LETTER

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The Get1/2 transmembrane complex is an endoplasmic-reticulum membrane protein insertase

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Hundreds of tail-anchored proteins, including soluble *N*-ethylmaleimide-sensitive factor attachment receptors (SNAREs) involved in vesicle fusion, are inserted post-translationally into the endoplasmic reticulum membrane by a dedicated protein-targeting pathway[1–4]. Before insertion, the carboxy-terminal transmembrane domains of tail-anchored proteins are shielded in the cytosol by the conserved targeting factor Get3 (in yeast; TRC40 in mammals)[5–7]. The Get3 endoplasmic-reticulum receptor comprises the cytosolic domains of the Get1/2 (WRB/CAML) transmembrane complex, which interact individually with the targeting factor to drive a conformational change that enables substrate release and, as a consequence, insertion[8–11]. Because tail-anchored protein insertion is not associated with significant translocation of hydrophilic protein sequences across the membrane, it remains possible that Get1/2 cytosolic domains are sufficient to place Get3 in proximity with the endoplasmic-reticulum lipid bilayer and permit spontaneous insertion to occur[12,13]. Here we use cell reporters and biochemical reconstitution to define mutations in the Get1/2 transmembrane domain that disrupt tail-anchored protein insertion without interfering with Get1/2 cytosolic domain function. These mutations reveal a novel Get1/2 insertase function, in the absence of which substrates stay bound to Get3 despite their proximity to the lipid bilayer; as a consequence, the notion of spontaneous transmembrane domain insertion is a non sequitur. Instead, the Get1/2 transmembrane domain helps to release substrates from Get3 by capturing their transmembrane domains, and these transmembrane interactions define a bona fide pre-integrated intermediate along a facilitated route for tail-anchor entry into the lipid bilayer. Our work sheds light on the fundamental point of convergence between co-translational and post-translational endoplasmic-reticulum membrane protein targeting and insertion: a mechanism for reducing the ability of a targeting factor to shield its substrates enables substrate handover to a transmembrane-domain-docking site embedded in the endoplasmic-reticulum membrane.

We have previously found that elution of substrates from Get3 immobilized on a resin can be achieved in the absence of any membranes by the addition of an engineered heterodimer of Get1/2 cytosolic domains (miniGet1/2)[8]. At physiological protein concentrations, mini-Get1/2 enabled substrate elution in a manner that was dependent on the interactions of both Get1/2 cytosolic domains with Get3. Notably, substrate elution by miniGet1/2 was also dependent on the presence of an engineered tail-anchored trap derived from Sgt2, a transmembrane domain (TMD)-recognition factor that delivers newly synthesized tail-anchored proteins to Get3 (ref. 14). By chemical crosslinking between Get3 and substrate, we have subsequently found that the tail-anchored trap prevents apparent re-binding of substrates to Get3 (Extended Data Fig. 1a). Thus, in the simplest model for insertion, the only role of the Get1/2 TMD is to physically link the Get1/2 cytosolic domains so that they can work together to enable 'trapping' of substrate tail anchors by the nearby hydrophobic lipid bilayer. A more complex alternative to this spontaneous insertion model is that the Get1/2 TMD is an insertase that facilitates entry of substrate tail anchors into the lipid bilayer.

The spontaneous insertion model predicts that the insertion of Get3 substrates should be insensitive to genetic perturbations of the Get1/2 TMD, which mediates complex formation, as long as the function of the Get1/2 cytosolic domains is preserved. To avoid the potential for complex disruption by mutations in the six transmembrane segments (Get1 TM1–3 and Get2 TM1–3), we first engineered a single-chain version of the Get1/2 heterodimer (Get2-1sc) expressed from the endogenous *GET2* promoter in *Δget1* cells. The resulting protein fusion was functional (Extended Data Fig. 1b and Fig. 1a), as measured using a green fluorescent protein (GFP) cell reporter of heat-shock factor transcriptional activity[15], which is a good monitor of tail-anchored protein aggregation in the cytosol due to compromised Get1/2 function[6]. Get1/2 transmembrane segments were replaced with transmembrane segments from unrelated endoplasmic reticulum (ER) membrane proteins, either Sec61-β or Ost4 (Extended Data Fig. 2a). In addition, we mutated an absolutely conserved aspartic acid residue near the middle of Get2 TM3 (D271K) because replacement of this transmembrane segment severely destabilized Get2-1sc (Extended Data Fig. 2b and data not shown). All the mutations in the Get1/2 TMD resulted in the loss of Get2-1sc function, as evidenced by elevated heat-shock factor activity, with some alleles resulting in more apparent heat shock than others (Fig. 1a).

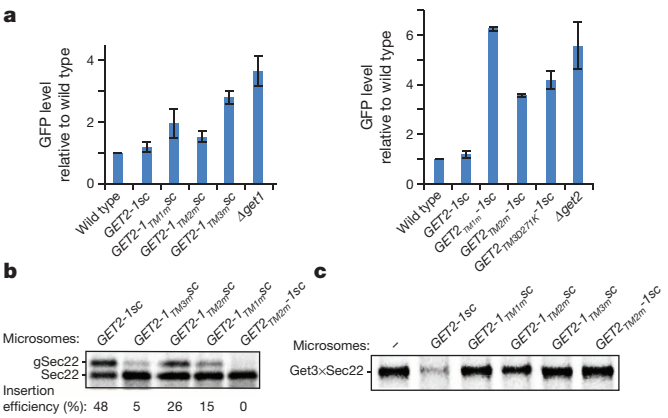

Figure 1 | *In vivo* and *in vitro* analysis of loss-of-function mutations in the Get1/2 TMD. a, The amount of GFP produced by the heat-shock reporter (Extended Data Fig. 1b) in the indicated strains was determined by flow cytometry analysis and plotted as the average of two independent experiments ($n = 2$). **b**, Affinity-purified Get3–Sec22 was incubated with the indicated microsomes at room temperature. Samples were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and visualized by autoradiography. The positions of unglycosylated and glycosylated Sec22 (gSec22) are indicated. Insertion efficiency is expressed as the percentage of Sec22 that is glycosylated. **c**, Affinity-purified Get3–Sec22 was incubated with the indicated microsomes or mock-incubated at room temperature followed by crosslinking with disuccinimidyl suberate (amine-reactive crosslinker). Samples were subjected to SDS–PAGE and visualized by autoradiography. The crosslinked product between Get3 and Sec22 is indicated (Get3 × Sec22).

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To measure the impact of TMD mutations on Get1/2 activity more directly, we first produced radiolabelled Sec22 (a SNARE tail-anchored protein that facilitates vesicle fusion in the early secretory system) by *in vitro* translation in a wild-type budding yeast cell extract. Next, we affinity-purified Get3–Sec22 and monitored insertion into ER-derived membranes (microsomes) by glycosylation at a carboxy (C)-terminal glycan attachment site. We observed that loss-of-function mutations in the Get1/2 TMD also caused a proportional decrease in Sec22 insertion (Fig. 1b and Extended Data Fig. 3a). Chemical crosslinking between Get3 and Sec22 revealed that reduced Sec22 insertion was due to reduced substrate release from Get3 (Fig. 1c and Extended Data Fig. 3b). Even though Sec22 has a relatively hydrophobic TMD that might hinder its release from Get3, another tail-anchored protein, Sec61-β, which has a significantly less hydrophobic TMD, was similarly compromised by genetic disruptions of the Get1/2 TMD (Extended Data Fig. 3c).

Loss of Get1/2 TMD function would be expected to cause a defect in Get3 substrate release if the ER bilayer were by itself not an efficient 'trap' for TMDs delivered into its proximity by Get3 interactions with Get1/2 cytosolic domains. We performed two stringent tests of this idea by focusing on our strongest loss of function allele, $Get2_{TM2m}$-1sc, which did not apparently disrupt Get2-1sc ER protein targeting and membrane topology (Supplementary Discussion and Extended Data Figs 4 and 5). First, we took advantage of our previous observation that Get3 interacts more avidly with miniGet1/2 than with either miniGet1^{CDm}/2 or miniGet1/2^{CDm}, which contain point mutations in Get1 and Get2 cytosolic domains that abolish interactions with Get3 (ref. 8). This explains why Get3–Sec22 association with microsomes during a flotation assay depends on its interactions with both Get1/2 cytosolic domains[8]: avidity is necessary for targeting complexes to remain membrane-bound in the absence of Get3 re-binding during the 3 h in which microsomes float to the top of a density gradient. Consistent with our starting hypothesis, we found that Get3–Sec22 targeting complexes maintained a better association with $Get2_{TM2m}$–Get1sc microsomes relative to control Get2^{CDm}/Get1 microsomes (Fig. 2a). As a second test, we adapted our tail-anchored trap system to work on the surface of membranes by preparing proteoliposomes with three ingredients: a phospholipid mixture that approximated the composition of the ER membrane, trace amounts of a Ni-NTA (nitrilotriacetic acid) phospholipid for subsequent tethering of the His_6-tagged tail-anchored trap, and $Get2_{TM2m}$–Get1sc that we affinity purified from yeast in complex with endogenous Get3 (Fig. 2b and Extended Data Fig. 6a). In further support of our hypothesis, we observed that the tail-anchored trap increased substrate release from $Get2_{TM2m}$–Get1sc proteoliposomes (Fig. 2b). Importantly, the tail-anchored trap was not able to rescue the substrate release defect of Get2/Get1^{CDm} proteoliposomes (Fig. 2b and Extended Data Fig. 6b). In summary, our data demonstrate that the ER bilayer is by itself not an efficient 'trap' for tail-anchored proteins delivered to its proximity by Get3 interactions with the cytosolic domains of Get1/2. They also argue that the Get1/2 complex has a distinct insertase function that resides in its TMD and that can be genetically separated from the function of its cytosolic domains.

We hypothesized that Get1/2 interacts with substrate TMDs to couple the release of tail-anchored proteins from Get3 to the subsequent membrane insertion step. To test this idea, we needed a means to stabilize potential Get1/2–substrate interactions during the rapid insertion process. Our solution was to fuse an S-tag to the C terminus of Sec22 and add S protein, which binds to the S-tag to form a stable tertiary structure[16]: S protein selectively blocked insertion of full-length Sec22 with a C-terminal S peptide (Sec22s) (Fig. 3a) without compromising the ability of Get1/2 to drive release of the road-blocked substrate from Get3 (Fig. 3b). To establish if road-blocked Sec22s forms a defined complex with Get1/2, we monitored by chemical crosslinking the proximity of an engineered single cysteine in the Sec22 TMD (S192C) and single cysteines at a variety of Get1/2 transmembrane positions that do not perturb Get1/2 function *in vivo* (Extended Data Fig. 7a). When we used bis-maleimidohexane (BMH), which irreversibly crosslinks reactive thiols that are within 13 Å of one another, we detected crosslinks between Sec22 and several positions that lie along the same face of the Get1 TM1 α-helix (Fig. 3c and Extended Data Fig. 7b). We also detected several crosslinks between Sec22 and positions on Get1 TM2 and TM3, as well as a substrate-crosslinking position on Get2 TM3 (Extended Data Fig. 7c). Our data suggest that the road-blocked Sec22 interacts with a composite TMD-docking site that resides near the cytosolic side of the ER membrane, comprises transmembrane regions from both Get1 and

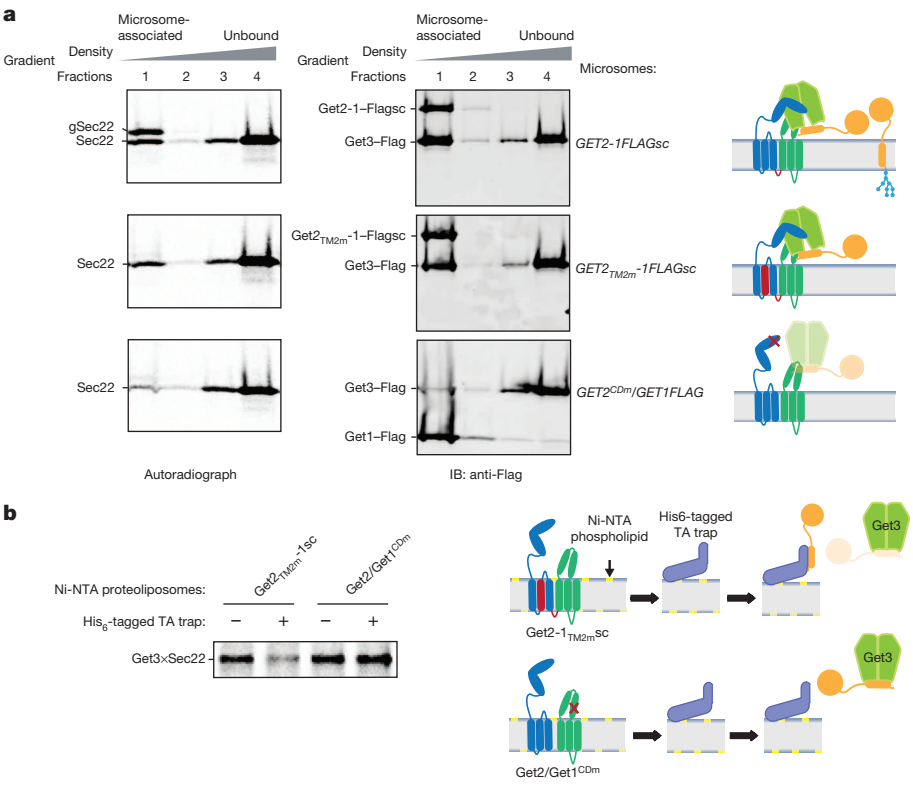

Figure 2 | Get1/2 has a novel insertase function that resides in its TMD. **a**, Affinity-purified Get3–Flag–Sec22 was incubated with the indicated microsomes at room temperature. Samples were overlaid with an Optiprep gradient and subjected to ultracentrifugation. Proteins precipitated from the four indicated gradient fractions were resolved by SDS–PAGE and visualized by autoradiography and immunoblotting. Non-glycosylated Sec22 that associated with *GET2-1FLAGsc*, *GET2$_{TM2m}$-1FLAGsc* and *GET2^{CDm}/GET1FLAG* microsomes corresponded to 11%, 10% and 2% of the summed Sec22 signal in all the fractions, respectively. **b**, Ni-NTA proteoliposomes reconstituted with the indicated mutant Get1/2 transmembrane complexes were incubated with tail-anchored (TA) trap or mock-incubated followed by crosslinking analysis as in Fig. 1c.

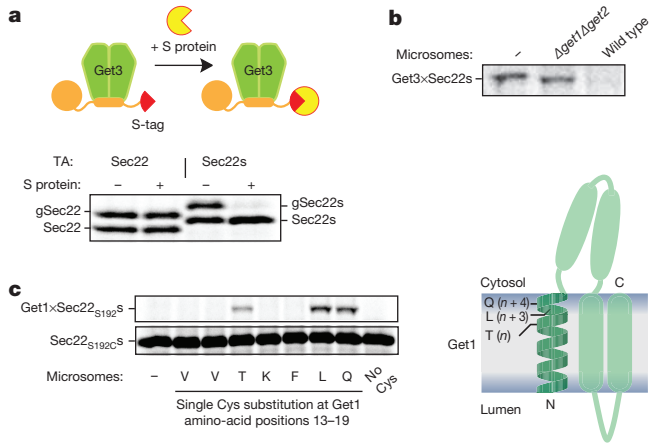

Figure 3 | The Get1/2 TMD binds a road-blocked tail-anchored protein released from Get3. **a**, Top: schematic showing attachment of S protein to the S-tag at the C terminus of a tail-anchored protein bound to Get3. Bottom: affinity-purified Get3–Sec22 or Get3–Sec22 (with S-tag) were incubated with S protein at room temperature or mock-incubated followed by Sec22 insertion analysis with wild-type microsomes as in Fig. 1b. **b**, Affinity-purified Get3–Sec22s was road-blocked with S protein and incubated with the indicated microsomes or mock-incubated at room temperature followed by crosslinking analysis as in Fig. 1c. **c**, Left: affinity-purified Get3–$Sec22_{S192C}$s was road-blocked with S protein and incubated with the indicated microsomes or mock-incubated at room temperature followed by BMH crosslinking on ice. Samples were subjected to SDS–PAGE and visualized by autoradiography. Uncrosslinked Sec22s and an approximately 60 kDa crosslink product between Get1 and Sec22s are indicated. Right: schematic showing the membrane topology of Get1 with crosslinking positions on TM1 indicated.

Get2, and is aqueous in nature, since BMH only reacts with thiols exposed to an aqueous environment[17].

Three lines of evidence argue that by road-blocking Sec22 we have stabilized a bona fide on-pathway (pre-integrated) intermediate. First, we detected no TMD crosslinking to an insertase-defective Get1/2 (Extended Data Fig. 8a, b). Second, as would be predicted for an arrested on-pathway intermediate, addition of S peptide after intermediate accumulation efficiently relieved arrest and resulted in efficient 'chasing' to the inserted product (Fig. 4a). Lastly, when we monitored the kinetics of Sec22 TMD crosslinking to two positions on Get1 TM1 during unimpeded integration, we observed the expected rapid and transient intermediate formation (Fig. 4b and Extended Data Fig. 8c). Importantly, Sbh1 (yeast Sec61-β homologue) followed a similar integration pathway to Sec22 (Extended Data Fig. 8d, e), arguing that we have uncovered a general mechanism for insertion of tail-anchored proteins into the ER.

Our work reveals that the Get1/2 transmembrane complex is an insertase that, compared with the Sec61 protein translocon in the ER membrane, provides an essential mechanistic insight into membrane protein insertion in general. Specifically, in the case of co-translational ER membrane protein insertion, the precise mechanism by which the signal recognition particle hands over its substrate to a binding site located within the Sec61 channel is not known but it involves GTP hydrolysis of the signal recognition particle and competitive binding between Sec61 and the signal recognition particle to their overlapping sites near the ribosome exit tunnel[18,19]. In the case of post-translational tail-anchored protein insertion into the ER, we have shown that Get1/2 cytosolic domains collaborate with the Get1/2 TMD to enable efficient substrate release from Get3. Speculatively, we suggest that substrate handover between Get3 and Get1/2 creates a facilitated pathway that overcomes the kinetic energy barrier between substrate release from the open conformation of Get3 and the thermodynamically favourable substrate state in

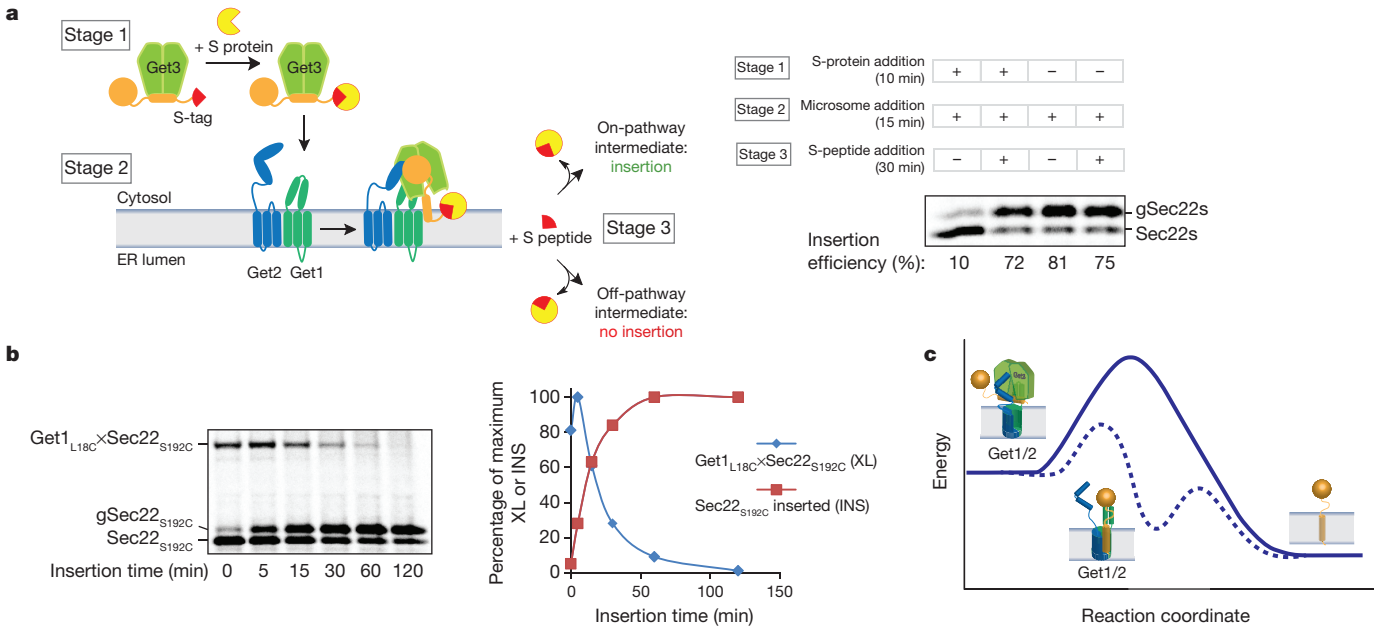

Figure 4 | Tail-anchored protein docking to the Get1/2 TMD is en route a facilitated integration pathway. **a**, Left: schematic illustrating two extreme outcomes of the S peptide 'chase' experiment. Right: affinity-purified Get3–Sec22s was split four-ways and subjected to the three stages of the experiment, as indicated, followed by Sec22s insertion analysis with wild-type microsomes as in Fig. 1b. **b**, Affinity-purified Get3–$Sec22_{S192C}$s was incubated with $Get1_{L18C}$ microsomes at room temperature for the indicated amounts of time. Samples were simultaneously subjected to Sec22 insertion and crosslinking analysis as in Figs 1b and 3c, respectively. On the right is a kinetic analysis plot of the gel data shown on the left. XL refers to the crosslinked product between Get1 and Sec22; INS, $Sec22_{S192C}$ inserted. **c**, Working model for the Get1/2 insertase mechanism. The cytosolic domains work together with a membrane-embedded TMD-docking site to afford a coupled mechanism that facilitates substrate release from Get3 and TMD partitioning into the phospholipid bilayer. We speculate that substrate binding to the TMD-docking site reduces the activation energy barrier (dotted line) that is apparently associated with spontaneous insertion of substrates delivered to insertase-defective Get1/2 (solid line). One way in which the TMD-docking site might afford an integration path around the hydrophilic headgroup barrier is in the form of an aqueous pocket lined on one side by phospholipid hydrocarbon chains, and on the other by a composite surface of Get1 and Get2 transmembrane helices.

which the TMD has partitioned into the lipid bilayer (Fig. 4c). Thus, the Get1/2 insertase mechanism, stripped away of the complexities associated with insertion coupled to co-translational protein translocation, reveals the fundamental molecular logic of membrane protein targeting and insertion: a mechanism for reducing the ability of a targeting factor to shield its substrates enables substrate handover to a binding site that is membrane-embedded within an insertion machine.

METHODS SUMMARY

Standard methods for monitoring Get1/2 function *in vivo* and *in vitro*, as well as *Saccharomyces cerevisiae* strains and plasmids used in this study, are described in detail in Methods.

For protein engineering, Get1 was linked to Get2 using the sequence ASGAGG SEGGGSEGGTSGAT, previously used to link the subunits of a hexameric AAA^+ ATPase[20]. S-protein attachment was achieved by mixing 2 μl of the appropriate affinity-purified Get3 targeting complexes with 0.4 μl of S-protein (EMD Millipore) in 8 μl of insertion buffer (22 mM HEPES-KOH pH 7.4, 1.5 mM $Mg(OAc)_2$, 120 mM KOAc, 2 mM DTT, 14% glycerol, 0.75 mM ATP, 25 mM creatine phosphate and 330 μg ml^{-1} creatine kinase) for 10 min at room temperature. In the experiment shown in Fig. 4a, the S-protein road-block was alleviated by the addition of synthetic S peptide (1 μg μl^{-1}) (KETAAAKFERQHMDS; Eton Bioscience).

To release tail-anchored substrate from Get3, 2 μl of affinity-purified Get3 targeting complex was mixed with 1 μl of microsomes (attenuance $D_{280\,nm} = 80$) in 8 μl of insertion buffer with ATP regenerating system and incubated for 30 min at room temperature (23 °C). This was followed by crosslinking with 0.5 mM disuccinimidyl suberate (Pierce) at room temperature for 30 min. Crosslinking was quenched by incubation with 50 mM Tris-HCl, pH 7.4 for 5 min at room temperature. Before monitoring Sec22 release from Get3 by proteoliposomes (Fig. 2a), we added 0.3 μg of tail-anchored trap to 1 μl of proteoliposomes and incubated for 20 min at room temperature. In one instance (Extended Data Fig. 1), membranes were substituted with miniGet1/2 and tail-anchored trap (0.1 μg μl^{-1}) or mock trap (0.1 μg μl^{-1}), as described previously[8].

For TMD docking to Get1/2, 2 μl of affinity-purified Get3 targeting complex was mixed with 1 μl of microsomes ($D_{280\,nm} = 80$) in 8 μl of insertion buffer at room temperature followed by crosslinking with 0.2 mM BMH (Pierce) on ice for 60 min. Crosslinking was quenched by incubation with 50 mM DTT for 15 min on ice.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions F.W. performed most of the experiments described in the study. C.C. analyzed Get1 cysteine N-ethylmaleimide (NEM) accessibility. N.R.W. performed cell microscopy experiments. F.W., C.C., N.R.W. and V.D. examined the data. V.D. conceived the project, guided the experiments, and wrote the paper with F.W. and input from C.C. and N.R.W.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to V.D. (vdenic@mcb.harvard.edu).

METHODS

***S. cerevisiae* strain construction.** Deletion strains were constructed in the BY4741 (mating type **a**) and BY4742 (mating type α) genetic backgrounds[21] by standard PCR-mediated gene knockout. A *3×FLAG::kan* cassette was used to modify the *GET1* gene locus by standard PCR-mediated epitope tagging as described previously[8].

GET2-1sc. To introduce *GET2-1sc* into the genome, *GET2* open reading frame was PCR-amplified and fused by overlap extension PCR to *GET1FLAG::kan* PCR-amplified from VDY9 (ref. 14). The overlapping primers were designed to fuse an amino-acid linker[20] in-frame with the flanking open reading frames. The resulting *GET2-1sc* PCR product was targeted to the *GET2* locus. Transmembrane/cysteine alleles were introduced into the *GET2-1sc* locus by standard *URA3*-mediated gene knockout followed by gene knock-in with the appropriate PCR products. All modifications to the *GET2-1sc* locus were confirmed by sequencing of the genomic DNA.

Heat-shock reporter. To introduce the heat-shock reporter into the genome, P_{CYC1}-*4×HSE-GFP::URA3* was PCR-amplified from a vector provided by O. Brandman and knocked into the *ura3* locus. The *URA3* marker was subsequently eliminated to enable genetic modifications of *GET1* and *GET2* gene loci by *URA3*-mediated gene knockout.

Tail-anchored protein aggregation reporter. To tag endogenous Sgt2 with mCherry into the genome, a *URA3* marker was integrated between nucleotides 1809 and 1810 of the *SGT2* coding sequence by standard PCR-mediated gene disruption. The *MCHERRY* gene was then amplified by PCR from a plasmid provided by K. Thorn to encode an SGGGSGGGSGGG–mCherry–SGGGPGGSGG cassette that was knocked into the *URA3*-disrupted *SGT2* locus.

TDH3 promoter swap. To replace the *GET2* promoter driving Get2-1sc expression with the strong *TDH3* promoter, we used a *URA3::*P_{TDH3} cassette provided by D. Breslow and standard PCR-mediated promoter replacement.

GET1cys/GET2cys alleles. To introduce *GET1/2cys* alleles into the genome, cysteine mutations were introduced at the desired positions in the *GET1/2* open reading frames by overlap extension PCR and knocked into the *URA3*-disrupted *GET1FLAG::kan* and *GET2* gene loci, respectively. To facilitate Get1/2 'double cysteine' strain construction, we used synthetic genetic analysis[22]: **a** mating-type strains with *GET1 cysFLAG::kan* alleles were crossed to α mating-type strains containing *Get2cys::nat* alleles and a 'magic marker' that enabled haploid selection following diploid sporulation. Notably, heat-shock reporter analysis revealed that marking of the *GET2* locus with the *nat* cassette did not disrupt Get2 function (data not shown).

Primer sequences for all strain constructions are available upon request.

DNA vector construction. Bacterial expression vectors. The vectors for bacterial expression of His_6-tagged versions of Get3–Flag, Get4–Get5, Sgt2ΔN (tail-anchored trap), Sgt2ΔC (mock trap) and miniGet1/2 have been previously described[8].

In vitro expression vectors. The vector for Sec61-β3F4 *in vitro* transcription was a gift from R. Hegde. The vector for Sec22opsin *in vitro* transcription was described previously[6]. Sec22 and Sec61-β truncations were constructed by PCR with the truncation position determined by the forward and reverse primers. S-tag, scrambled S-tag (encoding TEKAAAEFKHQRSDM) and cysteine TMD alleles were added during PCR amplification of Sec22 and Sbh1 to prepare DNA templates for *in vitro* transcription.

Primer sequences for all vector constructions are available upon request.

Recombinant protein expression and purification. His-tagged protein expression from pET vectors in BL21 DE3 *Escherichia coli* cells was induced by isopropyl-β-D-thiogalactoside followed by several purification steps as described previously[14].

Flow cytometry analysis of heat-shock reporter. Single yeast colonies were inoculated into a 96-well plate containing synthetic media and grown overnight at 30 °C with shaking. Saturated cultures (average $D_{600\,nm}$ was approximately 10 after about 16 h) were back-diluted to $D_{600\,nm} \sim 0.1$ into fresh synthetic media and grown at room temperature for 7 h. GFP cell fluorescence was measured with a LSRFortessa cell analyser (BD) and quantitated using FlowJo (version 10.0) software. To correct for any variation in cell size, cell fluorescence measurements were normalized by scatter.

***In vitro* transcription, translation and tail-anchored insertion into microsomes.** Capped mRNAs for *in vitro* translation of Sec22 and Sbh1 constructs were *in vitro* transcribed from PCR templates using a mMessage mMachine T7 kit (Ambion) as described previously[6].

In vitro translation of mRNAs encoding Sec22 and Sbh1 constructs was performed in the presence of [^{35}S]methionine in wild-type yeast cell extracts supplemented with Get3–Flag and Get4–Get5 as described previously[14]. Tail-anchored protein insertion into microsomes was monitored by glycosylation as described previously[14]. Insertion efficiency was determined by phosphorimager analysis using a Typhoon imaging system with ImageQuant TL software (GE).

[^{35}S]Sec61-β3F4 was produced by coupled *in vitro* transcription and translation in reticulocyte lysates (TNT SP6 Coupled Reticulocyte Lysate Systems; Promega) supplemented with Get3–Flag (100 ng μl^{-1}) and Get4–Get5 (200 ng μl^{-1}).

Microsome preparation and tail-anchored insertion conditions were described previously[8]. The standard tail-anchored insertion time at room temperature was 30 min unless otherwise indicated, and in the case of insertion of road-blocked substrate in Fig. 3c the insertion time was 15 min.

Affinity purification of Get3 targeting complexes. Targeting complexes were affinity-purified with anti-Flag resin and eluted with Flag peptide as described previously[8].

Membrane flotation analysis. Ten microlitres of microsomes ($D_{280\,nm} = 80$) were mixed with either 1 μl of 0.03 μg μl^{-1} recombinant Get3–Flag protein (Extended Data Fig. 4a) or 2 μl of affinity-purified Get3 targeting complex (Fig. 2a), and the final volume was brought up to 24 μl with binding buffer (20 mM HEPES-KOH pH 6.8, 5 mM $Mg(OAc)_2$, 150 mM KOAc, 250 mM sorbitol, and 2 mM ADP). After 20 min incubation at room temperature, samples were analysed by flotation in an Optiprep gradient as described previously[8].

TEV protease protection. One microlitre of microsomes ($D_{280\,nm} = 80$) was solubilized in microsome buffer (20 mM HEPES, 150 mM KOAc, 250 mM sorbitol, and 5 mM $Mg(OAc)_2$) containing 1% Triton X-100 or mock-treated on ice for 30 min. Treated microsomes samples were digested overnight with 0.5 μl of TEV protease (Invitrogen, 12575-015) at 4 °C with agitation.

Proteoliposome reconstitution. Large-scale anti-Flag affinity purification from digitonin-solubilized microsomes was performed as described previously[23]. Proteoliposomes were prepared by removal of detergent from purified membrane complexes with SM2 Biobeads (Bio-Rad) in the presence of synthetic phospholipids as described previously[23], with one notable modification: 10% 18:1 DGS-NTA(Ni) (Avanti Polar Lipids) was also included to enable subsequent tethering of His_6-tagged tail-anchored trap. The resulting proteoliposomes were resolved from non-membrane-associated material by the membrane flotation assay. The low-density membrane fraction was diluted into 1 ml of ice-cold water and spun at 20,000*g* for 20 min at 4 °C. The proteoliposome pellet was re-suspended in microsomes buffer.

Tail-anchored release from Get3. Two microlitres of affinity-purified Get3 targeting complex was mixed with 1 μl of microsomes ($D_{280\,nm} = 80$) or proteoliposomes in 8 μl of insertion buffer with ATP regenerating system (22 mM HEPES-KOH pH 7.4, 1.5 mM $Mg(OAc)_2$, 120 mM KOAc, 2 mM DTT, 14% glycerol, 0.75 mM ATP, 25 mM creatine phosphate and 330 μg ml^{-1} creatine kinase). This was followed by crosslinking with 0.5 mM disuccinimidyl suberate (Pierce) at room temperature for 30 min. Samples were quenched by incubation with 50 mM Tris-HCl, pH 7.4 for 5 min at room temperature. Before monitoring Sec22 release from Get3 by proteoliposomes, we added 0.3 μg of tail-anchored trap to 1 μl of proteoliposomes and incubated for 20 min at room temperature.

Confocal microscopy. Single yeast colonies were inoculated into synthetic complete media and grown with shaking at 30 °C to saturation. Cultures were back-diluted to $D_{600\,nm} = 0.2$ into fresh synthetic media and grown at 30 °C for 5 h with shaking. Live cells were then adhered to no. 1.5 22 mm × 30 mm coverslips (VWR) coated with concanavalin A as described previously[24] and washed with fresh synthetic complete media before imaging. Fluorescence images were acquired on an Eclipse Ti inverted microscope (Nikon) equipped with a CSU-X1-M1L-E laser scanning unit (Yokogawa) and a 594 nm laser line (Cobolt). Images (512 pixels × 512 pixels) were acquired with a 500 ms exposure at 16-bit resolution using a Plan Apo ×100/1.45 numerical aperture oil immersion objective lens (Nikon) and Metamorph 7.8.1.0 acquisition software (Molecular Devices). Linear contrast adjustment was performed uniformly across each image and identically on each image using ImageJ 1.43u (National Institutes of Health), and images were cropped around representative cells (out of 100 cells) in Adobe Photoshop CS4.

Denaturing immunoprecipitation. We used yeast wild-type cell extracts supplemented with Get3 (without the Flag-tag) and Get4–Get5 to translate Sec22s (Extended Data Fig. 7b) or Sbh1 (Extended Data Fig. 8e) *in vitro*. Following addition of S protein to the extracts, we removed DTT with a spin column, and then added microsomes for 15 min (Sec22) or 5 min (Sbh1) at room temperature, followed by BMH crosslinking. Samples were quenched, adjusted to 1% SDS, boiled for 5 min, and then diluted tenfold with ice-cold immunoprecipitation buffer (1% Triton X-100, 50 mM HEPES-NaOH pH 7.4 and 100 mM NaCl). Following immunoprecipitation with anti-Flag M2 resin (Sigma), samples were eluted with SDS–PAGE loading buffer.

Cysteine accessibility assay. Four microlitres of microsomes ($D_{280\,nm} = 40$) were incubated with 16 μl of 0.25 mM NEM in 50 mM HEPES-NaOH pH 7.4 for 1 h at 4 °C with agitation. Alkylation was quenched with 100 μl of ice-cold microsome buffer (100 mM NaCl, 2 mM $Mg(OAc)_2$, 50 mM HEPES-KOH pH 7.8) containing 5 mM DTT for 10 min at 4 °C. Membranes were collected by spinning at 20,000*g* for 15 min at 4 °C and washed with 50 μl of microsome buffer. Washed microsomes were re-suspended in 10 μl SDS-containing buffer (1% SDS, 0.25 mM TCEP, 50 mM HEPES-NaOH pH 7.4) and boiled for 5 min. Boiled samples were cooled down to 30 °C for 10 min before adding10 μl of 16 mM 5 kDa PEG-maleimide (Nanocs) in

50 mM HEPES-NaOH pH 7.4 and further incubation at 30 °C for 1 h with agitation. Alkylation was quenched with 0.4 µl of 1 M DTT at 30 °C for 20 min.

The fraction of Get2-1sc PEGylated (fPEG) was determined by ImageJ as Get2-1scPEG/(Get2-1sc + Get2-1scPEG) after subtracting from each Get2-1scPEG signal the background level observed at that position in the corresponding no cysteine control reaction. The NEM protection factor (NPF) of a given cysteine position was defined as NPF = $fPEG_{(+NEM)}/fPEG_{(-NEM)}$. The corresponding accessibility factor was defined as 1 − NPF.

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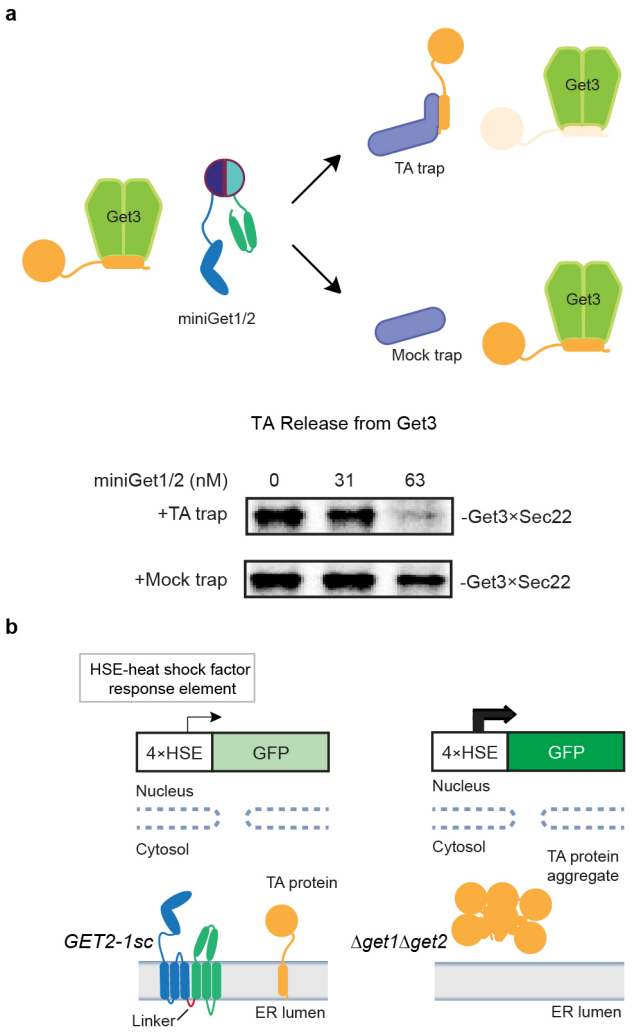
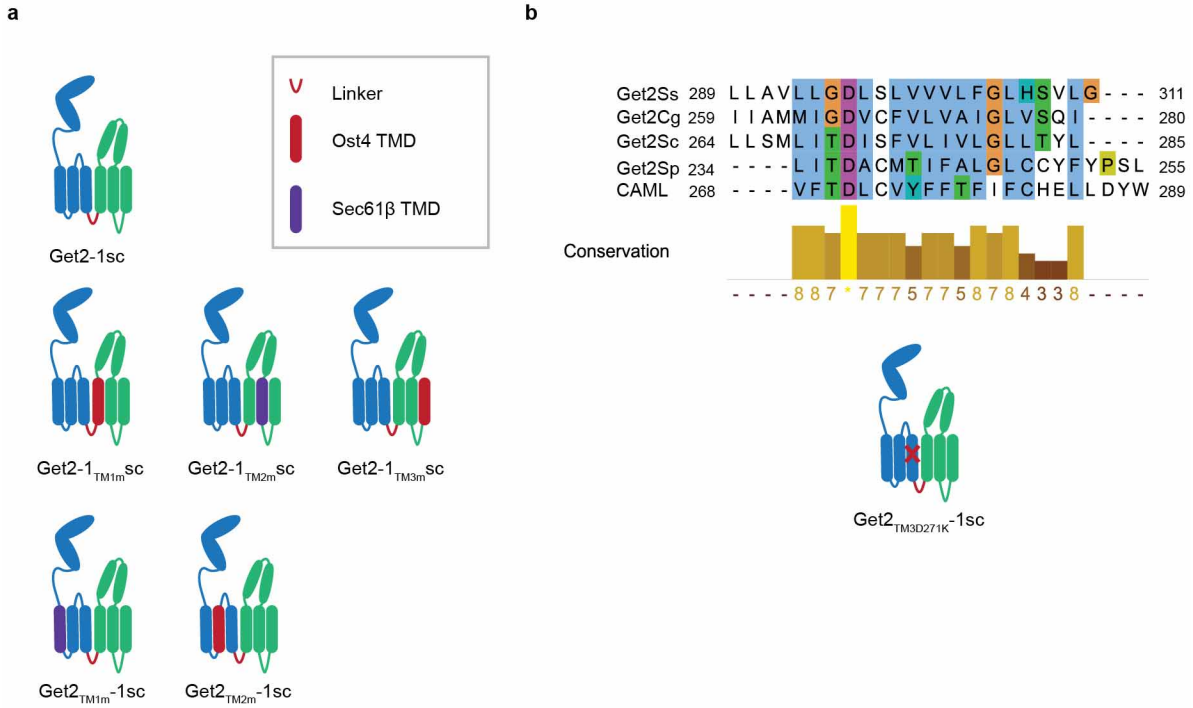
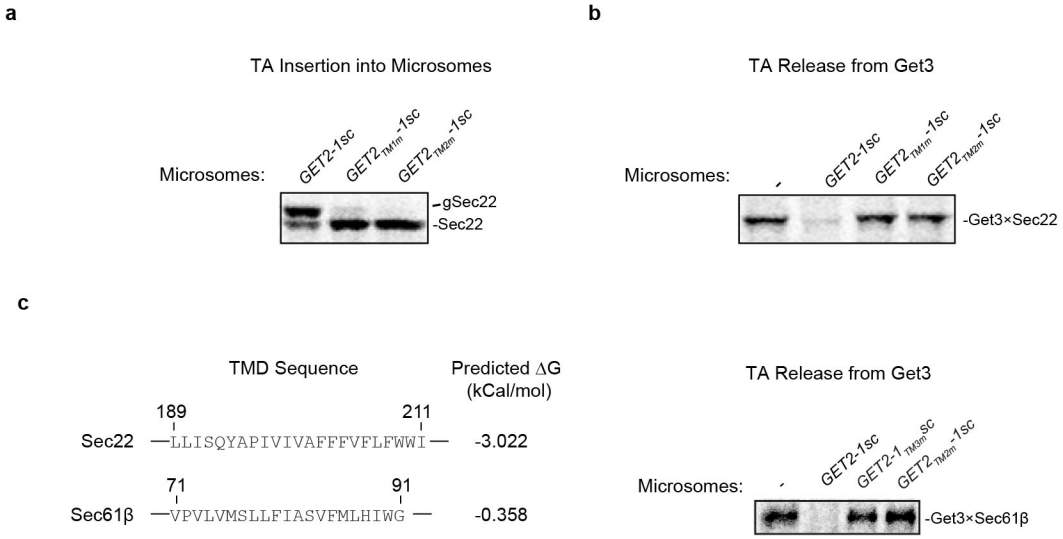


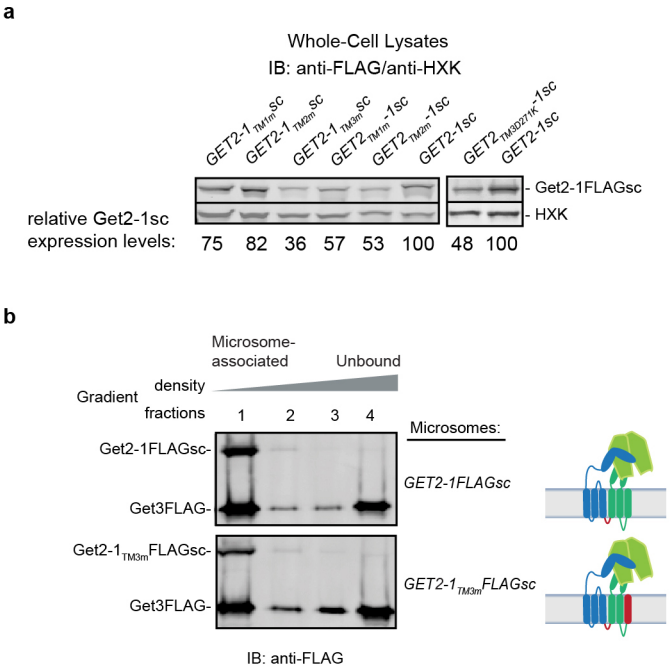
Extended Data Figure 1 | Get1/2 cytosolic domains and tail-anchored trap work together to prevent substrate re-binding to Get3. **a**, Top: schematic illustrating how tail-anchored trap but not mock trap drives substrate release from Get3 in the presence of miniGet1/2. Bottom: affinity-purified Get3–Sec22 was incubated at room temperature for 30 min with the indicated concentrations of miniGet1/2 in the presence of either excess tail-anchored trap (Sgt2ΔN) or mock trap (Sgt2ΔC)[8] followed by crosslinking as in Fig. 1c. **b**, Schematic showing how Get1/2 activity can be monitored *in vivo* using a transcriptional GFP reporter.



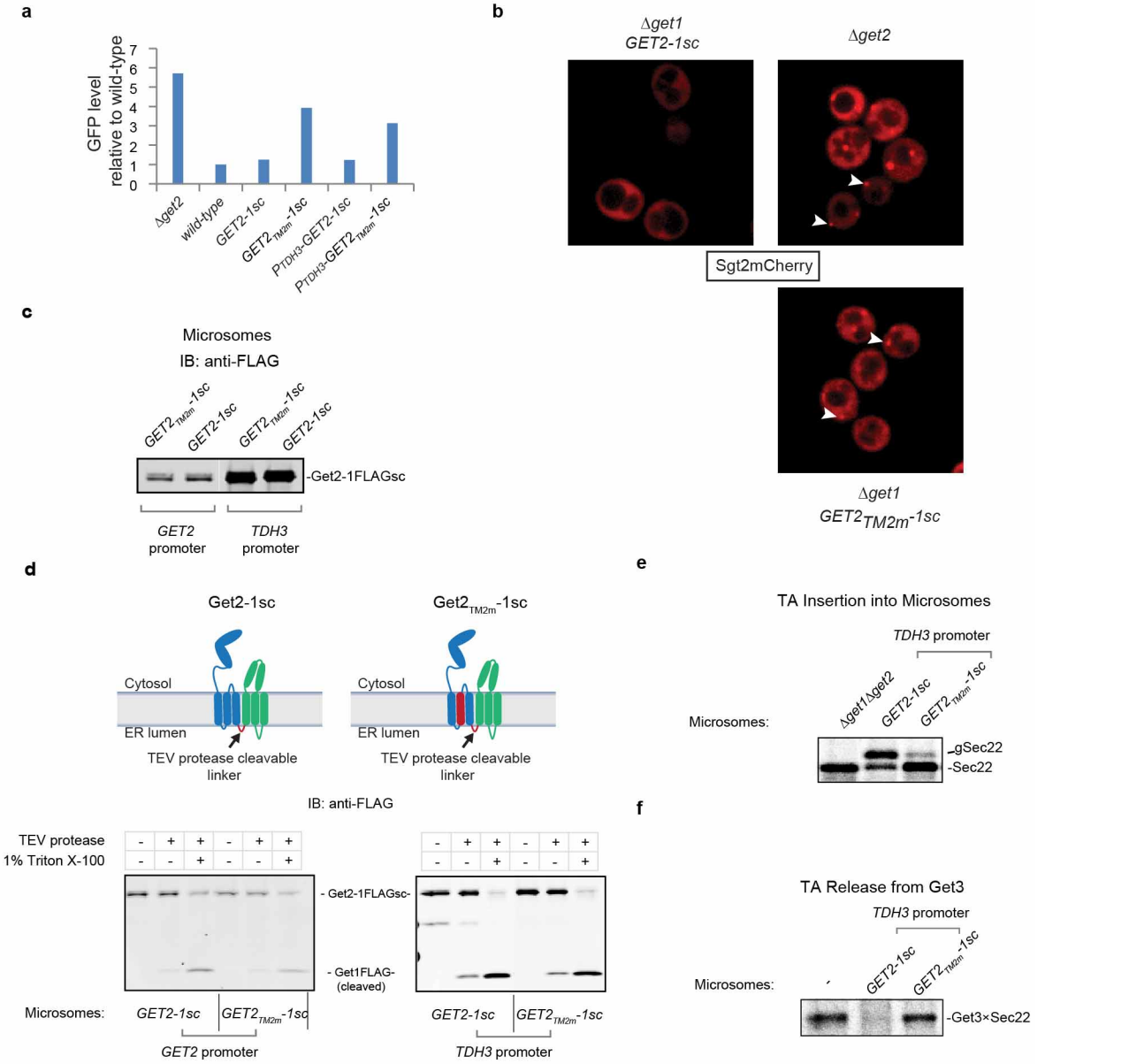
Extended Data Figure 2 | Get1/2 TMD mutations used in this study. **a**, Schematic of Get2-1sc and transmembrane swap mutants used in this study. Ost4 TMD (N_{lumen}-C_{cyto}) containing a mutation that abolishes its interaction with other components of the OST complex was used to replace Get1/2 transmembranes that have the same topology. Sec61-β TMD was used to replace the indicated Get1/2 transmembranes of opposite topology. **b**, ClustalW2 alignment of numbered amino-acid sequences corresponding to the Get2 TM3 (ΔG prediction server version 1.0) from the indicated fungal homologues (Sc, *Saccharomyces cerevisiae*; Cg, *Candida glabrata*; Ss, *Scheffersomyces stipites*; Sp, *Schizosaccharomyces pombe*) and human calcium-modulating cyclophilin ligand (CAML). Aligned positions were colour-coded by Jalview and the degree of conservation is shown below as a sequence consensus histogram. Asterisk indicates 100% conservation of Get2Sc D271.



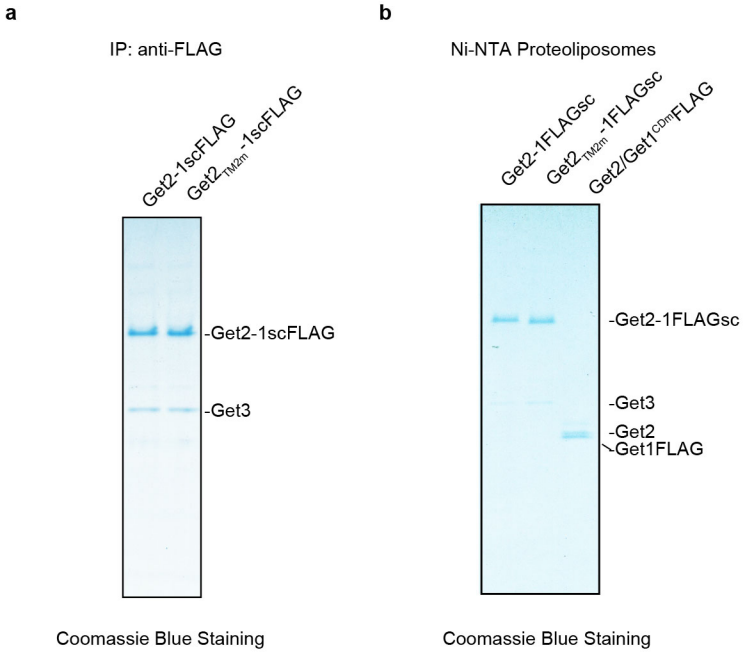
Extended Data Figure 3 | Genetic disruption of the TMD of Get1/2 causes a general defect in substrate release from Get3. a, Affinity-purified Get3–Sec22 was incubated with the indicated microsomes and analysed by Sec22 insertion analysis as in Fig. 1b. **b**, Affinity-purified Get3–Sec22 was incubated with the indicated microsomes or mock-incubated and analysed by crosslinking analysis as in Fig. 1c. **c**, Left: the hydrophobicity of the indicated TMD sequences was calculated using the ΔG prediction server version 1.0. Right: affinity-purified Get3–Sec61-β was incubated with the indicated microsomes followed by crosslinking analysis as in Fig. 1c.



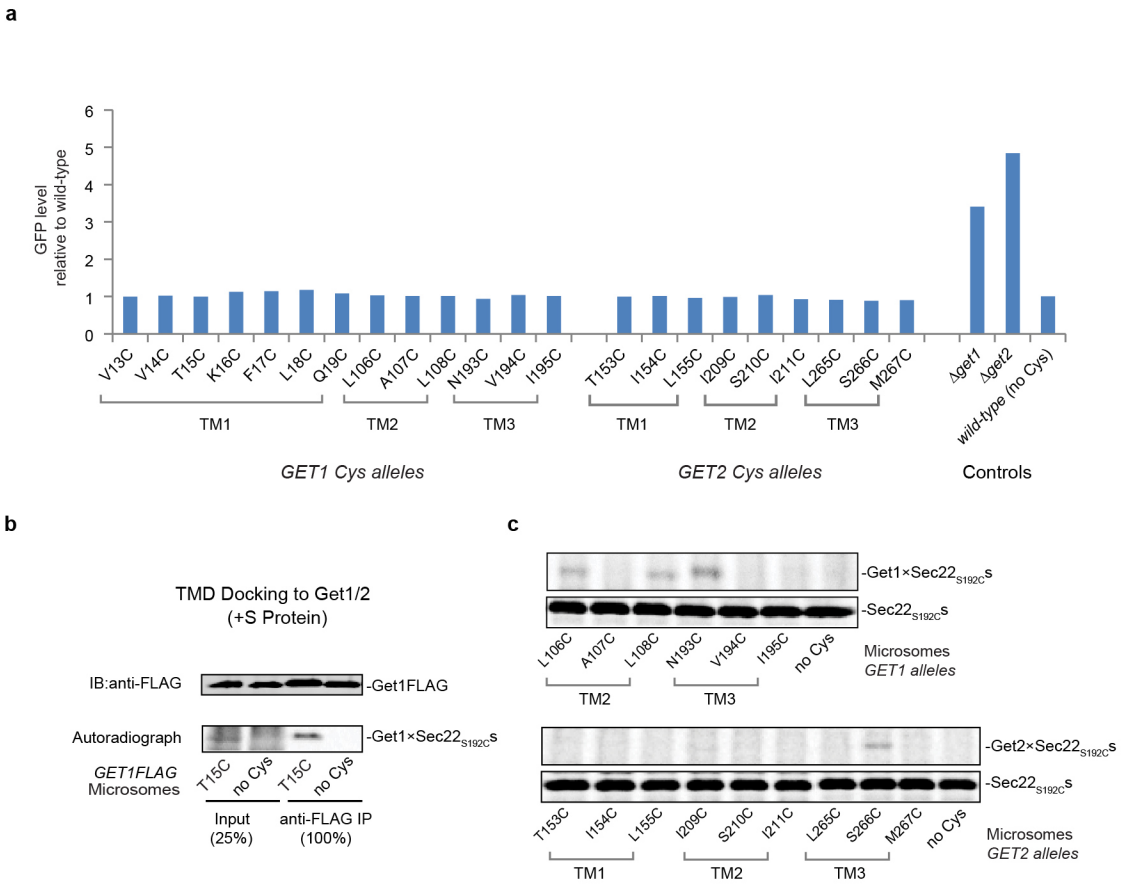
Extended Data Figure 4 | Get3 is efficiently recruited to microsomes with a severe genetic disruption of the Get1/2 TMD. **a**, Whole-cell lysates from the indicated yeast strains were subjected to SDS–PAGE analysis and visualized by immunoblotting. Hexokinase (HXK) was used as a loading control. **b**, Recombinant Get3–Flag was incubated with the indicated microsomes and analysed by membrane flotation analysis and immunoblotting as in Fig. 2a.



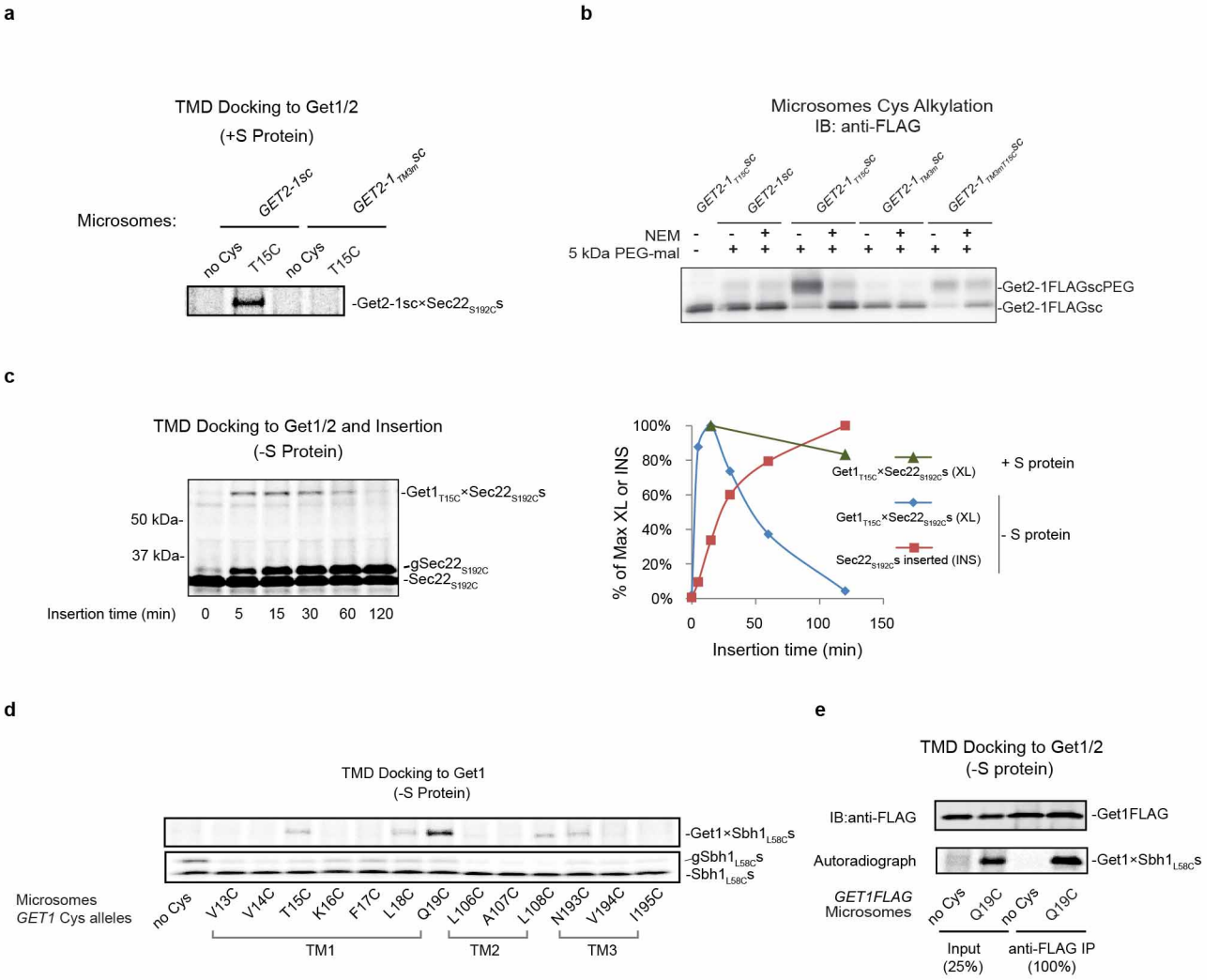
Extended Data Figure 5 | Further *in vivo* and *in vitro* analysis of mutant phenotypes associated with Get2$_{TM2}$-1sc. **a**, GFP expression of the heat-shock reporter in the indicated strains was measured by flow cytometry analysis as in Fig. 1a ($n = 2$). **b**, Representative confocal microscopy images of Sgt2–mCherry localization in the indicated strains. White arrowheads indicate the presence of cytosolic tail-anchored protein aggregates. **c**, Equal amounts of microsomes (normalized by $D_{280\,nm}$) were subjected to SDS–PAGE analysis and visualized by immunoblotting. **d**, The indicated microsomes were solubilized in Triton X-100 detergent or mock-solubilized on ice for 30 min. TEV protease digestion of samples, as indicated, was performed overnight at 4 °C followed by SDS–PAGE analysis and immunoblotting. **e**, Affinity-purified Get3–Sec22 was incubated with the indicated microsomes and analysed by Sec22 insertion analysis as in Fig. 1b. **f**, Affinity-purified Get3–Sec22 was incubated with the indicated microsomes or mock-incubated and analysed by crosslinking analysis as in Fig. 1c.



Extended Data Figure 6 | Biochemical characterization of Get1/2 transmembrane complexes and proteoliposomes used in Fig. 2b. **a**, The indicated samples were affinity-purified by anti-Flag immunoprecipitation and elution with Flag peptide, subjected to SDS–PAGE analysis, and visualized by Coomassie blue staining. **b**, Following proteoliposome reconstitution with the indicated affinity-purified proteins, membranes were separated from the rest of the material by flotation analysis followed by centrifugation of the low-density fraction. The resulting purified proteoliposomes were subjected to SDS–PAGE analysis and visualized by Coomassie blue staining.



Extended Data Figure 7 | Defining Get1/2 transmembrane Cys positions that interact with tail-anchored proteins. **a**, Cells expressing the indicated single-cysteine alleles of Get1 and Get2 from their endogenous genetic loci and the indicated control cells were subjected to heat-shock reporter analysis as in Fig. 1a ($n = 2$). **b**, Cell extracts containing Get3–Sec22$_{S192C}$ were incubated with S protein followed by the addition of the indicated microsomes for 15 min at room temperature and crosslinking analysis as in Fig. 3c. Samples were denatured with SDS (an ionic detergent) and diluted into immunoprecipitation buffer with Triton X-100 (a non-ionic detergent) before pull-down with anti-Flag resin. Eluted material was subjected to SDS–PAGE analysis and visualized by immunoblotting and autoradiography. **c**, Get3–Sec22$_{S192C}$s was road-blocked with S protein and incubated with the indicated microsomes followed by crosslinking analysis as in Fig. 3c.



Extended Data Figure 8 | Insertase-disrupting mutation prevents Get1/2 TMD interactions with pre-integrated tail-anchored proteins. **a**, Affinity-purified Get3–$Sec22_{S192C}$s was road-blocked with S protein and incubated with the indicated microsomes for subsequent crosslinking analysis as in Fig. 3c. **b**, NEM alkylation or mock treatment of the indicated microsomes was followed by NEM quenching and membrane solubilization in SDS. The denatured samples were subjected to PEG-maleimide alkylation, as indicated. Alkylation was quenched and samples were subjected to SDS–PAGE analysis and visualized by immunoblotting. Get2-1scPEG indicates the PEGylated form of Get2-1sc. Quantitation revealed that the cysteine accessibility factor of T15C was 0.97 for Get2-1sc and 0.52 for Get2-1_{TM3m}sc. Thus, the lack of $Sec22_{S192C}$ crosslinking to Get2-1_{TM3}sc (T15C) (Extended Data Fig. 8a) is unlikely owing to cysteine inaccessibility. **c**, Affinity-purified Get3–$Sec22_{S192C}$s was road-blocked with S protein for 10 min at room temperature or mock-treated before incubation with $Get1_{T15C}$ microsomes for the indicated amounts of time. Samples were simultaneously subjected to Sec22 insertion and crosslinking analysis as in Figs 1b and 3c, respectively. On the right is a kinetic analysis plot of the gel data shown on the left. XL indicates the amount of crosslinked product between Get1 and Sec22. **d**, Affinity-purified Get3–$Sbh1_{L58C}$s was incubated with the indicated microsomes at room temperature for 5 min. Samples were simultaneously subjected to substrate insertion and crosslinking analysis as in Figs 1b and 3c, respectively. **e**, Cell extracts containing Get3–$Sbh1_{L158C}$s were incubated with the indicated microsomes for 5 min at room temperature and crosslinking/immunoprecipitation analysis as in Extended Data Fig. 7b.