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RESEARCH ARTICLE

Myelin protein zero mutations and the unfolded protein response in Charcot Marie Tooth disease type 1B

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Abstract

Objective: To determine the prevalence of *MPZ* mutations that cause Charcot Marie Tooth neuropathy type 1B (CMT1B) and activate the unfolded protein Response (UPR). **Background**: CMT1B is caused by >200 heterozygous mutations in *MPZ*, the major protein in peripheral nerve myelin. Mutations Ser63del MPZ and Arg98Cys MPZ cause the mutant protein to be retained in the ER and activate the generally adaptive UPR. Treatments that modulate UPR activation have improved cellular and rodent models of CMT1B raising the possibility that other *MPZ* mutations that activate the UPR would also respond favorably to similar treatment. The prevalence of MPZ mutations that activate the UPR is unknown. **Methods**: We developed a dual luciferase reporter assay of Xbp1 splicing using stably transfected RT4 Schwann cells to assay the ability of cDNA constructs bearing 46 distinct MPZ mutations to activate the UPR. Constructs also carried an HA tag to permit detection of ER retention of mutant proteins. UPR activation and ER retention were correlated with clinical phenotypes. **Results**: Eighteen mutations demonstrated ER retention and UPR activation to a similar degree as Ser63del and Arg98Cys MPZ. Thirty-five of the mutations activated the UPR > 1.5 fold compared to that of wild-type MPZ. Correlation was high between firefly and Nano-luciferase reporters and between both reporters and ER localization. UPR activity did not correlate with clinical onset or severity. **Conclusion**: Many CMT1B causing mutations activate the UPR and may be susceptible to therapeutic efforts to facilitate UPR function.

Introduction

Charcot Marie Tooth disease type 1B (CMT1B) is the second most frequent form of CMT1 and is caused by mutations in myelin protein zero (*MPZ*). More than 200 different mutations that cause CMT1B have been identified and these present with one of three characteristic phenotypes depending on the mutation. One large group presents in infants or toddlers who have delayed early milestones, very slow nerve conduction velocities (NCV) and pronounced disability. A second large group, also classifiable as CMT2i, presents in adulthood with normal or near normal NCV, and a milder phenotype. A third, smaller group, present with slow NCV and a slowly progressive neuropathy, similar to the phenotype found in patients with the most common form of CMT, CMT1A.[1]

How particular *MPZ* mutations cause neuropathy is not known. However, several mutations cause the mutant protein to be retained in the endoplasmic reticulum (ER) rather than being transported to the cell membrane or myelin sheath. Examples include in vitro studies of MpzSer51delTrp57,[2] 506delT and 550del3insG[3] and in vivo reports of Ser63del and Arg98Cys MPZ mice.[4–6] ER retention in both mouse models activated a canonical unfolded protein response (UPR).[4,5] UPR activation reduces the load of unfolded proteins through upregulation of chaperones, attenuation of protein synthesis and

increased protein degradation.[7] Treatments directed at modulating UPR activation have led to improvement in both the Ser63del;[8] and Arg98Cys[9] MPZ mice. More recently, treatment with Sephin1, a selective inhibitor of the Gadd34 holophosphatase, prolonged eIF2α phosphorylation in the PERK arm of the UPR and prevented the molecular, motor and morphological abnormalities of the neuropathy of Ser63del Mpz mice.[10] Taken together these data suggest that manipulating UPR activation may be a viable therapeutic option for at least some patients with MPZ mutations. However, it is not known how many *MPZ* mutations cause ER retention of the mutant protein and/or activation of the UPR.

To determine this prevalence we developed an in vitro assay to identify how many CMT1B causing mutations resulted in ER retention and UPR activation in a group of 46 *MPZ* mutations found in patients evaluated by the Inherited Neuropathy Consortium (INC), a member of the Rare Disease Clinical Research Network (RDCRN). Clinical investigators in the INC had evaluated and reported on these patients and their respective symptoms, signs and physiology.[1] We report our findings in the present manuscript.

Material and Methods

Site directed mutagenesis and generation of mutant MPZ plasmids

In order to obtain wild-type human MPZ we performed 2 mm punch skin biopsies from a normal control, without CMT, using methods previously described[11] and the biopsy was immediately placed into RNALater (Cat. #:1017980, QIAGEN GmbH, Hilden, Germany). RNA was isolated with NucleoSpin RNA (REF #:740955 Macherey-Nagel GmbH & Co. KG, Duren, Germany) and reverse transcribed using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Cat. #: 11752, Invitrogen, Carlsbad, CA 92008). The PCR reaction to isolate MPZ was performed with Platinum Taq DNA Polymerase High Fidelity(Cat. #: 11304, Invitrogen, Carlsbad, CA 92008). The upstream primer was in the 5′UTR and the downstream primer was in the 3′UTR. After Sanger sequencing to verify the plasmid sequence, an HA tag was fused, in frame to MPZ

(hMPZwt-HA-pME) to generate the wild-type MPZ-HA plasmid for use in transfections. Site directed mutagenesis was then performed to generate the 46 mutant constructs to be used in subsequent transfections. Site directed mutagenesis was performed with overlap PCRs in which primers were designed bearing the respective mutation. Products underwent Sanger sequencing to verify the predicted mutation and the plasmids were then purified and used for the transient transfections described below. (HiPure Plasmid Filter Maxiprep Kit, Cat. #: K210016, Invitrogen Carlsbad, CA 92008 USA).

Stable Transfection to create Xbp1 reporter lines

A genomic region containing exons and introns of the mouse Xbp1 gene (mm10 chr11:5,520,995-5,524,904) was amplified using these primers:

F:CTCTATCGATAGCCCATATATGGAGTTC; R:TCTG AGCTCAACTAGAGGCTTGGTGTA. The product was digested with ClaI/SacI and cloned into two existing reporter constructs containing the CMV promoter and firefly luciferase, nano-luciferase, and puromycin resistance genes, each separated by 2a ribosome stuttering sequence, similar to previously described reporters.[12] The completed construct has the following structure: CMV-Xbp1-fLuc2aNluc2aPuro.

RT4 Schwann cells were transfected with a 2 μg plasmid using the Lipofectamine 3000 transfection kit (Invitrogen, Cat# L3000-008). After transfection, we selected and expanded stable clones using 1 μg/mL puromycin. Then, selected clones were transferred and assessed for expression of nano and firefly luciferase. The selected clones were allowed to grow for 24 h before introduction of Tunicamycin (Sigma, Cat#150028). XBP1 was cloned so that its coding sequence would come into frame with the reporters only if there was alternative splicing induced through activation of the IRE1α arm of the UPR.

Assaying UPR activity

Tunicamycin was added at 5 μg/mL, 10ug/mL, and Thapsigargin (Santa Cruz Biotech, Cat# SC 24017) was added at 1 μmol/L and 10 μmol/L to wells in 12-well plates containing the RT4 cell lines. RT4 cells in plain media not containing the luciferase reporters served as negative controls. Wells were incubated with tunicamycin and thapsigargin for 6 h, then the media was removed and each well was washed with 1xPBS before adding 150 μL of 1x passive lysis buffer. To assess firefly luciferase activity, we added 50 μL of cell lysate to a luminometer tube and then added 50 μl LARII (Promega, Dual luciferase reporter assay system, Cat# E1910). For the Nano assay, we added 50 μL of cell lysate to the luminometer tube then added 50 μL of Nano substrate solution (Promega, Nano-glo luciferase assay, Cat# N1110) before taking a measurement value with the luminometer (Monolight 3010).

Cell culture, transfection, and immunocytochemistry

RT4 Schwann cells were grown on 4-well chamber slides in Dulbecco's modified Eagle medium (DMEM) supplemented

with 5% bovine growth serum and 1% penicillin–streptomycin and then transfected with 2 μg MPZ plasmid DNA using Lipofectamine™ 3000 kit. 48 h after transfection, immunocytochemistry was carried out.[9] In brief, cells were rinsed twice in PBS for 5 min, fixed in 4% paraformaldehyde for 5 min and washed again three times in 1xPBS. Cells were then incubated with primary antibodies at 4°C overnight: Anti-hemagglutinin (anti-HA), 1:1000 (Cell Signaling Technology® Cat#2367 (mouse); #3724(rabbit)); anti-calnexin (marker for endoplasmic reticulum), 1:300 (Abcam, Cat#ab22595). Following three 10-min washes in 1xPBS and 1 h incubation with secondary antibodies (Cy3-conjugated donkey anti-rabbit IgG, Jackson Immuno Research,cat#711-165-152 or anti- mouse, Cat#711-165-1521; Fluorescein (FITC)-conjugated AffiniPure Donkey anti-mouse IgG, cat#712-095-151; or anti-rabbit IgG cat#711-095-152), the coverslips were mounted using Anti-Fade 4-6-diamino-2-phenylindole (DAPI) mounting media that labels cell nuclei (Invitrogen Cat# P36931).

To determine the percentage of cell expression with endoplasmic reticulum retention of wild-type or mutant MPZ, transfected RT4 cells were imaged under 40X objective lens of the microscope (Zeiss 710) for each MPZ mutant group; HA-tag labeled cells were counted as a positively transfected cell. The number of colocalized cells showing overlap of HA-MPZ and ER were divided by the total number of transfected cells to obtain percentage of colocalization.

Cell culture, cotransfection, luciferase assay analyses

RT4 Schwann cells were grown on a 12-well plate in Dulbecco's modified Eagle medium supplemented with 5% bovine growth serum and 1% penicillin–streptomycin and cotransfected with 1.9 μg MPZ and 0.1 μg CMV-lacz plasmid DNA using Lipofectamine™ 3000. Triplicate wells were run for each group. Twenty-four hours after transfection, firefly and nano luciferase assays were carried out as described above. Detection of β-galactosidase reporter enzyme was run following the instructions of Galacto-light plus β-galactosidase reporter gene assay system (Thermo Fisher scientific, Cat# T1007). The Firefly/Nano luciferase measurement value was divided by the β-galactosidase reporter enzyme measurement to obtain the final firefly/Nano luciferase value.

Results

RT4 Reporter Cells activate XBP1 splicing when incubated with tunicamycin or thapsigargin

We stably transfected firefly and Nano-luciferase reporters fused out of frame to XBP-1 into RT4 rat Schwann cells, which express relatively high levels of myelin genes. The two luciferase reporters are fused in frame with an intervening 2a ribosome stuttering sequence, and both are pulled into frame by XBP-1 splicing in the cytoplasm that occurs following activation of the IRE1-α arm of the UPR. We tested the ability of the transfected RT4 reporter cells to detect UPR activation by incubating them with either thapsigargin or tunicamycin, compounds known to induce the UPR in cells.[13] Both thapsigargin and tunicamycin significantly increased both firefly and nano luciferase expression in the cells suggesting that they were effectively activating the IRE1-α arm of the UPR (Fig. 1).

Ser63del and Arg98Cys MPZ activate the UPR in RT4 Reporter Cells

Ser63del MPZ[4] and Arg98Cys MPZ[5] activate the UPR in mice and cause CMT1B.[1] We therefore investigated whether these mutations would activate the UPR in our RT4 cell lines. cDNA constructs of the two mutations were transiently transfected into the RT4 cells and both firefly and Nano luciferase expression was compared to that of transfections with wild-type MPZ cDNA. The cells were all cotransfected with a plasmid containing the lacZ gene driven by the CMV promoter to normalize results for transfection efficiency. Both firefly and Nano luciferase levels were approximately twice that obtained from transfections with wild-type MPZ (Fig. 2A). In addition, we have placed an HA tag on the 3′ end of each of the MPZ constructs to visualize whether the mutant MPZ was retained within the ER, as we would predict if the mutations activated the UPR through ER stress. Expression of the HA tag was identified in the ER in a significantly higher number of cells transfected with Ser63del and Arg98Cys compared to cells transfected with wild-type MPZ (Fig. 2B).

Most CMT1B mutations activate the IREα arm of the UPR

To determine which CMT1B mutations cause UPR activation, we generated cDNA constructs for each of the 46 disease causing mutations evaluated by members of the INC. We selected these mutations because we had characterized and reported the clinical phenotype for 103 patients bearing these mutations in our clinics.[1] We then transfected the respective constructs into the RT4 reporter cell line and compared the luciferase levels to transfections with those from wild-type MPZ as we had done with Ser63del and Arg98Cys MPZ. Thirty five of the 46 (76%) mutations caused an increase in luciferase expression 1.5 fold higher than wild-type MPZ in at least one of the two reporters and 18 (39%) expressed equal or

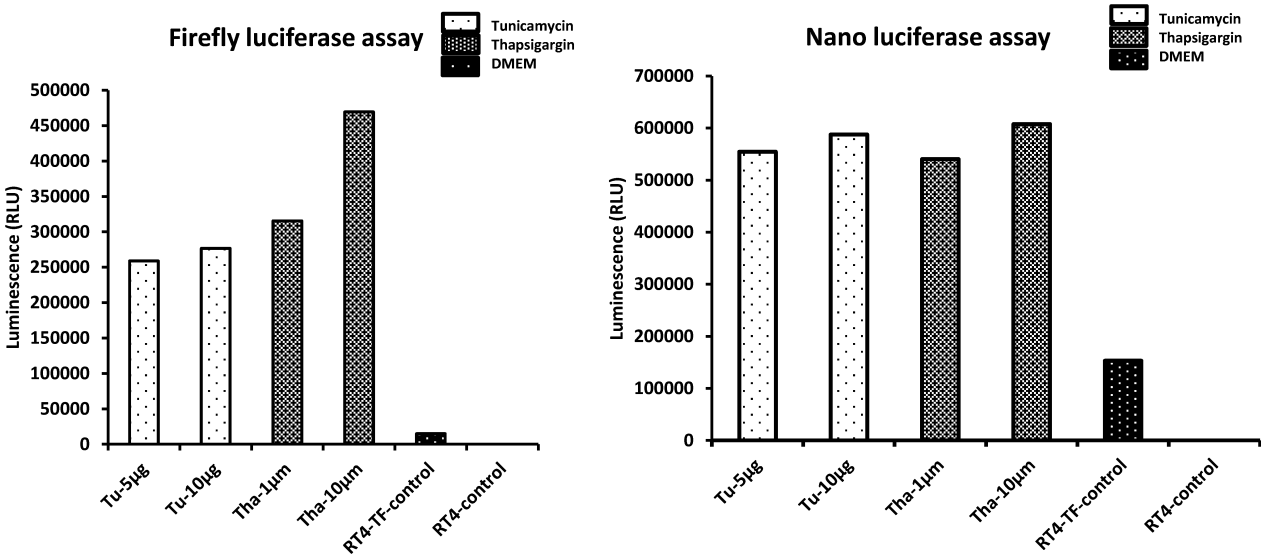

Figure 1. Thapsigargin and tunicamycin significantly increase firefly and nano luciferease expression in RT4 reporter cells. The transfected RT4 reporter cells were incubated for 6 h with thapsigargin (1 μmol/L and 10 μmol/L), tunicamycin (5 μmol/L and 10 μmol/L) or DMEM. Results showed that both thapsigargin and tunicamycin increased firefly and nano luciferease expression compared to controls demonstrating that the UPR was activated in these cells.

higher levels of luciferase than did either Ser63del or Arg98Cys MPZ, known activators of the UPR (Fig. 3).

We next compared firefly and nano luciferase expression following the transfections to determine whether characteristics of either reporter could be influencing our data. The correlation between the firefly and Nano-luciferase data was high (ρ =0.984, $P < 0.05$)(Fig. 4). Because each of the constructs contained an HA tag we also investigated the number of mutations causing ER retention compared to results from wild-type MPZ constructs. Virtually all mutations that increased luciferase expression caused ER retention of the transfected protein (Fig. 4, Table 1). The correlation between intracellular retention and luciferase activity was also high (ρ = 0.864 for firefly vs. IHC and 0.861 for Nano luciferase vs. IHC, $P < 0.05$ for both). The one outlier mutation (Val102 fs MPZ) differed from the other mutations because almost no transfected cells could be identified, presumably due to nonsense mediated decay of the truncated protein. The relatively rare transfected cells that we could find demonstrated HA labeling in the ER, so that the percentage of transfected cells with ER retention by IHC was high despite the paucity of transfected cells.

UPR activation level does not correlate with clinical phenotype

We next investigated whether UPR activity correlated with clinical phenotype based on our previous clinical evaluation of patients with the 46 different mutations.[1] We identified similar percentages of UPR activation for mutations associated with infantile, childhood or adult onset forms of CMT1B (Fig. 5). For example, both the Thr65Asp and Arg227Ser *MPZ* mutations activate the UPR more than twofold more than wild-type MPZ. However, our proband for the Thr65Asp mutation was a 13-year-old girl who could not walk independently until 19 months of age, was unable to run after 5 years of age and had median motor nerve conduction velocities of 6 meters/sec. Alternatively, the proband for the Arg227Ser mutation was a 44-year-old man who walked by a year, was a fast runner as a child playing on his varsity football team in high school, who did not develop symptoms until 40 years of age and had median MNCV of 32 met/sec.[1] We also identified no correlation between UPR activation and disease severity, as measured by the CMTNS (Fig. 6). Therefore, the degree of UPR activation did not correlate with disease severity or age of onset in patients with CMT1B.

Discussion

We have developed a dual luciferase reporter assay in RT4 cells to estimate the percentage of MPZ mutations causing CMT1B that activate the IRE1α arm of the UPR. We transfected cDNA constructs with 46 different MPZ mutations that cause CMT1B in patients we evaluate within the INC.[1] This permitted us to correlate UPR activation in our assays with the clinical presentations of the patients. Previous studies have shown that Ser63del and

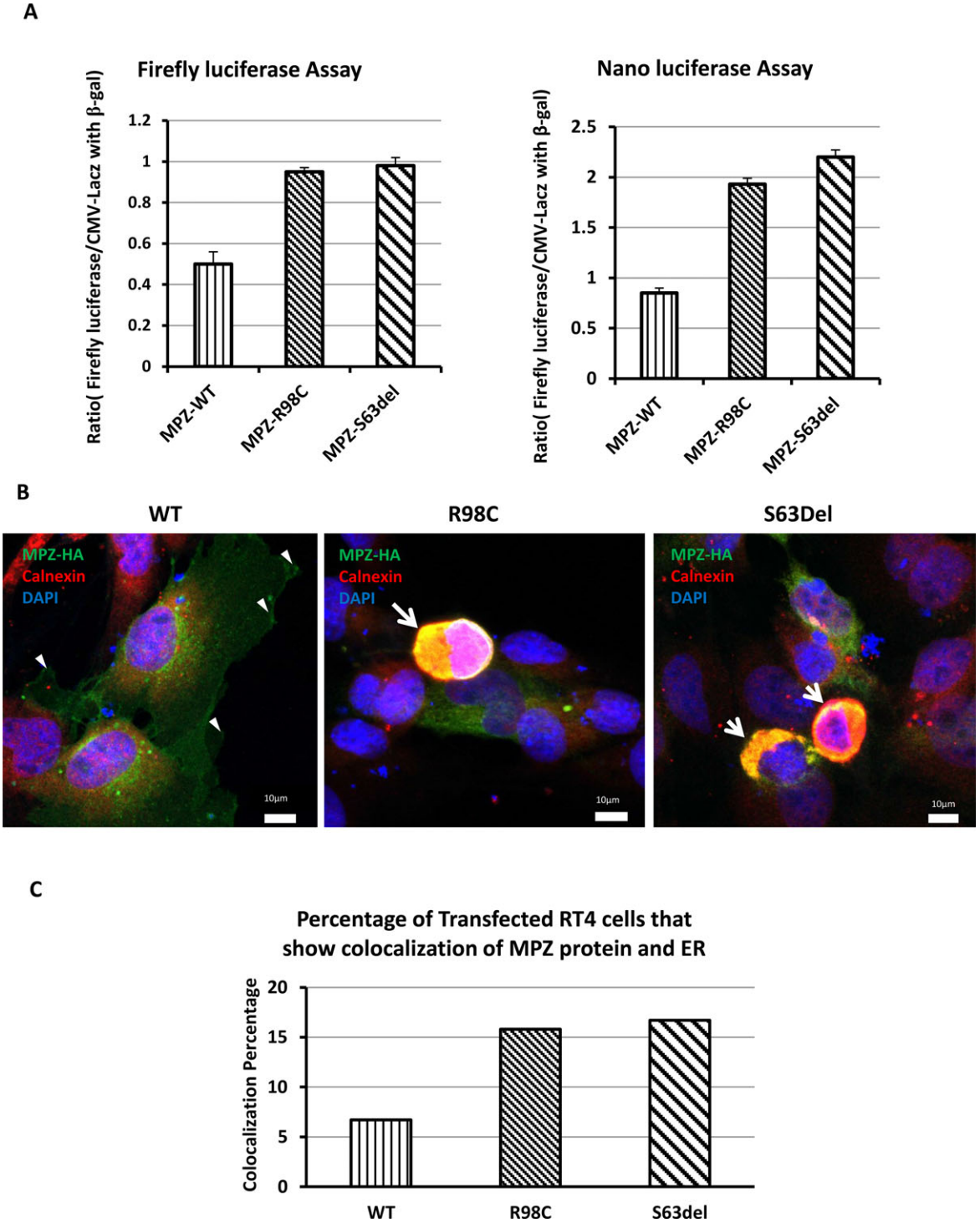

Figure 2. Ser63del(S63Del) and Arg98Cys(R98C) MPZ activate the UPR. Dual reporter RT4 cells were transiently transfected with cDNAs expressing wild-type, Ser63del(S63Del) or Arg98Cys(R98C) MPZ. Results showed that both firefly and Nano luciferase levels were approximately twice that obtained from transfections with wild-type MPZ (Figure 2A). WT MPZ transfected cells in merged confocal immunostaining images showed localization of MPZ protein to the cell membrance (arrow heads) with low ER retention. Mutant Ser63del(S63Del) and Arg98Cys(R98C) MPZ aggregates in the ER(arrows) and fail to localize to the plasma membrance (Figure 2B). The colocalization of immunostaining with the HA tag and ER retention was identified in significantly more cells transfected with Ser63del(S63Del) and Arg98Cys(R98C) compared to cells transfected with wild-type MPZ (Figure 2C).

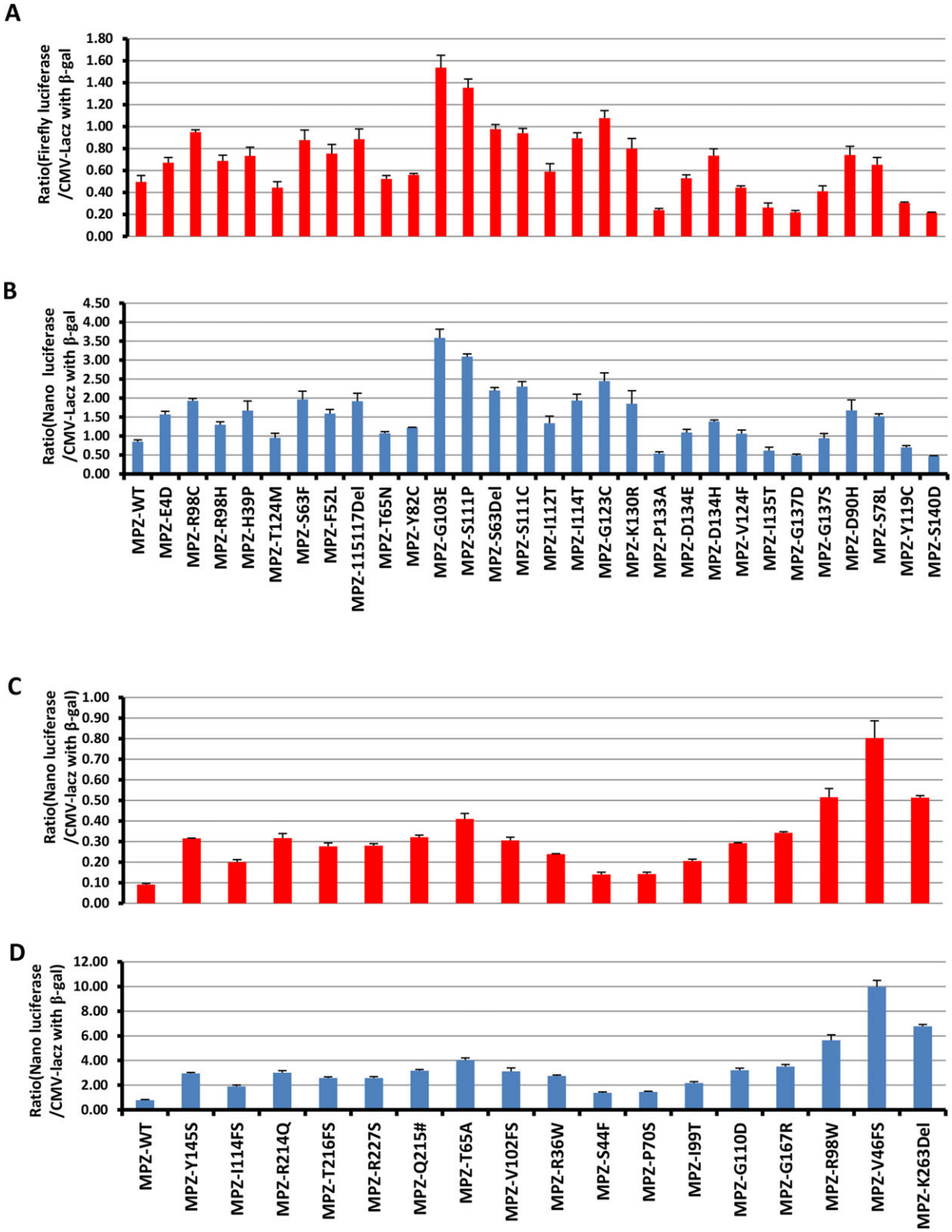

Figure 3. Most CMT1B mutations activate the IREα arm of the UPR. RT4 Reporter cells were cotransfected with cDNAs of wild type, or 46 different mutations in MPZ and results were normalized with the cDNA from the *lacZ* gene. Both firely and Nano luciferase levels were higher in most CMT1B mutation than wild-type MPZ (Figure 3A-D).

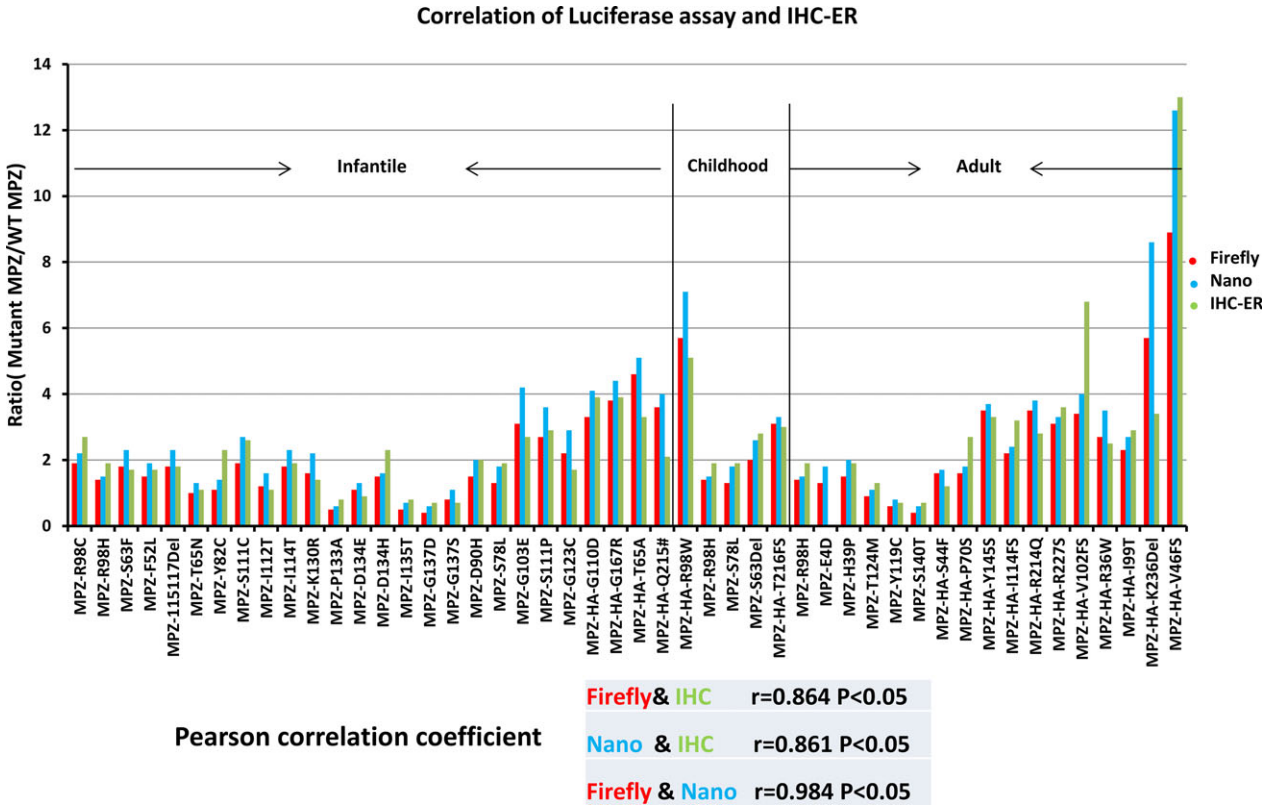

Figure 4. The comparison of the ratio of firefly and nano luciferase as well as the comparison of ER retention by immunohistochemisty to wild-type levels (Mutant MPZ/WT MPZ) are presented in bar graphs. Correlations between the firefly and nano-luciferase data (ρ = 0.984, $P < 0.05$) were significant. Correlations between intracellular ER retention and luciferase activity was also significant (ρ =0.864 for firefly vs. IHC and 0.861 for nano luciferase vs. IHC, $P < 0.05$ for both).

Arg98Cys MPZ cause UPR activation and that tuning of the UPR results in improvement of cellular and rodent disease models in mice with these mutations.[4,5,8–10] Therefore, we believed it appropriate to consider any values higher than those observed from Ser63del and Arg98Cys MPZ as significantly activating the UPR. Eighteen of the 46 (39%) of the mutations caused an elevation at least 1.8 fold higher than wild MPZ in our assay, the values obtained for Ser63del and Arg98Cys MPZ mutations. If we reduced the threshold to 1.5 times wild-type activity, 76% (35/46) mutations caused activation of the UPR. Taken together, our results demonstrate that UPR activation is present in a large number of MPZ mutations that cause CMT1B.

We were not surprised that many mutations in MPZ activate the UPR considering that Schwann cells must function as cellular "factories" to generate myelin. Myelinating Schwann cells increase their plasma membranes several thousand fold to generate a myelin sheath which requires a marked upregulation of protein and lipid levels.[14] MPZ itself comprises up to 50% of all myelin proteins[15] and approximately 2% of all Schwann Cell transcripts during the peak of myelination.[16] Normally, newly synthesized MPZ is folded and post-translationally modified into its native conformation by ER-protein quality control pathways (ERQC) that consist of various chaperones and other molecules.[17,18] Subsequently MPZ is processed through the Golgi, packaged into vesicles and transported to the myelin sheath.[19] If MPZ cannot be properly folded the protein is not transported to the Golgi but instead is targeted to ER associated degradation (ERAD) pathways that dispose of the abnormal protein through various degradation processes such as ubiquitin proteasome or lysosomal proteolysis pathways. When the level of misfolded proteins like mutant MPZ exceed the capacity of the ER to fold or degrade the proteins through standard ERAD pathways the UPR is then activated as an adaptive and protective process for the cell and ER to deal with the misfolded proteins.[20] Thus, the UPR is a protective adaptive process for cells in stress. When cells are in persistent stress, such as with neurodegenerative disorders like CMT1B, a transient UPR

Table 1. Summary of luciferase assay and Immunohistochemistry.

Mut-MPZ	Firefly[1]	Nano[2]	IHC-ER[3]	Mut-MPZ	Firefly	Nano	IHC-ER	Mut-MPZ	Firefly	Nano	IHC-ER
MPZ-G137D	0.4	0.6	0.7	MPZ-F52L	1.5	1.9	1.7	MPZ-S111P	2.7	3.6	2.9
MPZ-S140D	0.4	0.6	0.7	MPZ-D134H	1.5	1.6	2.3	MPZ-HA-R36W	2.7	3.5	2.5
MPZ-I135T	0.5	0.7	0.8	MPZ-D90H	1.5	2	2	MPZ-G103E	3.1	4.2	2.7
MPZ-P133A	0.5	0.6	0.8	MPZ-K130R	1.6	2.2	1.4	MPZ-HA-T216FS	3.1	3.3	3
MPZ-Y119C	0.6	0.8	0.7	MPZ-HA-S44F	1.6	1.7	1.2	MPZ-HA-R227S	3.1	3.3	3.6
MPZ-G137S	0.8	1.1	0.7	MPZ-HA-P70S	1.6	1.8	2.7	MPZ-HA-G110D	3.3	4.1	3.9
MPZ-T124M	0.9	1.1	1.3	MPZ-I114T	1.8	2.3	1.9	MPZ-HA-V102FS	3.4	4	6.8
MPZ-T65N	1	1.3	1.1	MPZ-S63F	1.8	2.3	1.7	MPZ-HA-Y145S	3.5	3.7	3.3
MPZ-Y82C	1.1	1.4	2.3	MPZ-115117Del	1.8	2.3	1.8	MPZ-HA-R214Q	3.5	3.8	2.8
MPZ-D134E	1.1	1.3	0.9	MPZ-S111C	1.9	2.7	2.6	MPZ-HA-Q215#	3.6	4	2.1
MPZ-I112T	1.2	1.6	1.1	MPZ-R98C	1.9	2.2	2.7	MPZ-HA-G167R	3.8	4.4	3.9
MPZ-E4D	1.3	1.8	NA	MPZ-S63Del	2	2.6	2.8	MPZ-HA-T65A	4.6	5.1	3.3
MPZ-S78L	1.3	1.8	1.9	MPZ-G123C	2.2	2.9	1.7	MPZ-HA-R98W	5.7	7.1	5.1
MPZ-R98H	1.4	1.5	1.9	MPZ-HA-I114FS	2.2	2.4	3.2	MPZ-HA-K263Del	5.7	8.6	3.4
MPZ-H39P	1.5	2	1.9	MPZ-HA-I99T	2.3	2.7	2.9	MPZ-HA-V46FS	8.9	12.6	13

[1,2]: Luciferase ratio (mutant MPZ luciferase/wt MPZ luciferase)
[3]: IHC ratio (mutant MPZ ER aggregate/wt ER aggregate)
NA: No-HA tag labeling
Highlight color: based on the data of firefly luciferase.
0–1.5 Yellow.
1.6–3.9 Orange.
4–5.9 Green.
6–9 Blue.

response to facilitate ERAD processes becomes inadequate to deal with chronically expressed high levels of mutated proteins such as MPZ that cannot be readily folded or processed for degradation. In these circumstances UPR signaling may either activate apoptotic pathways, resulting in death of the cell or in signaling that alters the normal phenotype of the cell. For example Schwann cells in Arg98Cys MPZ mice develop a novel phenotype that is distinct from that of both myelinating and premyelinating Schwann cells.[5] This again is an adaptive response by the cell to protect it from conditions of extreme ER stress.

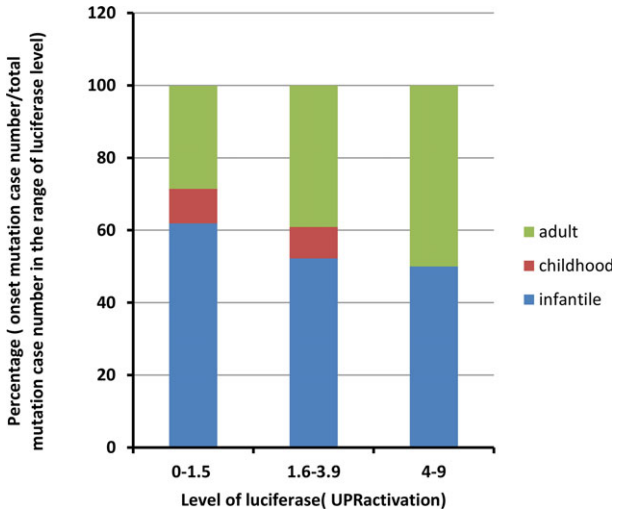

Figure 5. Lack of correlation between disease onset and UPR activation. Luciferase levels do not correlate with infantile, childhood or adult onset CMT1B. The distribution of luciferase levels compared to wild-type MPZ is similar in infantile, childhood and adult CMT1B.

While our data demonstrate that many MPZ mutations activate the stress response of the UPR, this activation is not sufficient to allow normal myelination as patients with CMT1B all develop peripheral neuropathy which in many cases is severe. The UPR therefore requires further assistance to more efficiently process misfolded proteins in these patients. Sephin1 prolongs eIF2α phosphorylation in the protein-kinase RNA-like endoplasmic reticulum kinase (PERK) arm of the UPR, largely rescuing the phenotype of Ser63del MPZ mice.[10] In contrast, blocking eIF2α phosphorylation severely worsens the neuropathy at least in the Ser63del MPZ model,[21] as is also the case when one ablates XBP1.[22] Taken together these data suggest that boosting the UPR further with a compound like Sephin1 may be an effective approach to improve myelination in other cases of CMT1B in which the UPR is active. Prolonging eIF2α phosphorylation in the PERK pathway leads to overall attenuation of translation. While it may seem counter-intuitive to consider this as a therapeutic strategy, translational attenuation will ultimately result in reduced levels of mutant protein in the ER

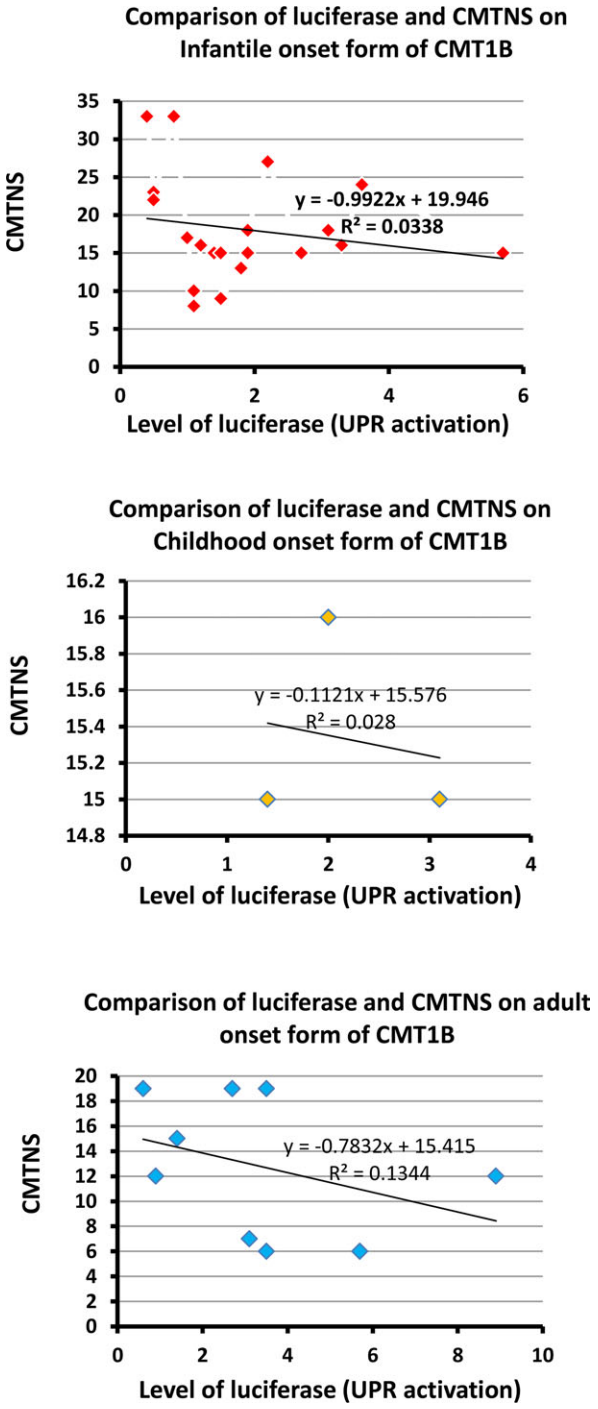

Figure 6. Lack of correlation between disease severity and UPR activation. Correlations between CMT severity, defined by the CMTNS, and UPR activation, measured by luciferase levels, are not significant and do not differ between cases of infantile, childhood or adult onset of neuropathy.

which may therefore allow chaperones and foldases to better perform their normal function of proper folding and delivery of wild-type MPZ and other myelin proteins such as PMP22 to myelin. In this situation the mutant, but not wild-type MPZ would be degraded by ERQC pathways.[23] Moreover, it has been shown that Ser63del MPZ acts as a dominant negative that also results in wild-type MPZ retention in the ER[24]; as such reducing S63del translation may permit more wild-type MPZ to be released to reach the myelin membrane. Fifty percent of normal MPZ is known to be adequate for normal myelin formation as haploinsufficiency of MPZ does not cause a clinical neuropathy in patients.[25,26] Whether less than 50% of normal MPZ will also permit compact myelin formation is not yet known.

We have demonstrated in this study that mutations causing early, traditional and late onset neuropathies can all activate the UPR. Moreover, many prior reports have outlined other potential pathogenic mechanisms including disruption of compaction,[27] myelin packing,[28] signaling from the cytoplasmic domain,[29] disruption of adhesion,[2] or disruption of glycosylation.[30] Therefore, we recognize that UPR activation by itself cannot be the sole cause of the phenotypes of CMT1B and that these other mechanisms likely participate as well. Our hypothesis is that reducing protein toxicity alleviating the ER-stress can potentially improve the neuropathy caused by these different mutations potentially by increasing the capacity of the UPR to process the mutant MPZ and allowing increased levels of wild-type MPZ to reach myelin.

Finally, we recognize that our studies were performed in an in vitro system that may have limitations. In preliminary studies, we have demonstrated UPR activity in vivo by detecting CHOP activity in the nuclei of myelinating Schwann cells from skin biopsies of patients with Ser63del MPZ (Wrabetz and Shy unpublished). We have also obtained similar results from patients with Thr124Met mutations, which fell below the level of Ser63del or Arg98Cys MPZ in our assays, suggesting that our 1.8 X WT level threshold may be somewhat artificial. We will continue these studies in human biopsies to determine how these results correlate with our in vitro assays although this will take time to complete. Unfortunately, antibodies to XBP1 have not worked well in human tissue so that we are unable to compare our in vitro results with XBP1 directly in patients. Nevertheless, we believe that our data suggest that many MPZ mutations activate the UPR and that therapies directed at modifying UPR activity many benefit patients with many different mutations. We also believe that our assay using permanently transfected RT4 cells could be used to identify candidate drugs that would reduce the UPR in small molecule screens. Moreover, given the strong correlation between luciferase expression and ER localization, testing assays could be designed to measure levels of mutant protein aggregation within the ER using fluorescent tags.

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Conflict of Interest

No author had a conflict of interest to declare for any of the experiments in this manuscript.

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