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New Concepts

Mutations of Peripheral Myelin Protein 22 Result in Defective Trafficking through Mechanisms Which May Be Common to Diseases Involving Tetraspan Membrane Proteins†

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ABSTRACT: Phenotypes of several heritable disorders including forms of hearing loss, myelin diseases, hypomagnesemia, and cataracts are linked to missense mutations in single alleles encoding membrane proteins having four transmembrane spans. In some cases, the mutant proteins exhibit dominant negative or gain-of-function behavior whereby heterozygous coexpression of mutant and wild-type genes leads to more serious pathology than is the case for individuals in which only a single wild-type allele is expressed. An example is found in the relationship of peripheral myelin protein 22 (PMP22) to Charcot−Marie−Tooth disease (CMTD) type 1A. A number of disease-linked PMP22 mutants fail to undergo normal trafficking beyond the endoplasmic reticulum or intermediate compartment to reach the cell surface. Moreover, recent evidence suggests that pathology resulting from this mistrafficking-based loss of function may also be augmented by the ability of some mutants to disrupt normal trafficking of the product of the wild-type PMP22 allele. The basis for this phenomenon appears to be the heterodimerization of trafficking-incompetent mutants with wild-type PMP22, such that both the wild-type protein and the mutant forms are retained early in the secretory pathway. The full cellular and structural biological details of these observations remain to be elucidated. However, the model suggested by the existing data regarding the relationship of PMP22 to CMTD may be useful to explain phenotypes of several other diseases involving other tetraspan membrane proteins and to facilitate predictions regarding previously undetected disease−protein linkages.

Protein misfolding and mistrafficking can be associated with human pathology through several mechanisms. At one end of the spectrum are pathologies which are induced solely by the loss of function normally carried out by the protein in question. An example is found in the etiology of cystic fibrosis, where recessive mutations lead to loss of function of the CFTR[1] protein in airway epithelial cells (*1*−*3*). At the other end of the spectrum are "gain-of-function" proteins such as amyloid-forming proteins where the misfolded protein is itself toxic (*4*−*6*). Here, we examine molecular mechanisms of disease which combine elements of both cases—namely, autosomal dominant diseases which result from loss of function of a protein and/or accumulation of a misfolded protein, but in which misfolding of the mutant protein promotes loss of function of the *wild-type* form encoded by the second allele. The underlying single-allele

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mutations are referred to as "dominant negative" or "gain-of-function" because the resulting disease phenotype cannot be simply explained by the loss of function of the protein encoded by the single mutated allele. In this case, the mutant protein product somehow disrupts the functionality of the wild-type protein encoded by the normal allele (dominant negative) and/or may directly produce toxic effects (gain-of-function) such as inducing cellular apoptosis.

In this contribution, we summarize the emergent body of knowledge regarding the molecular basis for dominant negative/gain-of-function type pathology associated with peripheral myelin protein 22 and Charcot−Marie−Tooth disease type 1A. Furthermore, evidence is presented in support of the concept that PMP22/CMT1A represents a paradigm which may be extended to phenotypes of other diseases associated with mutations in tetraspan membrane proteins.

PMP22 Is a Critical Component of Myelin in the Peripheral Nervous System

PMP22, a 160 residue tetraspan membrane glycoprotein, is a component of mammalian PNS myelin where it comprises 2−5% of the total protein (*7*). PMP22 is also found in other cells at lower levels, particularly epithelial cells (*7*). The PNS myelin sheath is generated by Schwann cells, from which the myelin radiates and wraps around adjacent axons (*8*). Along with the glycoprotein P_0 (*9*), PMP22 is found primarily within compact myelin, where layers of plasma membrane are separated only by thin alternating layers of the Schwann cell cytoplasm and ectoplasm. PMP22 is found in this membrane, having a putative topology as illustrated in Figure 1 (*10*).

PMP22 and P_0 are considered to be absolutely essential to PNS myelin. The principal role of P_0 appears to be adhesive, "gluing" the layers of myelin together through trans homophilic interactions between molecules on juxtaposed membranes separated by ectoplasmic space (*8, 12, 13*). PMP22 is thought to directly associate with P_0 and/or itself both in trans fashion across two juxtaposed membranes and also in cis fashion within the same membrane plane (*14*). Such interactions may enhance adhesion between layers of compact myelin and may contribute to PMP22's most critical function, which is thought to be as a regulator of Schwann cell proliferation and myelin production (*7, 13, 15*).

The manner in which PMP22 regulates myelin production is not well understood. However, clues regarding possible molecular mechanisms may be gleaned by surveying what is known about potentially related tetraspan membrane proteins, as summarized in Table 1. A number of these proteins are components of myelin (*16*). General themes are as follows:

(i) These proteins tend to be found in specialized membranes, particularly membranes which undergo direct interactions with juxtaposed membranes, as in myelin, gap, and tight junctions.

(ii) Some of these proteins have been documented to form oligomers within the bilayer, with the hexameric oligomeric state in more than one case being specifically documented.

(iii) These proteins are often involved in trans homo- or heterophilic interactions between juxtaposed membranes, with the connexins representing an especially well-characterized example for which a low-resolution 3-D structure is available (*25*).

(iv) Some are involved in transport, in some cases forming hemichannels which span two membranes across an intervening extracellular space.

(v) Some of these proteins undergo and may help direct distinctive membrane trafficking leading to specialized membrane domains (*23, 24, 27, 29−30*).

(vi) A number of these proteins are known or suspected to be directly involved in the etiology or pathology of certain heritable diseases.

PMP22 and Charcot−Marie−Tooth Disease Type 1A

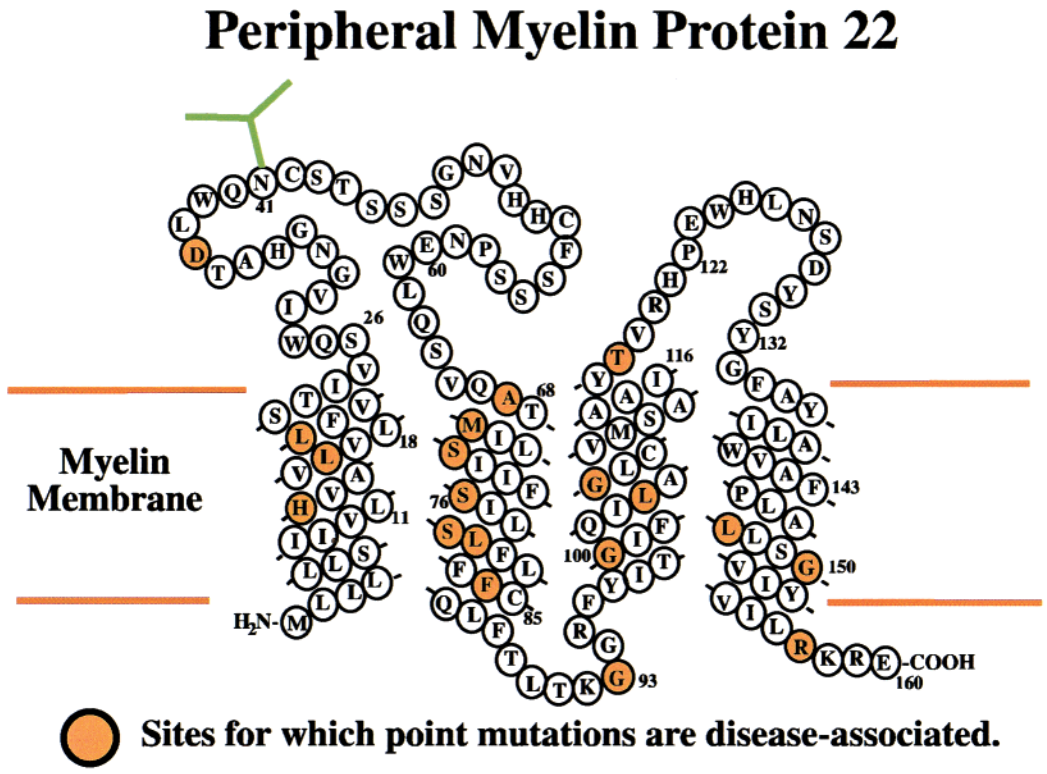
CMTD is a hereditary neuropathy of the peripheral nervous system which afflicts about 1 in 2500 humans (*32*). There are multiple forms of CMTD, with differences in phenotype being based both upon the mutated gene (PMP22, P_0, or Connexin-32) and on the nature of the mutation (*32, 33*). Here, we shall discuss the type 1A phenotypes of CMTD which are associated with defects in PMP22. The most common form of CMT1A is caused by the presence of a third PMP22 allele and the subsequent overproduction of the protein. A very mild form of the disease, HNPP, is caused by the absence of one allele (WT/null) and the subsequent underproduction of PMP22. This is in contrast to the case where both alleles are missing; this does not appear to have been observed in humans, suggesting lethality, while PMP22-(null/null) mice exhibit severe disease symptoms and highly defective PNS myelin (*13, 34*).

Both classical CMT1A and the milder HNPP phenotypes are linked to a variety of single missense mutations in the coding regions of one PMP22 allele (*15, 38*). Other missense mutations have been associated with severe forms of CMTD such as Dejerine−Sottas syndrome, manifesting dramatic symptoms and profound myelin defects. In these conditions, heterozygous expression of both mutant and WT forms of PMP22 leads to a higher degree of myelin dysfunction than does the expression of a single normal allele in the complete absence of the second allele (*15, 38*). In these heterozygous conditions, the mutated protein must be actively promoting disease beyond its mere failure to function. Known PMP22 sites which are mutated as a result of disease-associated missense mutations are illustrated in Figure 1. Remarkably, most sites are located within the putative transmembrane regions of the protein.

Disruption of Wild-Type PMP22 Trafficking by Dominant Mutants

Wild-type PMP22 protein folds and trafficks to the plasma membrane in both model cell lines and in Schwann cells in vivo with a low efficiency of ca. 20% (*35*). Most nascent

[1] Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator protein; CMTD, Charcot−Marie−Tooth disease; CMT1A, Charcot−Marie−Tooth disease type 1A; CNS, central nervous system; Cx, connexin; DM20, short splice variant of the central nervous system myelin proteolipid protein; DSS, Dejerine−Sottas syndrome; ER, endoplasmic reticulum; HNPP, hereditary neuropathy with liability to pressure palsies; IC, intermediate compartment between the endoplasmic reticulum and the Golgi apparatus; PLP, long splice variant of the central nervous system myelin proteolipid protein; PMP22, peripheral myelin protein 22; PNS, peripheral nervous system; TM, transmembrane; VDCC-γ, voltage-dependent calcium channel gamma subunit; WT, wild type.



Transmembrane Segment 3 of Disease-Related Tetraspan Proteins:

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Connexin-50     KGTKKFRLEGTLLRTYICHIIFKTLFEVGFIVGHYFLYGFRILPLY
Connexin-26     IKTQKVRIEGSLWWTYTSSIFFRVIFEAAFMYVFYVMYDGFSMQRL
Connexin-32     VKRHKVHISGTLWWTYVISVVFRLLFEAVFMYVFYLLYPGYAMVRL
PLP             CLGKWLGHPDKFVGITYALTVVWLLVFACSAVPVYIYFNTWTTCQSI
PMP22           CQLFTLTKGGRFYITGIFQILAGLCVMSAAAIYTVRHPEW
Stargazin       ASEFYKTRHNIILSAGIFFVSAGLSNIIGIIVYISANAGDPSKSDSK
Claudin-16      LPDEPYIKVRICFVAGATLLIAGTPGIIGSVWYAVDVYVERS
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Homologous Region of Extracellular Loop for Connexins and PMP22:

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Cx-50   loop to TM4   PLYRCSRWPCPNVVDCFVSRPTE   KTIFILFMLSVAS
Cx-26   loop to TM4   LYVKCNAWPCPNTVDCFVSRPTE   KTVFTVFMIAVSG
Cx-32   loop to TM4   RLVKCDVYPCPNTVDCFVSRPTE   KTVFTVFMLAASG
PMP22   loop to TM2   QWQNCSTSSSGNVHHCFSSSPNEWLQSVQATMILSILSL
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Color Key for Aligned Sequences

Black:	Probable Extramembrane Sites
Red:	Probable Transmembrane Sites
Yellow:	Conserved Site
Blue:	Site at which Mutation is Linked to Disease
Green:	Site of N-Glycosylation

FIGURE 1: Top: Probable membrane topology of PMP22 showing known sites of CMTD-associated point mutations (*15*, *33*). A recent paper (*11*) proposed an alternate topology in which the 2nd and 3rd transmembrane segments are not transmembrane, but instead lie at or above the extracellular face of the membrane. Middle: An example of the sequential relationships among disease-associated tetraspan membrane proteins. Homology is apparent between some pairs of sequences, but not between others. Bottom: Previously unrecognized sequence motif shared by PMP22 and the connexins. This motif is almost certainly directly involved in trans interactions between juxtaposed connexins located on an adjacent membrane which form dodecameric hemichannels connecting two cells (see *25*). This suggests that this motif in PMP22 may be involved in analogous trans protein−protein interactions. However, it is interesting to note that while mutations for sites in this motif have been identified as being disease-linked for the connexins, this is not the case for PMP22 (at least not yet). This suggests that these sites are less essential for PMP22's correct folding/trafficking and/or for its critical function than they are for connexins.

Table 1: Tetraspan Membrane Proteins Which May Have Some Relationship to PMP22[a]

protein	homology to PMP22?[b]	other close homologues?[b]	no. of residues	location	possible function	oligomeric state	associated disease?
PMP22 (PAS-II, GAS-3, SR13, CD25)	NA	MP17−20, LIM2, EMP1−3, TMP, XMP, YMP, CL-20, PERP/PIGPC1	160	PNS myelin and other locales, especially epithelial cells (*7*)	see text	hexamers or octamers suggested (*17*)	CMT1A, DSS, HNPP[c]
claudin-16 (paracellin)	modest	claudin family (*18*)	305	tight junctions of epithelial cells (*19*)	probable structural protein of tight junctions involved in trans membrane adhesion, involved in formation of paracellular Mg(II) channels (*20*)	ND[d]	hypo-magnesemia (*20*)
stargazin (VDCC-γ-2)	modest	VDCC-γ subunit family (*21, 22*)	323	brain	escort for protein trafficking to cell surface or synapse (*23, 24*)	ND	murine epilepsy (*22*)
connexin-32	weak	connexin family[c]	283	PNS myelin junctions[c]	hemichannel formation at gap junctions	trans dimer of cis hexamers (see *25*)	CMTD type X[c]
connexin-26	weak	connexin family[c]	226	gap junctions	hemichannel formation at gap junctions	trans dimer of cis hexamers	hereditary deafness[c]
connexin-50	weak	connexin family[c]	432	gap junctions	hemichannel formation at gap junctions	trans dimer of cis hexamers	congenital cataracts[c]
CNS proteolipid proteins (splice variants PLP and DM20)	no	lipophilin family (M6-A, M6-B, rhombex-29)	PLP: 277 DM20: 247	myelin of CNS	structural protein of compact CNS myelin, may form channels	probable cis hexamers (*26*), possible trans dimerization of hexamers	Palizaeus-Merzbacher and related diseases[c]
plasmolipin, MVP17, VIP17, MAL, BENE	no	small family of closely related proteins	ca. 180	epithelial cells and myelin, high levels in brain (*27*)	associated with glycolipid rafts and apical sorting, possible active role in trafficking (*27*)	ND	NI[d]

[a] Not included in this table are the following tetraspan membrane proteins which may also be related to PMP22, but where the relationship is less certain and/or interesting: claudin-11 (oligodendrocyte-specific protein) and other claudins, other gamma subunits of the voltage-dependent calcium channel, other connexins, subunit c of the vacuolar ATPase (ductin), occludin, peroxisomal membrane protein 22 (MPV17), and the tetraspanin family (including the misfolding-based ocular disease-associated proteins peripherin and rom). [b] Based on unpublished BLAST sequence homology searches and analyses. The "weak" homology between PMP22 and the connexin family is not detected using standard BLAST algorithms (*28*), but is detected only by subjective manual alignment, as summarized in Figure 1. The modest sequence homologies observed between PMP22 and members of the claudin family are characterized by Expect "*E*" values as low as 0.00005, while *E* values as low as 0.08 are observed between members of the VDCC-γ and close homologues to PMP22. [c] See text for references. [d] ND: not determined; NI: none yet identified.

PMP22 molecules never reach maturity beyond the endoplasmic reticulum but are recognized as misfolded by the "quality control" machinery in the ER and are ultimately polyubiquinated and transported to proteosomes (*36*). In this regard, WT PMP22 is similar to WT CFTR, which folds and trafficks to the cell surface with only modest efficiency even under normal (nondisease) conditions (*2, 3*). The large number and scattered distribution of disease-associated missense mutations in the sequences of both CFTR and PMP22 may reflect the delicate balance between folding and misfolding/degradation pathways for these proteins in the ER, a balance which is evidently easy to tip to almost exclusively favor misfolding (*37*).[2]

The trafficking of some eight different dominant CMT1A disease-associated point mutant forms of PMP22 has been examined in transfected cell lines (*38*−*40*). Seven out of eight of these mutants exhibited levels of cell surface expression which are less than 10% that observed for the wild-type protein under homozygous conditions. Most of the nascent mutants never advance beyond the ER or IC. This suggests that most disease-associated missense mutants of PMP22 exhibit loss of function because of retention in the ER/IC, as opposed to possible perturbations of PMP22 function in otherwise correctly folded/trafficked protein. This result does not, of course, explain why associated disease phenotypes are often more severe for heterozygotes than for the WT/null hemizygote. This critical issue has been illuminated by additional detailed studies involving two PMP22 mutants.

[2] The effect of a given mutation in PMP22 upon the trafficking of this protein could be due to either or both of two distinct classes of perturbations. First, the mutation might lie in a sequential motif which is specifically recognized by components of the protein trafficking machinery of the secretory pathway. In this case, the mutation leads to a failure in molecular recognition and/or association which might not in any other way perturb PMP22's folding, stability, conformation, or dynamics; only its interactions with specific proteins would be changed. Second, the mutation might perturb PMP22's folding/unfolding rates, stability, conformation, quaternary structure, or dynamics in a way which results in the protein being identified as aberrant by components of the protein folding quality control apparatus of the ER. Once recognized as aberrant, its trafficking to the cell surface would be prohibited. A protein whose trafficking has been altered as a result of this second mechanism would be referred to as being "misfolded" in this paper. Thus, the term "misfolding" is here used broadly, encompassing a number of different types of possible structural perturbations. In all likelihood, mutations in PMP22 which lead to mistrafficking are the result of misfolding, since disease-associated sites of mutation are numerous and found throughout the full sequence of the protein, and since these sites are generally not part of established protein sorting/trafficking recognition motifs.

The mutant G150D and L16P forms of PMP22 are each associated with CMT1A, as well as with CMT1A mouse models known as Trembler (Tr) and Trembler-J (Tr-J), respectively (*7*). CMT1A symptoms in humans and mice heterozygotes harboring one of these mutant alleles are more severe than the mild symptoms associated with the WT/null hemizygote. It has been shown not only that the Tr and Tr-J mutants of PMP22 fail to exit the ER/IC, but also that coexpression of the mutant with wild-type PMP22 in model cell lines also dramatically reduces the amount of wild-type protein that reaches the cell surface (*40, 41*). These observations indicate that the Tr and Tr-J mutant forms of PMP22 interfere with the trafficking of the wild-type protein to the cell surface. While the extendibility of this conclusion to myelinating Schwann cells is not without dispute (*42*), these observations may at least partially explain the relatively severe phenotypes associated with these mutations: the amount of nascent PMP22 which actually reaches its functional state is much lower in the WT/Tr or WT/Tr-J heterozygotes than in the case of WT/null.

There is now evidence which may provide insight into how mutant PMP22 interferes with the trafficking of wild-type PMP22 (*41*). Wild-type PMP22 has been shown to form dimers and possibly higher oligomers in the secretory pathway (*17*). Moreover, in model cell lines coexpressing both the wild-type protein and the L16P (Tr-J) mutant, heterodimers of the two proteins have been detected in immunoprecipitation experiments (*41*). This may explain how mutant forms of PMP22 can lower the efficiency by which the wild-type protein trafficks to the cell surface. Such mutants retain the ability to dimerize with the wild-type protein. However, either prior to or during the process of association with wild-type protein, some molecular event or events occur which results in the failure of the mutant protein and its heteromeric complexes to traffic beyond the ER/IC.[2] A corollary of this model is that the amount of wild-type protein which is degraded as a result of heterodimer formation depends on the relative affinities of mutant/wild-type and wild-type/wild-type interactions (*41*). This may help to explain why different dominant missense mutations lead to a spectrum of disease phenotypes. The least severe phenotypes would be those in which the PMP22 mutant fails to form tight complexes with the WT protein, such that WT monomers oligomerize primarily with other WT molecules to form complexes which traffic with normal (ca. 20%) efficiency to the cell surface.

The above model for how PMP22 mutants perturb trafficking of the wild-type protein suggests an interesting paradox—namely, that from a disease standpoint the most devastating PMP22 mutations may *not* be those which result in severely misfolded protein. Instead, the most problematic mutants may be those which attain a conformation similar enough to that of the wild-type protein to form heterodimers and yet which are in some way defective in that they fail to traffic beyond the ER/IC.[2] The fact that most of the disease-associated point mutations in the PMP22 protein fall within putative transmembrane segments also raises the interesting question of whether these sites are directly involved in oligomerization or whether they play roles in monomer folding or stability which may impact oligomerization only indirectly. In either case, the disease-relevance of the transmembrane sites of PMP22 highlights a broadening appreciation for the roles of such sites in membrane protein structure, stability, and folding (cf. *43, 44*).

Finally, it should be noted that the fate of the trapped WT/Tr-J heterodimers observed in the IC is not clear. It *has* been shown that the substantial fraction of WT PMP22 which misfolds under normal homozygous conditions is degraded in proteosomes (*36*). Whether this is also the fate of the mistrafficked WT/Tr-J heterodimers, and whether there are "gain-of-function" cytotoxic effects which could result if the heterodimers are not efficiently degraded, is not yet clear.

Are Other Diseases Linked to Tetraspan Membrane Proteins through Analogous Mechanisms of Mistrafficking?

Several proteins proposed to be related to PMP22 (Table 1) are linked to heritable diseases, and it is reasonable to contemplate whether some phenotypes of these diseases result from the same class of oligomerization-based mistrafficking defects described for PMP22. In some cases, existing disease/protein linkage data are sparse, but it is possible to predict that dominant negative phenotypes may ultimately be documented. For example, the role of missense mutants of claudin-16 in recessive hypomagnesemia was only recently established, and to date only a handful of sites of the disease-associated mutation have been identified (*20*). It is therefore remarkable that when the sequences for the third transmembrane segment (TM3) of PMP22 and claudin-16 are aligned, two of the seven known hypomagnesemia-linked sites of claudin-16 align perfectly with sites of PMP22 which are linked to CMT1A disease (Figure 1, middle). This strongly suggests that some of their associated disease phenotypes may share some common mechanisms involving defects in protein trafficking. Furthermore, these same two Gly sites of PMP22 and claudin-16 are also conserved in TM3 of stargazin (Figure 1). While the only stargazin mutation identified to date as being disease-related is the recessive null mutant (*22*), the observation that these two sites in PMP22 and claudin-16 are disease-linked may be a predictor that missense mutants involving these sites of stargazin may eventually be linked to disease phenotypes of epilepsy. Indeed, engineered missense mutations that alter the Gly at these positions may be useful starting points for structure/function studies of the novel stargazin/VDCC-γ family.

In the case of the tetraspan CNS myelin PLP protein, the actual degree of primary sequence homology between this protein and PMP22 is negligible (see Figure 1). The gene for PLP is located on the X chromosome, so that only a single active allele is normally present in each cell. Phenotypes of Palizaeus−Merzbacher and related diseases associated with PLP sometimes exhibit "gain-of-function" characteristics such that disease symptoms are more severe for missense mutants of PLP than for PLP null mutants (*45*−*47*). Part of the explanation for this appears to be that some mistrafficked forms of PLP accumulate and exhibit varying degrees of cellular toxicity, perhaps by triggering apoptosis (*48*−*50*). This is perhaps not surprising given the very high level of PLP which is produced in CNS oligodendrocyte cells (>50% of myelin protein); inefficient trafficking of this highly expressed protein could easily overload the ER quality control machinery and its associated degradation pathways, thereby leading to accumulation of protein (*48*−*50*). How-

ever, a second likely contributing factor to gain-of-function phenotypes associated with PLP may be an interesting variation of the theme exemplified by PMP WT/Tr-J heterodimerization. Following transcription of the PLP gene, two PLP mRNA splice variants are generated (*51*). One encodes the full-length PLP protein, while the other encodes the smaller DM20 protein, missing residues 116−150 of PLP. The folding and trafficking of DM20 to the cell surface has been shown to be much more tolerant of some of the same mutations which are known to result in inefficient trafficking of PLP (*45*, *51*, *52*). Unlike the WT/Tr-J PMP22 case, coexpression of mutant PLP known to mistraffic with DM20 does not result in reduced DM20 trafficking to the cell surface (*52*). However, trafficking of *both* wild-type and mutant forms of PLP is more efficient in the presence of DM20 than in its absence (*52*, *53*), suggesting DM20 can serve as a folding/trafficking escort for PLP, rescuing PLP which might otherwise not be able to leave the ER. It is possible that some PLP/DM20-associated disease phenotypes may reflect the effect of mutations that limit the ability of DM20 to serve as a PLP escort without necessarily affecting its own ability to fold and traffic to the cell surface.

Diseases involving connexins include X-linked CMTD. In this case, it is unclear whether there are gain-of-function phenotypes for the associated missense mutants of Cx32 (*33*). However, it is known that some of the many missense mutant forms of Cx32 misfold and fail to traffic to the membrane surface (*54*, *55*). Moreover, since different connexin subtypes are sometimes expressed in the same cell and form heteromers which function as hemichannel connexons (*54*, *56*, *57*, *59*), it will not be surprising if some X-linked Cx32 mutants are eventually observed to induce mistrafficking of *other* connexin subtypes in a manner analogous to that in which mutant forms of PMP22 induce mistrafficking of WT PMP22.

Other disease-linked connexins are autosomally encoded, such as Cx26, Cx30, Cx31, and Cx50. Heterozygous missense mutations involving these connexins are associated with disease phenotypes including certain types of hearing loss, cataracts, and skin disorders (*58*, *59*). While not as well-characterized as the Tr and Tr-J mutants of PMP22, a number of autosomal disease-associated connexin mutants have been shown to undergo defective trafficking (*58*, *60*, *61*). Given that connexins appear to oligomerize early in the secretory pathway (*57*, *61*), it will be surprising if some of the known dominant mutants are not ultimately demonstrated to be involved in a PMP22-like mechanism of mistargeting the wild-type allele product which involves oligomerization in the ER.

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