1996). Connexins oligomerize into hemichannels (connexons), which dock with another hemichannel on an apposed cell membrane to form a complete channel (Musil and Goodenough, 1993; Kumar et al., 1995). Myelinating Schwann cells express Cx32 (Scherer et al., 1995), which is localized to particular regions of the myelin sheath in which gap junctions have been identified by electron microscopy (Meier et al., 2004). Gap junctions formed by Cx32 probably provide a shortcut for the diffusion of small molecules and ions directly across the myelin sheath, short circuiting the circumferential cytoplasmic pathway (Balice-Gordon et al., 1998).

More than 260 different *GJB1* mutations have been described, affecting most portions of Cx32 (http://www.molgen.ua.ac.be/CMTMutations/). Two of these mutations affect a potential prenylation motif: a Cys to Gly substitution at 280 (C280G) and a Ser to stop mutation at 281 (S281x). Prenylation covalently adds either farnesyl (15-carbon) or geranylgeranyl (20-carbon) isoprenoid lipids to cysteine residues near the C termini of proteins (Zhang and Casey, 1996). Prenylation occurs on two different motifs, the CC/CXC and CaaX. The CC/CXC motif is used by the *Rab* family (small GTP-binding) of proteins and is exclusively modified by the addition of a geranylgeranyl moiety. For proteins terminating in a CaaX motif (C stands for cysteine, "a" stands for any amino acid, but usually an aliphatic amino acid, and X is the final amino acid of the protein), a farnesyl or a geranylgeranyl isoprenoid is covalently added to the cysteine residue. Prenylation causes cytosolic proteins to become membrane associated; this is probably required for their function, because on-

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Table 1. Summary of transgenic lines

Mutant	Founder	Germ-line transmission	RT-PCR	Immunostaining in wild-type mice	Crossed with *Gjb1*-null mice
C280G	15	12	6 (7M15, 7M20, 7M33, 7M37, 7M39, 7M45)	6 (7M15*, 7M20, 7M33, 7M37, 7M39, 7M45)	3 (7M33, 7M37, 7M39)
S281x	16	10	4 (8M25, 8M34, 8M35#, 8M39)	3 (8M25, 8M34*, 8M39*)	1 (8M25)

Approximately one-half of the transgenic founders with germ-line transmission in wild-type background (the numbers of lines are indicated) were screened by immunohistochemistry and RT-PCR, thereby identifying lines that expressed Cx32 in all myelin sheaths and human Cx32 mRNA (typically at levels that were higher than endogenous/mouse mRNA), respectively. We excluded three lines (*) that showed non-uniform expression by immunostaining and one line (#) in which there was less human/mutant Cx32 than endogenous mouse Cx32 mRNA. The male transgenic (hemizygous) mice from some of these lines were bred with *Gjb1*-null female mice; the resulting male offspring were used for additional analysis.

cogenic mutants of *Ras* lose their transforming ability if their prenylation is inhibited (Hancock et al., 1989; Jackson et al., 1990; Kato et al., 1992). Thus, it is not obvious why Cx32, an intrinsic membrane protein, should be prenylated. To investigate this issue, we metabolically labeled transfected COS7 cells with a precursor of isoprenyl groups and found that wild-type Cx32 is prenylated, whereas the C280G and S281x mutants are not. When expressed in myelinating Schwann cells, the C280G and S281x mutants were properly localized and delayed the onset of demyelination in *Gjb1*-null mice; similar results have been reported for mice expressing wild-type human Cx32 (Scherer et al., 2005). These results indicate that prenylation of Cx32 is not required for its proper trafficking, and perhaps not even for certain aspects of its function, in myelinating Schwann cells.

Materials and Methods

Site-directed mutagenesis. For the prenylation study, the C280G and S281x mutations were introduced into the open reading frame of the human Cx32 cDNA (subcloned into pBlueScript vector; Stratagene, La Jolla, CA) by QuickChange site-directed mutagenesis (Stratagene). Oligonucleotide mutagenic primers were designed and incorporated using *Pfu*-Turbo DNA polymerase. The PCR products were digested by *Dpn*I endonuclease to eliminate the parental DNA template. The resulting DNA was used to transform *XL-1* Blue bacteria, and minipreps were made from single colonies and sequenced. Inserts were isolated by double digestion with *Bam*HI and *Kpn*I and subcloned into pREP9 (Invitrogen, Carlsbad, CA). A large-scale plasmid preparation was made from a single colony, and the *Gjb1*/Cx32 sequence was confirmed by direct sequencing. To generate Cx32 transgenic mice, the two point mutations C280G and S281x were introduced to the P0Cx32WT (see Fig. 3*B*) construct by using QuikChange II XL site-directed mutagenesis kit (Stratagene). The program used for mutagenesis was as follows: 94°C for 2 min; 18 cycles of 95°C for 50 s, 63°C for 50 s, 68°C for 18 min; and then 68°C for 7 min. Oligonucleotide mutagenic primers were incorporated using *PfuUltra* high-fidelity DNA polymerase. The other steps are the same as above, except the DNA was used to transform *XL10*-Gold ultracompetent cells. The whole insert was sequenced, and clones containing undesired mutations were discarded until a clone with desired mutation was found.

Metabolic labeling. An expression vector containing the human Cx32 open reading frame (pREP9-hCx32), or the mutations C280G or S281x, was mixed together with a plasmid encoding the mevalonate transporter gene (pMev; American Type Culture Collection, Manassas, VA) and transfected into confluent COS7 cells by using Lipofectamine2000 (Invitrogen) following the manual of the manufacturer. Control COS7 cells were transfected with pMev plasmid and an "empty" pREP9 vector. After 24 h, transfected cells were incubated for 20 h in medium containing 10% FBS, 150 μCi/ml mevalonolactone (20 Ci/mmol specific activity), *RS*-[5-^{3}H(N)] (American Radiolabeled Chemicals, St. Louis, MO), and 20 μM compactin (Sigma-Aldrich, St. Louis, MO). The cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (10 mM sodium phosphate, pH 7.0, 150 mM sodium chloride, 2 mM EDTA, 50 mM sodium fluoride, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS) and precleared with recombinant protein G agarose (Invitrogen) for 1 h at 4°C. After pelleting insoluble material, supernatants were removed and incubated overnight with a mouse monoclonal antibody against Cx32 (Sigma-Aldrich) on a rotating platform at 4°C. Protein G agarose beads were added for 1 h, and then the beads were washed three times in RIPA buffer, resuspended in electrophoresis sample buffer (62.5 mM TRIS, pH 6.8, 20% glycerol, 2% SDS, and 100 mM DTT), and separated by electrophoresis in a 12% SDS-PAGE gel. Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) for 1 h at 4°C using a semidry apparatus (Fisher Scientific, Pittsburgh, PA). The membrane was sprayed with En^{3}Hance, exposed to X-OMAT AR-2 (Eastman Kodak, Rochester, NY) film for 2 weeks at −80°C, stripped of fluorography enhancer by rinsing with methanol and TBS containing 0.5% Tween 20, blocked for 1 h with 5% powdered skim milk–0.5% Tween 20 in TBS, and incubated with rabbit anti-Cx32 (1:1000; Zymed, San Francisco, CA) at 4°C overnight. After several washes, the blots were incubated in peroxidase-coupled secondary antibodies against rabbit (1:10,000 dilution; Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature, washed several times, revealed using ECL Plus (Amersham Biosciences, Piscataway, NJ), and exposed to X-OMAT AR-2 film (Eastman Kodak).

Generation of Cx32 mutant mice. The generation and initial characterization of *Gjb1*-null mice has been described previously (Nelles et al., 1996; Anzini et al., 1997; Scherer et al., 1998). Animals were generated from our colony at the University of Pennsylvania and genotyped by PCR analysis of genomic DNA isolated from tail clips (Anzini et al., 1997). The rat *Mpz* promoter-human *GJB1* construct has been described previously (Abel et al., 1999; Scherer et al., 2005). The C280G and S281x mutations were introduced by site-directed mutagenesis (see Fig. 3*B*). A 6.4 kb transgene cassette for each clone was released from vector sequences by digestion with *Apa*I and *Nsi*I, purified using Elutip minicolumns (Schleicher & Schuell, Keene, NH) and sent to the transgenic facility of the University of Pennsylvania. The purified DNA was microinjected into the male pronucleus of fertilized eggs obtained from B6SJL/F1 mice (The Jackson Laboratory, Bar Harbor, ME), according to standard protocols (Brinster et al., 1985). Eggs that survived microinjection were then placed into pseudopregnant foster mice for gestation. Transgenic progeny were identified by PCR with transgene-specific primers as described previously (Abel et al., 1999; Scherer et al., 2005). In this way, we identified lines that transmitted the transgene to their progeny: 12 lines for the C280G and 10 for S281x.

Analysis of transgene expression. The general strategy for selecting the transgenic lines for final study was shown in Table 1. For reverse transcription (RT)-PCR, total RNA was isolated from snap-frozen sciatic nerves of 1-month-old mice (crushed in a mortar and pestle on dry ice) using the RNeasy Mini Kit (Qiagen, Valencia, CA) protocol for small amounts of tissue and quantitated by spectrophotometry. RT-PCR was performed using Superscript first-strand synthesis system reverse transcriptase kit with random hexamer primers (Invitrogen) from 1 μg of total RNA. cDNA was amplified with primers that recognize the identical sequences in human and mouse *GJB1/Gjb1* (Scherer et al., 2005). For each sample, the same amount of amplified DNA was digested with *Msc*I or *Hha*I or left uncut, and the relative levels of the transgenic/human to endogenous/mouse mRNA was compared in ethidium bromide-stained gels.

Protein was isolated from snap-frozen sciatic nerves of adult mice (crushed in a mortar and pestle on dry ice), suspended in Tris-buffered SDS lysis buffer (50 mM Tris, pH 7.0/1% SDS/0.017 mg/ml phenylmethyl sulfonyl fluoride), and sonicated (Sonic Dismembrator; Fisher Scientific). Samples were spun at 4°C to pellet insolubles, and the supernatant was measured by modified Lowry assay (Bio-Rad, Hercules, CA). A total

Table 2. Comparison of abnormally myelinated axons between TG^+ and TG^- mice

Line	Mouse	Genotype	De-	Re-	Total	Proportion abnormal	Odds ratio	*p* values	Robust SE	z	95% CI lower to upper
7M37 C280G	372	TG^-	6	159	1160	0.142					
	373	TG^-	2	127	1126	0.115					
	374	TG^+	0	46	1123	0.041					
	375	TG^+	1	8	1151	0.0078	5.95	0.002	3.47	3.07	1.90 to 18.60
7M33 group1 C280G	3311	TG^+	0	3	1129	0.0027					
	3312	TG^+	0	17	1193	0.014					
	3315	TG^-	6	253	1165	0.222					
7M33 group2 C280G	331	TG^+	0	2	1063	0.002					
	332	TG^+	0	9	1172	0.0077					
	334	TG^-	4	132	1086	0.125	31.07	0.000	14.93	7.15	12.11 to 79.7
7M39 C280G	395	TG^-	5	125	1069	0.122					
	396	TG^+	0	2	1167	0.0017	80.64	0.000	57.57	6.15	19.90 to 326.76
8M25 S281x	252	TG^-	3	155	1145	0.138					
	253	TG^+	0	1	1131	0.0009					
	254	TG^+	1	2	1160	0.0026	91.53	0.000	38.69	8.9	39.97 to 209.60

This table gives the number of demyelinated and remyelinated axons, as well as the total (normal, demyelinated, and remyelinated) number of myelinated axons in the femoral motor branch from four lines of mice at P158. The proportion of abnormally (De-, demyelinated; Re-, remyelinated) myelinated axons is shown, and a statistical comparison (odds ratio) between TG^+ and TG^- animals is shown for each line.

of 50 μg of protein per lane was electrophoresed in 12% SDS-PAGE gels, transferred to the PVDF membrane, blocked for 1 h with 5% powdered skim milk–0.5% Tween 20 in TBS, and incubated with rabbit anti-Cx32 (1:100 dilution; Chemicon, Temecula, CA) or rabbit anti-HSP 90α/β (1:12,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. After several washes, the membranes were incubated in peroxidase-coupled secondary antibodies against rabbit (1:10,000 dilution; Jackson ImmunoResearch) for 1 h at room temperature, washed, and revealed using ECL Plus (Amersham Biosciences) and exposed to X-OMAT AR-2 film (Eastman Kodak).

Immunohistochemistry. Because fixation in paraformaldehyde reduces Cx32 immunoreactivity (Scherer et al., 1995), we teased nerve fibers from unfixed nerves; these were dried on glass slides (SuperFrost Plus; Fisher Scientific), postfixed for 10 min with acetone, blocked at room temperature for at least 1 h in 5% fish skin gelatin containing 0.5% Triton X-100 in PBS, and incubated 12–24 h at 4°C with various combinations of primary antibodies. We used a mouse monoclonal antibody against rat Cx32 (1:1 dilution; 7C6.C7), which recognizes an epitope in the C-terminal tail (Li et al., 1997). The anti-Cx32 antibodies were usually combined with a rat monoclonal antibody against E-cadherin (1:50 dilution, DECMA; Sigma), a chicken antibody against nonphosphorylated neurofilament heavy (1:250 dilution; Chemicon) or rabbit antisera against myelin-associated glycoprotein (MAG) [1:100 dilution (Pedraza et al., 1990)], claudin-19 [1:600 dilution (Miyamoto et al., 2005)], or Cx29 [1:300 dilution (Altevogt et al., 2002)]. After incubating with the primary antibodies, the sections were washed and incubated with the appropriate fluorescein-, rhodamine-, and cyanine-5-conjugated donkey anti-rabbit, -mouse, -chicken, and/or -rat secondary antibodies (Jackson ImmunoResearch). These preparations were imaged with epifluorescence tetramethylrhodamine isothiocyanate and FITC optics on a Leica (Nussloch, Germany) DMR light microscope equipped with a cooled Hamamatsu (Bridgewater, NJ) camera, followed by image manipulation with Adobe Photoshop (Adobe Systems, San Jose, CA). When appropriate, the images were made with comparable exposure times to allow better comparison between mice of different genotypes.

Light microscopy. We examined litters containing *Gjb1*$^{-/Y}$ mice at postnatal day 158 (P158). Mice were deeply anesthetized with chloral hydrate and then perfused with 0.9% NaCl, followed by 3% glutaraldehyde in 0.1 M phosphate buffer (PB). The sciatic and femoral nerves were removed and placed in fresh fixative overnight at 4°C, rinsed in PB, postfixed in 2% OsO_4 in PB, dehydrated in an ascending series of ethanol, and embedded in epoxy. Semithin sections of the femoral motor and sensory branches and sciatic nerve were stained with toluidine blue, examined by light microscopy, and imaged with Leica DMR light microscope equipped with a cooled Hamamatsu camera, followed by image manipulation with Adobe Photoshop.

Statistical analysis. For a quantitative analysis of demyelinated and remyelinated axons, digital images (OpenLab software; Improvision, Lexington, MA) were made of single transverse semithin sections femoral motor nerve. All demyelinated, remyelinated, and normally myelinated axons were counted by the same observer (S.S.S.) without knowledge of the animal's genotype. Axons larger than 1 μm in diameter without a myelin sheath were considered demyelinated. Axons with myelin sheaths that were <10% of the axonal diameter as well as myelinated axons that were surrounded by "onion bulbs" (circumferentially arranged Schwann cell processes and extracellular matrix) were considered remyelinated. The rest of the myelinated axons were considered to be normally myelinated. The proportion of demyelinated and remyelinated axons in transgenic TG^+ versus TG^- animals was compared statistically using odds ratios (Tables 2, 3) as described previously (Scherer et al., 2005).

Results

CaaX motif is conserved during evolution

Two mutations at the C terminus, C280G and S281x (in this case, "x" stands for a stop codon), drew our attention to a putative CaaX motif in Cx32 (in this case, "X" is the final amino acid of a protein). To determine whether the CaaX motif was unique to Cx32 and whether it was conserved, we searched GenBank for all known connexin genes. Cx32 was the only cloned mammalian connexin with a CaaX motif. Retrieved sequences of highest homology (Fig. 1), likely to be Cx32 orthologs, were found in numerous mammalian species, as well as in chicken (*Gallus gallus*) and frogs (*Xenopus laevis* and *X. tropicalis*); all had a C-terminal CaaX sequence. In teleost fish, a CaaX motif is found in one of two connexins reported in the red seabream (*Pagrus major*) but not in any zebrafish (*Danio rerio*) or pufferfish (*Fugo rubripes*) connexins. Thus, the CaaX motif of Cx32 is a phylogenetically conserved attribute of this protein, originating before the last common ancestor of amphibians and mammals and perhaps earlier.

Cx32 is prenylated

Nearly all prenylated proteins are predicted to be cytoplasmic by their primary structure. Approximately 200 proteins in the human genome are predicted to contain a CaaX motif (Fivaz and Meyer, 2003), but <10 are likely to be intrinsic membrane proteins (http://www.ensembl.org/Homo_sapiens/). To demonstrate whether Cx32 is prenylated, COS7 cells were transiently transfected with an eukaryotic expression vector containing human *GJB1/Cx32*. Twenty-four hours later, radioactive [^{3}H]MVA

Table 3. Comparison of abnormally myelinated axons between transgenic lines

Line	TG$^+$	Odds ratio versus line 90	TG$^-$	Odds ratio versus line 90
90 (WT)	0.1% (3 of 2495; $n = 3$)		9.8% (217 of 2223; $n = 3$)	
		20.6		1.36
7M37 (C280G)	2.4% (55 of 2274; $n = 2$)	$p = 0.001$	12.9% (294 of 2286; $n = 2$)	$p = 0.19$
		5.69		1.97
7M33 (C280G)	0.7% (31 of 4557; $n = 4$)	$p = 0.040$	17.5% (395 of 2251; $n = 2$)	$p = 0.048$
		1.43		1.28
7M39 (C280G)	0.2% (2 of 1167; $n = 1$)	$p = 0.66$	12.2% (130 of 1069; $n = 1$)	$p = 0.27$
		1.45		1.48
8M25 (S281x)	0.2% (4 of 2291; $n = 2$)	$p = 0.67$	13.8% (158 of 1145; $n = 1$)	$p = 0.081$

The average proportion (%) of abnormally myelinated (demyelinated and remyelinated) axons in P158 femoral motor nerves is shown for individual transgenic lines. The data from line 90 [express wild-type human Cx32 as a transgene (WT)] are from Scherer et al. (2005); the data for the other lines are derived from Table 2. For each line, the proportion of abnormally myelinated axons is compared with that in line 90 by an odds ratio test, separately for TG$^+$ and TG$^-$ mice. The odds ratio of 20.6 means that the odds of an abnormal myelinated axon (vs a normally myelinated axon) in TG$^+$ in line 7M37 was 20.6 times the odds of an abnormal myelinated axon in TG$^+$ in line 90.

[a metabolic precursor of isoprenoids (James et al., 1993)] and compactin (to inhibit endogenous isoprenoids/cholesterol synthesis) were added, and Cx32 was immunoprecipitated after another 20 h. The cells were cotransfected to express a mevalonate transporter (to enhance cellular uptake the [^{3}H]MVA) and either wild-type human Cx32 or one of the mutants. Immunoprecipitated Cx32 was separated by SDS-PAGE and exposed to film to detect [^{3}H]isoprenoid-labeled Cx32. As shown in Figure 2*A*, wild-type Cx32 incorporated [^{3}H]MVA, but the C280G and S281x mutants did not. Subsequent immunoblotting of the same membrane revealed that Cx32 was expressed in all transfected cells, and that Cx32 mutants were expressed at even higher levels than wild-type Cx32. Thus, despite their higher levels of expression, [^{3}H]MVA incorporation was not detected in these mutants, indicating that they were not prenylated (Fig. 2*B*). We repeated this experiment several times with similar results. COS7 cells transfected with the empty pREP9 vector did not express Cx32, and a band corresponding to metabolically labeled Cx32 was not detected (Fig. 2).

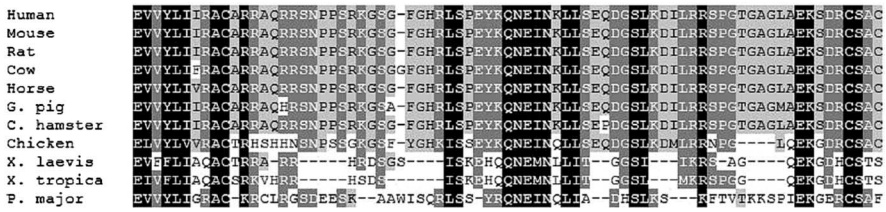

Figure 1. Alignment of Cx32 orthologs. The C termini (amino acids from 208 to 283 in human Cx32) of the following putative Cx32 orthologs were aligned using GeneDoc: human (NP_000157), mouse (NP_032150), rat (NP_058947), cow (NP_776494), horse (AAQ20110), guinea pig (BAC07263), Chinese hamster (AAP37453), chicken (NP_989702), *Xenopus laevis* (CAA68745), *Xenopus tropicalis* (NP_001001240), and *Pagrus major* (BAA90669). Black, 100% similarity; gray, 80–90% similarity; light gray, 60–70% similarity.

Generation of C280G and S281x transgenic mice

It is interesting that some Cx32 mutants form functional gap junctions in transfected cells, including C280G and S281x (Castro et al., 1999; Abrams et al., 2001; Yum et al., 2002), yet cause CMT1X in humans. Perhaps these mutants do not traffic properly in myelinating Schwann cells, just as certain rhodopsin mutants do not traffic properly in rods (Sung et al., 1994), or perhaps they form channels with abnormal properties (Castro et al., 1999; Abrams et al., 2001; Liang et al., 2005). To address this issue, we used a rat *Mpz* promoter to drive the expression of the human *GJB1* gene (Fig. 3). Other derivates of this construct have been used successfully to drive the expression of transgenes in myelinating Schwann cells, including *GJB1* (Abel et al., 1999; Pot et al., 2002; Leone et al., 2003; Scherer et al., 2005). We generated 15 founders for C280G and 16 founders for S281x mice (Table 1). Lines with no germ-line transfer of the transgene were eliminated. TG$^+$ mice in the remaining lines appeared behaviorally normal and transmitted the transgene in a Mendelian manner; no excessive mortality was found (data not shown).

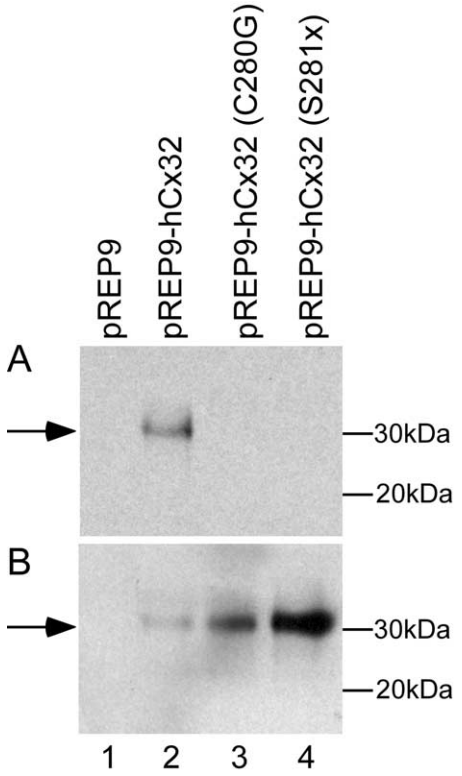

Figure 2. Wild-type human Cx32 is prenylated, but CaaX mutants are not. COS7 cells were transiently transfected with expression constructs for a mevalonate transporter (pMev; lanes 1–4) and human Cx32 (pREP9-hCx32; lanes 2–4). After 24 h, cell culture medium was removed and replaced with labeling medium, and cells were returned to the incubator for 20 h. Cx32 was immunoprecipitated, and the immunoprecipitates were separated on a 12% SDS-PAGE gel and transferred to a PVDF membrane. The membrane was exposed to film for 14 d (***A***) and then immunoblotted for Cx32 with a rabbit antiserum (***B***). All Cx32 constructs showed the band of the correct size (arrow).

Human/transgenic Cx32 DNA expression

To select a smaller number of lines for additional analysis, we performed RT-PCR on sciatic nerves from 1-month-old TG$^+$

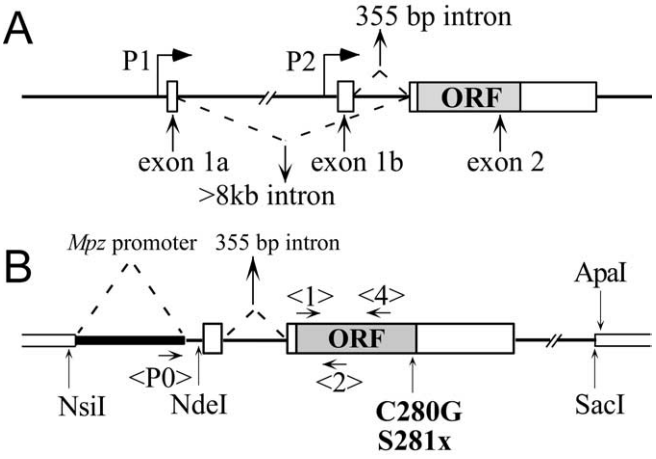

Figure 3. Schematic transgenic cassette. ***A***, Schematic structure of the human *GJB1* gene (not to scale). Exons are depicted as boxes; introns are depicted by lines. The open portions of the boxes depict untranslated regions; the gray portion depicts the open reading frame (ORF), which is entirely contained within the second exon and is included in transcripts initiated at either the P1 or the P2 promoters. In myelinating Schwann cells, Cx32 transcripts are initiated from the P2 promoter; in the liver, transcripts are initiated from the P1 promoter (Neuhaus et al., 1995, 1996; Söhl et al., 1996). ***B***, Schematic map of the *Mpz-GJB1* transgene construct P0Cx32WT. The 5.3 kb *Nde*I/*Sac*I fragment of *Gjb1* was cloned 3′ to the 1.1 kb rat *Mpz/P0* promoter fragment. *Nde*I cuts 7 bp 3′ the P2 promoter TATAA box, which is thus excluded from the transgene construct. Flanking vector sequences are shown as open-ended boxes on either side of the construct. The transgene cassette is isolated by an *Nsi*I and *Apa*I double digest. Primers <P0> and <2> were used for PCR genotyping, and primers <1> and <4> were used for RT-PCR analysis. The sites of the C280G and S281x mutations are marked (arrows).

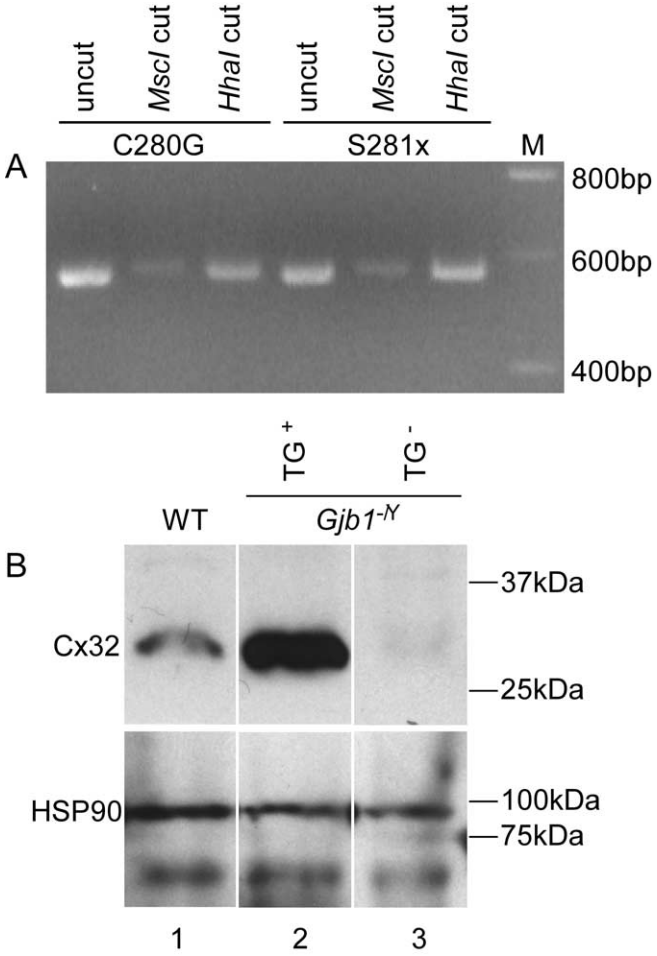

Figure 4. Expression of Cx32 mRNA and protein in peripheral nerve. ***A***, RT-PCR analysis of transgene expression in sciatic nerves from 1-month-old mice expressing either the C280G (line 7M39) or S281x (line 8M25) mutations in a wild-type background. All lanes have a 553 bp PCR product amplified by a primer pair that recognizes an identical sequence in both human and mouse Cx32. For each nerve, the same amount of PCR product was loaded in every lane, but some of the samples were digested with *Msc*I or *Hha*I. *Msc*I cuts only the human PCR product; the residual 553 bp (uncut) band is the endogenous/mouse Cx32 cDNA. *Hha*I cuts only the mouse PCR product; the residual 553 bp (uncut) band is transgenic/human Cx32 cDNA. The amount of transgenic/human Cx32 mRNA is greater than the amount of endogenous/mouse Cx32 mRNA. M, Molecular DNA markers. ***B***, Immunoblot analysis of Cx32 in sciatic nerve from TG $^{+}$ and TG $^{-}$ *Gjb*$^{-/Y}$ adult littermates as well as adult wild-type mice. Fifty micrograms of protein were loaded in each lane and electrophoresed in a 12% SDS-PAGE gel. After transfer, the membrane was split into two pieces; one was probed with a rabbit antiserum against Cx32 (1:100 dilution; top), and the other was probed with a rabbit antiserum against heat shock protein 90 (HSP90) (1:12,000 dilution; bottom). Note that there is relatively more Cx32 in TG $^{+}$ *Gjb1*$^{-/Y}$ mice than in wild-type mice.

mice with primers that recognize nucleotide sequences that are identical in both human and mouse *Cx32/cx32* (Fig. 3*B*, <1>, <4>). Restriction digestion of the amplified 553 bp product allowed a qualitative determination of the relative amounts of transgenic/human and endogenous/mouse Cx32 DNA in ethidium bromide-stained gels. *Hha*I uniquely cut the mouse product, whereas *Msc*I uniquely cut the human product. As expected (Scherer et al., 2005), double digestion cut all of the PCR product (data not shown). For six of six C280G lines, and three of four S281x lines, the levels of transgenic/human Cx32 were higher than that of endogenous/mouse Cx32. Examples are shown in Figure 4*A*. The overexpression of transgenic/human compared with endogenous/mouse Cx32 is a consistent finding with the *Mpz-GJB1* construct (Scherer et al., 2005) (L. J. B. Jeng and S. S. Scherer, unpublished observation).

The localization of C280G and S281x mutants in a wild-type background

In wild-type mice, Cx32 is mainly localized to incisures and paranodes (Bergoffen et al., 1993; Scherer et al., 1995; Chandross et al., 1996; Meier et al., 2004). To determine the localization of transgenic/mutant Cx32, we immunostained unfixed teased fibers from adult TG $^{+}$ mice in a wild-type background, from 10 different lines (six lines C280G and four lines S281x). In all lines examined, there was prominent Cx32 immunostaining of paranodes and incisures, including aggregates that were much larger than those seen in wild-type nerves (Fig. 5*A–D*). Three lines (one C280G and two S281x) showed variable staining of individual myelin sheaths and were not analyzed further. Thus, the localization and overexpression of the C280G and S281x mutants is strikingly similar to that of wild-type human Cx32 expressed with the same transgenic construct (Scherer et al., 2005). We conclude that prenylation is not required for proper targeting of Cx32 in myelinating Schwann cells.

We also examined the localization of E-cadherin, Cx29, MAG, and claudin-19, which are individually localized to various aspects of incisures, paranodes, inner and outer mesaxons, and the juxtaparanodal Schwann cell membrane (Scherer et al., 2004). In lines expressing either C280G and S281x, aggregates of Cx32 were colocalized with strands of E-cadherin staining at outer mesaxons (Fig. 5*A*, *B*) (Fannon et al., 1995), Cx29 was localized correctly in incisures and in the juxtaparanodal region (Fig. 5*C*,*D*) (Altevogt et al., 2002; Li et al., 2002), and MAG and claudin-19 were properly localized (data not shown) (Trapp et al., 1989; Miyamoto et al., 2005). Thus, the expression (and even the overexpression) of

the C280G and S281x mutants did not appear to disrupt the organization of other molecules expressed in the same regions of the myelin sheath.

The effects of C280G and S281x mutants in a *Gjb1*-null background

To evaluate the possibility that the normal localization of the C280G and S281x mutants might owe to their association with the endogenous/mouse Cx32, we examined the localization of these mutants in a *Gjb1*-null background (three lines C280G and one line S281x). Female $Gjb1^{-/-}$ mice were crossed with hemizygous TG^+ male mice; the male offspring were $Gjb1^{-/Y}$, and equal proportions were TG^+ and TG^-. Immunostaining teased fibers from TG^+ mice demonstrated that the localization of Cx32 was similar to that in wild-type mice: Cx32 was localized to the incisures, paranodes, and outer mesaxons but appeared to be expressed at higher levels than in wild-type mice (Fig. 6*A–D*). As in a wild-type background (see above), the localization of E-cadherin, Cx29 (Fig. 6*A–D*), MAG (data not shown), and claudin-19 (supplemental Fig. 1, available at www.jneurosci.org as supplemental material) were unaffected. Thus, the prenylation motif is not required for the proper trafficking of Cx32 in myelinating Schwann cells.

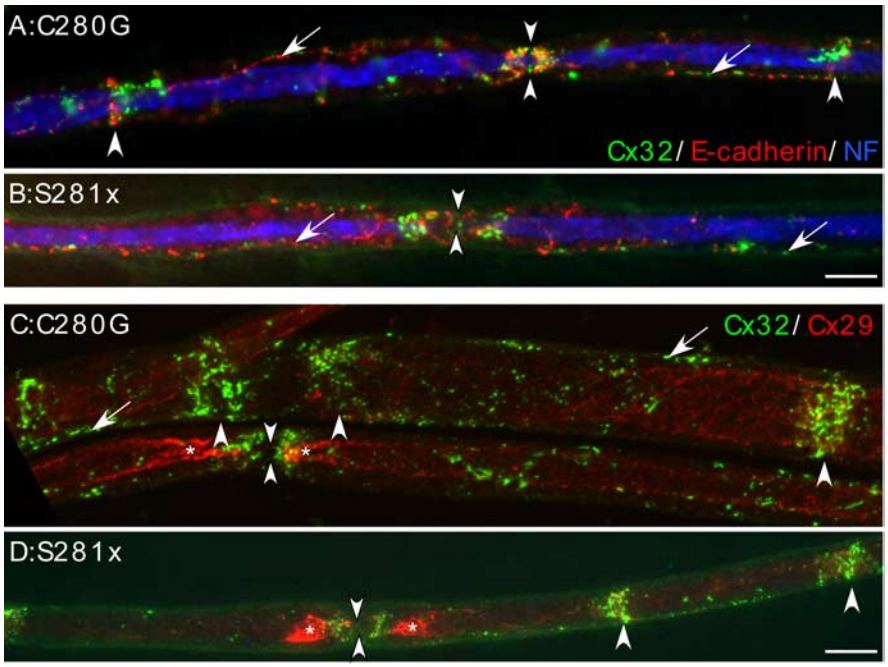

Figure 5. C280G and S281x mutants are localized to noncompact myelin in a wild-type background. These are images of unfixed teased fibers from lines 7M33 (C280G; ***A***, ***C***) and 8M25 (S281x; ***B***, ***D***). The fibers were immunostained with a mouse monoclonal antibody against Cx32 (green), a rat monoclonal antibody against E-cadherin (red; ***A***, ***B***), a chicken antiserum against neurofilament (blue; ***A***, ***B***), or a rabbit antiserum against Cx29 (red; ***C***, ***D***). In ***A*** and ***B***, note that Cx32 is localized to incisures (arrowheads), paranodes (the regions that flank the nodes of Ranvier; apposed arrowheads), and outer mesaxons (arrows); overlapping the distribution of E-cadherin. In ***C*** and ***D***, note that Cx32 colocalizes with Cx29 in incisures (arrowheads) but not in the juxtaparanodal region (asterisks). Scale bars, 10 μm.

We directly compared the levels of Cx32 protein in TG^+ and TG^- $Gjb1^{-/Y}$ mice for one line expressing the C280G mutation by immunoblotting. As shown in Figure 4*B*, the amount of Cx32 was higher in TG^+ nerves than in wild-type nerves (Scherer et al., 2005). Cx32 was not detected in TG^- *Gjb1*-null nerves. These results demonstrate that Cx32 is overexpressed in myelinating Schwann cells of TG^+ mice compared with wild-type mice.

C280G and S281x mutants rescue the phenotype of *Gjb1*-null nerves

To determine whether expressing the C280G and S281x mutants in myelinating Schwann cells prevents demyelination in $Gjb1^{-/Y}$ mice, we analyzed semithin sections of the sciatic and femoral nerves from TG^+ and TG^- $Gjb1^{-/Y}$ littermates (three lines C280G and one line S281x). We killed the mice at P158 because demyelination becomes prominent by this age (Anzini et al., 1997; Scherer et al., 1998; Scherer et al., 2005). Because myelinated motor fibers are much more affected than are myelinated sensory fibers in $Gjb1^{-/Y}$ mice, we focused on the femoral motor nerve because it is smaller and contains a higher proportion of motor axons than does the sciatic nerve (Boyd and Davey, 1968; Swett et al., 1986). These results are summarized in Table 2, and examples are shown in Figure 7 and supplemental Figure 2 (available at www.jneurosci.org as supplemental material). In all lines examined, TG^- mice had more demyelinated and remyelinated axons than did their TG^+ littermates. Similar results were found in the sciatic nerve (supplemental Fig. 3, available at www.jneurosci.org as supplemental material).

To substantiate this point, we performed a statistical analysis of the femoral motor branch. We counted all of the demyelinated, remyelinated, and normally myelinated axons in a single transverse section, combining the counts from the left and right nerves of individual animals. For each line, the odds of having an abnormally myelinated axon (demyelinated or remyelinated) in TG^+ mice was compared with that in TG^- mice. As summarized in Tables 2 and 3, the odds ratios were significantly different in all lines. Two of the lines (7M39 and 8M25), moreover, had similar odds ratio to line 90 (Table 3), which express wild-type human Cx32 (Scherer et al., 2005). These results indicate that the expression of either C280G or S281x can significantly reduced demyelination of motor axons of $Gjb1^{-/Y}$ mice, even comparable with that of wild-type human Cx32.

To determine whether C280G and S281x mutants can prevent demyelination comparable with wild-type human Cx32, we compared the proportion of abnormally myelinated fibers in TG^+ animals in a *Gjb1*-null background at P158. The difficulty with making such comparisons include that the experiments were done at separate times (several years apart), that each line was a distinct genetic entity, and that all of the sample sizes were small. As summarized in Table 3, there was no significant difference in the proportion of abnormally myelinated axons in one C280G (7M39) and one S281x (8M25) line compared with line 90. The two other lines that expressed C280G (7M37 and 7M33) had a significantly higher proportion of abnormally myelinated axons than did line 90. The proportion of abnormally myelinated axons in the TG^- mice was also significantly different in some lines, however, so the effects of the *Gjb1*-null gene may not be comparable between the different groups. Nevertheless, these data indicate that the expression of a C280G or S281x mutant can prevent the onset of demyelination until at least P158, comparable with the effect of expressing wild-type human Cx32. Whether this effect is sustained at older ages, and why there is variability be-

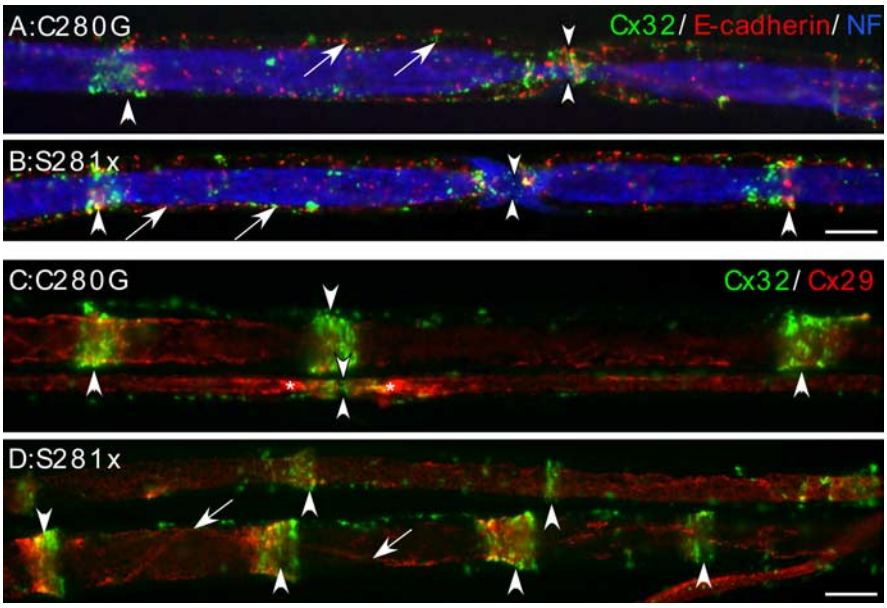

Figure 6. C280G and S281x mutants are localized to noncompact myelin in *Gjb1*-null mice. These are images of unfixed teased fibers from TG$^+$ *Gjb1*-null mice line 7M37 (C280G; ***A***, ***C***) and 8M25 (S281x; ***B***, ***D***). The fibers were immunostained with a mouse monoclonal antibody against Cx32 (green), a rat monoclonal antibody against E-cadherin (red; ***A***, ***B***), a chicken antiserum against neurofilament (blue; ***A***, ***B***), or a rabbit antiserum against Cx29 (red; ***C***, ***D***). In ***A*** and ***B***, note that Cx32 is localized to incisures (arrowheads), paranodes (the regions that flank the nodes of Ranvier; apposed arrowheads), and outer mesaxons (arrows); overlapping the distribution of E-cadherin. In ***C*** and ***D***, note that Cx32 colocalizes with Cx29 in incisures (arrowheads) but not in the juxtaparanodal region (asterisk) or inner mesaxon (arrows). Scale bars, 10 μm.

tween different lines that express the same mutant, remains to be determined.

Discussion

Cx32 is prenylated

Of the cloned mammalian connexins (Willecke et al., 2002), only Cx32 has a CaaX motif. Its conservation in Cx32 orthologs of mammals, birds, amphibians, and possibly some teleost fishes indicates that its function is conserved in evolution. As we show here, human Cx32 can be prenylated, and two CMT1X mutations are associated with disrupted prenylation. These are the first examples of CaaX mutations that are associated with an inherited disease. Mutations in the Rab escort protein-1 (*REP-1*), which encodes a component of geranylgeranyltransferase type II, cause choroideremia (Seabra et al., 1995). Because REP-1 mutations should disrupt the prenylation of a group of Rab proteins, this represents a different kind of prenylation defect. Whether Cx32 is prenylated in myelinating Schwann cells, the proportion that is prenylated, and whether prenylated Cx32 has a different cellular distribution and/or function remain to be shown. In a study examining the prenylation in myelin-related proteins from brain, a prenylated 21–27 kDa protein was described but not characterized; it could have been Cx32 (Sepp-Lorenzino et al., 1994). Prenylation of Cx32 remains to be demonstrated in mice or humans.

Why should Cx32 be prenylated? For cytosolic proteins, prenylation enhances their association with cell membranes, but the functional consequences are known only in a few examples besides mammalian *Ras*. Farnesylation increases the affinity of yeast *Ras2* for adenylate cyclase by ~100-fold (Kuroda et al., 1993); the γ subunit of trimeric G-proteins must be prenylated to interact with the other subunits (Iniguez-Lluhi et al., 1992; Casey, 1994; Kisselev et al., 1995), and the assembly of the large antigen of hepatitis δ virus is prenylation dependent (Glenn et al., 1992; Bordier and Glenn, 2004). How prenylation might affect intrinsic membrane proteins is less clear. Of the 200 proteins in human genome predicted to contain a CaaX motif (Fivaz and Meyer, 2003) (http://www.ensembl.org/Homo_sapiens/), <10 are likely to be intrinsic membrane proteins, and only one, a prostacyclin receptor, has been shown to be prenylated (Hayes et al., 1999). For this receptor, prenylation was required for the efficient coupling to its effectors (adenylate cyclase and phospholipase C) but had no effect on ligand-binding properties. Prenylation of Cx32 should cause its C terminus to become membrane associated; the functional consequences of this are unknown but do not include detectable alterations in its electrophysiological properties (Castro et al., 1999). It is possible that prenylation enables Cx32 to interact with other proteins in myelinating Schwann cells, but the localization of components of tight junctions (claudin-19) and adherens junctions (E-cadherin) as well as Cx29 and MAG were not disrupted in TG$^+$ mice, or in *Gjb1*-null mice for that matter (Arroyo et al., 1999).

Genotype-phenotype correlations

More than 260 different *GJB1* mutations have been described (http://www.mol-gen.ua.ac.be/CMTMutations/), affecting every domain of the Cx32 protein, the promoter and 3′ untranslated region, as well as deletions of the entire gene. None of the mutations that alter the amino acid sequence have been reported to be polymorphisms. Missense mutations alone affect 139 of 283 amino acids, emphasizing the importance of each amino acid. However, despite the large number of different mutations, the clinical severity caused by different mutations appears to be relatively uniform in affected men, including those with a deleted gene. Some mutations appear to cause exceptionally severe phenotypes, typically with early onset: R22stop (Ionasescu et al., 1996; Birouk et al., 1997), the double-mutation R22Q and V63I (Silander et al., 1998), V136A (Choi et al., 2004), 147 frameshift (Meggouh et al., 1998), C201R (Sillen et al., 1998), F235C (Lin et al., 1999), and the deletion nucleotides 265–273 deletion/frameshift (Ionasescu et al., 1996). E102G, N175D, and T191A have been reported to be associated with a milder phenotype (Silander et al., 1998; Kobari et al., 2000; Abrams et al., 2003). If these clinical observations are correct, then it will be of interest to establish the basis for both milder and more severe phenotypes. For the F235C mutation, for example, "leaky hemichannels" may be the cause of a more severe phenotype (Liang et al., 2005).

The similar clinical phenotypes of most CMT1X patients suggest that most *GJB1* mutations cause a loss of function. This issue has been analyzed by expressing mutants in heterologous cells, in which many mutants do not form functional channels (Abrams et al., 2000) and typically do not traffic to the cell membrane (Yum et al., 2002). Other mutants form functional channels with altered biophysical characteristics; two of these (S26L and M34T) maintain electrical coupling but have an apparent reduction in their pore diameter, which may prevent the diffusion of small molecules (Oh et al., 1997). These studies also demonstrate that the nature of the mutation may be important, because the R15W and H94Q mutants form normal channels, whereas R15Q and

H94Y do not (Abrams et al., 2001). Conversely, several disease-related mutants, including C280G and S281x, form fully functional channels (Castro et al., 1999; Abrams et al., 2001).

Why then do patients with the C280G and S281x mutations develop CMT1X? When we began these studies, we favored the possibility that these mutants would not traffic normally in myelinating Schwann cells, just as certain rhodopsin mutants that cause retinitis pigmentosa (inherited retinal degenerative diseases of diverse genetic causes) function normally in transfected cells but do not traffic properly in rods (Sung et al., 1994). Instead, we found that the C280G and S281x mutants appear to traffic normally and could even rescue/delay demyelination in motor axons of *Gjb1*-null mice. Other Cx32 mutants are expressed and localized differently: 175 frameshift cannot be detected (Abel et al., 1999), and R142W accumulates in the Golgi (Bone and Scherer, unpublished). Thus, the effects of the C280G and S281x mutants are different from these other mutants but are similar to the effects of expressing wild-type human Cx32 in myelinating Schwann cells of *Gjb1*-null mice (Scherer et al., 2005). Thus, why the C280G and S281x mutants cause CMT1X remains to be determined. It should be noted that it is difficult to compare the findings of mice with those in humans because humans have longer nerves and live much longer, and there is no information about the pathological changes in nerves from children with CMTX. Prenylation could affect Cx32 in some important way; possibilities include a decreased half-life in the myelin sheath or interactions with other proteins.

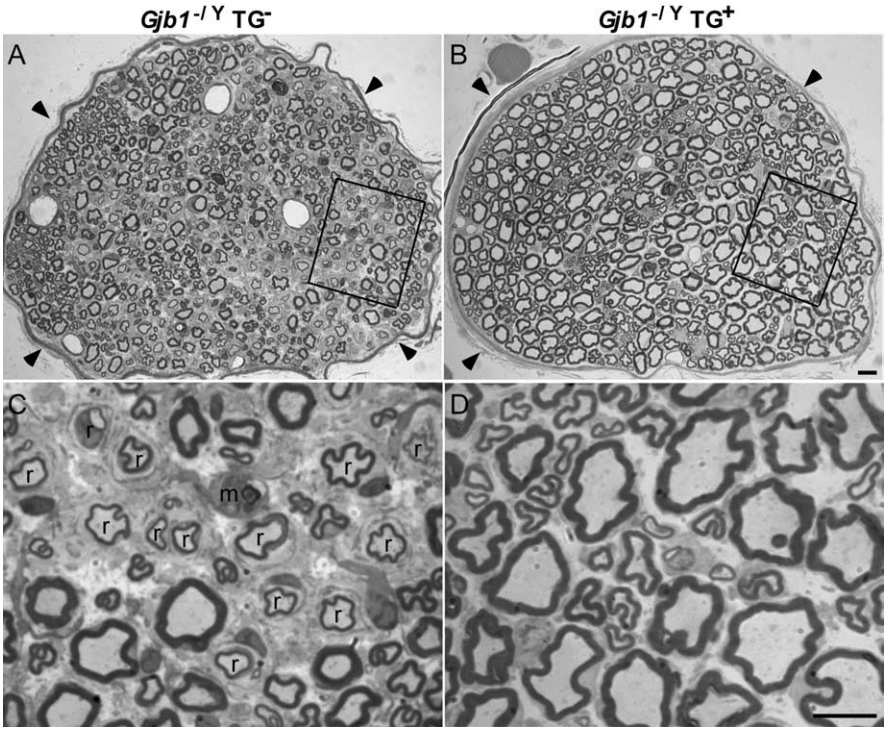

Figure 7. Transgenic expression of C280G mutant protein rescues demyelination in *Gjb1*-null mice. These are images of semithin sections of the femoral motor branch from TG^+ or TG^- $Gjb1^{-/Y}$ littermates from line 7M33 (at P158). The myelinated axons appear normal in the TG^+ nerves, whereas the TG^- nerve has remyelinated (r) axons and macrophages containing myelin debris (m). The areas outlined by the rectangles in ***A*** and ***B*** are enlarged in ***C*** and ***D*** (***C*** and ***D*** are rotated in opposite directions). Scale bars, 10 μm.

Based on their effects in *Gjb1*-null mice, one might anticipate that patients with the C280G or S281x mutations would have a milder phenotype, but this does not seem to be the case. Dr. Peter De Jonghe (University of Antwerp, Antwerp, Belgium) kindly provided some clinical information on a male patient with the S281x mutation. As a child, he could not run as fast as his peers and had problems with fine hand movements. He used orthoses at age 30 because of severe weakness in ankle plantar extension (weakness in the tibialis anterior muscle results in "foot drop"). Clinical exam at age 40 demonstrated severe weakness and atrophy of muscles below the knees and in the hands, typical of CMT1X (Hahn et al., 1990). The patient's affected mother and the affected sister remain only slightly symptomatic (personal communication). Thus, these clinical data do not support the idea that the S281x mutation results in a milder phenotype because it causes a partial loss of function.

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