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SUPPLEMENTARY DISCUSSION

Our systematic mutagenesis of the Get2-1sc transmembrane domain resulted in the reduction of total protein Get2-1sc level ranging from 82% to ~50% (Fig. S4a), depending on the mutant, with one exception: the abundance of Get2-1_{TM3} was approximately one third of Get2-1sc. Nonetheless, microsomes prepared from this particular mutant were still able to recruit Get3, suggesting that Get2-1_{TM3}sc has normal cytosolic domains function (Fig. S4b). We looked further into the possibility that phenotypes we observed *in vivo* and *in vitro* are due to some combination of insufficient protein expression, and gross protein misfolding resulting in inefficient ER targeting and/or incorrect membrane topology. First, we replaced the native *GET2* gene promoter with that of *TDH3*, which drives the expression of one of the most abundant yeast proteins[21]. Next, we examined the effect of this promoter swap on the heat shock reporter and observed no significant suppression of the mutant phenotype (Fig. S5a). This observation is consistent with our interpretation that the apparent heat shock in this mutant background is a reflection of TA protein aggregation in the cytosol rather than Get2-1_{TM3}sc misfolding, which we confirmed by monitoring the cell localization of Sgt2 (Fig. S5b), a known marker of cytosolic TA protein aggregates[22]. Two additional lines of *in vitro* evidence support our conclusion that the Get3 substrate release defect caused by mutations in the Get1/2 transmembrane domain is due to defective insertase function rather than trivial explanations. First, when we compared microsomes with either natively expressed or overexpressed Get2_{TM2}-1sc (Fig. S5c), we found in both cases that the TEV site engineered in the luminal linker became accessible to TEV cleavage in the presence of a detergent (Fig. S5d). These data are consistent with our finding that Get2_{TM2}-1sc

microsomes effectively recruit Get3 targeting complexes (Fig. 2a), as well as with the ability of the TA trap to drive substrate release by $Get2_{TM2}$-1sc proteoliposomes (Fig. 2b). Second, overexpression of $Get2_{TM2}$-1sc did not significantly rescue Sec22 release from Get3 (Fig. S5e) and, as a consequence, Sec22 insertion remained defective (Fig. S5f). In summary, our work demonstrates that genetic mutations in the Get1/2 transmembrane domain can cause selective loss of Get1/2 insertase function without perturbing the ability of Get1/2 cytosolic domains to reduce Get3's affinity for its substrates. Future unbiased genetic screening of Get1/2, as well as, ideally, high-resolution structure-based mutagenesis, should facilitate more surgical disruption of Get1/2's insertase function.