Supporting Information

Ion Mobility – Mass Spectrometry Reveals the Role of Peripheral Myelin Protein in Neuropathic Disease

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PMP22 released from C12E8 Micelles				
	Expected	Observed	Expected	Observed
	Monomer		Dimer	
PMP22 WT	19276	19407 ± 6	38552	39139 ± 21
PMP22 S22F	19336	19340 ± 13	38672	38734 ± 19
PMP22 A67T	19306	19310 ± 9	38612	38685 ± 33
PMP22 T118M	19306	19306 ± 6	38612	38689 ± 30
PMP22 G93R	19375	19378 ± 22	38750	38815 ± 28
PMP22 L16P	19260	19265 ± 8	38520	38800 ± 16
PMP22 H12Q	19267	19268 ± 15	38534	38635 ± 14
PMP22 G150D	19334	19345 ± 5	38668	38698 ± 18

PMP22 released from SCOR Bicelles				
	Expected	Observed	Expected	Observed
	Monomer		Dimer	
PMP22 WT	19276	19292 ± 9	38552	38575 ± 31
PMP22 L16P	19260	19480 ± 12	38520	38998 ± 10
PMP22 G150D	19334	19399 ± 14	38668	38797 ± 18

Table S1. Mass analysis of PMP22 variant monomers and dimers. Expected (Exp) and observed (Obs) are listed for all complexes used in analysis in Daltons (Da). Note that due to the presence of single amino acid additions related to variations in strep tags, the signals corresponding to the lowest mass to charge at each charge state were chosen for mass analysis. More information on the additions can be found in Figure SI2.
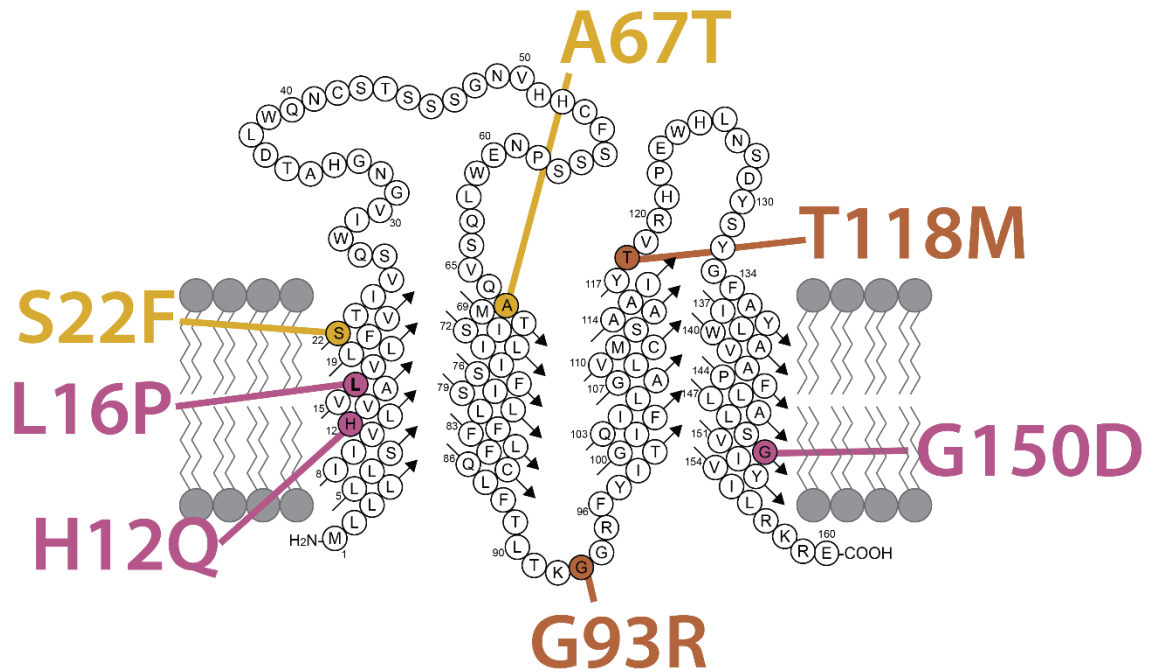
Figure S1. Sequence location of the tetraspan PMP22 mutations studied in this work, *figure adapted from Schlebach et al 2015.* In addition to WT PMP22, two mutations associated with the mild neuropathy HNPP (A67T and S22F), two mutations associated with the moderate neuropathy CMT1 (T118M and G93R), and three mutations associated with the severe neuropathy DSS (L16P, H12Q, and G150D) were characterized in this work.
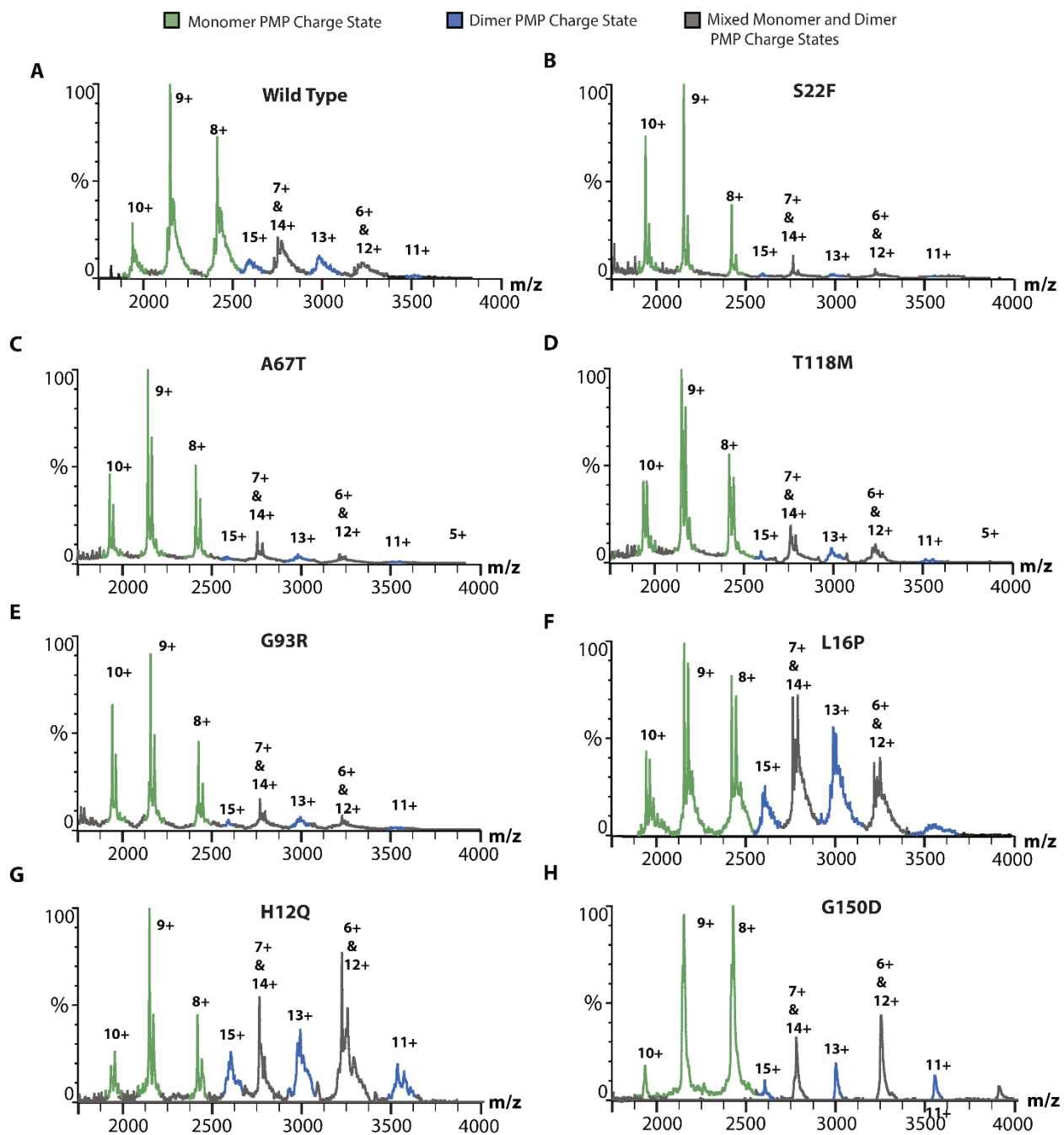
Figure S2. Mass spectra of PMP22 variants released from C12E8 detergent micelles. For all variants released from detergent micelles, charge states associated with monomer (green) and dimer (blue) were observed. Based on the charge states envelope, some signals were determined to contain indistinguishable amounts of both monomer and dimer (gray). These signals were excluded from percent dimer calculations. Spectra shown here are at 80 V of trap accelerating potential and background subtracted for visualization purposes, but data de-noising for further analysis is only performed as described in Figure S7. Any dimers observed are likely specific, as they appear stable throughout the range of 5 V – 80 V with negligible amounts of dissociation into monomers.
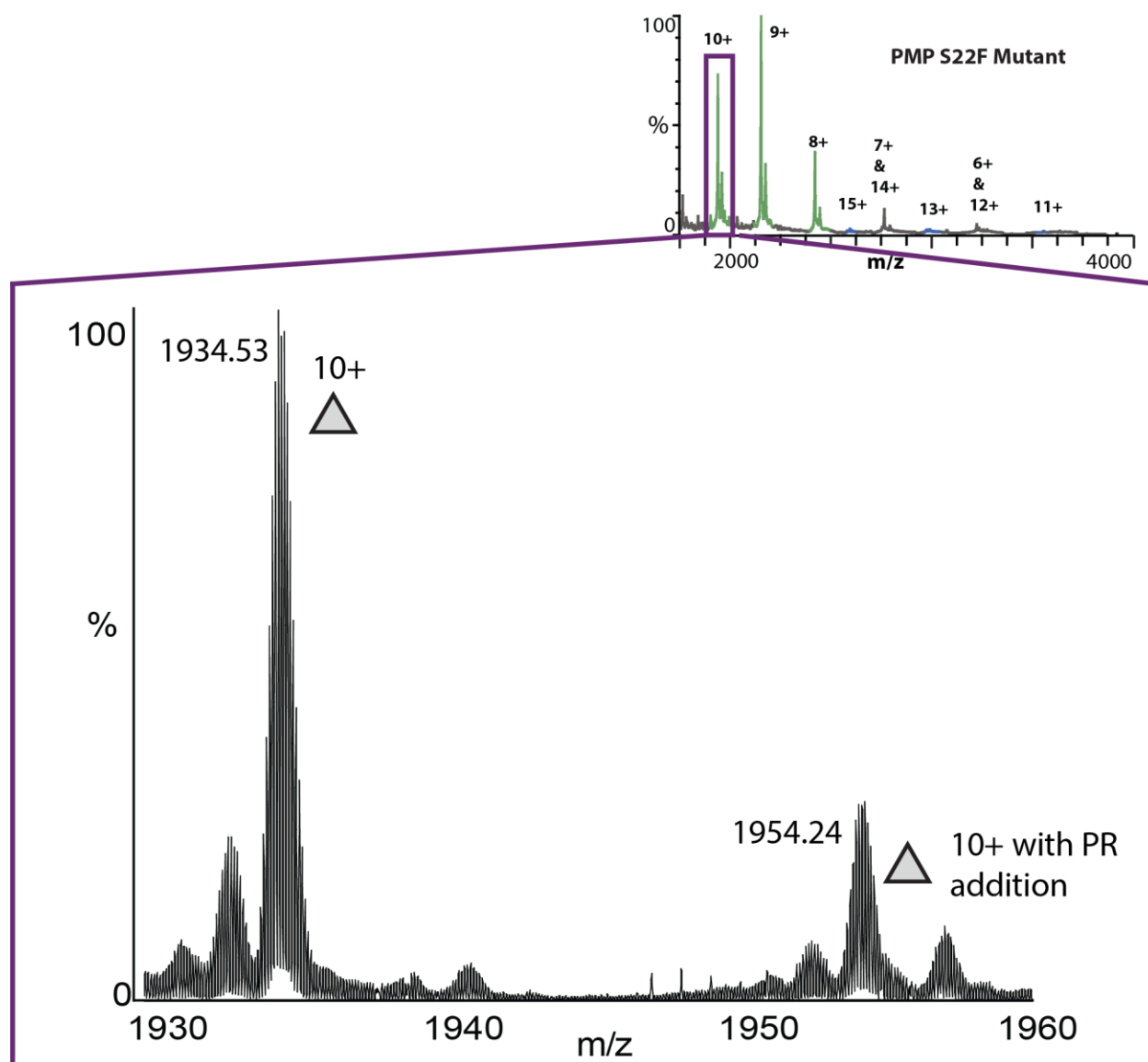
Figure S3. High resolution mass spectrometry identifies Strep-tag related additions to PMP22. PMP22 S22F without any additions can be observed at the major peak of 1934.53 Th. PMP22 S22F with an additional two residues, proline and arginine, can be observed at the minor peak of 1954.24 Th. The presence of the additional residues is likely a result of promiscuous cleavage at the site of the streptavidin tag. Present in all micelle samples except G150D, which were prepared using a different cleaving enzyme. CIU of data extracted with narrow windows from each individual amino acid addition exhibited no significant differences, so to maximize signal intensity, all CIU include the base protein and additions.
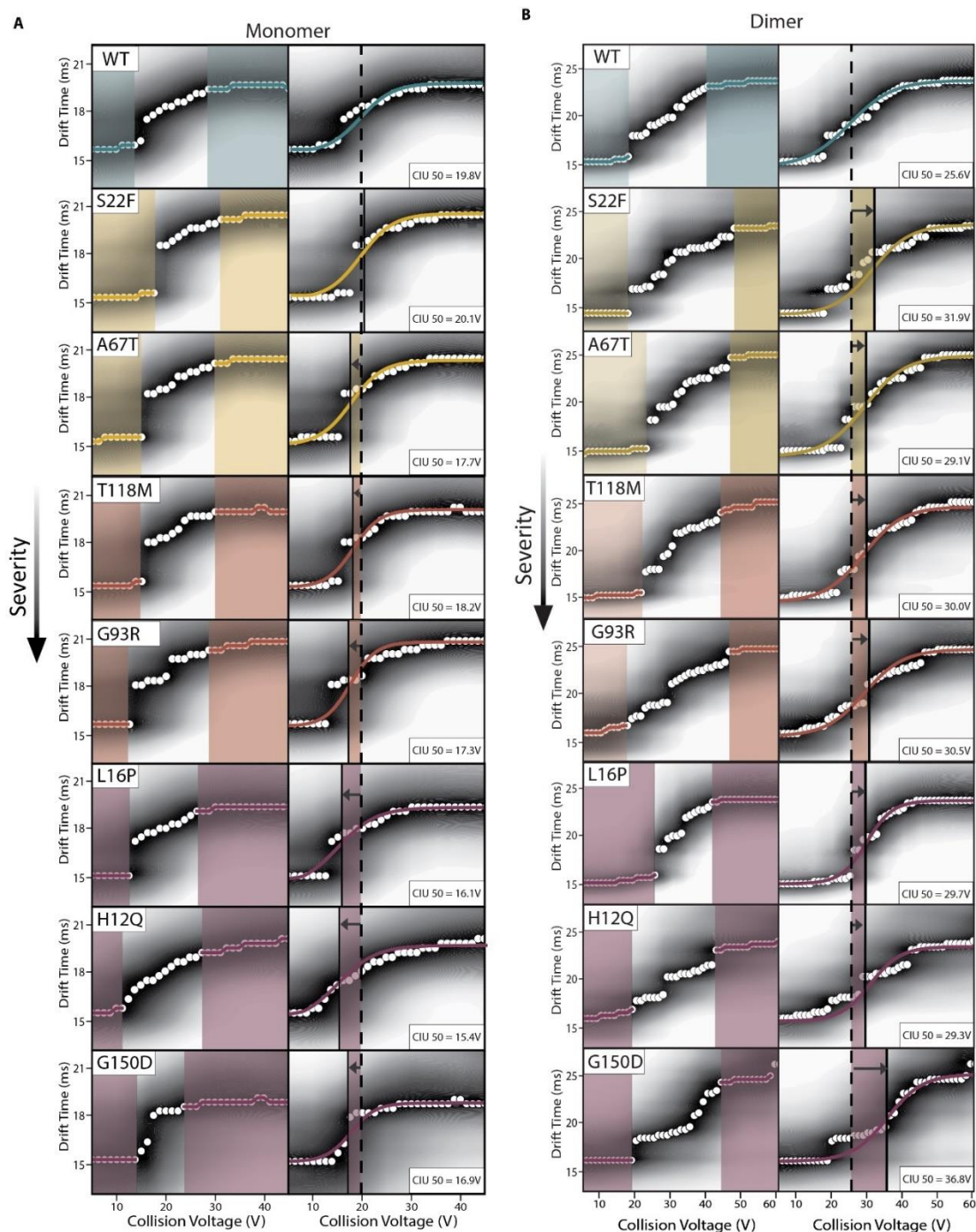
Figure S4. 9+ monomer and 13+ dimer CIU fingerprints for all variants studied in this work. Averaged, N=3. **A.** Automated CIU feature detection (left) and CIU50 sigmoidal transitions (right) for monomeric PMP22 reveal that all PMP22 mutations except for S22F show some degree of destabilization relative to WT. **B** Automated CIU feature detection (left) and CIU50 sigmoidal transitions (right) for dimeric PMP22 reveal that all PMP22 mutants are stabilized relative to WT.
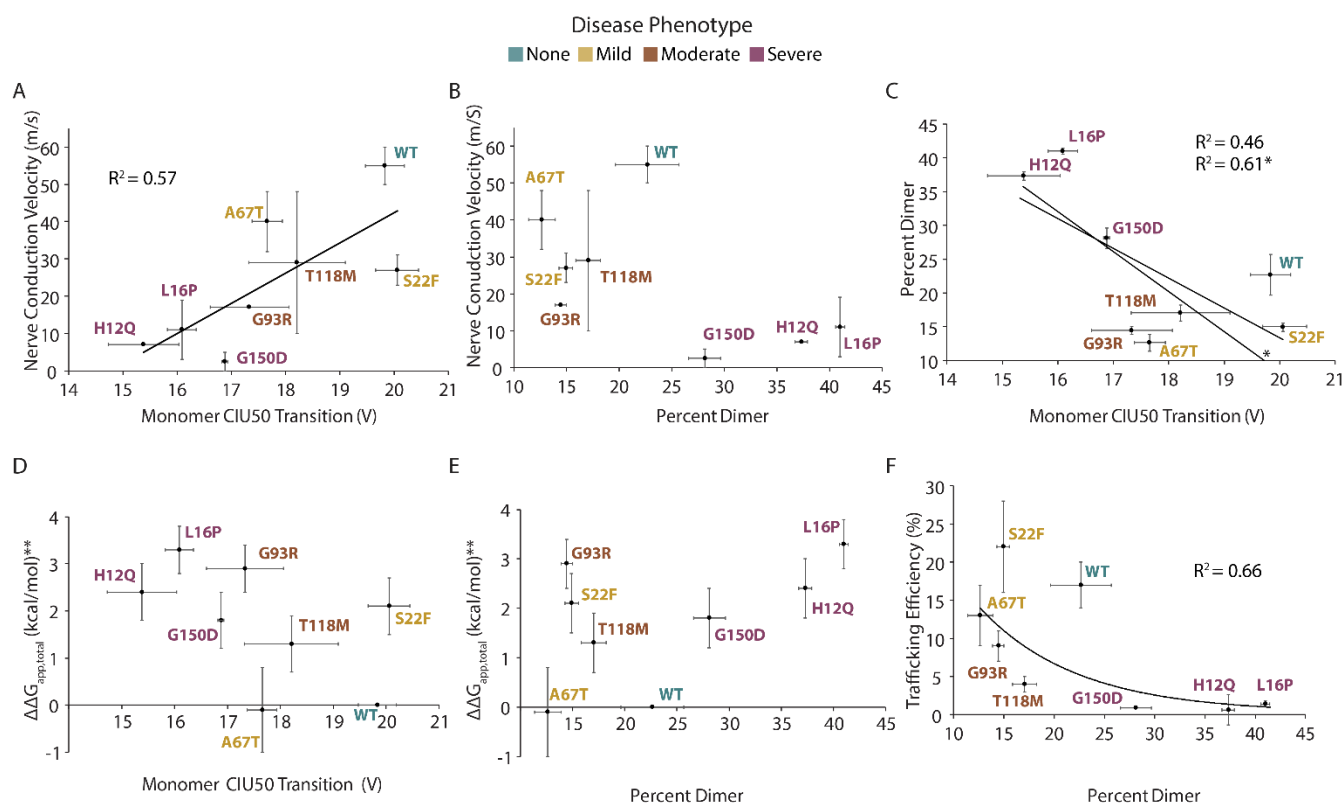
Figure S5. Correlative plots. Nerve conduction velocities (NCV), cellular trafficking efficiencies, and $\Delta\Delta G_{app,total}$ from Schlebach et al. All error bars represent the standard deviation. **A, B.** NCVs (m/s) versus monomeric PMP22 CIU50 transition values (V) and percent dimer. Linear regression analysis of NCV versus CIU50 yields $R^2 = 0.57$. No significant relationship between NCV and percent dimer is observed **C.** Monomer CIU50 transition (V) versus percent dimer shows a linear inverse correlation with $R^2 = 0.48$ when all data is included. *When the WT PMP22 data point is removed from this data $R^2 = 0.61$. **D,E.** $\Delta\Delta G_{app,total}$ (kcal/mol) versus monomer CIU50 transition (V) and percent dimer show no significant correlations. **$\Delta\Delta G_{app,total}$ is a measure of Zn(II) binding free energy associated with protein folding. **F.** Percent dimer versus trafficking efficiency. An exponential fit yields $R^2 = 0.66$.
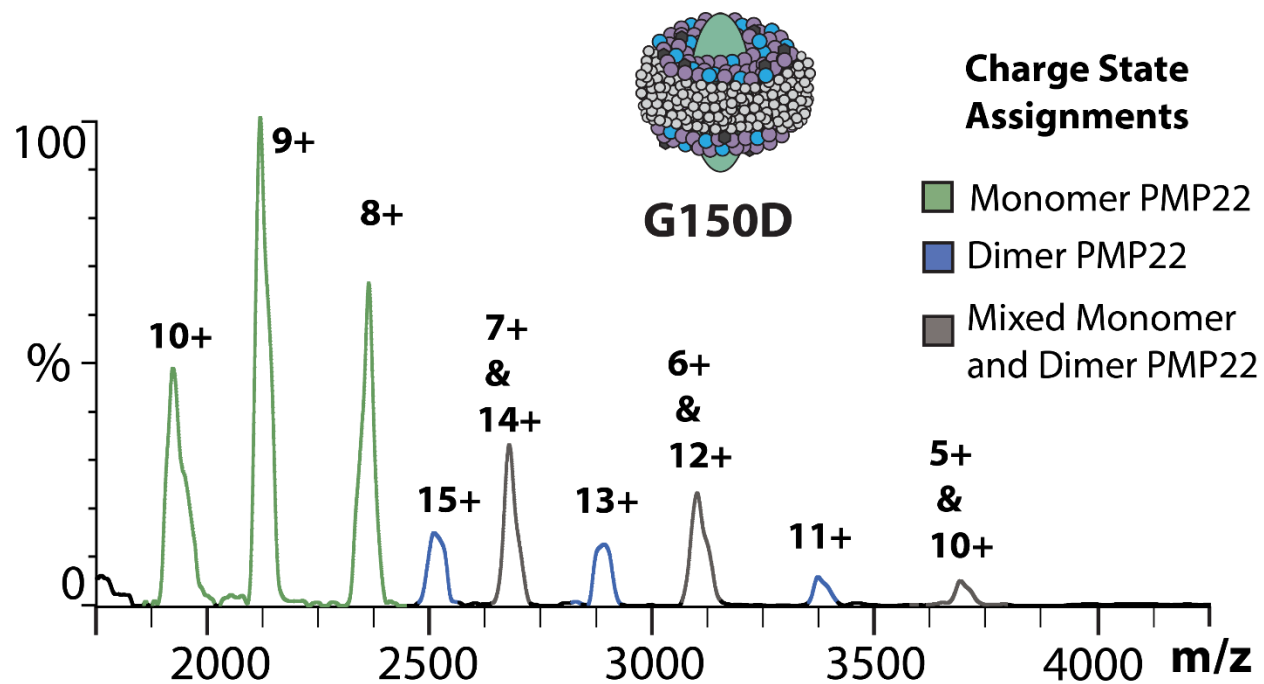
Figure S6. Mass spectra of G150D PMP22 released from SCOR bicelles. Signals corresponding to monomer (green), dimer (blue) and mixed monomer and dimer signals (gray) are observed. Data collected at 80 V of trap accelerating potential and was manually extracted from Driftscope then smoothed twice with a Savtizky-Golay window of three m/z.
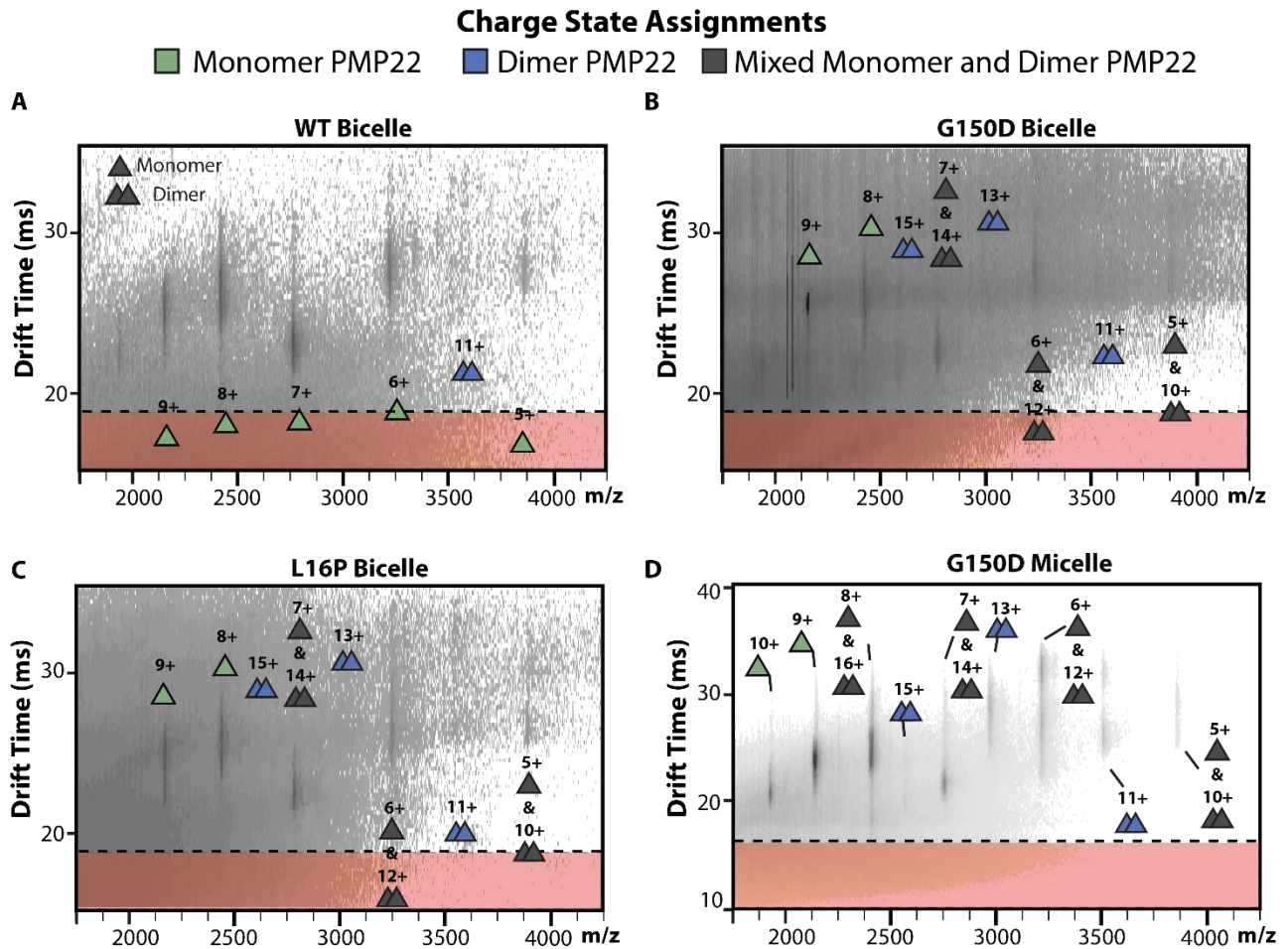
Figure S7. Denoising of bicelle and G150D micelle data (trap = 80 V, exponential intensity scale). **A, B, C.** Mass to charge versus arrival time plots of PMP22 variants liberated from SCOR bicelles exhibited noise signals associated with lipid and detergent clusters in the lower arrival time and mass to charge space. To minimize any bias towards monomeric PMP22 in the percent dimer analysis, the data extract was parameterized to extract only the arrival time distribution starting at 18 ms. **D.** G150D micelle samples were prepared and stored in glycerol, which led to a similar clustering issue in the low mass to charge range. G150D micelle arrival time distribution data was extracted starting at 17 ms. Note, this noise was not present in other micelle samples as there was no glycerol present, so no de-noising was performed.

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Supplemental Methods

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Preparation of SCOR bicelles

The **s**phingolipid and **cho**lesterol-**r**ich (SCOR) bicelle used in this study is composed of a lipid mixture consisting of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), egg sphingomyelin (eSM), and cholesterol in a mole ratio of 4:2:1, and the detergent b-n-dodecylmelibioside (DDMB), such that the q ratio (lipid-to-detergent mole ratio) is 0.33. Powdered samples of DMPC (CardenPharma), egg SM (NOF Corporation), and cholesterol (Sigma-Aldrich) necessary to make a 10-mL stock solution of 6% (w/v) SCOR bicelles were first weighed out into a 15-mL glass vial and the mixture was dissolved in chloroform by vortexing. The solvent was then evaporated at 45°C in a Smart Evaporator C1 (BioChromato) until a gel-like substance remained. The lipid mixture was then lyophilized overnight to remove the remaining solvent. The lyophilized mixture was resuspended in 15% (w/v) stock solution of DDMB (Anatrace) in milliQ water at a volume necessary to bring the q ratio to 0.33. The volume of the mixture was brought to 10 mL by adding milliQ water. The resulting mixture was then subjected to a series of freeze-thaw cycles consisting of freezing in liquid nitrogen, followed by thawing in warm sonication bath, and extensive vortexing before freezing again, until it turned clear (usually at least three cycles). The formation of SCOR bicelle was confirmed by dynamic light scattering.

Expression of PMP22

Wild type and mutant PMP22 (i.e S22F, A67T, T118M, G93R, L16P and H12Q) proteins were expressed in *E. coli* BL21(DE3) as described.[1] Briefly, the plasmid containing cDNA encoding recombinant human PMP22 was transformed in the *E. coli* BL21 star (DE3) cells and grown in minimal M9 media at 20 °C with agitation. 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added when OD_{600} was ~0.8, to induce the expression of the PMP22 protein. Cells were harvested by centrifugation ~22 hours after induction and the cell pellets were flash frozen until use in purification.

Purification of human PMP22 in detergent micelles:

Recombinant PMP22 proteins (i.e. WT, S22F, A67T, T118M, G93R, L16P and H12Q) were purified on Ni-NTA column (Qiagen) as described with some modifications. To refold the protein, the EMPIGEN BB detergent (N,N-Dimethyl-N-dodecylglycine betaine, N-(Alkyl C_{10}-C_{16})-N,N-dimethylglycine betaine, Sigma-Aldrich) was exchanged with Decyl-Maltoside (DM, Anatrace) by rinsing PMP22 bound to Ni-NTA resin with 200 ml of 50 mM Tris buffer (pH 8.0) contained 0.5% DM and 1mM TCEP (Tris(2-carboxyethyl)phosphine, COMPANY). PMP22 and PMP22 mutants were eluted with 300 mM Imidazole dissolved in refolding buffer (5mM Imidazole, 50mM Tris buffer pH 8.0, 0.15% DM and 1mM TCEP). Finally, after overnight thrombin cleavage the 50 ml protein solution was passed over a second Ni-NTA column that was pre-equilibrated with 50mM Tris buffer (pH 8.0) containing 0.15% DM and 1mM TCEP. Flow through was discarded and bound PMP22 was eluted with 15 mM Imidazole dissolved in 50mM Tris buffer pH 8.0, 0.15% DM and 1mM TCEP. The PMP22 proteins were concentrated to 1-2 mg/ml and stored at -80°C for use in mass spectrometry studies. The final protein concentration

was estimated by measuring the absorbance at 280 using an extinction coefficient of 44,900 $M^{-1}cm^{-1}$.

Purification of PMP22 into SCOR Bicelles

Cells containing overexpressed PMP22 were resuspended in lysis buffer (75 mM Tris-HCl (pH 7.5), 300 mM NaCl, 0.2 mM EDTA) at 20 mL buffer per gram of cell pellet. Lysozyme (0.2 mg/mL), DNase (0.02 mg/mL), RNase (0.02 mg/mL), phenylmethanesulfonyl fluoride (PMSF, 1 mM), and magnesium acetate (5 mM) were added and the mixture was tumbled for at least 30 minutes at 4°C, followed by probe-sonication on ice for 10 minutes. To dissolve the protein from the membrane and inclusion bodies, lauryl betaine (empigen detergent, BOC Sciences) to a final concentration of 3%, as well as glycerol (15%) and tris(2-carboxyethyl)phosphine (TCEP, 1 mM), were added, and the mixture was rotated at 4°C for at least an hour. The insoluble cell debris were then removed by centrifugation of the lysate at 20,000 rpm and 4°C for 30 minutes. The supernatant was collected and mixed with 0.5 mL of pre-equilibrated HisPur^{TM} Ni-NTA Superflow Agarose resin (Thermo Scientific) per gram of cell pellet. The resulting mixture was rotated at 4°C for ~14 hours.

The resin was then packed into a chromatography column and washed first with 5 column volumes (CV) of purification buffer (40 mM HEPES (pH 7.5), 300 mM NaCl) containing 15% glycerol, 3% empigen, and 1 mM TCEP, followed by purification buffer containing 40 mM imidazole, 15% glycerol, 1.5% empigen, and 1 mM TCEP, until the measured absorbance at 280 nm reaches the baseline, indicating elution of weakly bound impurities (typically ~15 CV). The empigen detergent was then exchanged with n-tetradecylphosphocholine (TDPC, Anatrace) by washing the resin with 12 CV of 25 mM sodium phosphate buffer (pH 7.2) containing 0.2% TDPC and 1 mM TCEP. The PMP22 fusion protein was then eluted from the resin using 50 mM Tris buffer (pH 8) containing 500 mM imidazole, 0.2% TDPC and 1 mM TCEP.

The eluted protein was buffer exchanged twice with 20 mM sodium dihydrogen phosphate buffer (pH 7.5) containing 1 mM TCEP to get rid of the imidazole. Each buffer exchange cycle consisted of concentrating the solution to ~1 mL by centrifugation at 3,000 x g using a 10K MWCO Amicon Ultra-15 centrifugal filter device (Millipore) and diluting back to ~15 mL. The final solution was then concentrated to ~5 mL before loading into a pre-equilibrated HiTrap SP FF cation exchange column (GE Healthcare) for cation exchange chromatography. The cation exchange chromatography serves two purposes: to remove a common *E. coli* stress protein YodA that cooverexpressed and coeluted with the PMP22 fusion protein, and to replace the membrane mimetic from TDPC to SCOR bicelles. After loading the sample, the column was then washed with at least 30 CV of 20 mM sodium dihydrogen phosphate buffer (pH 7.5) containing 1 mM DTT, 0.2% SCOR bicelle (q = 0.33), and 0.3 mM b-n-dodecylmelibioside (DDMB, Anatrace) to exchange the membrane mimetic. The PMP22 fusion protein in SCOR bicelles was eluted by running a gradient where the concentration of NaCl is increased from 0 to 1 M over the course of 14 CV.

Cleavage of the fusion partner of PMP22 was carried out by adding 1000 units of thrombin (Recothrom) to ~0.8 mg/mL of purified PMP22 fusion protein adjusted to pH 8.0 by addition of 20 mM Tris buffer (pH 8.0) prior to cleavage. The solution was rotated gently at

room temperature overnight. The His tag-containing fusion partner, as well as uncleaved PMP22 fusion protein, were separated from the cleaved PMP22 by performing another Ni affinity chromatography. Pre-equilibrated Ni-NTA resin (at 1 mL per 10 mg fusion protein) and 20 mM imidazole were added to the cleavage reaction mixture before rotating for at least an hour at 4°C. The resin was packed into a chromatography column and the flow through containing most of the cleaved PMP22 was collected. Successive washes with 20 mM Tris buffer (pH 8.0) containing 0.2% SCOR bicelle (q = 0.33), 0.3 mM DDMB, 1 mM TCEP, and increasing concentrations of imidazole (20 mM, 30 mM, 40 mM, 250 mM) were carried out. The solution containing the cleaved PMP22 was concentrated before loading into a pre-equilibrated size exclusion column (GE Superdex 200 Increase 10/300 GL). Size exclusion chromatography was performed using 10 mM acetate buffer (pH 5.0) containing 100 mM NaCl, 0.2 % SCOR bicelle (q = 0.33), 1 mM EDTA, 5 mM TCEP, and 0.3 mM DDMB to remove empty bicelles and to exchange the buffer. Fractions containing PMP22 were combined and concentrated using 10K MWCO Amicon concentrator for use in native ion mobility-mass spectrometry experiments. The final concentration of PMP22 in SCOR bicelles was determined by measuring the absorbance at 280 nm using an extinction coefficient of 41,940 M^{-1} cm^{-1}.

References:

1. **Reversible Folding of Human Peripheral Myelin Protein 22, a Tetraspan Membrane Protein.**
Jonathan P. Schlebach, Dungeng Peng, Brett M. Kroncke, Kathleen F. Mittendorf, Malathi Narayan, Bruce D. Carter, and Charles R. Sanders. *Biochemistry* **2013** *52* (19), 3229-3241