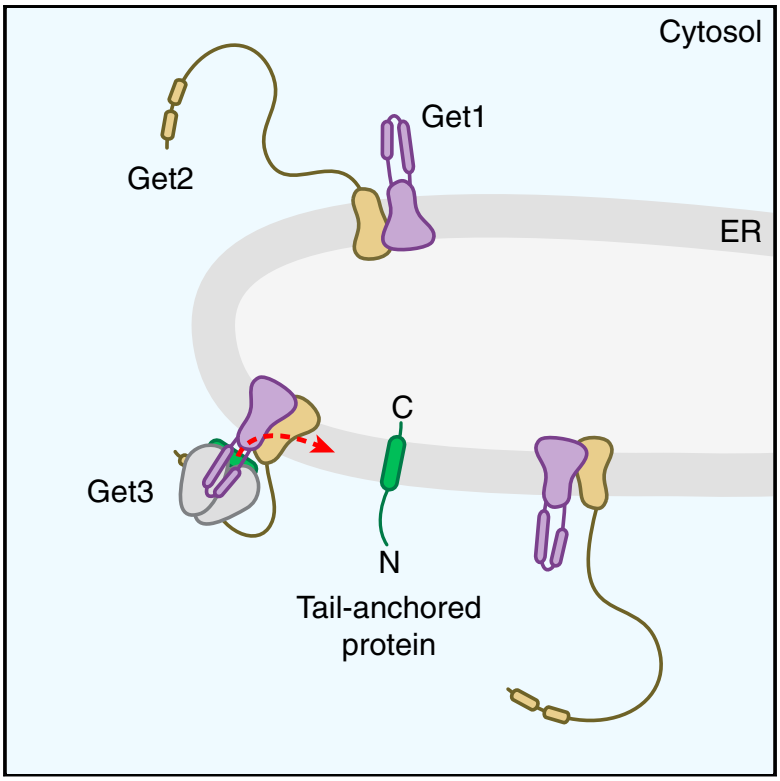
Cell Reports

Tail-Anchored Protein Insertion by a Single Get1/2 Heterodimer

Graphical Abstract



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In Brief

Tail-anchored membrane proteins are inserted into the endoplasmic reticulum via the post-translational GET pathway. Zalisko et al. combine single-molecule and bulk fluorescence measurements with quantitative in vitro insertion assays to define the architecture of the heterodimeric Get1/2 insertase and its engagement with the soluble chaperone Get3.

Highlights

- The minimum functional unit for TA protein insertion is a Get1/2 heterodimer
- Get3 binds a single Get1/2 complex, even when multiple complexes are present
- Get1 and Get2 bind simultaneously to opposite sides of post-hydrolysis Get3 dimers

Zalisko et al., 2017, Cell Reports *20*, 2287–2293
September 5, 2017
http://dx.doi.org/10.1016/j.celrep.2017.08.035

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Tail-Anchored Protein Insertion by a Single Get1/2 Heterodimer

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http://dx.doi.org/10.1016/j.celrep.2017.08.035

SUMMARY

The Get1/2 transmembrane complex drives the insertion of tail-anchored (TA) proteins from the cytosolic chaperone Get3 into the endoplasmic reticulum membrane. Mechanistic insight into how Get1/2 coordinates this process is confounded by a lack of understanding of the basic architecture of the complex. Here, we define the oligomeric state of full-length Get1/2 in reconstituted lipid bilayers by combining single-molecule and bulk fluorescence measurements with quantitative in vitro insertion analysis. We show that a single Get1/2 heterodimer is sufficient for insertion and demonstrate that the conserved cytosolic regions of Get1 and Get2 bind asymmetrically to opposing subunits of the Get3 homodimer. Altogether, our results define a simplified model for how Get1/2 and Get3 coordinate TA protein insertion.

INTRODUCTION

Tail-anchored (TA) proteins, defined by a single carboxy-terminal transmembrane domain (TMD) and a cytosolic-facing amino-terminal domain (Kutay et al., 1993), are post-translationally inserted into the endoplasmic reticulum (ER) membrane via the evolutionarily conserved guided-entry of TA protein (GET) pathway (Denic et al., 2013; Hegde and Keenan, 2011; Stefanovic and Hegde, 2007). In yeast, the membrane targeting factor is a homodimeric ATPase called Get3, which changes conformation in a nucleotide-dependent manner (Bozkurt et al., 2009; Hu et al., 2009; Mateja et al., 2009; Suloway et al., 2009; Yamagata et al., 2010) to bind TA proteins in the cytosol and release them at the ER membrane. The Get1/2 transmembrane complex (Schuldiner et al., 2008) recruits the Get3-TA targeting complex (Mateja et al., 2015) to the ER, coordinates TA protein release and insertion, and mediates ATP-driven recycling of Get3 to the cytosol (Mariappan et al., 2011; Stefer et al., 2011; Wang et al., 2011, 2014).

Get1 and Get2 are the only integral membrane components required for TA protein insertion (Mariappan et al., 2011; Wang et al., 2011), and specific functions for the individual subunits have been defined based on a series of functional and structural studies (Kubota et al., 2012; Mariappan et al., 2011; Stefer et al., 2011; Wang et al., 2011). The long N-terminal cytosolic domain of Get2 facilitates initial recruitment of the targeting complex, while the cytosolic coiled coil of Get1 drives TA protein release. Following release, the TMDs of both Get1 and Get2 contact the TA protein as it inserts into the bilayer (Wang et al., 2014), and ATP binding enhances dissociation of Get3 from the Get1 coiled coil, facilitating Get3 recycling to the cytosol.

Despite these mechanistic insights, how full-length Get1 and Get2 function together to coordinate events at the membrane remains unclear. Although Get1/2 complexes can be isolated with Get3 from yeast rough microsomes (Auld et al., 2006; Jonikas et al., 2009), the quaternary structure of the Get1/2/3 complex is undefined. Crystal structures of the cytosolic Get1 or Get2 fragments bound symmetrically to different nucleotide states of homodimeric Get3 have led to closely related models involving a heterotetrameric Get1/2 assembly of two Get1 and two Get2 subunits (Figure 1A) (Mariappan et al., 2011; Stefer et al., 2011); such an assembly might exist constitutively, or it might form dynamically in the presence of Get3 (Figure 1B). Simpler models are also plausible, including a heterodimeric Get1/2 assembly with only one copy of each subunit (Figure 1C).

Defining the oligomeric state of the functional Get1/2 complex is critical for understanding its molecular mechanism. For example, the number of subunits present in the functional complex likely dictates whether the Get1 and Get2 cytosolic domains bind competitively or simultaneously to the same or opposite sides of the Get3 homodimer at various stages along the pathway (Figure 1) (Mariappan et al., 2011; Stefer et al., 2011). Likewise, the number of subunits has important implications for how the Get1/2 TMDs guide TA substrates into the bilayer (Wang et al., 2014) and whether conformational changes in Get3 can be coupled to these TMDs during insertion (Denic et al., 2013; Hegde and Keenan, 2011; Stefer et al., 2011). Thus, a rigorous description of how Get1/2 coordinates key steps at the membrane requires knowledge of its quaternary structure. Here we show that the minimal functional unit of the full-length Get1/2 complex is a heterodimer, which drives the insertion of TA proteins by binding to opposites sides of the Get3 homodimer.

RESULTS

To gain insight into the organization of the Get1/2 complex, we developed a bulk fluorescence resonance energy transfer (FRET) assay in proteoliposomes that reports on changes in

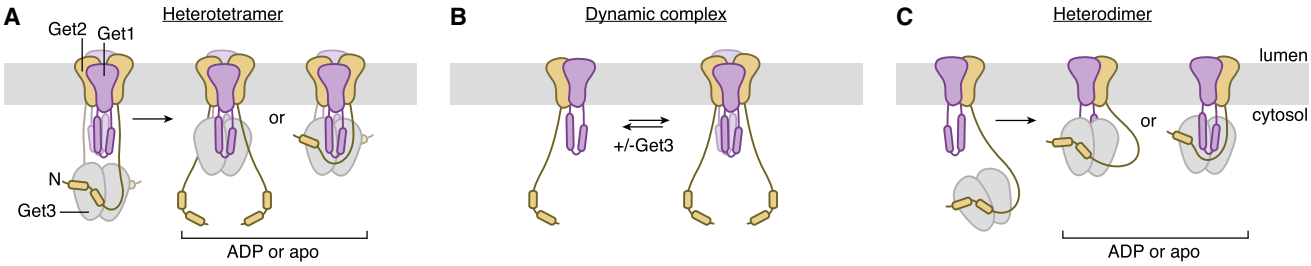

Figure 1. Alternative Models for the Get1/2 Architecture

(A) In static heterotetramer models, based on structural and biochemical studies (Mariappan et al., 2011; Stefer et al., 2011), Get1/2 exists as a constitutive complex comprising two copies of each subunit. Accordingly, the Get3-TA protein complex is captured by two copies of Get2, which bind on opposite sides of the symmetric Get3 dimer. Subsequently, the ADP or apo form of the Get3-TA complex is handed off to Get1, which displaces each Get2 subunit such that two Get1 subunits bind on opposite sides of Get3. Alternatively, Get1 only partially displaces Get2, such that two Get1 and two Get2 subunits are bound to Get3. All heterotetramer models predict that two copies of Get1 and/or Get2 bind simultaneously to Get3 at different steps in the pathway. In such models, conformational changes in Get3 could be coupled to the transmembrane domains of Get1/2 by rigid interactions mediated by the coiled coil (not shown).

(B) In a dynamic model, Get3 binding drives the transient assembly of two heterodimeric Get1/2 complexes into a single heterotetrameric complex.

(C) In a static heterodimer model, the Get3-TA complex is initially captured by a single copy of Get2 bound to one side of the Get3 dimer; Get1 then engages the ADP-bound or apo form of the Get3-TA complex by partially displacing Get2 or by binding to the opposite side of the Get3 dimer.

the proximity of Get1 and/or Get2 subunits upon binding to Get3. We introduced single cysteines at membrane-proximal (Get1-A95C; Get2-E220C) or cytosolic (Get1-S77C; Get2-S28C) positions within Get1 and Get2 (Figure 2A). After purification (Figure S1), the individual subunits were labeled with FRET donor (Cy3) or acceptor (Cy5) fluorophores. Cytosolically or membrane-proximal-labeled Get1 and Get2 subunits were then reconstituted into proteoliposomes in different donor-acceptor combinations: Get1^{Cy3}-Get1^{Cy5}-Get2, Get1^{Cy3}-Get2^{Cy5} (membrane proximal), Get1^{Cy5}-Get2^{Cy3} (cytosolic), and Get1-Get2^{Cy3}-Get2^{Cy5}. These proteoliposomes are reconstituted at a high protein-to-lipid ratio (12:10,000) such that they contain multiple copies of Get1/2. This allows for an unbiased analysis of Get3 binding to different oligomeric states of Get1/2. After verifying the insertion activity of the different proteoliposomes (Figure S2A), we monitored changes in FRET as a function of binding to different nucleotide states of Get3.

We first explored whether Get3 binding drives Get1/2 toward a higher oligomeric state, as would be expected in the dynamic model (Figure 1B). When proteoliposomes containing different combinations of membrane-proximal labels were incubated with Get3, we observed no significant FRET increase, regardless of which subunits were labeled or the nucleotide state of Get3 (Figure 2B; Figure S2B). Thus, consistent with static models, Get3 binding does not drive assembly of a higher-order oligomer of Get1/2.

Next, we used proteoliposomes containing different combinations of cytosolically labeled Get1 and Get2 to explore how they engage Get3. The cytosolic coiled coil of Get1 and the long (150-residue) unstructured N terminus of Get2 do not interact with each other but instead bind to overlapping sites present on each of two sides of the Get3 homodimer (Mariappan et al., 2011; Stefer et al., 2011). These cytosolic regions are expected to FRET most efficiently when brought into proximity by simultaneous binding to Get3. Studies with the isolated cytosolic fragments show that the Get2-Get3 interaction is insensitive to nucleotide but that the Get1-Get3 interaction is weakened by ADP and completely disrupted by ATP (Mariappan et al., 2011; Rome et al., 2014; Stefer et al., 2011; Wang et al., 2011).

When Get3 was incubated with cytosolically labeled Get1^{Cy3}-Get1^{Cy5}-Get2 or Get1-Get2^{Cy3}-Get2^{Cy5} proteoliposomes, no significant FRET increase was observed, regardless of the nucleotide state (Figure 2B; Figure S2B). Likewise, no significant FRET increase was observed when ATP-bound Get3 was incubated with Get1^{Cy5}-Get2^{Cy3} proteoliposomes. However, when ADP-bound or nucleotide-free Get3 was incubated with Get1^{Cy5}-Get2^{Cy3} proteoliposomes we observed a strong FRET increase (Figure 2B; Figure S2B). When cytosolically labeled Get1^{Cy5}-Get2^{Cy3} proteoliposomes (10 nM) were titrated with Get3, we observed a linear FRET increase that became saturated at one equivalent of Get3 (10 nM homodimer) (Figure 1C) and could be reversed by ATP (Figure 2C). These data are consistent with Get3 binding with sub-nanomolar affinity to a single Get1/2 heterodimer.

The quantitative and reversible Get3-dependent FRET increase observed with cytosolically labeled Get1^{Cy5}-Get2^{Cy3} proteoliposomes provides direct evidence for simultaneous binding of full-length Get1 and Get2 to the nucleotide-free and ADP-bound states of Get3. Moreover, the absence of Get3-dependent FRET increases with cytosolically labeled Get1^{Cy3}-Get1^{Cy5}-Get2 or Get1-Get2^{Cy3}-Get2^{Cy5} proteoliposomes suggests that Get3 does not bind concomitantly to two Get1 and/or two Get2 subunits in any nucleotide state, consistent with a heterodimeric Get1/2 complex.

To recapitulate the result with single Get1/2 heterodimers per liposome, we repeated the bulk FRET experiment with all donor-acceptor combinations reconstituted at a lower protein-to-lipid ratio (1.2:10,000). Consistent with the presence of ~1 Get1/2 heterodimer per liposome, we observed negligible FRET in Get1^{Cy3}-Get1^{Cy5}-Get2 and Get1-Get2^{Cy3}-Get2^{Cy5} proteoliposomes but clear FRET for both cytosolically labeled Get1^{Cy5}-Get2^{Cy3} and membrane-proximal Get1^{Cy3}-Get2^{Cy5} proteoliposomes (Figure S2B). Get3 binding did not significantly change FRET between membrane-proximal-labeled Get1^{Cy3}-Get2^{Cy5}

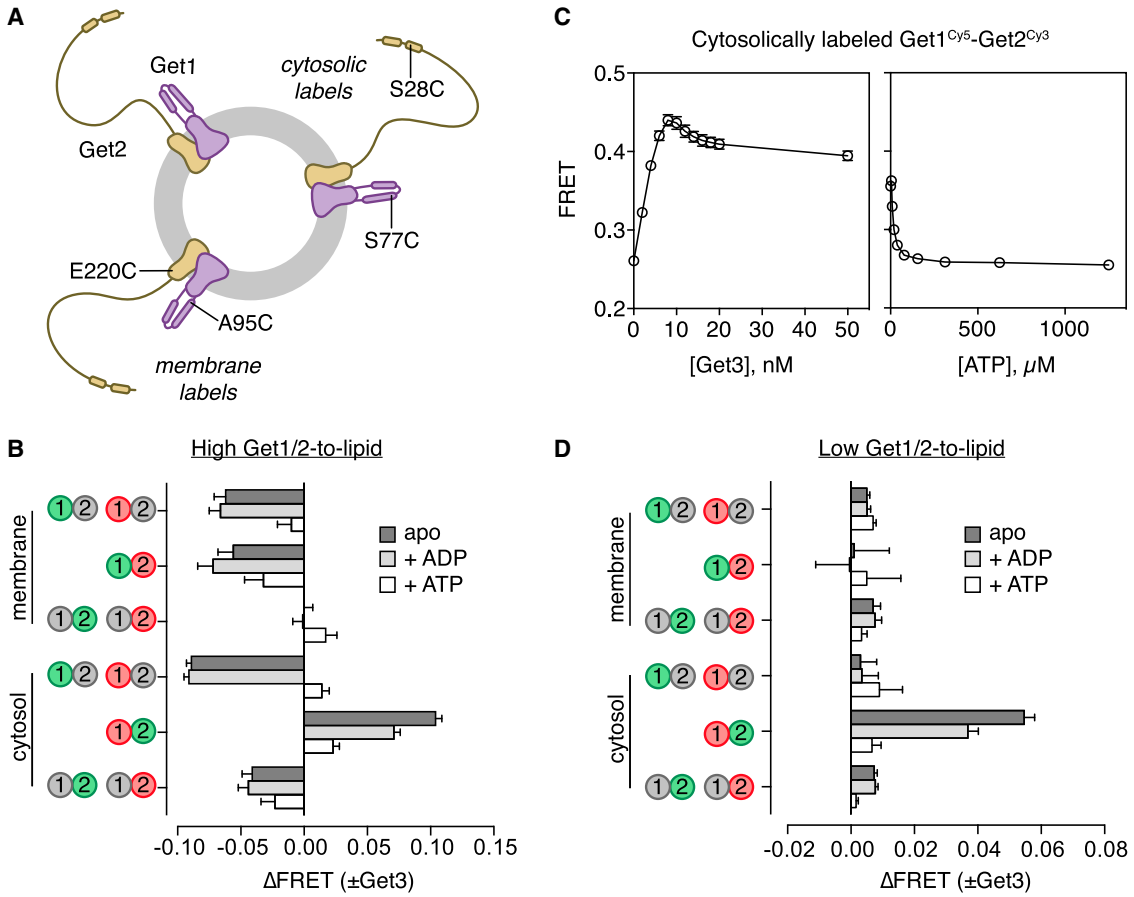

Figure 2. Bulk FRET Measurements of Get3 Binding to Get1/2 Complexes in Proteoliposomes

(A) Get1 and Get2 subunits were labeled with FRET donor Cy3 (green) or FRET acceptor Cy5 (red) fluorophores at membrane-proximal or cytosolic positions and then reconstituted into proteoliposomes in different donor-acceptor combinations.

(B) Proteoliposomes were reconstituted at 12 Get1/2 molecules per 10,000 lipids ("High Get1/2-to-lipid"). The histogram shows the FRET change after addition of 50 nM Get3 (dark gray), Get3 + ADP (gray), or an ATPase-deficient Get3-D57N mutant + ATP (white) to each of the six proteoliposomes (10 nM Get1/2). A significant Get3-dependent FRET increase is only observed when the donor-acceptor pairs are located on the cytosolic positions of Get1 and Get2 subunits. All samples show a non-specific FRET component in the absence of Get3 due to co-reconstitution of multiple donors and acceptors in the same proteoliposomes (Figure S2B); we interpret a Get3-dependent FRET decrease as an increase in the average distance between labeled Get1/2 heterodimers due to steric hindrance caused by Get3 binding. This decreased FRET is eliminated in proteoliposomes reconstituted at lower protein-to-lipid ratios (D and Figure S2B).

(C) At left, FRET-based titration of cytosolically labeled $Get1^{Cy5}$-$Get2^{Cy3}$ proteoliposomes (10 nM Get1/2) with Get3-D57N. At right, disruption of the Get1-Get2-Get3 interaction in the same proteoliposomes, monitored by the change in FRET upon titration with ATP.

(D) As in (B), but after reconstitution at lower Get1/2-to-lipid ratios (1.2 Get1/2 molecules per 10,000 lipids; "Low Get1/2-to-lipid").

See also Figures S1 and S2.

(Figure 2D), indicating that the Get3-dependent FRET decreases observed at higher protein-to-lipid ratios (Figure 2C) result from the presence of multiple copies of labeled Get1/2 in the same proteoliposome. However, as with the higher protein-to-lipid ratio reconstitutions, the addition of ADP-bound or nucleotide-free Get3 increased FRET between cytosolically labeled $Get1^{Cy5}$-$Get2^{Cy3}$ (Figure 2D). While these bulk FRET data are consistent with a model in which Get3 binds to a heterodimeric Get1/2 complex comprising a single copy of each subunit, they do not formally exclude the possibility that Get1/2 functions as a heterotetrameric or higher-order complex.

To rigorously test this heterodimeric Get1/2 model, we sought to quantify the number of Get1/2 complexes required for TA protein insertion into membranes. If the minimal functional unit is a Get1/2 heterodimer, then proteoliposomes containing a single Get1/2 heterodimer would be expected to have the same specific activity as proteoliposomes containing multiple Get1/2 heterodimers.

To prevent dissociation of the Get1 and Get2 subunits during reconstitution, we took advantage of an engineered single-chain Get1/2 (Get2-1sc) construct shown previously to be functional in yeast (Wang et al., 2014). We expressed and purified Get2-1sc from *E. coli* and verified its activity in vitro. Like native Get1/2, Get2-1sc, but not its variants containing inactivating mutations in the cytosolic fragments of Get1 (R73E) or Get2 (R17E), is functional for TA protein insertion in proteoliposomes (Figure 3A).

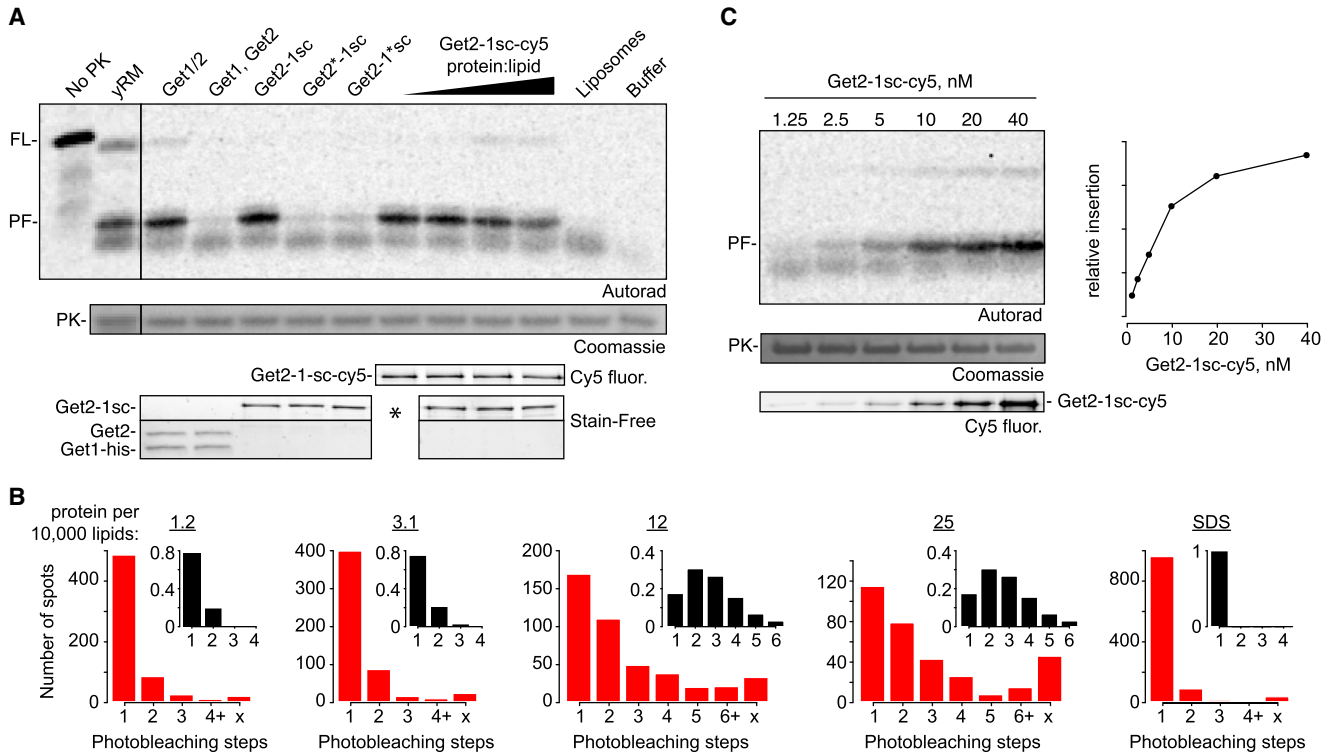

Figure 3. Single Get1/2 Heterodimers Mediate TA Protein Insertion

(A) Yeast rough microsomes (yRMs) or the indicated proteoliposomes were tested for insertion of radiolabeled TA protein, Sec61β (FL), by a proteinase K protection assay. The appearance of a protected fragment (PF), which is diagnostic for insertion, was quantified by SDS-PAGE and autoradiography. Co-reconstituted Get1 and Get2 subunits (Get1/2) show equivalent specific activity to Get2-1sc proteoliposomes. Mixtures of Get1-only and Get2-only proteoliposomes (Get1, Get2) and single-chain constructs containing inactivating point mutations in either the Get1 (Get2-1*sc; R73E in Get1) or the Get2 (Get2*-1sc; R17E in Get2) cytosolic domains show no activity. The four Get2-1sc-Cy5 samples correspond to the four protein-to-lipid ratio reconstitutions in (B), at increasing protein-to-lipid ratios. All reconstitutions were diluted with empty liposomes to a final concentration of 10 nM Get1/2. The normalized insertion activity is independent of the number of Get2-1sc-Cy5 molecules in each proteoliposome. Coomassie-stained proteinase K (PK) was used as a loading control. Before performing the insertion assay, proper Get2-1sc-Cy5 normalization was confirmed by Cy5-imaged SDS-PAGE. For completeness, the concentration of Get2-1sc-Cy5 samples was also compared with unlabeled Get1/2 samples by stain-free SDS-PAGE; no sample was run for the most dilute Get2-1sc-Cy5 sample (marked with an asterisk), because of high lipid content, low protein concentration, and the large amount of sample required for stain-free detection.

(B) Single-molecule photobleaching analysis of proteoliposomes from (A), reconstituted with Cy5-labeled single-chain Get1/2 (Get2-1sc-Cy5) at different protein-to-lipid ratios. The number of photobleaching steps per labeled proteoliposome is shown in red; x represents discarded traces. SDS-solubilized Get2-1sc-Cy5 serves as a monomeric control. The inset (black) shows the calculated proportion of Get2-1sc-Cy5 found in different oligomeric states, as described in the Supplemental Experimental Procedures.

(C) In parallel with the assay shown in (A), Get2-1sc-Cy5 proteoliposomes (reconstituted at a ratio of 12 proteins per 10,000 lipids) were diluted to the indicated final concentrations, and TA protein insertion was quantified by autoradiography. This control experiment demonstrates that the assay is linear up to a total Get2-1sc-Cy5 concentration of ~10 nM and is not limited by the active targeting complex.

See also Figures S1 and S3.

Next, we purified and fluorescently labeled a Get2-1sc construct harboring the Get1-S77C mutation (Figure S1). By varying the protein-to-lipid ratio during reconstitution, the average number of Get2-1sc-Cy5 molecules per liposome could be adjusted. This was directly quantified by single-molecule photobleaching using total internal reflection fluorescence (TIRF) microscopy (Figure 3B; Figures S3A–S3D). At the lowest protein-to-lipid ratio tested, more than 80% of Get2-1sc-Cy5 was incorporated into liposomes containing only one Get2-1sc-Cy5 molecule. At the highest protein-to-lipid ratios, we observed a corresponding increase in the number of molecules per liposome, with as much as 80% of Get2-1sc-Cy5 incorporated into liposomes containing two or more Get2-1sc-Cy5 molecules. Thus, proteoliposomes reconstituted at the highest protein-to-lipid ratio contain about four times as many Get2-1sc-Cy5 molecules—that could, in principle, oligomerize—than proteoliposomes reconstituted at the lowest protein-to-lipid ratio.

To measure the specific insertion activity of the different proteoliposome reconstitutions, we normalized the total Get2-1sc-Cy5 concentration in each sample by adding the appropriate volume of empty liposomes. If oligomerized Get2-1sc-Cy5 is required for insertion, photobleaching analysis predicts that proteoliposomes reconstituted at the lowest protein-to-lipid ratio should show at least 4-fold-lower insertion activity than Get2-1sc-Cy5 reconstituted at the highest protein-to-lipid ratio. We found that the specific insertion activity was essentially

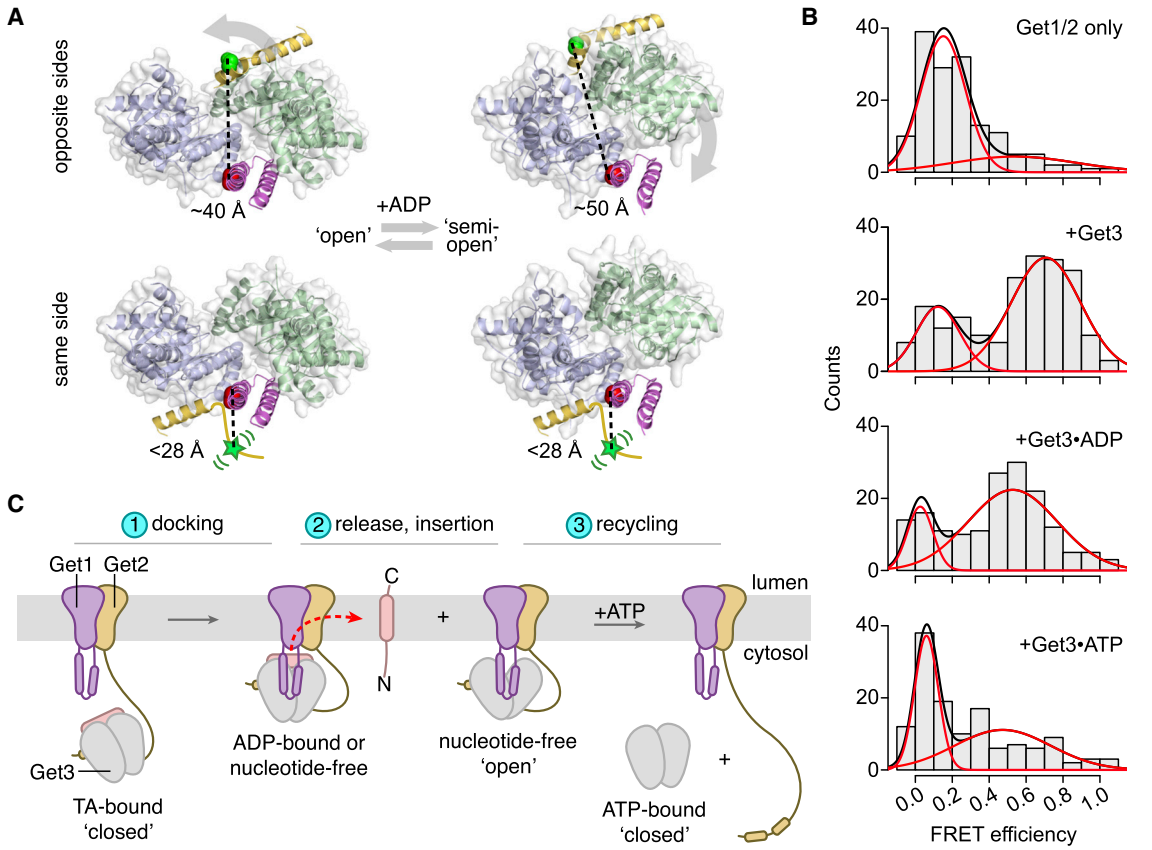

Figure 4. Get1 and Get2 Bind on Opposite Sides of Get3 in Its Post-hydrolysis States

(A) Models of Get1 (magenta) and Get2 (yellow) bound to the same or opposite sides of the nucleotide-free (open; PDB: 3ZS8) and ADP-bound (semi-open; PDB: 3VLC) conformations of the Get3 homodimer (blue, green). The models are aligned on one Get3 subunit (blue) to highlight the pseudo-rigid-body swivel (gray arrows) of the other subunit (green) that accompanies ADP binding. When bound to opposite sides of Get3, Get1 and Get2 move apart in response to ADP binding; thus, the distance between donor and acceptor fluorophores on Get1-S77C-Cy5 (red) and Get2-S28C-Cy3 (green) is expected to increase when ADP is added. In contrast, when bound to the same side of Get3, the relative positions of Get1 and Get2 are fixed, because the primary contacts are made to the same subunit (blue). Thus, the distance between fluorophores is expected to remain constant as Get3 changes conformation in response to ADP binding.

(B) Distributions of single-molecule FRET efficiencies (gray histograms) in nanodiscs containing cytosolically labeled Get1-S77C-Cy5 and Get2-S28C-Cy3 heterodimers during incubation with different nucleotide states of Get3. The solid black curves are the sums of the individual Gaussian functions (red lines) used to fit the raw data. In contrast with the constitutively high FRET efficiencies (>99%) predicted for same-side binding (Figure S4E), intermediate FRET efficiencies are observed in the nucleotide-free and ADP-bound states. Moreover, the FRET efficiency of the ADP-bound state (53% ± 4%) is less than in the nucleotide-free state (71% ± 2%), as expected for an increase in the distance between fluorophores as Get3 changes conformation from the semi-open to an open state.

(C) Model of the conformation-specific interactions between Get3 and the heterodimeric Get1/2 complex. See text for details.

See also Figure S4.

independent of the number of copies of Get2-1sc-Cy5 per liposome; robust insertion was observed in proteoliposomes containing the largest proportion of single Get2-1sc-Cy5 molecules (Figures 3B and 3C; Figures S3E–S3I). Thus, we conclude that a single Get1/2 heterodimer is minimally required for TA protein insertion.

Finally, we sought structural information on how the two subunits of Get1/2 simultaneously contact Get3. Previous nuclear magnetic resonance (NMR) studies showed that isolated cytosolic fragments of Get1 and Get2 can bind simultaneously to the same side of the Get3 dimer (Figures 1C and 4A) (Stefer et al., 2011). In this configuration, the two subunits are close, with Get1 displacing the second helix of Get2 (Figure 4A; Figure S4E). Because Get1 and Get2 are bound to the same subunit of Get3, structural modeling predicts that their relative position will remain fixed irrespective of the Get3 conformational state (Figure 4A). Alternatively, Get1 and Get2 might bind on opposite sides of the Get3 homodimer. In this case, the two subunits should be farther apart. Moreover, because Get1 and Get2 are bound to different subunits of Get3, the distance between them is expected to increase as the Get3 dimer changes conformation from its open to a semi-open state in response to ADP binding (Figure 4A) (Kubota et al., 2012; Mariappan et al., 2011; Stefer et al., 2011).

To distinguish between these two possibilities, we used single-molecule FRET as a qualitative measure of the distance between Get1 and Get2 bound to different conformational states of Get3. Here we reconstituted cytosolically labeled $Get1^{Cy5}$-$Get2^{Cy3}$

into nanodiscs, yielding a more homogeneous population of single Get1/2 heterodimers (Figure S4) and providing a cleaner system for use in TIRF flow cells. After verifying the heterodimeric Get1/2 composition of the nanodiscs by single-molecule photobleaching, we showed that they are functional for TA protein release from Get3 (Figures S4C and S4D).

Next, we measured the distribution of FRET efficiencies between the Get1 and the Get2 cytosolic domains bound to different nucleotide states of Get3 and fit each dataset to two Gaussian distributions (Figure 4B). In the absence of Get3, we observed a broad distribution of low FRET states corresponding to long inter-dye distances, as expected for non-interacting cytosolic domains. Similarly, in the presence of ATP-bound Get3, we observed a broad distribution of low FRET states, consistent with the inability of Get1 to interact with ATP-bound Get3. However, when ADP-bound Get3 was added, an intermediate FRET state was observed at 53% ± 4%, and this shifted to a higher FRET state of 71% ± 2% in the presence of nucleotide-free Get3.

The magnitude of the observed FRET efficiencies is inconsistent with simultaneous Get1 and Get2 binding on the same side of Get3, which would be expected to give rise to high FRET (~99%) in the nucleotide-free and ADP-bound Get3 samples. Moreover, the observed shift from intermediate FRET in the ADP-bound state to higher FRET in the nucleotide-free state is consistent with the expected changes if Get1 and Get2 bind on opposite sides of the Get3 homodimer. Given the Förster distance of the Cy3 and Cy5 FRET pair (60 Å) (Murphy et al., 2004) and assuming that the fluorophores freely rotate at the labeling site (i.e., $\kappa^2 = 2/3$), the relative change in distance between fluorophores in the nucleotide-free and ADP-bound states (~7 Å) is in qualitative agreement with the expected distance change based on structural modeling (~10 Å) (Figure 4A; Figure S4E). These data are most consistent with a model in which Get1 and Get2 bind simultaneously to opposite sides of the Get3 homodimer.

DISCUSSION

The previously undefined nature of the oligomeric state of the Get1/2 transmembrane complex has limited our understanding of how Get1, Get2, and Get3 coordinate TA protein insertion. Here, using full-length Get1 and Get2 in lipid bilayers, we show that the minimal functional unit of Get1/2 is a heterodimer comprising a single copy of each subunit. Even when presented with multiple complexes in the same membrane, Get3 engages only a single Get1/2 heterodimer. We also show that the cytosolic domains of Get1 and Get2 bind simultaneously to opposite sides of the post-hydrolysis Get3 homodimer.

The bulk and single-molecule FRET studies described here were performed in the absence of TA protein, because we lack an experimental means to trap a post-hydrolysis Get3-TA targeting complex that does not release TA protein to Get1/2. Nevertheless, we qualitatively extend our conclusions regarding the architecture of the Get1/2/3 complex to the targeting complex, because previous structural and biophysical analyses have shown that TA protein binding stabilizes closed conformations resembling those sampled by ATP- and ADP-bound Get3 (Mateja et al., 2009, 2015). Thus, we propose a simplified model for how docking, TA protein release and insertion, and Get3 recycling are coordinated by a Get1/2 heterodimer (Figure 4C).

Following release from Get4/5, the Get3-TA complex arrives at the membrane in a closed conformation. Because the Get1 binding site is only partially accessible in this conformation (Mateja et al., 2009, 2015; Stefer et al., 2011), the targeting complex is captured first by Get2, bringing it close to Get1 (Mariappan et al., 2011; Rome et al., 2014; Stefer et al., 2011; Wang et al., 2011). Once ATP has been hydrolyzed, Get1 binds to the opposite side of ADP-bound or nucleotide-free Get3-TA, driving it from a partially destabilized closed conformation into an open conformation that disrupts the hydrophobic TA protein binding site (Mariappan et al., 2011; Stefer et al., 2011). This large conformational change in Get3 is likely decoupled from the TMDs of the Get1/2 heterodimer by the long, flexible cytosolic domain of Get2. Following release, the TA protein is guided into the membrane by the TMDs of Get1 and Get2 (Wang et al., 2014). Finally, Get3 is recycled by ATP binding, which disrupts the Get1-Get3 interaction (Mariappan et al., 2011; Stefer et al., 2011); subsequent dissociation from Get2 may be facilitated by Get4/5 (Rome et al., 2014). Future studies are needed to obtain high-resolution structural information for each step along the pathway.

EXPERIMENTAL PROCEDURES

Protein Expression, Purification, and Labeling

Full-length, 6xHis-tagged Get1, Get2, Get3, and Get2-1sc constructs were expressed in *E. coli* and purified via Ni-nitrilotriacetic acid (NTA) affinity- and size-exclusion chromatography. Single-cysteine containing variants were labeled with maleimide fluorophores Cy3 or Cy5 (GE Healthcare), and free dye was removed by size-exclusion chromatography.

Proteoliposome Reconstitutions

Liposomes were prepared at 20 mg/mL via 100 nm extrusion in 50 mM HEPES (pH 7.5), 15% glycerol, and 2 mM DTT. Get1/2 was reconstituted at 4°C by diluting protein to 90 μL in deoxy big chap (DBC) buffer on ice. Then, 10 μL liposomes was added and incubated 15 min. DBC was removed by overnight incubation with biobeads.

Insertion Assay

Radiolabeled Get3-TA protein targeting complexes were prepared by cell-free synthesis of TwinStrep-tagged Sec61β in the presence of ^{35}S-methionine and 25 μM Get3, followed by Streptactin affinity purification. Proteoliposomes were normalized with empty liposomes, diluted to 10 nM Get1/2, and incubated with ~200 nM targeting complex for 30 min at 32°C. After 2 hr of incubation on ice with 2 mg/mL proteinase K, the reaction was quenched with PMSF followed by hot SDS. Samples were separated by 12% Tris-Tricine SDS-PAGE and visualized by Coomassie staining and autoradiography.

Fluorescence Measurements

Bulk fluorescence was recorded using a Synergy Neo plate reader with excitation filters at 540/25 and emission filters at 590/35 and 680/30. Single-molecule measurements were made with a custom TIRF microscope employing 532 and 633 nm lasers; emissions were filtered at 585/40 and 692/40. Single-molecule TIRF videos were recorded with a 200 ms exposure over 500 frames. Proteoliposomes for single-molecule photobleaching were adhered to freshly plasma-cleaned glass coverslips. Get1/2 nanodiscs with biotinylated membrane scaffold protein (MSP) were attached to glass coverslips via glass-adhered neutravidin and casein blocking.

Get1/2 Nanodisc Reconstitutions

Purified MSP1E3D1 was biotinylated with NHS-PEG_4-Biotin (Thermo). Mixed micelles were prepared by sonication of lipid and excess n-undecyl-β-d-maltoside (UM) detergent until clear. Get2 and 6xHis-tagged Get1 were diluted into UM buffer on ice, followed by addition of mixed micelles and MSP. After 1 hr of incubation, detergent was removed by overnight incubation with biobeads. Nanodiscs were then purified by Ni-NTA affinity- and size-exclusion chromatography.

Statistical Methods

Errors are reported ± SEM. The bulk FRET experiments in Figure 2 and Figure S2 combine data from three independent proteoliposome reconstitutions for each Get1/2 fluorophore combination. The single-molecule photobleaching and insertion data in Figure 3 are from the same reconstitution, one Get2-1sc-Cy5 proteoliposome reconstitution for each condition, and are representative of multiple experiments (>3). Figure S3 shows variance of the insertion assay by splitting each reconstitution into triplicates. The single-molecule FRET distributions in Figure 4 were fit to two Gaussian curves using the mle package in R.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.08.035.

AUTHOR CONTRIBUTIONS

B.E.Z., V.D., R.S.R., and R.J.K. designed the research; B.E.Z. and C.C. performed the research; B.E.Z. and R.J.K. wrote the initial draft; and all authors edited the manuscript.

ACKNOWLEDGMENTS

We thank members of the Denic, Rock, and Keenan laboratories for technical assistance and helpful discussions, and we thank E. Perozo and F. Bezanilla for sharing equipment. This work was supported by NIH R01 GM086487 (to R.J.K.), GM078450 and GM109863 (to R.S.R.), and GM099943 (to V.D.). B.E.Z. was supported by an NIH Chemistry and Biology Interface training grant (T32 GM008720).

Received: March 13, 2017
Revised: June 27, 2017
Accepted: August 7, 2017
Published: September 5, 2017

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Cell Reports, Volume *20*

Supplemental Information

Tail-Anchored Protein Insertion by a Single Get1/2 Heterodimer

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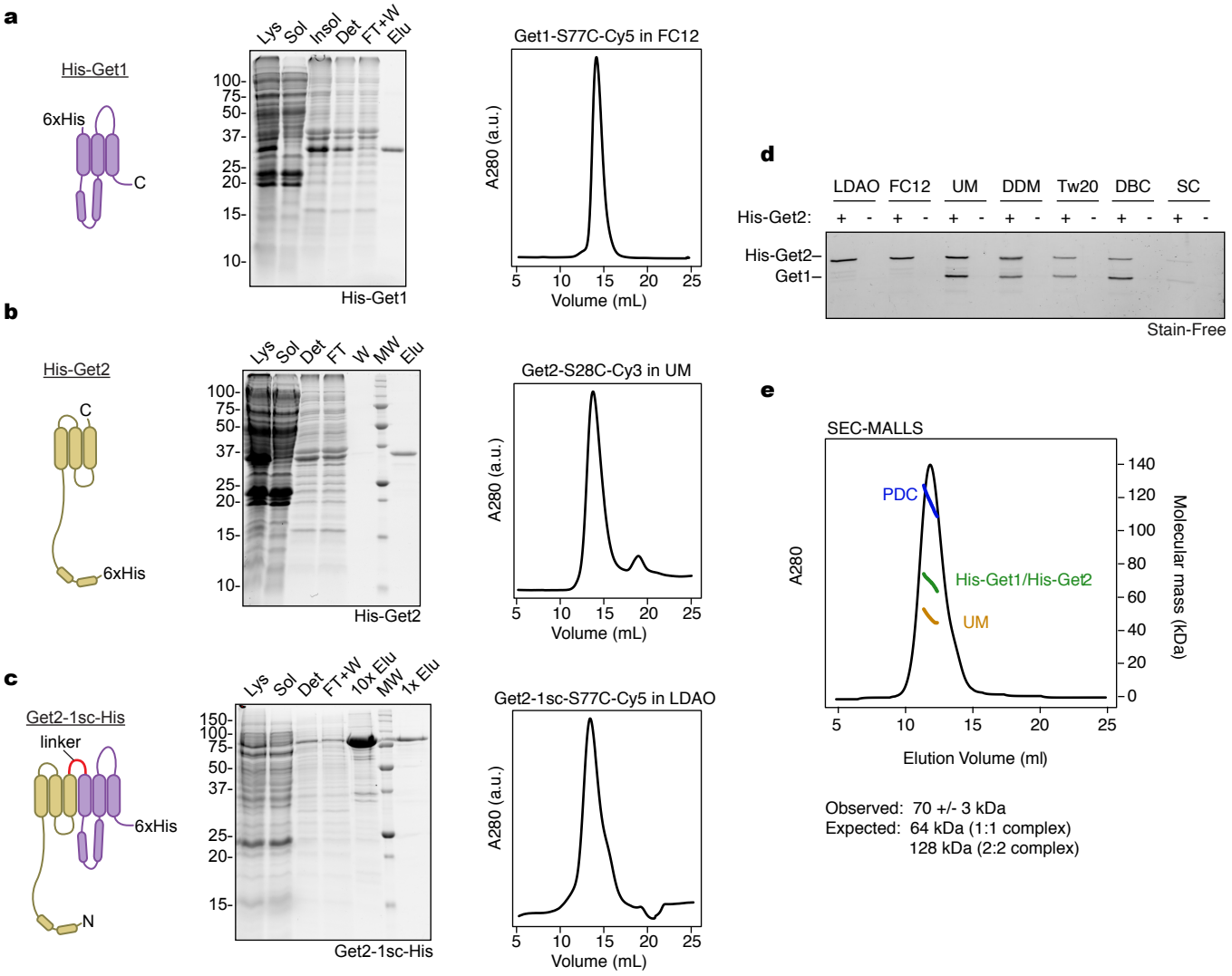

Figure S1, related to Figures 2 and 3. Preparation of recombinant Get1/2 constructs. (**a-c**) Individual His-Get1 and His-Get2 subunits and a single-chain Get2-Get1 fusion protein (Get2-1sc-His) were expressed in *E. coli* and purified by Ni-NTA affinity chromatography and gel filtration. Fractions from representative purifications were analyzed by SDS-PAGE and Coomassie blue staining; Lys = total lysate, Sol = crude lysate supernatant, Insol = resuspended lysate pellet, Det = detergent solubilized supernatant, FT = flow-through, W = wash, Elu = imidazole elution, MW = markers. After fluorescent labeling the cysteine mutants, the samples were further purified by gel filtration in the indicated detergent-containing buffers. (**d**) Purified Get1 (in FC12) was added to a 2-fold molar excess of His-Get2 (in UM), diluted at least 10-fold into the indicated detergents, subjected to Ni-NTA pull-down, and the elution fraction was analyzed by stain-free SDS-PAGE. Get1 is only recovered in the presence of His-Get2, and only in mild detergents. In SC, the subunits are not completely soluble, while in harsh detergents including LDAO and FC12, the Get1-Get2 interaction is disrupted. LDAO = 0.1% n-Dodecyl-N,N-Dimethylamine-N-Oxide, UM = 0.1% n-Undecyl-β-D-Maltopyranoside, DDM = 0.1% n-Dodecyl-α-D- Maltopyranoside, Tw20 = 0.1% Tween 20, DBC = 0.1% Deoxy Big Chap, SC = 1% Sodium Cholate. (**e**) SEC-MALLS analysis of an equimolar mixture of purified His-Get1 and His-Get2 after exchanging into UM. The observed molecular mass of the complex (70 +/- 3 kDa) is consistent with a 1:1 Get1/2 complex (His-Get1 = 30 kDa; His-Get2 = 34 kDa). The observed molecular masses of the protein-detergent complex ('PDC') and the UM micelle are 119 +/- 5 kDa and 49 +/- 3 kDa, respectively. All reconstitutions were performed in DBC (proteoliposomes) or UM (nanodiscs), as described in the Supplemental Experimental Procedures.

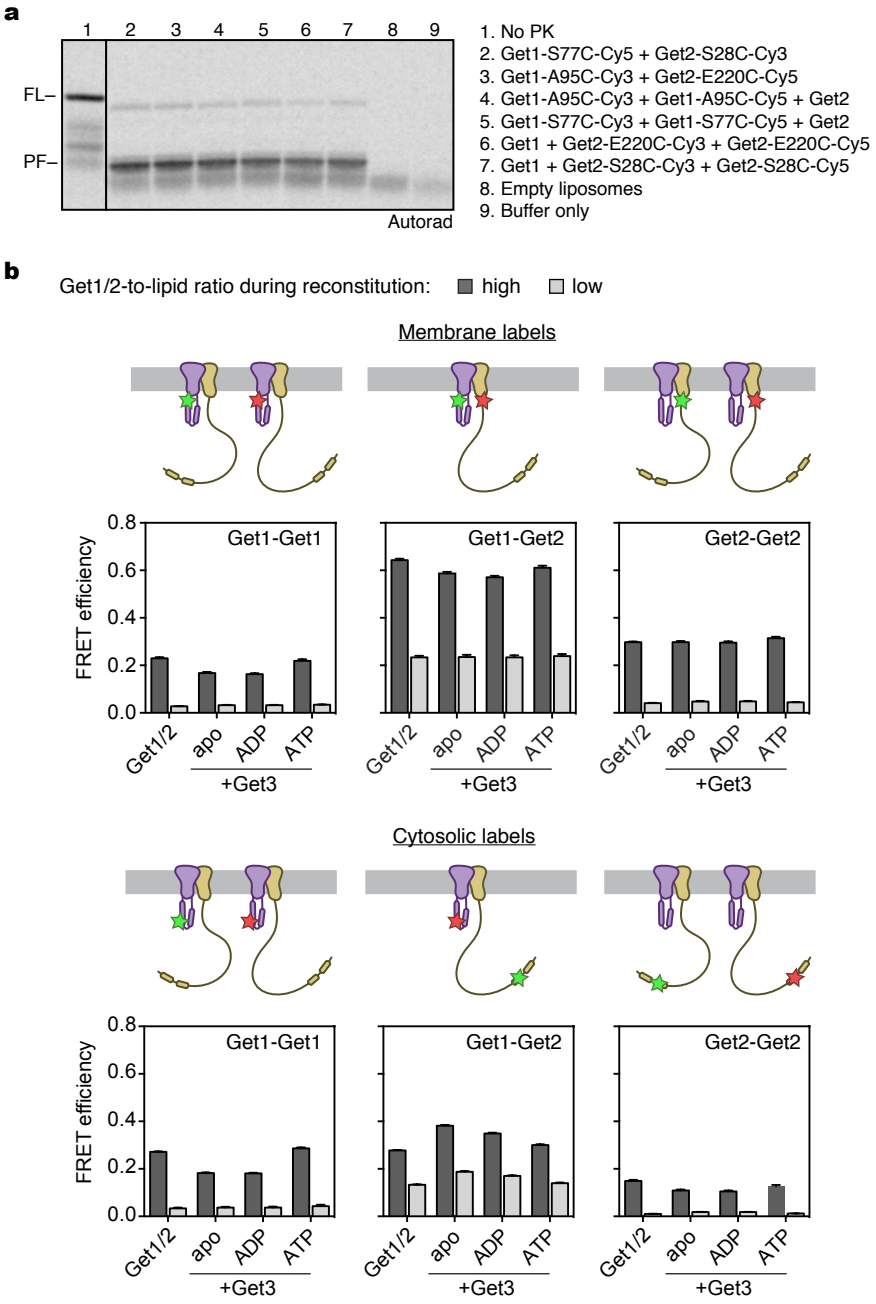

Figure S2, related to Figure 2. Additional details for the bulk FRET experiments. (**a**) Proteoliposomes reconstituted with the indicated labeled Get1 and Get2 subunits at a protein-to-lipid of 12:10,000 were tested for insertion of radiolabeled TA protein, Sec61β (FL), by a PK protection assay. The appearance of a protected fragment (PF) is diagnostic of insertion. Full-length, undigested Sec61β (FL) is shown; empty liposomes and buffer only samples were included as negative controls. (**b**) Absolute FRET efficiencies before and after addition of Get3 for proteoliposomes containing the indicated combinations of membrane-proximal- or cytosolically-labeled Get1 and Get2 subunits, reconstituted at 'high' (12:10,000; dark grey) or 'low' (1.2:10,000; grey) protein-to-lipid ratios. Reconstitutions were done in triplicate, and are reported ±SEM. Proteoliposomes with high protein-to-lipid ratios show a non-specific FRET component (~15-30%) in the absence of Get3 due to the presence of multiple Get1/2 complexes labeled with donor and acceptor fluorophores in the same liposome. We interpret Get3-dependent decreases in this FRET signal as an increase in the average distance between Get1/2 heterodimers arising from steric hindrance caused by the recruitment of Get3 to the membrane (see also Fig. 2).

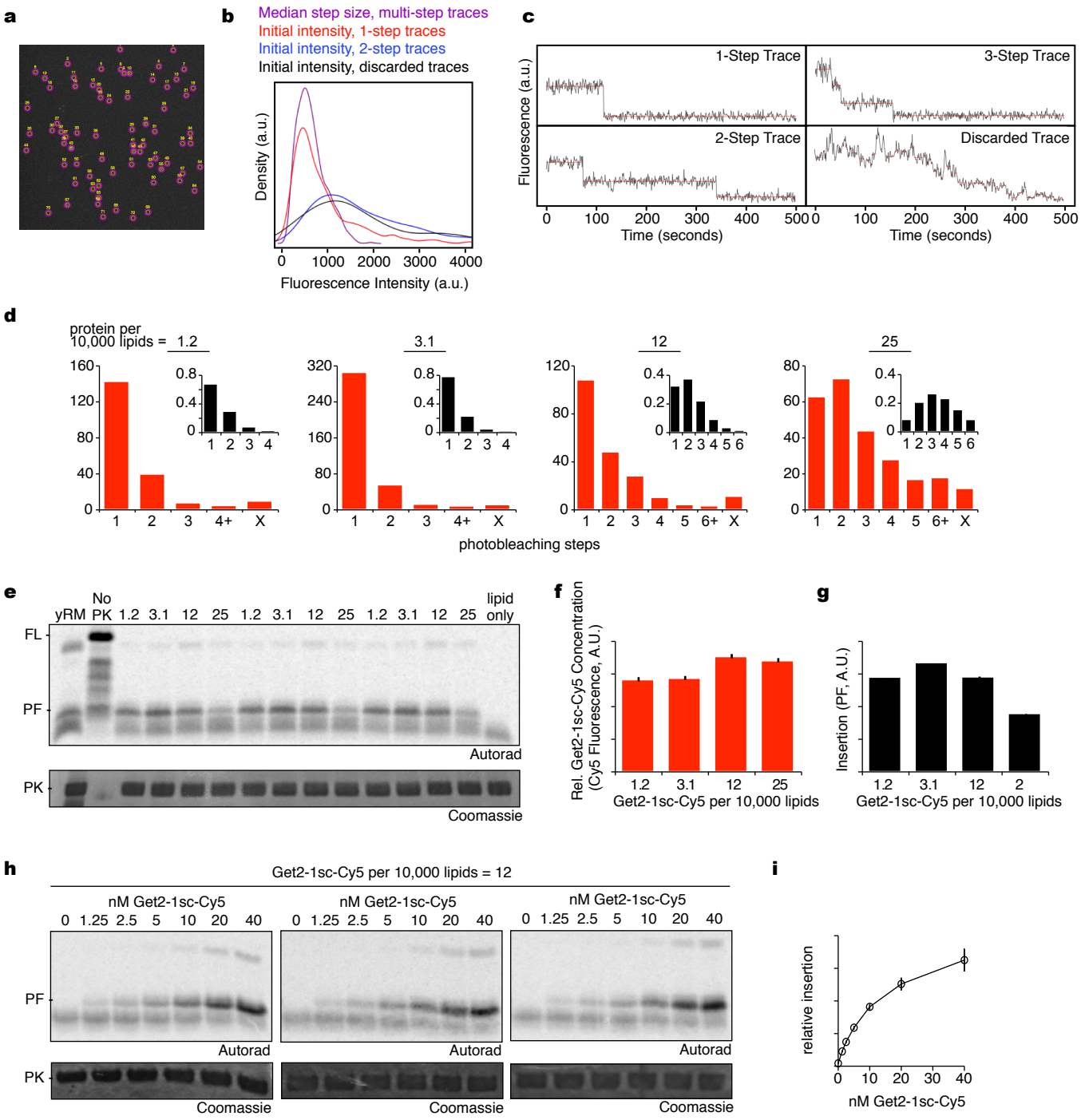

Figure S3, related to Figure 3. Single-molecule photobleaching data analysis. (**a**) Regions of interest (ROIs, yellow circles) were selected using an automated method of fit. ROIs were discarded if within 540 nm (purple circles) of another ROI, or the edge of the image. This filter was used as an unbiased means of preventing additional photobleaching steps from an adjacent ROI. (**b**) Fluorescence intensity varies by location in the field of view and is therefore not as accurate a means of determining stoichiometry as counting photobleaching steps. However, the average intensity of sorted photobleaching steps can be used to confirm accurate counting (Jain et al., 2014). The initial intensity or step size of traces for all reported photobleaching analyses are shown here as a kernel density function. Single step photobleaching traces (red) have the same average, initial intensity as the

median step size of multi-step traces (magenta), and half the average, initial intensity of two-step traces (blue). (**c**) Representative fluorescent intensities of ROIs are plotted in black; step traces, determined using the 'changepoint' package in R, are shown in red. (**d-i**) An independent replicate of the single-molecule photobleaching and quantitative insertion analysis shown in Fig. 3. Proteoliposome were reconstituted with Cy5-labeled single-chain Get1/2 ('Get2-1sc-Cy5') at different protein-to-lipid ratios. The number of photobleaching steps per labeled proteoliposome is shown in red; 'X' represents discarded traces. The inset (black) shows the calculated proportion of Get2-1sc-Cy5 found in different oligomeric states, as described in Supplemental Experimental Procedures. (**e**) Yeast rough microsomes ('yRM'), Get2-1-sc-Cy5 proteoliposomes from panel (d), or empty liposomes (negative control) were tested for insertion of radiolabeled TA protein, Sec61β (FL), by a proteinase K protection assay, in triplicate. The appearance of a protected fragment (PF), which is diagnostic for insertion, was quantified by SDS-PAGE and autoradiography. Coomassie-stained PK serves as a loading control. (**f**) All Get2-1sc-Cy5 proteoliposomes were diluted with empty liposomes to a final concentration of 10 nM Get2-1sc-Cy5, and equivalent concentrations were confirmed via Cy5 fluorescence of these dilutions in 1% SDS. (**g**) Insertion activity quantified from the PF bands in panel (d) and displayed with standard errors of the mean. As shown in Fig. 3, specific insertion activity does not decrease as proteoliposomes are enriched for single copies of Get2-1sc-Cy5 per liposome. The small decrease in insertion activity seen for the proteoliposomes reconstituted at the highest protein-to-lipid ratios may reflect overcrowding in liposomes containing more than ~4 copies of Get2-1sc-Cy5. (**h**) As in Fig. 3c, Get2-1sc-Cy5 proteoliposomes reconstituted at 12 proteins per 10,000 lipids were diluted to the indicated concentration using empty liposomes and subjected to an insertion and protease protection assay, in triplicate. (**i**) Plot showing insertion activity quantified from the PF bands for each concentration in (h), with standard errors of the mean. This control experiment demonstrates that the assay is linear up to a total Get2-1sc-Cy5 concentration of ~10 nM, and is not limited by active targeting complex (see also Fig. 3).

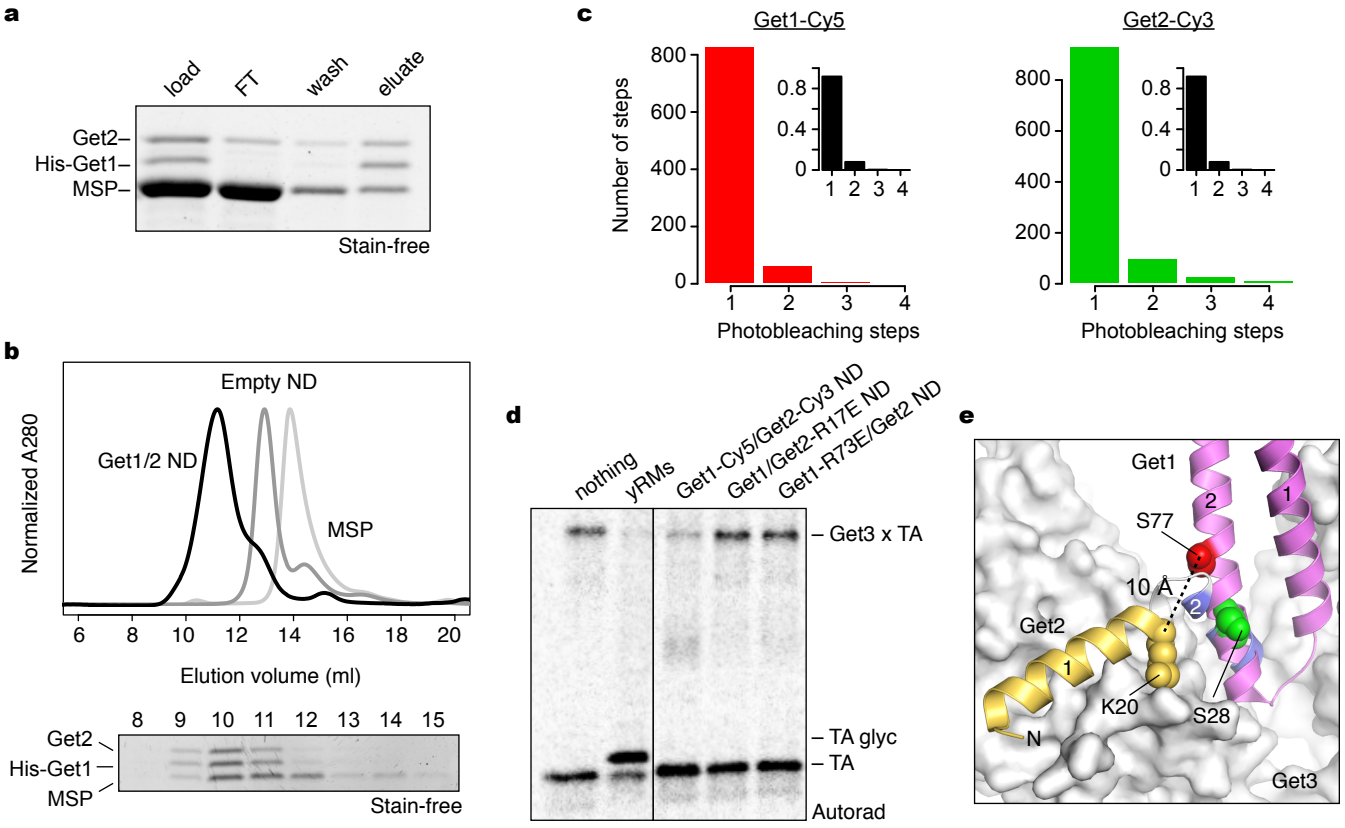

Figure S4, related to Figure 4. Reconstitution and functional analysis of heterodimeric Get1/2 nanodiscs. (**a**) Representative Ni-NTA purification of Get1/2 nanodiscs, analyzed by stain-free SDS-PAGE. (**b**) SEC of Ni-NTA purified Get1/2 nanodiscs (black); empty nanodiscs (grey) and MSP (light grey) shown for comparison. Peak fractions were analyzed by stain-free SDS-PAGE, which allows for direct protein quantification based on the number of tryptophan residues (His-Get1 [8], Get2 [5] and MSP [3]). ImageJ analysis of band intensities are consistent with a 1:1:2 ratio of Get1:Get2:MSP after purification. (**c**) Single-molecule photobleaching analysis of labeled Get1/2 nanodiscs. Insets show the proportion of each subunit found in different oligomeric states, as described in the Supplemental Experimental Procedures; this analysis shows that more than 90% of Get1 and Get2 are present in nanodiscs containing only one copy of each subunit, as expected for nanodiscs containing single Get1/2 heterodimers. (**d**) Get3-TA protein complexes were incubated with yeast rough microsomes (yRMs) or the indicated Get1/2 nanodiscs, and release of radiolabeled TA protein from Get3 was monitored by chemical cross-linking using DSS. Incubation with yRMs results in loss of Get3-TA crosslinks and appearance of glycosylated TA protein via its C-terminal opsin tag. Incubation with labeled Get1/2 nanodiscs (30 nM) also results in loss of Get3-TA crosslinks. In contrast, Get1/2 nanodiscs harboring inactivating mutations (Get2-R17E or Get1-R73E) fail to drive TA release from Get3. (**e**) An NMR titration of nucleotide-free Get3 (grey surface) bound to the isolated cytosolic Get2 fragment with the isolated cytosolic Get1 coiled coil reveals that the Get2 helix 2 (blue) becomes displaced by Get1 (magenta), while Get2 helix 1 (yellow; residues 4-20) is unperturbed (Stefer et al., 2011); the labeling sites on Get1 (S77C; red) and Get2 (S28C; green) are indicated for reference. If full-length Get1 and Get2 bind simultaneously to the same side of Get3, the donor fluorophore at position S28 of Get2 will be displaced. We estimate the average distance between the displaced Get2-S28C-linked donor and the Get1-S77C-linked acceptor fluorophores by accounting for the fact that residues 21-28 of Get2, but not the helix 1 residues 4-20, become displaced. Assuming this segment acts as a wormlike chain with a persistence length of unfolded polypeptide (Schwaiger et al., 2004), the RMS distance between K20 and S28 is ~18 Å (Howard, 2001). Combining this with the observed distance between K20 and S77 (10 Å; dotted line), the expected distance between fluorophores should be 28 Å or less, depending on which direction the displaced chain points. Given the Cy3-Cy5 R_0 ~60 Å (Murphy et al., 2004), same-side binding of Get1 and Get2 should give rise to a high (>99%) FRET state.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

DNA Constructs

Full-length Get1, Get2, and Get3 were subcloned into a pET28 derivative containing a tobacco etch virus (TEV) cleavage site between an N-terminal 6xHis tag and the polylinker, essentially as described previously (Mariappan et al., 2011; Mateja et al., 2009). A single-chain Get2-Get1 construct described previously (Wang et al., 2014) was modified for bacterial expression by fusing Get1 to Get2 with a 27-residue linker (LGAGGSEGGENLYFQSGSEGGTSGATS), and subcloned into pET29b in-frame with a C-terminal 6xHis tag. The plasmid for in vitro translation of Sec61β in the PURE system was based on the PURExpress DHRF control template (NEB). The DHFR open reading frame was replaced with an open reading frame encoding an N-terminal Twin-Strep tag, Sec61β, and a C-terminal 3F4 epitope. Site-directed mutants were obtained by QuickChange mutagenesis.

Protein Expression and Purification

Get3 was expressed and purified as described previously (Mateja et al., 2009). Get1 and Get2 were individually expressed in E. coli Ros2(DE3)/pLysS (Novagen) cells. All growth media was supplemented with 50 μg/mL kanamycin (TCI) and 34 μg/mL chloramphenicol (EMD). Fresh, single colonies from LB/agar plates were grown in 3 mL TB (Fisher) precultures until OD_{600}=0.5-1, and then 1 mL of this preculture was used to inoculate 500 mL of prewarmed, homemade TB autoinduction medium (Studier, 2005) in a 2.8 L baffled glass flask. After 18 hr at 37° C and 250 rpm, cells were harvested in a JLA-8.1 rotor at 6,000 x g for 15 minutes, and the pellet was stored in a 50 mL falcon tube at -80° C.

For purification, the frozen pellet was resuspended at 4° C in 50 mL Buffer A (50 mM Hepes, pH 8.0, 200 mM NaCl, 5% glycerol) supplemented with 10 mM imidazole, 5 mM β-mercaptoethanol (BME), 1 mM PMSF (Sigma), 25 μg/mL DNase (Sigma), and 2 mM $MgAc_2$). The resuspended pellet was subjected to 10 passes with a PTFE/glass homogenizer, and lysed by two passes through a microfluidizer (Emulsiflex-C5, Avestin). After a 40 minute spin in a Ti45 rotor at 35,000 rpm at 4° C, the pellet was gently resuspended with a paintbrush in 50 mL Buffer A supplemented with 10 mM imidazole, 5 mM BME, and 1% DDM. After incubating on a gently rotating wheel at 4°C for 2 hr, the suspension was spun for 40 minutes in a Ti45 rotor at 35,000 rpm at 4°C. The detergent-soluble supernatant was batch purified by gently incubating with a 3 mL bed volume of Ni-NTA resin (Qiagen) at 4° C for 40 minutes. After removing the flow-through, the Ni-NTA resin was exchanged into a new detergent by washing with 20 column volumes of Buffer A supplemented with 20 mM imidazole and 0.1% n-Undecyl-β-D-Maltopyranoside (UM, Anatrace) for Get2 or 0.1% Fos-Choline-12 (FC12, Anatrace) for Get1. The protein was eluted from the resin with 5 column volumes of the same buffer supplemented with 200 mM imidazole. For cysteine-containing mutants, wash and elution buffers were supplemented with 1 mM TCEP. After elution, 1 mM EDTA was added, and the elution was concentrated in an Amicon centrifugal filter (Millipore) (30 kDa MWCO for Get1 in FC12, and 50 kDa MWCO for Get2 in UM) Concentrated protein was further purified by gel filtration using a Superdex 200 10/300 column (GE Healthcare) equilibrated in 50 mM Hepes-KOH, pH 7.5, 200 mM NaCl, 5% glycerol, 0.1% detergent, and 1 mM TCEP. Peak fractions were concentrated to 50-100 μM for Get1, and 20-50 μM for Get2. Aliquots were flash frozen in liquid nitrogen and stored at -80°C. Typical yields were between 5 and 20 mg of purified protein per liter of culture.

Single-chain Get2-Get1 (Get2-1sc) was expressed in LOBSTR-BL21(DE3)-RIL cells, a gift from Thomas U. Schwartz (MIT) (Andersen et al., 2013). All growth media was

supplemented with 50 μg/mL Kanamycin and 34 μg/mL Chloramphenicol. A single colony was transferred to a 50 mL TB preculture shaking at 250 rpm at 37° C. The TB for Get2-1_{sc} expression was specially prepared with 50 g/L Fisher LB and 0.5% glycerol. After autoclaving, cooling, and immediately before use, 100 mL/L of 10X TB salts (170 mM potassium phosphate and 720 mM dipotassium phosphate) was added along with antibiotics. When the preculture OD_{600}=1.5, 9 mL of the preculture was transferred to 1 L of pre-warmed TB shaking at 250 rpm at 37° C in a 2.8 L non-baffled Nalgene polycarbonate flask. When OD_{600}=0.5, flasks were transferred to a 17° C shaker at 180 rpm for 1 hr. Cells were then induced with 0.4 mM IPTG and grown for 17 hr at 17° C and 180 rpm. Cells were harvested by JLA8.1 rotor at 4,000 rpm for 20 minutes and stored at -80° C. Pellet is typically 5 g/L of culture.

For purification, the frozen pellet from 4 L of culture was resuspended at 4° C in 100 mL Buffer B (50 mM Hepes, pH 8.0, 500 mM NaCl, 10% glycerol) supplemented with 10 mM imidazole, 5 mM BME, 1 mM PMSF, 25 μg/mL DNase, and 2 mM $MgAc_2$. The suspension was subjected to 10 passes with a PTFE/glass homogenizer and lysed by 5 passes through a microfluidizer. Unlysed cells were removed by a slow spin at 10,000 x g at 4° C for 20 minutes. The supernatant was spun at 40,000 rpm in a Ti45 rotor at 4° C for 1 hr. The pellet was gently resuspended with a paintbrush in 50 mL DDM buffer (Buffer B supplemented with 10 mM imidazole, 5 mM BME, and 1% DDM) and gently rotated on a wheel at 4° C for 2 hr. This suspension was spun for 1 hr in a Ti45 rotor at 40,000 rpm at 4° C. The supernatant was applied to 1.5 mL bed volume of Ni-NTA resin (Qiagen) and gently rotated on a wheel at 4° C for 40 minutes. After removing the flow-through, the column was successively washed with 10 mL of Buffer B supplemented with: (i) 0.3% DDM, 5 mM BME, and 10 mM imidazole; (ii) 300 mM NaCl and 25 mM imidazole; (iii) 10 mL the same buffer but with, 150 mM NaCl, and 40 mM imidazole. The protein was eluted with 8 mL of Buffer B supplemented with 0.3% DDM, 5 mM BME and 200 mM imidazole. After elution, 1 mM EDTA and 0.1% n-Dodecyl-N,N-Dimethylamine-N-Oxide (LDAO, Anatrace) was added to the eluted protein, which was then concentrated in a 50 kDa MWCO Amicon and purified by gel filtration using a Superdex 200 10/300 column equilibrated with 50 mM Hepes-KOH, pH 7.5, 200 mM NaCl, 5% glycerol, 0.1% LDAO, and 1 mM TCEP. Peak fractions were concentrated, and aliquots were flash frozen in liquid nitrogen and stored at -80° C. Typical yields were between 1 and 3 mg of purified protein per liter of culture.

Get1/2 pull-down in different detergents

His-tagged Get2 (0.5 nmol) was added to 1 nmol of untagged Get1, and diluted into 100 μL with buffer (50 mM Hepes-KOH, pH 7.6, 200 mM NaCl, 5 mM BME) supplemented with 0.1% respective detergent. After incubating for 10 min at room temperature, the dilutions were added to 10 μL bed volume of Ni-NTA resin and rotated 30 min on a wheel at 4° C. The resin was loaded on a 96-well Nunc filter plate, and the flow-through was collected via a 30 second gentle swing-bucket centrifuge spin that did not dry the resin. The Ni-NTA resin was washed twice with 100 μL the samples' respective buffer supplemented with 20 mM imidazole. The protein was eluted with 100 μL of its respective buffer supplemented with 250 mM imidazole.

Multi-angle laser light scattering

The absolute molecular mass of the Get1/2 complex in UM was determined by static multi-angle laser light scattering (MALLS). 10 nmol of His-Get1 and His-Get2, purified in FC12 and UM respectively, was diluted into 3 mL UM buffer (50 mM Hepes, pH 8, 200 mM NaCl, 0.1% UM),

diluting the FC12 left from the His-Get1 stock 30x. The sample was concentrated to 200 µL in a 50 kDa MWCO Amicon, and then diluted again with UM buffer to a final volume of 3 mL. After concentrating to 100 µL, the sample was loaded into a Superdex 200 10/300 column equilibrated with UM buffer. The column was coupled to an online UV detector (UPC-900, GE Healthcare), static light scattering detector (Dawn HELEOS II, Wyatt Technology), and a refractive index detector (Optilab rEX, Wyatt Technology). Complex mass and protein conjugate analysis was calculated using ASTRA software (Wyatt Technology). ExPasy was used to calculate extinction coefficients; dn/dc for UM (0.1506 mL/g) is from Anatrace, and the dn/dc for Get1/2 (0.1872) was calculated based on its sequence(Zhao et al., 2011).

Maleimide labeling of cysteine mutants

Purified proteins, free of imidazole and BME, were labeled on ice at pH 7.5 in the presence of 1 mM TCEP. Solid Cy3 or Cy5 maleimide (GE Healthcare) was dissolved immediately before use in 10mM Hepes pH 7.0, and the concentration of fluorophore was determined using fluorophore absorbance. The protein was serially incubated with 1 equivalent of dye for 1 hr, an additional 1 equivalent of dye for another hour, and an additional 2 equivalents of dye for two hours. The reaction was quenched with 10 mM BME, and free dye was removed by PD10 or Superdex 200 10/300 equilibrated in 50 mM Hepes, 200 mM NaCl, 5% glycerol, 1mM DTT, and 0.1% detergent. SDS-PAGE was used to confirm the complete removal of free dye. Labeling of Get1 and Get2 without cysteine mutations showed less than 5% nonspecific labeling under these conditions. Labeling efficiencies, determined by NanoDrop using ExPASy and GE Healthcare extinction coefficients for proteins and fluorophores respectively, are listed below.

Protein	Cysteine Mutation	Cy5 Labeling Efficiency	Cy3 Labeling Efficiency
Get2-1sc	S77C (on Get1)	80%	N/A
Get1	S77C	70%	71%
Get1	A95C	49%	46%
Get2	S28C	67%	66%
Get2	E220C	59%	71%

Liposome Preparation

Liposomes were prepared by extrusion. Egg-PC (Avanti) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (PE, Avanti) lipids were mixed as chloroform stocks at a 4:1 ratio by weight. 1 mg solid DTT was added per 1 mL of chloroform mixture. The chloroform was removed under a stream of dry nitrogen followed by at least 5 hr on high vacuum (<0.1 torr) at room temperature to remove residual chloroform. The lipid film was resuspended to a concentration of 20 mg/mL in buffer (50 mM Hepes-KOH, pH 7.5, 15% glycerol, 1 mM DTT) by incubation on a wheel at room temperature and intermittent, thorough vortexing. When homogeneously cloudy, the suspension was subjected to three freeze-thaw cycles in liquid nitrogen followed by 25 passes through an extruder and polycarbonate membrane with 100 nm pores. Liposomes were flash frozen in aliquots and stored at -80°C.

Proteoliposome reconstitutions

Proteoliposome reconstitutions were prepared by diluting protein to 1 μM in reconstitution buffer (50 mM Hepes-KOH, pH 7.5, 150 mM KAc, 10% sucrose, 0.01% NaN_3, 1 mM DTT, 0.225% DBC) and incubating on ice for 30 minutes. This protein mixture was further diluted to the desired concentration into 90 μL of cold reconstitution buffer in a 0.2 mL PCR tube. 10 μL of liposomes (20 mg/mL) was immediately added and gently mixed by pipette. After 15 minutes on ice, 25-30 mg of activated biobeads (Bio-Rad) were added, followed by a very brief spin on a tabletop centrifuge. After gently revolving on a wheel at 4° C overnight, the supernatant was removed from the biobeads by pipette and centrifuged at 20,000 x g for 20 minutes at 4° C to remove any aggregate. A successful reconstitution contained no visible pellet. Final protein concentration in proteoliposomes was determined by comparison to purified Get1 and Get2 on SDS-PAGE and typically show 50-80% protein recovery. Proteoliposomes were stored at 4° C and found to be competent for insertion for up to two weeks. However, all insertion assays, single-molecule photobleaching, and FRET experiments reported here were all performed within 36 hr of proteoliposome recovery.

For bulk FRET measurements, labeled Get1 and Get2 subunits were reconstituted into proteoliposomes in triplicate at 320 nM protein (protein-to-lipid ratio of 12:10,000), shown by photobleaching to contain multiple copies of each subunit per liposome. Fluorescently labeled Get1/2 containing a single Cy3 or Cy5 fluorophore for every cysteine mutant, as well as empty liposomes, were also reconstituted for use in fluorescence bleed-through and background subtraction.

For the quantitative photobleaching and activity assays, the number of Get2-1sc-Cy5 molecules per liposome was modified by using different protein-to-lipid ratios (1.2×10^{-5}, 3.1×10^{-5}, 1.2×10^{-4}, and 2.5×10^{-4}) during the proteoliposome reconstitution. This was achieved by reconstituting different concentrations of Get2-1sc-Cy5 (32 nM, 80 nM, 320 nM, and 640 nM respectively); detergent and liposome concentrations were held constant.

Bulk FRET in Proteoliposomes

Each set of labeled proteoliposomes was diluted to a final concentration of 10 nM Get1/2 in a 96-well plate with insertion buffer supplemented with 5% glycerol. Samples were supplemented with buffer only, 50 nM Get3, 50 nM Get3 + 2 mM ADP, or 50 nM Get3(D57N) + 2 mM ATP. Fluorescence was recorded using a Synergy Neo plate reader with excitation at 540/25 and emission filters at 590/35 and 680/30. FRET was calculated as described below for smFRET.

Single-Molecule Photobleaching

Glass cover slips were cleaned successively with water and 70% ethanol three times. After being dried under a nitrogen stream, covers slips were plasma cleaned and used the same day. Flow cells were prepared for TIRF microscopy using glass slides and coverslips separated by double-sided tape and sealed with epoxy to minimize drift. Proteoliposomes or nanodiscs containing fluorescently labeled protein were diluted into 50 mM Hepes, 150 mM KAc, 1 mM DTT buffer and incubated in the 15 μL flow cells for 3 minutes followed by a 50 μL wash with buffer containing 10 mM DTT and 2 mM partially oxidized trolox as triplet state quenchers, as previously described (Cordes et al., 2009). Samples were imaged using Total Internal Reflection Fluorescence (TIRF) microscopy, and 500 frame videos were recorded using a 200 ms exposure.

Fluorescent spots were selected from the first video frame by applying a Laplacian of Gaussian filter with a scale parameter of 150 nm. Peaks were identified by thresholding and non-

maximum suppression over a 360 nm^2 area. Peaks that were within 540 nm of each other, or within 540 nm of the image edge, were discarded. The fluorescence intensity of these regions of interest (ROIs) was then recorded for the length of the video.

Steps were counted manually using the "changepoint" function in R as a guide. To ensure objectivity, a homemade script was used to randomly display proteoliposome and nanodisc traces without sample identification to blind the user during manual step assignment. Fluorescence traces that were too noisy for step counting or contained fewer than 3 frames of fluorescence were discarded, as indicated in Fig. 3b and Fig. S3d (photobleaching steps = 'x'). The average intensity of the highest step and the median step size for every trace was also recorded and plotted as a kernel density plot (Fig. S3b) to confirm that steps were accurately counted, similar to previously reported photobleaching controls(Jain et al., 2014).

Distributions of photobleaching step counts were converted into corrected estimates of protein stoichiometry in three steps: First, to correct for miscounted extra steps, SDS-solubilized Get2-1sc was used as a monomeric control. Under these conditions, the number of two-step counts (miscounted steps or nonspecific labeling) was 8.9% of the one-step counts. Therefore, the experimental samples were corrected by removing a portion of each multi-step count equal to 8.9% of the count with one fewer steps. Second, a poisson distribution was fit to this distribution to find the value of lambda, which was then used with the calculated labeling efficiency to produce a poisson distribution that includes both labeled and unlabeled protein. Third, the step-count populations were multiplied by the number of steps they represent because 2-step complexes contain twice as much protein as 1-step complexes, and 3-step complexes contain 3 times as much, etc.

Yeast Microsomes

Yeast microsomes were prepared essentially as described previously (Mariappan et al., 2011). A Get3 knock-out strain of S. cerevisiae (Open Biosystems) was grown at 32° C in YPD supplemented with 15 µg/mL Kanamycin to a final OD_{600}= 5. Cells were harvested at 2,880 x g for 5 minutes. All future steps were performed at 4° C with cold buffers. The pellet was washed by successive resuspension and pelleting in 300 mL water, then twice in 200 mL lysis buffer (20 mM Hepes-KOH, pH 7.5, 100 mM KAc, 2 mM $MgAc_2$, 1 mM DTT). The pellet was resuspended to a total volume of 50 mL in lysis buffer supplemented with a PiC protease inhibitor tablet (Roche) and 1 mM PMSF. The suspension was split between two 50 mL falcon tubes. 45 g of chilled glass beads were added to each, and the tubes were shaken up and down twice per second over a 50 cm pathlength for 1 minute followed by 3 minutes on ice. This was performed three times for each 25 mL tube. Approximately 50% cell lysis was observed by microscope. Glass beads were removed by straining through a cheesecloth. Unlysed cells were removed by a 20 minute centrifugation at 10,000 x g. The supernatant was loaded on top of a 14% glycerol cushion (in lysis buffer) in a Ti45 centrifuge tube and spun at 40,000 rpm (186,000 x g) for 35 minutes. The supernatant was removed and the pellet was resuspended in 15 mL lysis buffer by pipette and homogenized by a glass/PTFE douncer. This suspension was added on top of a 5 mL 14% glycerol cushion and spun in a Ti70 rotor for 2 hr at 38,000 rpm (148,600 x g). The pellet was resuspended in 2 mL of fresh glycerol cushion buffer and homogenized with a 2 mL glass douncer 10 times. The final material had an A280 of 190, as determined using a Nanodrop after dilution in 1% SDS. Aliquots were flash frozen and stored at -80° C.

Quantitative insertion assay

Targeting complexes composed of purified Get3 and radiolabeled Sec61β were obtained by translating a plasmid encoding TwinStrep-Sec61β-3F4 in a 250 µL PURExpress reaction in the presence of 15 µL ^{35}S-Methionine and 25 µM purified Get3. After 2.5 hr at 37° C, the reaction was incubated with 50 µL Streptactin resin. The flow-through was collected via spin filter and reapplied to the resin 3 times. The resin was washed 4 times with 200 µL buffer (50mM Hepes-KOH, pH 7.5, 500 mM KAc, 7 mM $MgAc_2$, 20% glycerol, and 2 mM DTT) and eluted with 150 µL the same buffer supplemented with 10 mM biotin. The concentration of radiolabeled Get3-Sec61β was estimated to be 500 nM by comparison to Get3 standards via SDS-PAGE.

Proteoliposome samples were normalized to a final concentration of 32 nM Get1 and Get2, or OD_{280} 30 for yeast microsomes. Samples were diluted with empty liposomes that had been subjected to the same reconstitution procedure to ensure equal lipid and buffer content. To confirm equal protein concentrations in the normalized samples, Get2-1sc-Cy5 proteoliposomes were run on SDS-PAGE and imaged by their Cy5 fluorescence (Figure 2b). To ensure equal concentrations with unlabeled Get1/2 samples, stain-free SDS-PAGE was used, however this method is less sensitive than Cy5 fluorescence and requires higher sample loads. These samples were normalized with buffer rather than lipid, and the high lipid content of the most dilute Get2-1sc-Cy5 reconstitution precluded it from being included in this gel (Figure 2b).

The normalized proteoliposomes were then diluted 2x with insertion buffer (50 mM Hepes-KOH, pH 7.5, 150 mM KAc, 7 mM $MgAc_2$, 2 mM DTT). 10 µL aliquots of each dilution was added to a 0.2 mL PCR tube. To each aliquot was added 5 µL of 500 nM radiolabeled Sec61β in complex with Get3 and supplemented with 3 mM fresh ATP (Acros Organics) and gently mixed. Samples were immediately incubated at 32° C. After 30 min, the tubes were transferred to ice. After 2 min on ice, 2 µL of 5 mg/mL proteinase K (PK, Roche) was added and gently mixed. After 2 hr on ice, 0.2 µL of 500 mM PMSF in DMSO was added to each reaction and gently mixed. 15 µL of this sample was then rapidly added and mixed to 95° C 2X loading dye (2:1:1 4X LDS loading dye, 50% glycerol, 1% SDS) and incubated at 95° C for 10 min to ensure all PK was quenched. Samples were run on a 12% Tris-Tricine SDS-PAGE, coomassie stained, dried, and exposed by phosphor screen.

Get1/2 in Nanodiscs

The pMSP1E3D1 plasmid encoding the N-terminal 6xHis-tagged construct of membrane scaffold protein (MSP) was purchased from Addgene (plasmid 20066). MSP was expressed, purified, and TEV cleaved as described previously (Alvarez et al., 2010). Cleaved protein was dialyzed against buffer (50 mM Hepes, pH 8.5, 150 mM NaCl) and then biotinylated using 4 equivalents of NHS-PEG_4-Biotin (Thermo) for 1 hr at room temperature. The reaction was quenched with 10 mM Tris (pH 6.8), dialyzed against buffer (50 mM Hepes, pH 8.0, 200 mM NaCl, 1 mM DTT) and concentrated to 50-100 µM in a 30 kDa MWCO Amicon. Aliquots were flash frozen and stored at -80° C.

Mixed micelles were prepared by combining lipid and removing chloroform as with the liposome preparation. After resuspending the lipid film in 50 mM Hepes, pH 8.0, 200 mM NaCl, the suspension was sonicated for at least 1 hr in a room temperature bath until the suspension appears homogeneous, a translucent milky white. Next, two equivalents of UM detergent were added followed by 30 minutes of sonication at room temperature. Successively, 0.2 eq of UM was added followed by 10 minutes of sonication until the solution was completely clear. This solution was diluted with buffer to a final lipid concentration of 10 µM and stored in aliquots at -80° C. The concentration of UM in this stock is typically 25-30 mM.

Nanodiscs were reconstituted as described previously (Ritchie et al., 2009). His-tagged Get1 and 1.2 equivalents of untagged Get2 were diluted into buffer (50 mM Hepes, pH 8.0, 200 mM NaCl, 0.1% UM, 1 mM DTT) and incubated on ice for 30 minutes. His-Get1 in FC12 was diluted at least 10x by volume into the Get2 UM dilution to ensure that at least 90% of the final detergent was UM. The mixed micelles were diluted in buffer (50 mM Hepes, pH 8.0, 200 mM NaCl, 1 mM DTT), cooled on ice, and then the Get1/2 dilution was added. After 10 minutes, MSP was added to the mixture followed by 1 hr incubation on ice. The scale and ratios of the reconstitution components were calculated to allow for a lipid:MSP ratio of 60:1, an MSP:Get1/2 ratio of 50:1, a final lipid concentration of 2-4 mM, and a final volume that nearly fills the sample container, a strategy to reduce agitation during mixing.

Next, biobeads, in an amount weighing 10% of the total reconstitution volume, were added to the reconstitution. The mixture was gently revolved overnight on a wheel at 4° C. After removing the biobeads by pipette the supernatant was spun at 20,000 x g for 10 minutes at 4° C to remove any aggregated protein or lipid; no pellet should be observed. Reconstituted nanodiscs were purified by Ni-NTA chromatography via the 6xHis tag on Get1. The supernatant was incubated with Ni-NTA resin (100 µL Ni-NTA bed volume per nmol of Get1/2) for 1 hr on a wheel at 4° C. After removing flow-through, the resin was washed with 20 column volumes of wash buffer (50 mM Hepes, pH 8.0, 200 mM NaCl, 20 mM imidazole, 1 mM DTT), and eluted with minimal wash buffer supplemented with 250 mM imidazole. The eluted material was further purified by gel filtration using a Superdex 200 10/300 column equilibrated with buffer (50 mM Hepes-KOH, pH 7.5, 200 mM NaCl, 1 mM DTT) and shows a single peak that contains His-Get1, Get2, and MSP in a ratio of 1:1:2 (quantified by stain-free SDS-PAGE and ImageJ). Nanodiscs were used immediately after reconstitution or flash frozen in aliquots after dialysis against buffer containing 10% sucrose.

TA Substrate Release Assay

The vector for Sec22-opsin (Sec22op) in vitro transcription was described previously (Schuldiner et al., 2008). The expression and purification of Get3-FLAG and Get4/5 complex was described previously (Wang et al., 2010). Capped mRNA for in vitro translation of Sec22op was transcribed using T7 RNA polymerase for 1 hr at 37˚ C from purified PCR product containing a T7 promoter at the 5' end and termination codon at the 3' end (Stefanovic and Hegde, 2007).

Sec22op mRNA (unpurified) was in vitro translated in the presence of ^{35}S-methionine in rabbit reticulate lysate supplemented with 160 ng/µL of recombinant Get3-FLAG and 80 ng/µL of recombinant Get4/5. Rabbit reticulate lysate was prepared as previously described (Stefanovic and Hegde, 2007). Get3-FLAG-Sec22-opsin complexes were affinity-purified with α-FLAG resin and eluted with FLAG peptide as described previously (Wang et al., 2011).

The substrate release assay was performed as previously described(Wang et al., 2014). In brief, 2 µL of affinity-purified Get3-FLAG-Sec22-opsin targeting complex was mixed with 30 nM Get1/2 nanodiscs or microsomes and incubated at room temperature for 30 minutes. Samples were then incubated with 0.5 mM disuccinimidyl suberate (Pierce) at room temperature for an additional 30 minutes. As a positive control for Sec22-opsin release for Get3-FLAG, 2 µL of $GET1_{E166C}$-FLAG microsomes (OD_{280} = 40) was incubated with targeting complex.

Single-Molecule Cy3/Cy5 FRET in Get1/2 Nanodiscs

Freshly plasma-cleaned coverslips were used to make flow cells. Neutravidin was incubated in the flow cells for 5 minutes followed by 1 mg/mL β-casein for 10 minutes. All successive flow cell buffers contain 0.1 mg/mL β-casein to ensure complete blocking. Get1/2 nanodiscs with biotinylated MSP were diluted and incubated 3 minutes in the flow cell. After being washed with 5 flow-cell volumes of trolox buffer containing the indicated Get3 and nucleotide condition, the flow cell was sealed with epoxy. Videos were recorded under four conditions with at least three slides per condition: 1) buffer only; 2) 100 nM Get3; 3) 100 nM Get3 + 2 mM ADP; 4) 1 μM Get3-D57N + 2 mM ATP

For each TIRF microscopy field of view, a single image of direct Cy5 excitation by 633 nm laser was first recorded for ROI selection and to ensure proper focus. Next, 500 frame videos were recorded with 532 nm laser excitation and 200 ms exposure. ROIs were picked manually with ImageJ based on direct excitation of Cy5, and these were translated onto the video to record donor and acceptor traces.

Bleed-through of Cy3 into the red channel was determined to be 11% using Cy3-only samples; this was removed from the experimental traces. No correction was needed for Cy5, since no direct excitation of Cy5 was detected when excited by the 532 nm laser. The ϒ efficiency, E, was determined as described previously (Roy et al., 2008), to be 1.5 +/- 0.09, and FRET was calculated frame by frame using the following equation in which "D" and "A" represent the intensity of the donor and acceptor fluorophores respectively: $FRET = 1/(1+\Upsilon*(A/D))$

As with photobleaching analysis, traces were analyzed manually with the user blind to the sample identity. An average of frames after both fluorophores have bleached was used for background subtraction. Only traces containing single photobleaching steps and anticorrelated fluorophore intensities were analyzed as described previously (Roy et al., 2008). The median FRET value of frames during a FRET state of at least 3 frames was recorded as the FRET value for that ROI. FRET regions were selected, and the median value was recorded as the FRET efficiency for that ROI.

Distances were estimated from FRET measurements using the relation: $E = 1/(1+(R/R_0))^6$, assuming rapidly rotating fluorophores (i.e., $\kappa^2 = 2/3$); E is FRET efficiency, R is the distance between fluorophores, and R_0 is the Forster radius for Cy3/Cy5 (60 Å) (Murphy et al., 2004). Two Gaussian functions were fit to the raw FRET efficiencies by finding the global minimum log likelihood and using the mle package in R to find standard errors.

Miscellaneous

SDS-PAGE gels were digitized using a ChemiDoc MP Imaging System (Bio-Rad) and analyzed using Image Lab 4.0 software (Bio-Rad) and ImageJ. Phosphor screens were digitized using a Typhoon Variable Mode Imager (Amersham Biosciences) and accompanying imaging software. 15% Tris-glycine or 12% Tris-tricine gels were hand-cast and used for SDS-PAGE. Stain free SDS-PAGE gels contained 0.5% 2,2,2-trichloroethanol (TCE). Figures were assembled using Adobe Illustrator and Autodesk Graphic software. Unless otherwise noted, all errors are reported as standard errors.

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