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TAIL ANCHORED PROTEIN INSERTION BY A GET1/2 HETERODIMER

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For my parents and all teachers, everywhere. The greatest education is to educate others.

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ABSTRACT

The post-translational GET pathway in yeast targets and inserts tail-anchored membrane proteins to the ER. These substrates are first shuttled from the ribosome to the soluble Get3 chaperone, forming a targeting complex. This complex interacts with the multimeric Get1/2 transmembrane insertase, stimulating release of substrate, followed by inserted into the ER membrane. The mechanism for the Get3 interaction with Get1/2 and the fundamental stoichiometry and structure of the Get1/2 complex have been elusive, and competing models have been proposed. Using numerous fluorescence tools, such as bulk FRET, single-molecule FRET, and single-molecule photobleaching, we show that Get3 interacts simultaneously with one of each Get1/2 subunits on opposite sides of Get3. We further explore the role of nucleotide state in regulating the GET pathway. Most importantly, we robustly demonstrate that the minimum functional unit required for tail-anchored protein release from Get3 and insertion into lipid bilayers is a Get1/2 heterodimer. Due to this project, we have developed insights into single-molecule photobleaching techniques and established multiple methods of reconstitution and manipulation of membrane proteins into proteoliposomes and nanodiscs, advancing work to determine the structure of the Get1/2 complex. These techniques have been further applied to the GET pathway in *Arabadopsis*, identifying the tail-anchored protein, SYP72.

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I joined the University of Chicago Chemistry Department literally the day after I graduated from Elmhurst College, eager to become a synthetic organic chemist and ultimately, a chemistry professor at a liberal arts college. Nearly 8 years later, my path has significantly diverged from that expectation. My exposure to the Biological Sciences at the University of Chicago and my smooth transition from the Chemistry Department is largely thanks to the CBI (Chemistry Biology Interface) Training Program. Thank you to the CBI organizers and Joe Piccirilli, my pro-forma advisor and long leash from the Chemistry Department.

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CHAPTER 1

AN INTRODUCTION TO THE GET PATHWAY

This chapter reviews the prior research used in identifying the components and mechanism of the GET pathway to introduce the basis for conflicting models addressed in this work.

Approximately 30% of all proteins are 'membrane proteins', directed to the endoplasmic reticulum (ER) or the bacterial plasma membrane[1,2]. Due to their exposed hydrophobic domains, nearly all membrane proteins require specific targeting to the proper organelle as well as chaperones to maintain solubility before they are inserted into the lipid bilayer[3]. For over 30 years, the vast majority of membrane proteins have been known to be targeted and inserted into the membrane via the co-translational SRP/Sec translocon pathway[1]. In this pathway, the N-terminal signal sequence on the nascent polypeptide emerges from the ribosome, causing the signal recognition particle (SRP) to engage the nascent polypeptide/ribosome complex during translation of the peptide[1,2]. SRP then binds to the SRP receptor at the membrane, docking the ribosome's exit channel to the SEC translocon (Sec61p in eukaryotes and SecYEG in prokaryotes)[1,2]. Translation resumes, and the membrane protein is driven through the translocon and into the membrane[1,2].

While co-translational targeting and insertion has been well studied, a mysterious class of membrane proteins was known to be inserted via a different mechanism, independent of SRP or the SEC translocon[4-7]. The reason for the independence of this class of proteins was found to lie in their common topology[4,6,8]. Such proteins contain a single transmembrane domain (TMD) at

the far C-terminus of the peptide[4,6,9]. Without more than 50-60 residues beyond the C-terminal TMD, the hydrophobic signal sequence does not emerge from the ribosome before the protein is completely translated and SRP has time to target the ribosome to the ER[1,4,9]. Referred to as 'tail-anchored' (TA) proteins, they include 3-5% of all eukaryotic membrane proteins[4,6,10]. TA proteins are involved in a variety of functions such as protein targeting, transport, apoptosis, and most notably, vesicle fusing SNAREs [4,5,8,9]. Until recently, the mechanism for TA protein targeting and insertion into the ER was unknown, but over the last ten years, the key components of an independent, post-translational insertion pathway, known as the GET pathway in yeast, has been identified[10-12].

First identified was the soluble chaperone of TA proteins, Get3 (TRC40 in mammals)[9,13-15]. This protein binds specifically to the TMD of TA proteins after release from the ribosome, and targets TA-proteins to the ER membrane[9,13]. Get3 forms a stable, soluble complex with TA-proteins, known as the 'targeting complex'[9,16]. Soluble pre-targeting machinery, involved in upstream loading of TA-proteins onto Get3 from the ribosome, were also identified as Sgt2, Get4, and Get5 in yeast (SGTA, Ubl4A, GdX, and the Bag6 complex in mammals)[17-19]. Finally, an ER receptor was identified, composed of Get1 and Get2 in yeast (WRB and CAML in mammals)[20,21]. The Sgt2/Get4/Get5 pre-targeting complex, the Get3 chaperone, and the Get1/2 receptor were shown to be necessary and sufficient to post-translationally target, shuttle, and insert TA proteins into the ER bilayer[10,21-23].

A number of structural and functional studies have shed light on the mechanism of the GET pathway and the structure of its components. Get3 is a homodimeric ATPase with a coordinated Zinc 'hinge' at the dimer interface[9,14,24]. In the 'closed conformation, a hydrophobic groove spans the dimer interface and engages the TMD of TA proteins[13,15]. This conformation is

favored upon coordination to nucleotide and stabilized by binding to TA proteins[13,15,16,25]. In the 'open' conformation, the hydrophobic region is disrupted and nucleotide is unbound[15,23]. On opposing sides of the dimer interface, Get3 features partially-overlapping binding sites to Get4, Get1, and Get2[23,26]. These binding partners regulate the nucleotide state and conformation of Get3 at each step along the GET pathway[23,25,27]. Whether the Get4, Get2, and Get1 binding partners bind cooperatively, simultaneously, or competitively to Get3 is unknown.

The Get1/2 complex is composed of two peptides, Get1 and Get2, each predicted to have three TMDs[15,21-23]. Get1 and Get2 associate in the membrane and in mild detergents via their transmembrane regions[23]. These TMDs form a vestibule composed of both subunits that contacts TA proteins on their way into the membrane, indicating that Get1/2 is an active insertase rather than a mere recruiter of Get3 to the ER membrane[28].

Get1 and Get2 each contain cytosolic domains that do not interact with each other, but interact with Get3[23]. The cytosolic domain of Get2 consists of a long (110 residue) disordered chain ending in a short, Get3-binding coil[23]. The Get1 cytosolic domain is a coiled-coil, smaller and more rigid domain than Get2[23,26]. Studies with Get1 and Get2 fragments of the cytosolic domains reveal comparable affinities to the 'open' conformation of Get3 (Get1/3 K_D = 17 nM[15], 51 nM[23], and 55 nM[27]; Get2/3 K_D = 150nM, 190 nM[23], and 470 nM[27]). While the Get2/3 interaction is relatively unaffected by Get3 conformation or nucleotide state, the Get1/3 interaction is highly dependent on these factors[15,23,27]. The Get1/3 interaction is disrupted upon binding to ADP and completely abolished upon ATP binding[15,23,27]. The Get1 preference for binding the 'open' conformation of Get3 has also been shown to drive release of TA protein from Get3, whereas Get2 has no effect on TA protein release[23,26]. However, this release requires non-physiologically high concentrations of Get1 fragment or a hydrophobic sink for released

TA-proteins[22,23]. Release can be achieved at physiological concentrations by tethering the Get1 and Get2 cytosolic fragments together[22].

These data have contributed to the idea that Get2 initially captures the 'closed' targeting complex, followed by Get1 driving Get3 into the open state to release the TA protein cargo[22,23,26]. Subsequent binding of ATP to open Get3 abolishes the interaction with Get1, facilitating recycling of Get3 to the cytosol, however this likely requires Get4/5 to further compete away Get3 from Get2[23,27].

These mechanistic insights have also informed conflicting models of the pathway. For example, conflicting models of the stoichiometry and architecture of the Get3/TA-protein targeting complex have been proposed[13,16,17,23,25,29]. Crystal structures of the archaeal homologue of Get3 show an apparent Get3 tetramer with a hydrophobic pocket oriented perpendicular to the hydrophobic groove observed in the Get3 homodimer[29]. Coexpression of Get3 and TA proteins often gives a complex mixture of different oligomeric states, all of which appeared to be competent for TA protein insertion[16,25]. For years, it appeared equally plausible that the disordered helices of the Get3 hydrophobic interface could coordinate tetramerization or stabilize the interaction between TA proteins and a Get3 dimer[13,29]. This ambiguity was resolved when the crystal structure of Get3 was finally solved in complex with TA proteins in a 2:1 ratio[16]. Thus, the Get3 dimer was the functional unit.

In parallel with the ambiguous stoichiometry of the targeting complex was the stoichiometry of Get1/2, key to understanding the mechanism of Get3 engagement and TA protein insertion[22,23,27]. Crystal structures of Get3 bound to cytosolic fragments of Get1 or Get2 each show a 2:2 stoichiometry[15,23,26]. This symmetry was the primary inspiration for the Get1/2 tetramer model, in which the targeting complex first engages two copies of Get2[23]. Two copies

of Get1 then displace (partially or fully) the Get2 cytosolic domains and drive the release of TA proteins[23,26]. It was further hypothesized that the symmetric engagement of two, rigid coiled-coil domains of Get1 by Get3 could drive TA protein insertion by distorting the Get1/2 TMDs[26]. Other models proposed that higher-order complexes of Get1/2 could be required to disassemble a tetrameric targeting complex[27].

Many of the techniques used in this research build upon previously reported work, but required extensive development and adaptation. Protease protection assays are critical to testing insertion activity, distinguishing aggregated substrates from properly insertion and orientated TA proteins[8,9]. Get3/TA-protein targeting complex preparation has been previously optimized[16,23,30]. Release Assays were performed by our collaborators Charlene Chen and Vlad Denic as previously reported[22,28]. Bulk FRET and TIRF-based single-molecule FRET experiments built upon techniques developed by the Ha lab[31]. Our Get1/2 proteoliposome reconstitutions build upon previous techniques[23]. Nanodisc technology was developed by the Sligar Lab[32].

The research reported in this thesis demonstrates a far simpler model for TA protein insertion than many of these previous models. We show that Get3 preferentially engages distinct Get1/2 heterodimers. We show that the Get1/2 heterodimer is the minimum functional unit for TA Protein insertion and release from Get3. Using single-molecule FRET, we report low-resolution structural information, demonstrating that full-length, reconstituted Get1/2 engages Get3 with one of each Get1/2 subunits on opposite sides of Get3. The Get1/2 cytosolic domains bind cooperatively to multiple conformations and nucleotide states of Get3. However, these conformational changes are likely decoupled from Get1/2 TMDs. These results significantly contribute to our mechanistic understanding of the GET pathway and membrane protein insertion in general. They also clarify the ambiguities of previously reported structural and functional

studies and demonstrate the importance of the more physiologically relevant membrane environment for the study of membrane protein structure and function.

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CHAPTER 2

TAIL-ANCHORED PROTEIN INSERTION BY A SINGLE GET1/2 HETERODIMER

This chapter contains the entirety of a manuscript submitted to Cell Reports. Following reviews of this manuscript, modest revisions are underway pending resubmission.

Authors of this manuscript are Benjamin E. Zalisko, Charlene Chan, Vladimir Denic, Ronald S. Rock, and Robert J. Keenan

Abstract

The Get1/2 transmembrane complex drives the insertion of tail-anchored 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. Taken together, 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[1], are post-translationally inserted

into the ER membrane via the evolutionarily conserved GET pathway[2-4]. In yeast, the membrane targeting factor is a homodimeric ATPase called Get3, which changes conformation in a nucleotide-dependent manner[5-9] to bind TA proteins in the cytosol and release them at the ER membrane. The Get1/2 transmembrane complex[10] recruits the Get3-TA targeting complex[11] to the ER, coordinates TA protein release and insertion, and mediates ATP-driven recycling of Get3 to the cytosol[12-15].

Get1 and Get2 are the only integral membrane components required for TA protein insertion[12,14], and specific functions for the individual subunits have been defined based on a series of functional and structural studies[12-14,16]. 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[15], 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[17,18], 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[12,13] (Fig. 1a); such an assembly might exist constitutively or it might form dynamically in the presence of Get3 (Fig. 1b). Simpler models are also plausible, including a heterodimeric Get1/2 assembly with only one copy of each subunit (Fig. 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[12,13] (Fig. 1). Likewise, the number of subunits has important implications for how the Get1/2 TMDs guide TA substrates into the bilayer[15] and whether conformational changes in Get3 can be coupled to these TMDs during insertion[3,4,13]. 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 FRET assay in proteoliposomes that reports on changes in 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 (Fig. 2a). After purification (Supplementary Fig. 1 and 2), the individual subunits were labeled with Cy3 donor (D) or Cy5 acceptor (A) fluorophores. Cytosolic or membrane-proximal labeled Get1 and Get2 subunits were then reconstituted into proteoliposomes in different donor-acceptor combinations: $Get1^{D}$-$Get1^{A}$-Get2, $Get1^{D}$-$Get2^{A}$ and Get1-$Get2^{D}$-$Get2^{A}$. Importantly, these proteoliposomes contain multiple copies of Get1 and Get2, allowing for an unbiased analysis of Get1/2/3 configurations in the membrane. After verifying the insertion activity of the different

proteoliposomes (Supplementary Fig. 3a), we monitored changes in FRET as a function of binding to different nucleotide states of Get3.

We first explored whether Get3 binding alters the oligomeric state of Get1/2, as would be expected in a dynamic model (Fig. 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 (Fig. 2b and Supplementary Fig. 3b). 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 cytosolic labels to explore how Get1 and Get2 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 bind to overlapping sites present on either side of the Get3 homodimer[12,13]. These cytosolic regions are expected to FRET most efficiently when brought into close proximity by simultaneous binding to Get3. Importantly, studies with the isolated cytosolic fragments show that the Get2-Get3 interaction is insensitive to nucleotide, but the Get1-Get3 interaction is weakened by ADP and completely disrupted by ATP[12-14,19].

When Get3 was incubated with cytosolically-labeled $Get1^{D}$-$Get1^{A}$-Get2 or Get1-$Get2^{D}$-$Get2^{A}$ proteoliposomes, no significant FRET increase was observed, regardless of the nucleotide state (Fig 2b and Supplementary Fig. 3c). Likewise, no significant FRET increase was observed when ATP-bound Get3 was incubated with $Get1^{A}$-$Get2^{D}$ proteoliposomes. However, when ADP-bound or nucleotide-free Get3 was incubated with $Get1^{A}$-$Get2^{D}$ proteoliposomes we observed a strong FRET increase (Fig 2b and Supplementary Fig. 3c). Importantly, when cytosolic-labeled $Get1^{A}$-$Get2^{D}$ proteoliposomes (10 nM) were titrated with Get3, we observed a

linear FRET increase that became saturated at one equivalent of Get3 (10 nM homodimer) (Fig. 1c), and could be reversed by ATP (Fig. 2c). These data are consistent with Get3 binding with subnanomolar affinity to a single Get1/2 heterodimer.

The quantitative and reversible Get3-dependent FRET increase observed with cytosolically-labeled $Get1^{A}$-$Get2^{D}$ 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 $Get1^{D}$-$Get1^{A}$-Get2 or Get1-$Get2^{D}$-$Get2^{A}$ proteoliposomes indicates that Get3 does not bind concomitantly to two Get1 and/or two Get2 subunits, in any nucleotide state. While these data do not formally exclude the possibility of heterotetrameric or higher order Get1/2 oligomers, the most parsimonious model is one in which Get3 binds to a heterodimeric Get1/2 complex comprising a single copy of each subunit.

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 liposomes containing a single Get1/2 heterodimer would be expected to have the same specific insertion activity as liposomes 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[15]. 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 (Fig. 3b).

Next, we purified and fluorescently labeled a Get2-1sc construct harboring the Get1-S77C mutation (Supplementary Fig. 1). 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 TIRF microscopy (Fig. 3a and Supplementary Fig. 4). At the lowest protein-to-lipid ratio tested, more than 80% of Get2-1sc-Cy5 was incorporated into vesicles containing only one Get2-1sc-Cy5 molecule. At the highest ratios, we observed a corresponding increase in the number of molecules per liposome, with as much as 80% of Get2-1sc-Cy5 incorporated into vesicles 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 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. Strikingly, we found that the specific insertion activity was essentially independent of the number of copies of Get2-1sc per liposome; indeed, robust insertion was observed in proteoliposomes containing the largest proportion of single Get2-1sc-Cy5 molecules (Fig. 3b). 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 NMR studies showed that isolated cytosolic fragments of

Get1 and Get2 can bind simultaneously to the same side of the Get3 dimer[13] (Fig.1c and 4a). In this configuration, the two subunits are adjacent to each other, with Get1 displacing the second helix of Get2 (Fig. 4a and Supplementary Fig. 5e). Notably, since 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 (Fig. 4a). Alternatively, Get1 and Get2 might bind on opposite sides of the Get3 homodimer. In this case, 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[12,13,16] (Fig. 4a).

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. To avoid the confounding contributions of FRET changes due to random co-localization of multiple Get1/2 complexes in proteoliposomes, we reconstituted Cy5-labeled Get1 (S77C) and Cy3-labeled Get2 (S28C) into nanodiscs using conditions that favor incorporation of single heterodimers (Supplementary Fig. 2). 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 (Supplementary Fig. 5).

Next, we measured the distribution of FRET efficiencies between the Get1 and Get2 cytosolic domains bound to different nucleotide states of Get3, and fit each dataset to two Gaussian distributions (Fig. 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 52.8 ± 3.9%, and this shifted to a higher FRET state of 70.7 ± 2.4% in the presence of nucleotide-free Get3.

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. Indeed, given the Förster distance of the Cy3 and Cy5 FRET pair (60 Å)[20] and assuming that the fluorophores freely rotate at the labeling site (i.e., $k^2 = 2/3$), 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) (Fig. 4a). These observations are also inconsistent with simultaneous Get1 and Get2 binding on the same side of Get3, which would be expected to give rise to constitutively high FRET (~99%) in the nucleotide-free and ADP-bound Get3 samples. Thus, we conclude that Get1 and Get2 bind simultaneously to opposite sides of the Get3 homodimer.

Discussion

The 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. Notably, 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 opposites sides of the post-hydrolysis Get3 homodimer.

These results define a simplified model for events at the membrane (Fig. 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[7,11,13], the targeting complex is captured first by Get2, bringing the targeting complex into close proximity with Get1[12-14,19]. Next, Get1 binds to the opposite side of ADP-bound or nucleotide-free Get3, driving it into an 'open' conformation that disrupts the hydrophobic TA protein binding site[12,13]. 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[15]. Finally, Get3 is recycled by ATP binding, which disrupts the Get1-Get3 interaction[12,13]; subsequent dissociation from Get2 may be facilitated by Get4/5[19]. Future studies are needed to obtain high-resolution structural information for each step along the pathway.

Acknowledgements

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Author Contributions

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

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Figures (Chapter 2 Appendix)

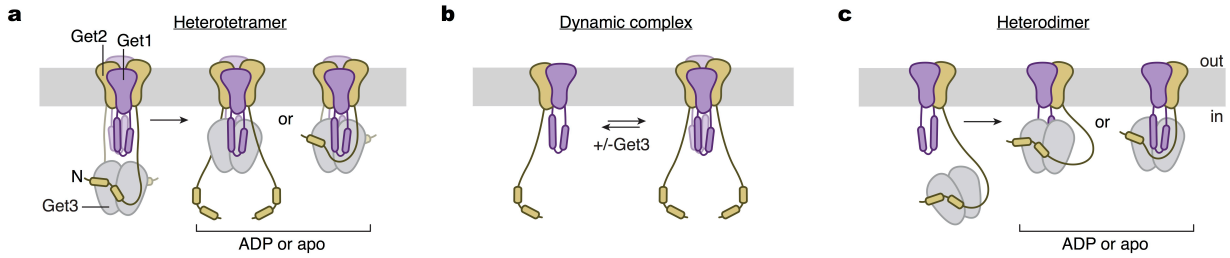


Figure 1. Alternative models for the Get1/2 architecture. (**a**) In static heterotetramer models, based on structural and biochemical studies[12,13], Get1/2 exists as a constitutive complex comprising two copies of each subunit. Accordingly, the Get3-TA substrate complex is captured by two copies of Get2, which bind on either side of the symmetric Get3 dimer. Subsequently, the ADP or apo form of Get3 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. Importantly, 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 dynamic models, Get3 binding drives the transient assembly of two heterodimeric Get1/2 complexes into a single heterotetrameric complex. (**c**) In static heterodimer models Get3 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 Get3 by partially displacing Get2, or by binding to the opposite side of the Get3 dimer.

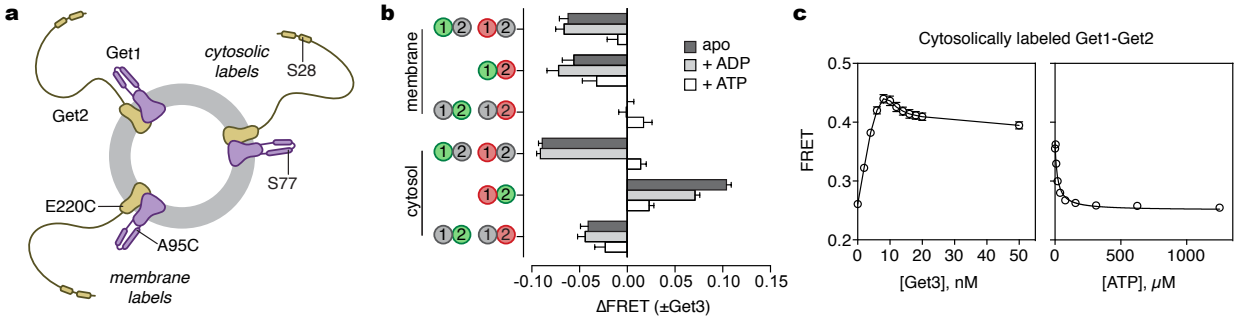


Figure 2. Bulk FRET measurements of Get3 binding to Get1/2 complexes in proteoliposomes. (**a**) Get1 and Get2 subunits were labeled with Cy3 (donor) or Cy5 (acceptor) fluorophores at the indicated positions, and then cytosolic or membrane-proximal labeled subunits were reconstituted into proteoliposomes in different donor/acceptor combinations: Get1^{D}-Get1^{A}-Get2, Get1^{D}-Get2^{A}, and Get1-Get2^{D}-Get2^{A}. (**b**) Change in FRET after addition of 50 nM Get3 (dark grey), Get3 + ADP (grey) or an ATPase-deficient Get3 mutant (D57N) + ATP (white) to each of the six different proteoliposomes (10 nM). 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 random co-localization of donors and acceptors in the proteoliposomes (Supplementary Fig. 3); we interpret Get3-dependent FRET decreases as a reduction in random co-localization due to steric hindrance caused by Get3 binding. (**c**) At left, FRET-based titration of cytosolically-labeled Get1-Get2 proteoliposomes (10 nM) 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.

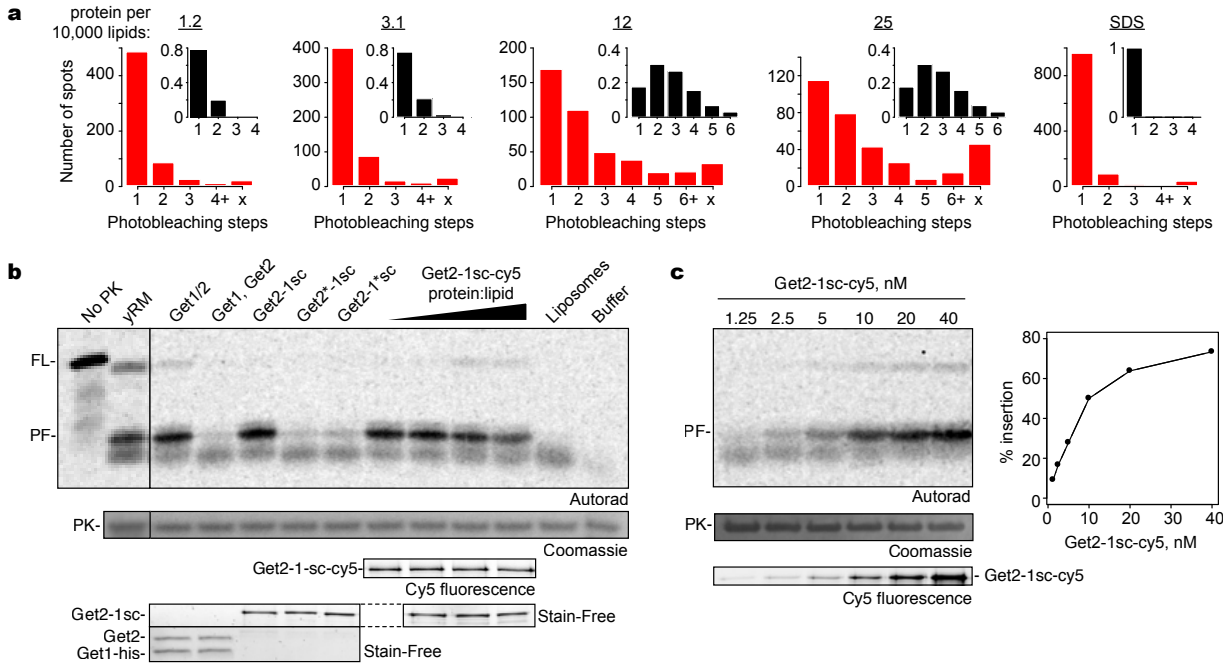


Figure 3. Single Get1/2 heterodimers mediate TA protein insertion. (**a**) Single-molecule photobleaching analysis of proteoliposomes reconstituted with Cy5-labeled single-chain Get1/2 ('Get2-1sc-Cy5') at different protein-to-lipid ratios. The number of photobleaching steps per labeled proteoliposome is shown in red; 'x' represents discarded traces. SDS-solubilized Get2-1sc-Cy5 serves as a monomeric control. The inset (black) shows the calculated distribution of protein stoichiometry, as described in Methods. (**b**) 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 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 panel (a). All protein was diluted with empty liposomes to a final concentration of 10 nM. Notably, the normalized insertion activity is independent of the number of Get2-1sc-Cy5 molecules in each proteoliposome. Loading controls for Get1/2 samples and proteinase K ('PK') are shown below via stain-free and/or Cy5-imaged SDS-PAGE. (**c**) In parallel with the assay shown in panel (b), Get2-1sc-Cy5 proteoliposomes (reconstituted at a ratio of 1.2 proteins per 10,000 lipids) were diluted to the indicated final concentrations, and TA protein insertion was quantified by autoradiography. Under these conditions, the assay is linear up to a total Get2-1sc-Cy5 concentration of ~10 nM.

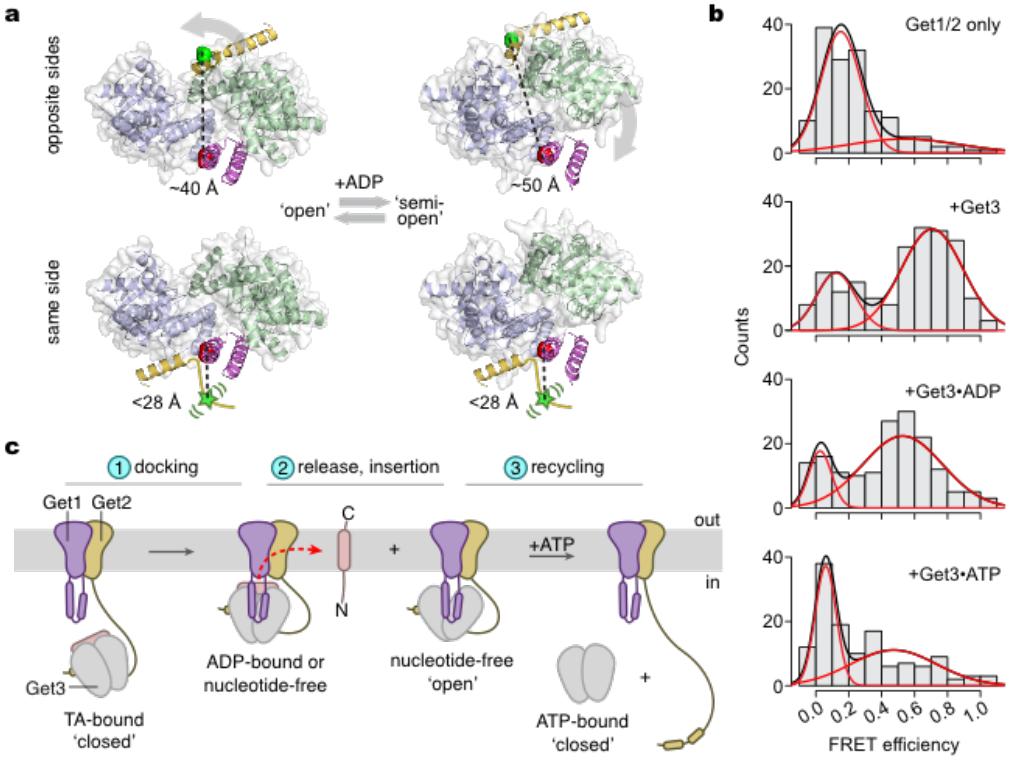
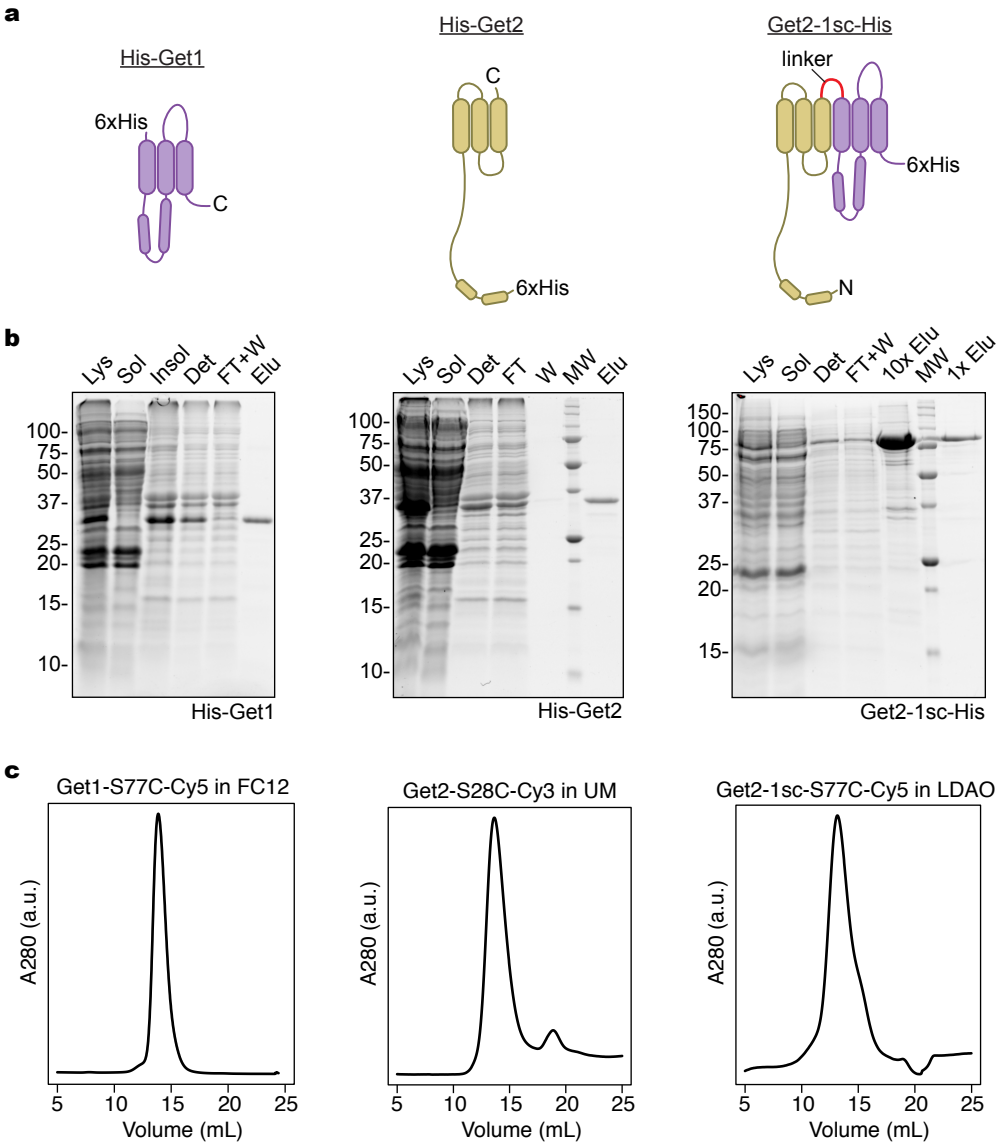
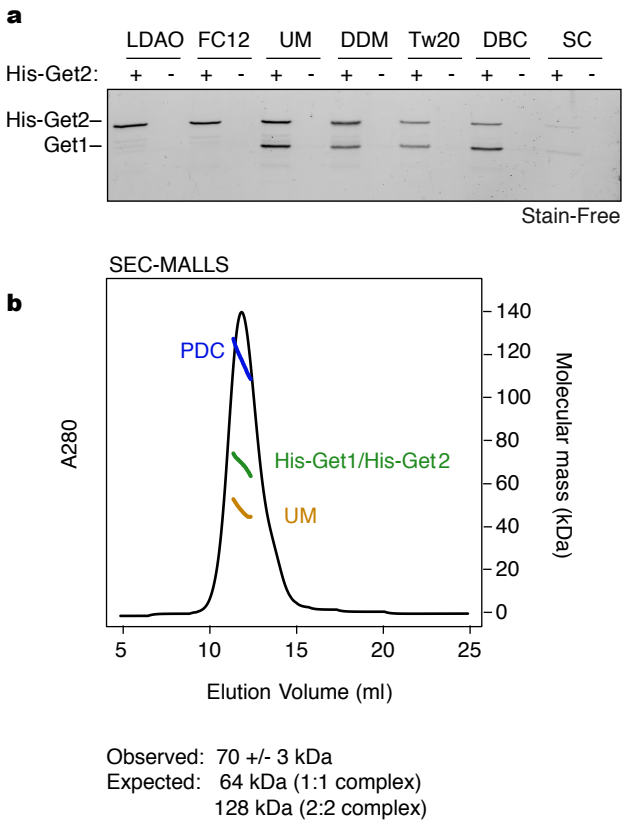


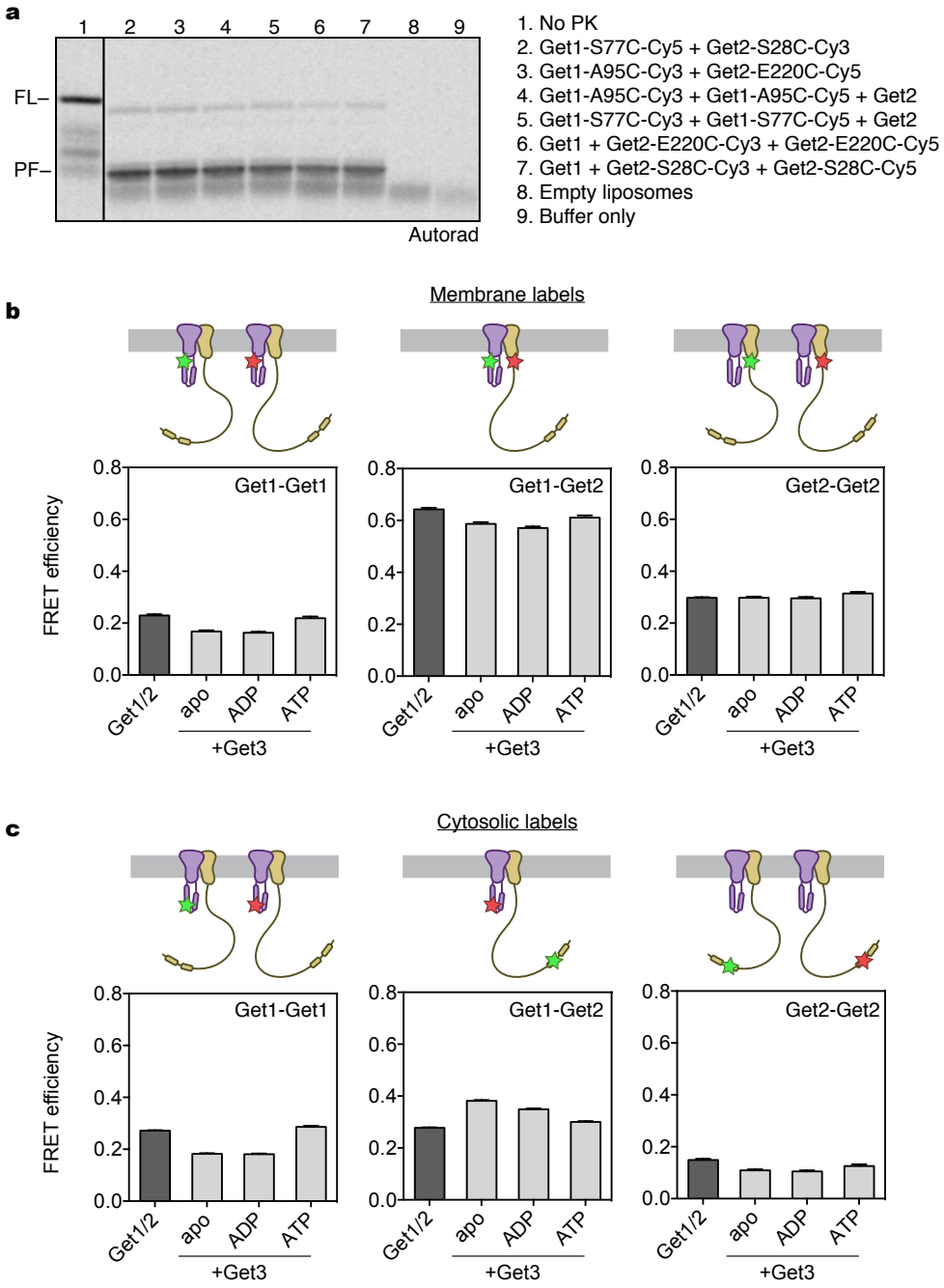
Figure 4. Get1 and Get2 bind on opposite sides of Get3 in its post-hydrolysis states. (**a**) Models of Get1 (magenta) and Get2 (yellow) bound to the same or opposite sides of the nucleotide-free ('open'; PDB 3ZS8) and ADP-bound ('semi-open'; PDB 3VLC) conformations of the Get3 homodimer (blue, green). The models are aligned on one Get3 subunit (blue) to highlight the pseudo-rigid-body 'swivel' (grey 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 (red) and Get2-S28C (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, since 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 (grey histograms) in nanodiscs containing cytosolically labeled Get1-S77C-Cy5 and Get2-S28C-Cy3 heterodimers after 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 (Supplementary Fig. 5e), intermediate FRET efficiencies are observed in the nucleotide-free and ADP-bound states. Moreover, the FRET efficiency of the ADP-bound state is less (53%) than in the nucleotide-free state (71%), 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.



Supplementary Figure 1. Preparation of recombinant Get1/2 constructs. (**a**) 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. (**b**) 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. (**c**) Gel filtration profiles of fluorescently labeled cysteine mutants in the indicated detergent-containing buffers.



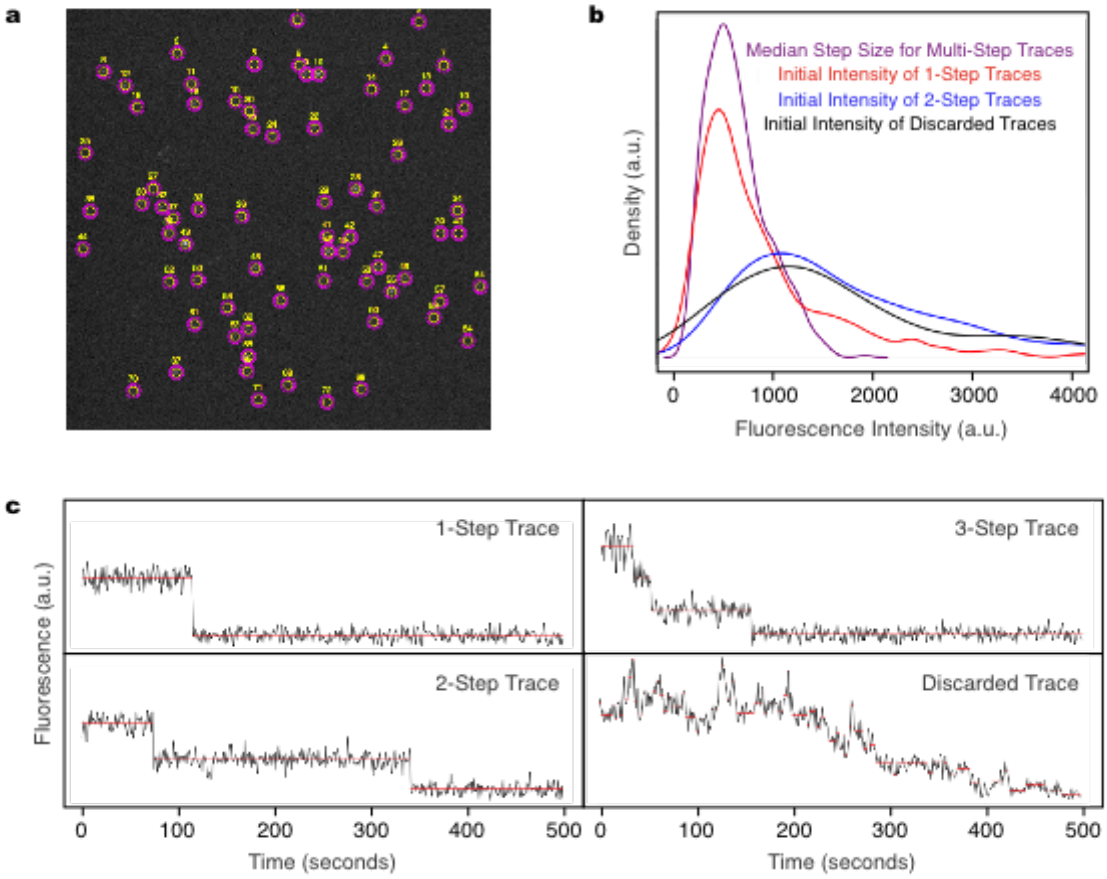
Supplementary Figure 2. Recombinant Get1-Get2 complex formation in detergent. (**a**) Purified Get1 (in FC12) was added to a 2-fold molar excess of His-Get2 (in UM), diluted at least 10-fold into the indicated detergents, subjected to Ni-NTA pull-down, and the elution fraction was analyzed by stain-free 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. (**b**) 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. Based on this result, all reconstitutions were performed in DBC (proteoliposomes) or UM (nanodiscs), as described in the Methods.



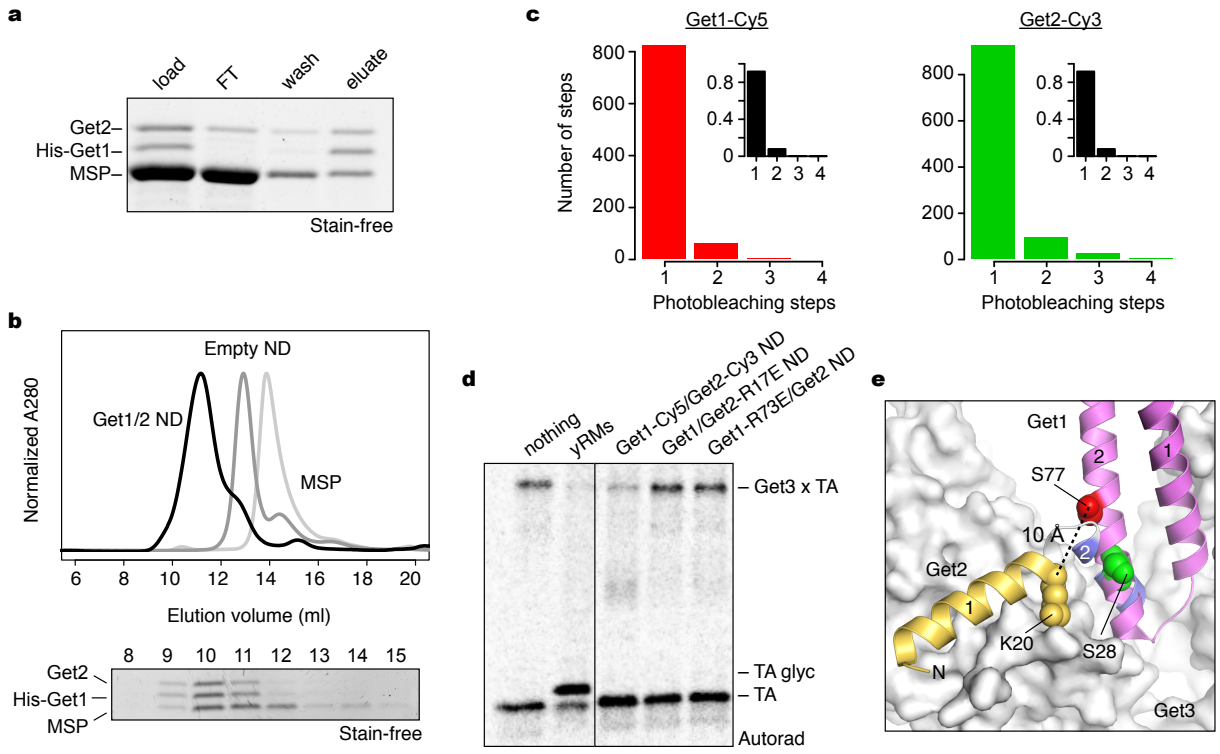
Supplementary Figure 3. Additional details for the bulk FRET experiments. (**a**) Proteoliposomes reconstituted with the indicated labeled Get1 and Get2 subunits were subjected to a proteinase K (PK) protection assay to verify their insertion activity. The appearance of a protected fragment (PF) is diagnostic of insertion. Full-length, undigested Sec61β (FL) is shown; empty liposomes and buffer only samples were included as negative controls. (**b**) Absolute FRET efficiencies for proteoliposomes containing the indicated combinations of membrane-proximal labeled Get1 and Get2 subunits.

Supplementary Figure 3 Continued

(**c**) As in panel (b), but for the cytosolically labeled proteoliposomes. All samples in panels (b) and (c) show a non-specific FRET component (~15-30%) in the absence of Get3 (dark gray) that presumably arises from random co-localization of donors and acceptors in the proteoliposomes. We interpret Get3-dependent decreases in FRET signal (see also, Fig. 2) as a reduction in random co-localization arising from steric hindrance; this effect is most pronounced when Get3 is bound to Get1 and Get2 (i.e., in the apo and ADP states).



Supplementary Figure 4. 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[25]. 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.



Supplementary Figure 5. Reconstitution and functional analysis of Get1/2 nanodiscs. (**a**) Representative Ni-NTA purification of Get1/2 nanodiscs, analyzed by stain-free SDS-PAGE. (**b**) Size-exclusion chromatography 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. Inset plots show the proportion of each subunit found in different oligomeric states, as described in the Methods; 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.

Supplementary Figure 5 Continued

(**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[13]; 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 can 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[30], the root-mean-squared distance between K20 and S28 is ~18 Å[31]. 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[20], same-side binding of Get1 and Get2 should give rise to a high (>99%) FRET state.

Methods

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[7,12]. A single-chain Get2-Get1 construct described previously[15] 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[7]. 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[21] 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)[22]. 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[23].

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 Bio-Beads (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 Bio-Beads 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 x

10^{-5}, 1.2 x 10^{-4}, and 2.5 x 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[24]. 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. 3a (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 (Supplementary Fig. 4) to confirm that steps were accurately counted, similar to previously reported photobleaching controls[25].

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[12]. 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[26]. 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 equivalents 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[27]. 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, Bio-Beads, 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 Bio-Beads 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-Protein Substrate Release Assay

The vector for Sec22-opsin (Sec22op) in vitro transcription was described previously[10]. The expression and purification of Get3-FLAG and Get4/5 complex was described previously[28]. 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[2].

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[2]. Get3-FLAG-Sec22-opsin complexes were affinity-purified with α-FLAG resin and eluted with FLAG peptide as described previously[14].

The substrate release assay was performed as previously described[15]. 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[29], 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[29]. 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., $k^2 = 2/3$); E is FRET efficiency, R is the distance between fluorophores, and R_0 is the Forster radius for Cy3/Cy5 (60 Å)[20]. 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.

CHAPTER 3

ADDITIONAL DATA AND DISCUSSION

This chapter discusses experiments and presents data that was not discussed in chapter 2 but are complimentary to that project.

The Get1/2 Heterodimer

In chapter 2, we demonstrated the detergent-dependent interaction of Get1 and Get2 via orthogonal His-tag pull-downs in various detergents. A similar experiment had been previously performed in a single detergent[1], which also demonstrated that Get1 and Get2 cytosolic fragments do not interact, leading to the conclusion that Get1 and Get2 interact solely via their transmembrane domains (TMD). To positively demonstrate this TMD interaction, we repeated the His-tag pull-down demonstrated in chapter 2, mixing HisGet1 and the isolated TMD fragment of Get2 in various detergents. This Get2TMD fragment was obtained by TEV cleavage at residue 130 (Fig. 5). We attempted to pull-down Get2TMD via His-Get1 in using Ni-NTA. We observed that His-Get1 and Get2TMD interact in moderate and mild detergents, just as with HisGet2 and Get1. This result directly demonstrates and supports the claim that Get1 and Get2 interact via their TMDs.

The bulk FRET experiment in Chapter 2 revealed that Get3 interacts with distinct Get1/2 heterodimers via one of each of the Get1/2 cytosolic domains. FRET between cytosolically-labeled Get1 and Get2 fluorophores increased upon addition of Get3, while other FRET combinations decreased (Fig. 2). This experiment used Get1/2 proteoliposomes that contained multiple copies of Get1/2, allowing Get3 to sample all possible combinations of interactions with

Get1/2. The cramming of multiple Get1/2 complexes into each liposome also complicated the interpretations of FRET data due to the close proximity of so many fluorophores. This leads to high background FRET between domains that might not interact, FRET that can both rise and fall as multiple Get1/2 complexes rearrange as they engage Get3. To simplify matters, we also performed this experiment using Get1/2 reconstituted with mostly isolated Get1/2 heterodimers per proteoliposome (Fig. 6b,e). As expected, both cytosolically-labeled and membrane-adjacent Get1-Get2 combinations constitutively FRET, but there is very little FRET between Get1-Get1 or Get2-Get2. This demonstrates that Get1/2 heterodimers are mostly isolated in single proteoliposomes. Engagement of Get3 produces the same pattern of FRET observed between Get1-Get2 in "oligomeric" proteoliposomes, demonstrating that Get3 engages isolated Get1/2 heterodimers. Get3 does not cause significant changes in Get1-Get1 and Get2-Get2 FRET combinations in the case of isolated Get1/2 heterodimers, implying that these observations in "oligomeric" proteoliposomes are artifacts of the high background FRET rather than intra-complex conformational changes.

The gradual titration of Get3 to each FRET combination also reveals interesting patterns that are not apparent with the endpoint assay (Fig. 2). The FRET increase between Get1-Get2 cytosolic domains increases steadily upon addition of Get3 (Fig. 2c, 6c-d). However, the decrease in FRET between the Get1-Get1 or Get2-Get2 cytosolic domains (Fig. 6c) is strongest after the addition of excess Get3 rather than the initial titration. This result implies that the reduction in FRET may be driven by a secondary interaction, perhaps of excess Get3 bound to individual Get1 or Get2 subunits without the increased avidity of a full Get1/2 complex. This binding of Get3 to a single copy of Get1 or Get2 would not lead to any increase in FRET, but would sterically occlude the area surrounding the complex, lowering FRET between non-

interacting complexes. This reduction can only be observed when multiple Get1/2 complexes are reconstituted per proteoliposome.

Get1/2 Engagement of the Targeting Complex

Studying the interaction of Get1/2 with the Get3/TA-substrate targeting complex is technically challenging because such an interaction drives the release of substrate from Get3. We were unable to trap the targeting complex in a way that would prevent release while maintaining the fidelity of the Get1/2 complex. Therefore, experiments reported in Chapter 2 (Fig. 2, 4) focused on the architecture of the Get1/2/3 complex without the presence of TA protein substrates.

Using the increase in FRET between Get1/2 cytosolic domains upon binding to Get3, we were able to obtain limited data of the Get3/TA-protein targeting complex as it engaged Get1/2. For these experiments, TA-protein pep12 was used because, when co-expressed with Get3-D57N in *E. coli*, large amounts of targeting complex in 2:1 ratios of Get3 to pep12 could be obtained. These complexes were dialyzed overnight in one of three nucleotide states (apo, ADP, or ATP) to analyze their different behaviors.

We first sought insight into the nucleotide state of the targeting complex as it first approaches Get1/2. No equilibrium measurement could be obtained, due to the release of substrate, but a FRET timecourse approximating a k_{on} was recorded. The increase in FRET between cytosolic domains of Get1/2 was used as a proxy for meaningful engagement of the targeting complex, and this FRET was monitored upon addition of exceptionally dilute targeting complex. Unsurprisingly, ATP-bound targeting complex does not drive an increase in FRET, as Get1 cannot bind ATP-bound Get3 (Fig. 7a). Both ADP-bound and apo (nucleotide-free)

targeting complexes could plausibly engage Get1/2. The essential question was whether or not ADP inhibited the engagement of targeting complex with Get1/2, which would be indicated by a slower rate of FRET increase in ADP than apo. In fact, no reduction in Get1/2 engagement was observed in the ADP sample, suggesting that ADP dissociation is not a rate-limiting step for engagement of Get1 and Get2 subunits, the key step in TA-protein release and insertion. The lower FRET value is most likely a result of the longer distance between Get1 and Get2 cytosolic domains across ADP-bound Get3 (Fig. 4).

Next, we demonstrated that post-release Get3 shows the same pattern of Get1/2 engagement as Get3 without substrate. Cytosolically-labeled Get1/2 was incubated with increasing concentrations of the Get3/pep12 targeting complex for 1 hour, allowing TA-protein to completely release and insert, leaving a post-release Get1/2/3 complex. Increasing concentrations of targeting complex produced an increase in FRET between Get1/2 cytosolic domains that saturates upon one equivalent of targeting complex (Fig. 7b), just as had demonstrated with Get3 alone (Fig. 2c). In the presence of ADP, there is a much less significant drop in FRET upon saturation than in the nucleotide-free sample. This may be a result of the ADP-weakened interaction between Get1 and Get3 that, while not inhibiting the Get1/2/3 interaction, weakens the interaction between Get3 and lone Get1, which would cause a drop in FRET via steric occlusion.

Additional Characterizations of Get1/2 Nanodiscs

Throughout the course of this project, various methods of analyzing the stoichiometry and activity of Get1/2 were developed. Proteoliposomes served as the best platform to test insertion activity with the protease protection assay. However, Get1/2 was difficult to isolate in

proteoliposomes without the dissociation of the subunits. The large amount of lipid and difficulty in further manipulating proteoliposomes made this a challenging scaffold. The use of the Get2-1sc fusion peptide solved many of these problems and drove the successful use of Get1/2 proteoliposomes as opposed to nanodiscs.

Nanodiscs served as a much easier scaffold with which to isolate Get1/2 and further purify and analyze the complex in membranes. Nanodiscs also improved the association of independent Get1/2 subunits with higher reconstitution concentrations that proteoliposomes and the ability to affinity purify via orthogonal tags. Unfortunately, we were not able to develop robust assays for substrate insertion into Get1/2 nanodiscs. However, we were able to robustly characterize the Get1/2 nanodiscs and successfully demonstrate the release and association of TA-proteins with Get1/2 nanodiscs.

Nanodisc reconstitutions were monitored and purified via size-exclusion chromatography (Fig 8a). Using numerous membrane scaffold proteins (MSP1D1, MSP1E3D1, and MSP2N2), an increase in size was observed upon reconstitution of lipid and lipid + protein. The smaller MSP1D1 nanodiscs have a smaller gel filtration profile, and therefore resolve a greater change in profile associated with the reconstitution of Get1 and Get1/2 (Fig. 8a). As additional components are reconstituted into the nanodisc, we see a corresponding shift to shorter gel filtration elution times.

In chapter 2, we report that Get1/2 nanodiscs are competent to release the TA-protein substrate from Get3. Although we were unable to test true insertion of TA Substrate into Get1/2 nanodiscs, we demonstrated the selective association of TA-protein (after release from Get3) with nanodiscs containing active Get1/2 (Fig. 8b). In this experiment, Get3 bound to Cy3-labeled, TA-substrate SUMO-Sec22-opsin was incubated with various Get1/2 nanodiscs for 30

min. Nanodiscs were then pulled down using streptavidin magnetic resin via the NHS-biotin-labeled membrane scaffold protein. After extensive washing to remove Get3, SDS-PAGE revealed that significantly more TA-protein was pulled down with nanodiscs containing Get1 and Get2. This assay does not confirm that substrate was inserted into the nanodisc bilayer, as it might have simply aggregated on the surface of the disc.

We robustly determined the stoichiometry of Get1/2 in proteoliposomes and nanodiscs using single-molecule photobleaching (Fig. 3a, Supplemental Fig. 5c and 4). However, we took advantage of the easily manipulated Get1/2 nanodisc to develop an orthogonal method of determining Get1/2 stoichiometry in nanodiscs. For this assay, Get1/2 complexes were subjected to pull downs via his tags on either Get1 or Get2. A trace amount of fluorescently labeled, tagless Get1 or Get2 subunit was also added, the same subunit as the one with a his tag. This labeled subunit could only be pulled down as a part of a higher order Get1/2 complex, so the extent to which this labeled subunit is recovered is the extent to which multiple Get1/2 heterodimers are in a single nanodisc. As a control, the pull down was also performed in a detergent in which Get1/2 is known to be a heterodimer (Supplementary Fig. 2b).

Nanodiscs were reconstituted at high MSP:Get1/2 ratios ("Dimeric ND") or low MSP:Get1/2 ratios ("Oligomeric ND"). Low MSP:Get1/2 ratios force multiple copies of Get1/2 into the same nanodisc. Indeed, in both detergent and the dimeric nanodisc reconstitutions, only trace amounts of untagged, fluorescent subunit is pulled down, indicating a reconstitution of primarily Get1/2 heterodimers. Significantly more fluorescent subunit is pulled down in the oligomeric nanodisc reconstitutions.

It is worth noting that oligomeric Get1/2 nanodiscs were exceptionally difficult to obtain. As the MSP to Get1/2 ratio was lowered, reconstitution efficiency dropped significantly. Longer

incubations (1-3hr) for the nanodisc reconstitutions improved this efficiency, but is appears that no more than 1-2 Get1/2 heterodimers could be reconstituted into a MSP1E3D1 nanodisc, despite there being room for more. This early result, reconstituting Get1/2 in membranes, raised doubts that higher order complexes of Get1/2 were physiologically relevant.

Nanodisc reconstitutions can sometimes produce large lipid aggregates that are even difficult to remove using size exclusion chromatography. Using negative stain EM, we demonstrated that the Get1/2 nanodiscs prepared for this project were homogeneous and of the expected size (Fig. 9). Work is underway, in partnership with Szymon Kordon and Claire Atkinson, to solve the structure of the Get1/2/3 complex using cryo electron microscopy.

References

1. Mariappan, M. *et al.* The mechanism of membrane-associated steps in tail-anchored protein insertion. *Nature* **477,** 61–66 (2012).

Figures (Chapter 3 Appendix)

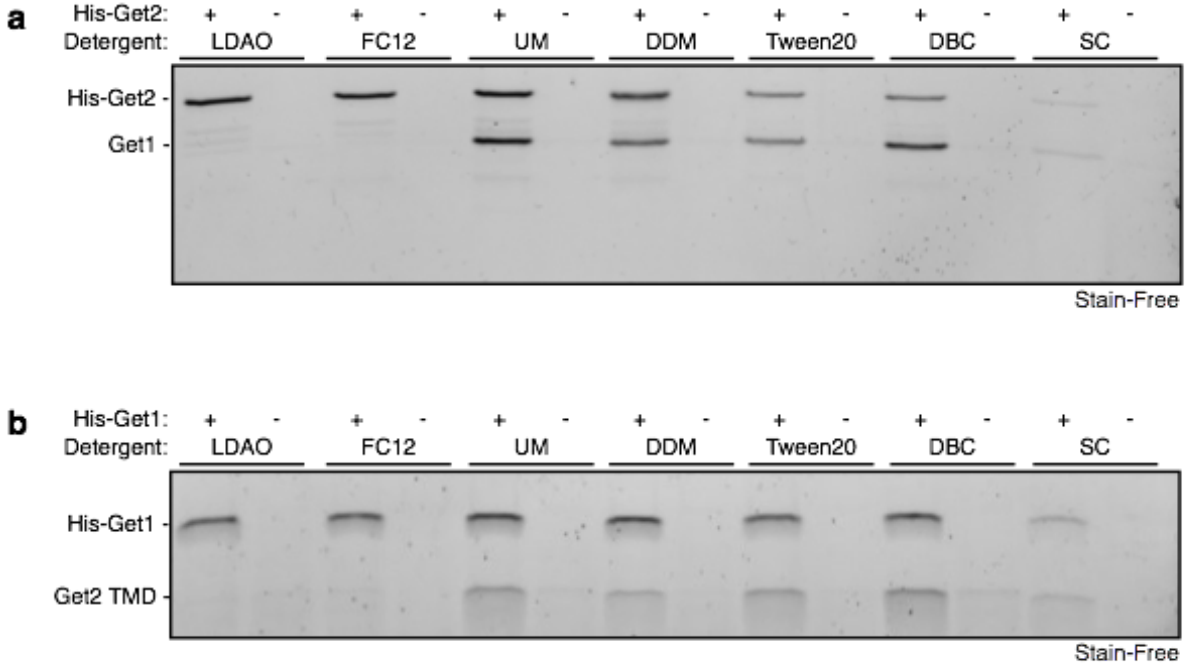


Figure 5. Get1-Get2TMD association in detergents. (a) Get1 and Get2-his were combined and diluted into various detergents and subjected to Ni-NTA pull down via a single subunit. "LDAO" = 0.1% n-Dodecyl-N,N-Dimethylamine-N-Oxide (Anatrace), "UM" = 0.1% n-Undecyl-β-D-Maltopyranoside (Anatrace), "DDM" = 0.1% n-Dodecyl-α-D-Maltopyranoside (Anatrace), "Tween20" = 0.1% Tween 20 (Fisher), "DBC" = 0.1% Deoxy Big Chap (Anatrace), "SC" = 1% Sodium Cholate (Affymatrix). Get1 is only pulled down in the presence of Get2-his and only in mild detergents. Get1 and Get2 do not interact in harsh detergents such as LDAO and FC12, but remain stable. In extremely mild SC, the complex is not completely soluble. (b) The same procedure was used, and the same results observed, for Get1-his and the transmembrane domains (TMD) of Get2, obtained by TEV cleavage at residue 130.

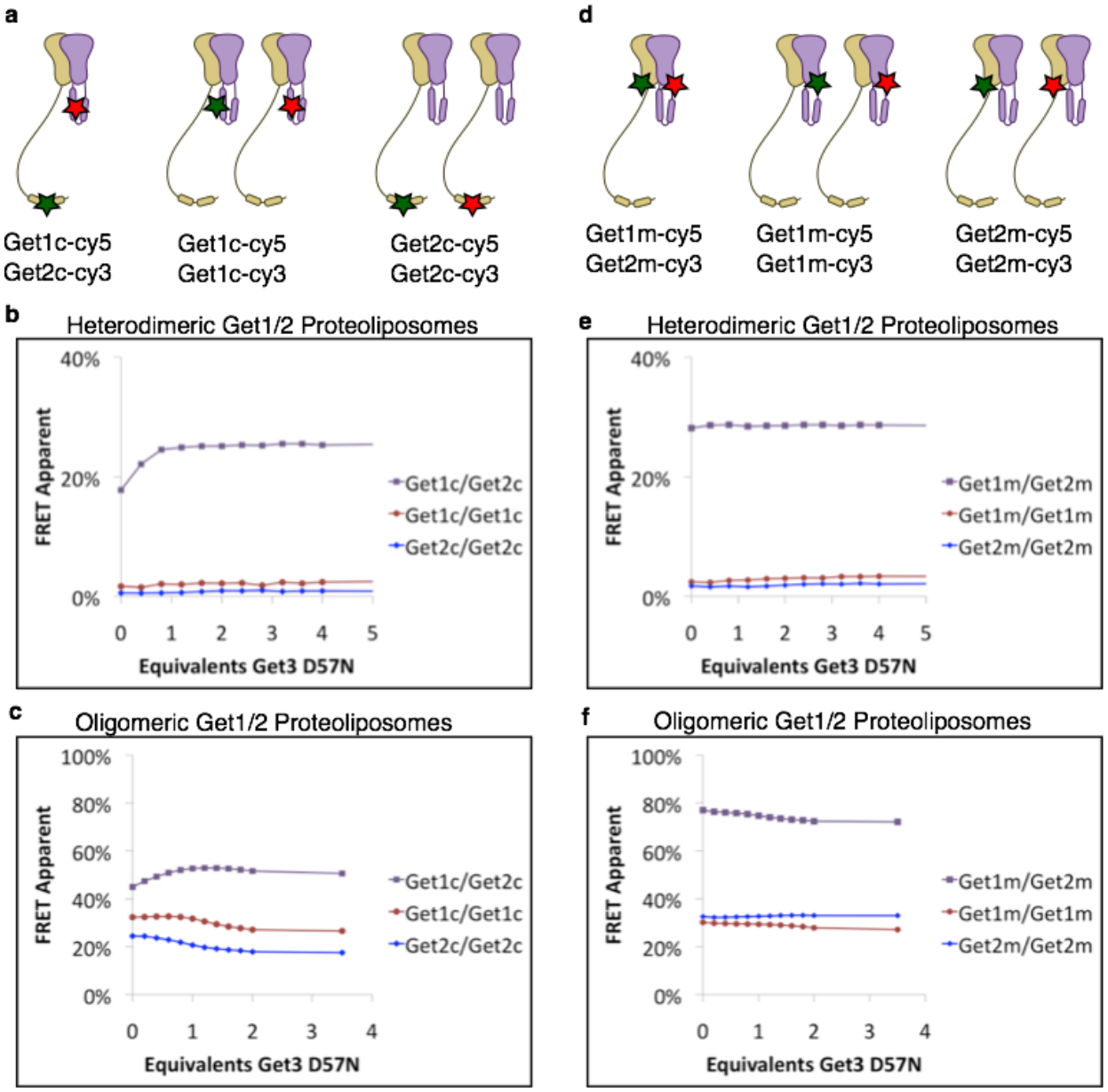


Figure 6. Bulk FRET in proteoliposomes. (a, b, c) Get1/2 fluorescent labels on the cytosolic domains show an increase in FRET only with differential labels on Get1 and Get2. The background FRET observed in "oligomeric" Get1c/Get1c and Get2c/Get2c is not observed when only a single Get1/2 heterodimer is reconstituted per liposome. (d, e, f) Get1/2 fluorescent labels on the membrane-adjacent domains show high levels of FRET with differential labels on Get1 and Get2. The background FRET observed in "oligomeric" Get1m/Get1m and Get2m/Get2m is not observed when only a single Get1/2 heterodimer is reconstituted per liposome. For isolated Get1/2 heterodimers, there is no significant change in FRET for any combination of fluorophores upon addition of Get3.

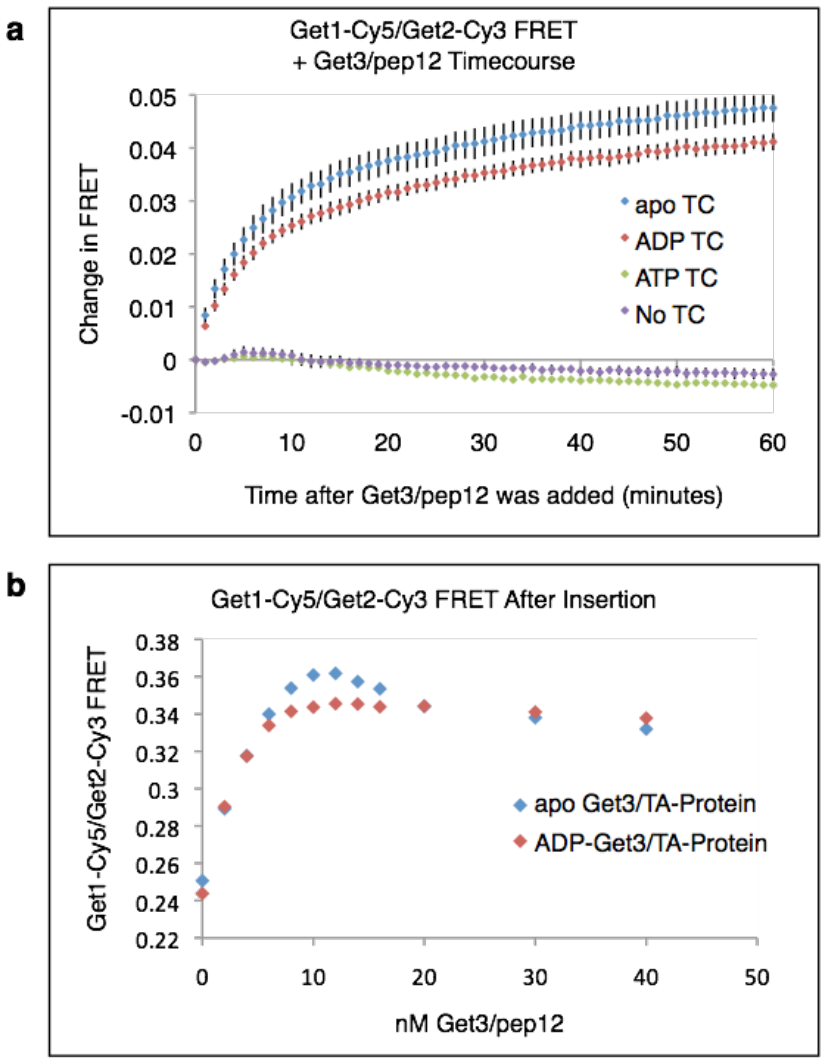


Figure 7. Titration of Get1-Cy5/Get2-Cy3 with Get3/pep12 targeting complex. (a) Cytosolically labeled Get1-Cy5/Get2-Cy3 in proteoliposomes (10 nM) is incubated with various concentrations of Get3/pep12 either without nucleotide or pre-incubated with ADP. The hump, or reduction in FRET, observed upon addition of excess apo Get3 is likely a result of Get3 binding to free Get1 and further causing steric separation between Get1/2 complexes in the same liposome. ADP reduces the affinity of Get3 for Get1, so reduces the prevalence of this Get1/3 interaction in the presence of ADP. (b) FRET timecourse following addition of Get3/pep12 to Get1-Cy5/Get2-Cy3 proteoliposomes in various nucleotide states. ADP produces a lower overall FRET increase, but does not reduce the rate of Get3/pep12 engagement with Get1/2

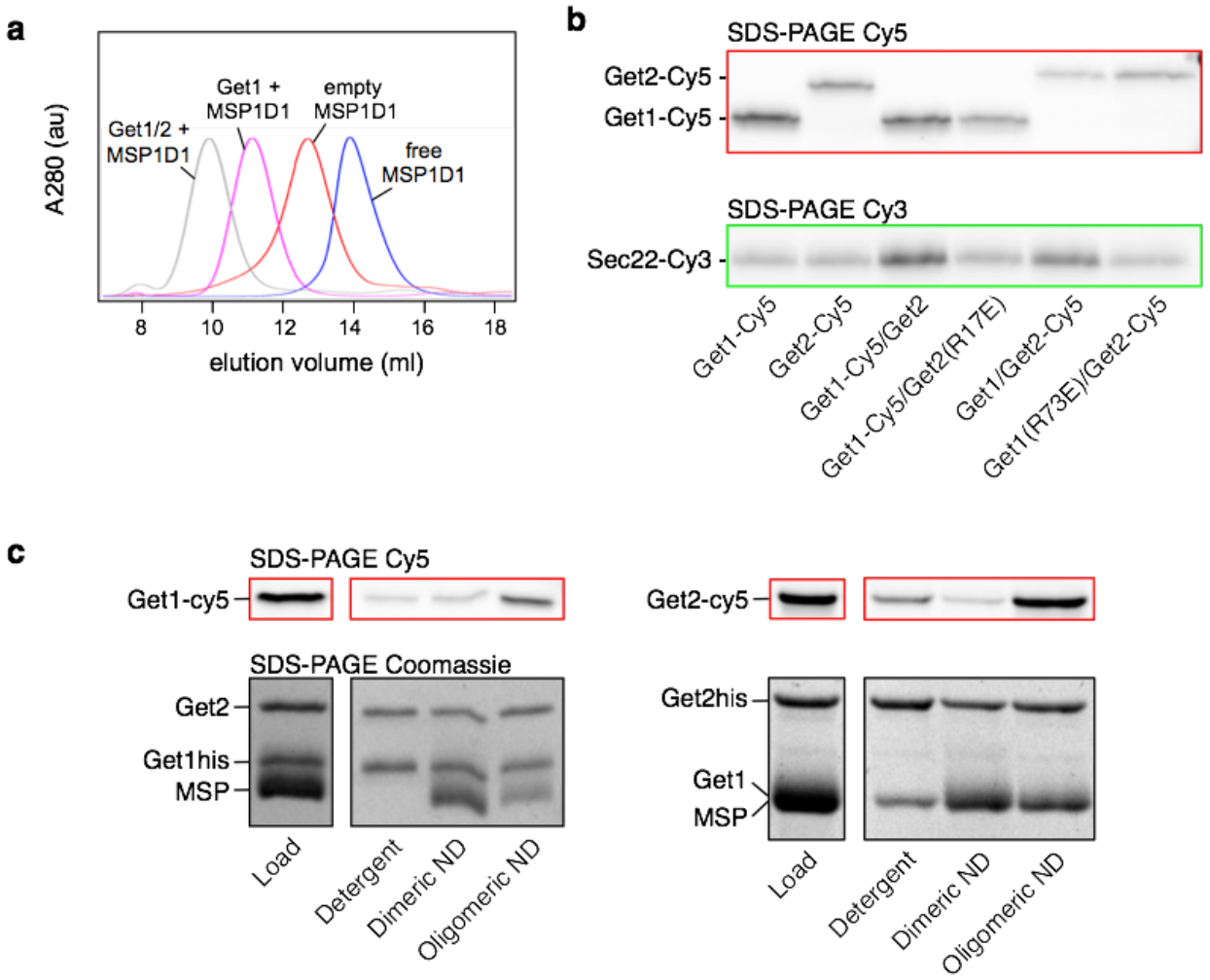


Figure 8. Nanodisc stoichiometry and activity. (a) Gel filtration profiles using a Superdex 10/300 column of nanodiscs demonstrating a shift toward larger size with an increasing number of reconstituted components. (b) Get1/2 nanodiscs, one subunit of which was fluorescently labeled with Cy5, was incubated with Get3/Sec22-Cy3. The nanodiscs were pulled down via biotinylated membrane scaffold protein, and run on SDS-PAGE, imaged by fluorescence. Significantly more Sec22-Cy3 is pulled down with Get1/2 nanodiscs containing two active subunits. Nanodiscs containing only one subunit or subunits with disabling R17E or R73E mutations pull down significantly less Sec22-Cy3. (c) Ni-NTA pull-downs test for the presence of higher order Get1/2 complexes. Only one subunit contains a his tag which pulls down it's complimentary subunit. A fluorescently labeled subunit without a tag is pulled down only in the case of higher order Get1/2 complexes. "Load" indicates the input to the Ni-NTA of the "oligomeric ND", though all samples contain the same concentrations of Get1 and Get2. The remaining three lanes indicate the respective elutions from the Ni-NTA

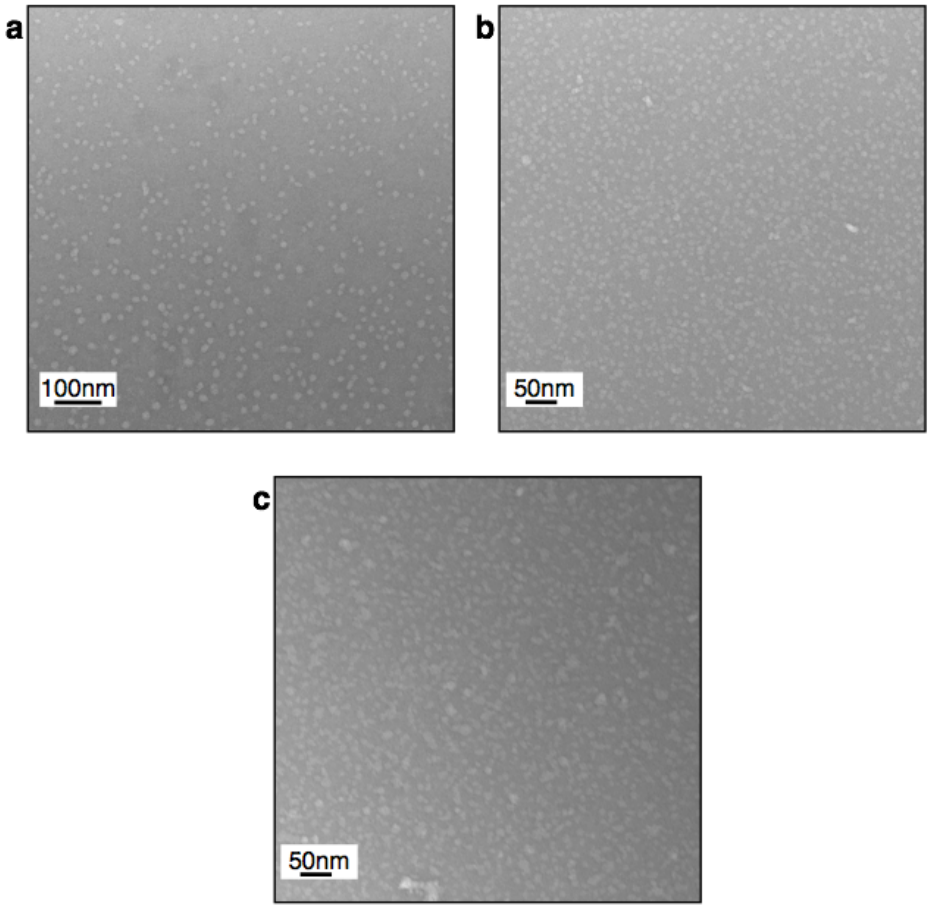


Figure 9. Negative stain cryo electron microscopy of Get1/2 and Get1/2/3 nanodiscs. All nanodiscs were reconstituted using MSP1D1, producing approximately 12nm nanodiscs (a) Get1/2 nanodiscs (b) Get1/2/3 nanodiscs (c) Get2-1sc/Get3 nanodiscs

CHAPTER 4

SINGLE MOLECULE PHOTOBLEACHING

In this chapter, I describe the lessons learned while optimizing the use of single-molecule photobleaching to determine protein stoichiometry. This chapter may be useful for those interested in using this technique.

In the study described in chapter 2, we sought to determine the minimum stoichiometry of Get1/2 that was competent to insert tail-anchored (TA) proteins into lipid bilayers. This was achieved by comparing the specific insertion activity of Get1/2 complexes reconstituted at various, defined stoichiometries. Robustly determining the stoichiometry of membrane proteins using single-molecule photobleaching required a significant technical investment. In this chapter, I discuss the techniques used and lessons learned while refining this method.

Using common biochemical tools, fundamental properties of peptides such as length, sequence, and even specific activity can often be easily determined. However, a property as fundamental as the complex's stoichiometry can be elusive. Robustly determining the number of specific peptides in a protein complex presents many challenges, but is key to understanding the mechanism and structure of the complex.

Multi-angle light scattering (MALS) serves as a robust means of estimating the molecular weight of a complex, from which stoichiometry can often be estimated. It can even be used to exclude non-protein conjugates such as detergent for the analysis of detergent-solubilized membrane proteins. However, detergents do not represent a physiologically relevant environment for membrane proteins, and can disrupt physiological interactions or produce

artificial, higher-order complexes. Crystallography and small-angle X-ray scattering can produce similarly non-physiological environments. It is often impossible or impractical to apply these techniques to membrane protein complexes in lipid bilayers.

Single-molecule photobleaching can be an effective tool for determining the stoichiometry of membrane proteins in lipid bilayers. It has even been used, under limited circumstances, to determine the stoichiometry of membrane proteins in living cells. When excited by a laser and imaged by total-internal reflection fluorescence (TIRF) microscopy, fluorophores eventually "bleach" or stop fluorescing at random intervals. By counting the number of distinct, step-wise decays in the fluorescence of an isolated, fluorescently-labeled protein complex, the number of labeled subunits in that complex can be directly counted. Measurement of hundreds of these complexes gives a distribution of photobleaching step-counts that is used to determine the stoichiometry of the membrane protein complex and even the distribution of heterogeneous stoichiometries.

The proper application of this technique requires that a number of technical challenges be overcome:

1. Fluorescent labeling of subunits that is highly specific and efficient
2. Accurate determination of labeling efficiency
3. Clear imaging of distinct fluorescent complexes
4. Unbiased image analysis to record fluorescent intensities of single complexes
5. Unbiased step counting of fluorescent traces
6. Quality control to control for systematic errors

Specific Labeling of Protein *in vitro*

The easiest way to fluorescently label recombinant proteins is by incorporating green-fluorescent protein (GFP) or a related fluorescent peptide into the sequence. Labeling is 100% efficient, which is highly advantageous in analyzing the final data. However, fluorescent proteins are not as resistant to photobleaching as small-molecule fluorophores, therefore GFP requires lower laser power, producing noisier fluorescent traces with which to count photobleaching steps. Fluorescent proteins are also much larger than small-molecule fluorophores and can affect the structure and function of proteins. We hoped to use our fluorescent Get1/2 complexes for FRET experiments, and these large, unstable, fluorescent proteins are impractical for this application. Many fluorescent proteins can even oligomerize, further complicating the determination of physiologically relevant stoichiometries. Finally, the expression of proteins can often be inhibited by the incorporation of fluorescent proteins like GFP, and our lab has been unsuccessful in expressing GFP-tagged Get1 or Get2. For these reasons, we resolved to label our recombinant proteins with commercially available, small-molecule, organic fluorophores.

The key to determining the stoichiometry of a protein complex using single-molecule photobleaching is specifically labeling each protein subunit with a single fluorophore. Therefore, non-specific labeling methods such as the use of N-HydroxySuccinimide (NHS) esters and its derivatives to label lysine residues are not appropriate. We found maleimide-functionalized fluorophores to be the most effective for specifically labeling cysteine residues that were introduced into our Get1 and Get2 constructs, both of which contain no native cysteine residues. Maleimide chemistry, often referred to as a kind of ‘click’ chemistry for its highly efficient and general use in reacting with thiols, reacts specifically with cysteine residues. Care must be taken to prevent the maleimide labeling of lysine residues, which tends to occur at high pH.

Though not used in any of the photobleaching or FRET experiments reported here, we also attempted to specifically label sites on Get3. Maleimide chemistry is not an option for labeling Get3 since the native sequence contains several cysteine residues. Furthermore, four cysteine residues in the Get3 homodimer coordinate a zinc ion that serves as the 'hinge' between the Get3 monomers. We suspected that this zinc ion would serve as a Lewis acid catalyst, increasing the reactivity of these cysteine residues. We therefore expressed Get3 while incorporation of the artificial amino acid azido-phenylalanine via amber codon suppression. We hoped to use the azide functionality as a handle for alkye-functionalized fluorophores via the Huisgen 1,3-dipolar cycloaddition reaction, catalyzed by copper. Unfortunately, Get3 was incredibly sensitive to the addition of copper and appeared to precipitate upon quantitative addition of copper sulfate to the buffer. We speculated that Get3 may be especially sensitive to copper ions due to the presence of the Zinc coordinated by Get3. The zinc ion may be displaced by copper, making Get3 highly unstable. We attempted to work around the problem by using fluorophores functionalized with strained alkynes, alkynes within an 8-carbon ring that can react with azides without a copper catalyst. This reaction was successful in that it produced stable, fluorescently labeled Get3. However, incubation of the strained alkyne fluorophores with native Get3 also resulted in a comparable degree of fluorescent labeling. Therefore, the Get3 labeled with strained-alkyne fluorophores was too non-specific to be useful. Get3 could be effectively labeled with NHS-ester fluorophores, despite not being site-selective. We found it critical that this NHS labeling of Get3 be performed in the presence of ATP, as the nucleotide-free, 'open' state of Get3 exposes lysine residues along the homodimer interface. Labeling of these Get3 lysine residues in the open state greatly disrupts the dynamic conformations and functions of Get3.

Non-specific maleimide labeling of Get1 and Get2 must be minimized to accurately determine stoichiometry using single-molecule photobleaching. The extent of non-specific labeling was initially determined by subjecting constructs both with and without cysteine mutations to the same labeling conditions, and then analyzing the reactions on SDS-PAGE to image the labeled protein separated from free dye. We used a pH of 7.5, recommended by the commercial protocol (GE), since higher pH results in greater lysine labeling, and lower pH reduces the reactivity of the cysteine residues. We evaluated labeling efficiency and non-specific labeling as a result of salt concentration, protein to dye ratios, detergent, and temperature. Most conditions had little effect on non-specific labeling, but room-temperature reactions had a much higher degree of non-specific labeling than reactions performed on ice. Optimized labeling conditions called for sequential, intermittent additions of 1 equivalent of dye up to 5 equivalents on ice at pH 7.5. Protein concentrations were always 1-5 mg/mL, and care was taken to eliminate all imidazole and thiols from buffers before labeling. Reducing agent tris(2-carboxyethyl)phosphine (TCEP) was present at 1 mM in all reaction buffers to prevent cysteine oxidation. This optimization of labeling the soluble domains of Get1 and Get2 (Get1-S77C and Get2-S28C) produced labeling efficiencies of 65-70% in their respective Fos-choline-12 (FC12) and undecyl-β-D-maltopyranoside (UM) buffers. Using nanodrop spectroscopy to determine labeling efficiency, the non-specific labeling of Get1 and Get2 without cysteines was estimated to be 5%. The concentration of non-specifically labeled protein was lower than the linear range of the fluorescence absorption, so the actual extent of non-specific labeling was likely lower than 5%.

Several commercially fluorophores were used to label Get1 and Get2. Cy3, Cy5, Atto655, Atto532, and Atto488 are all water soluble, and efficiently labeled the subunits with

negligible non-specific labeling. However, Cy3 and Cy5 were used most commonly due to their low cost and common, established use as FRET pairs. The water-solubility of these fluorophores proved to be key. Free dye was effectively removed from the labeled protein using PD10 size-exclusion, Superdex 10/300 size exclusion, or Ni-NTA purification. The removal of free dye was confirmed by SDS-PAGE. Samples were loaded without loading dye and alongside a sample containing free fluorophore dye. It is critical that free dye be completely removed from the labeled protein as there is no way to distinguish labeled protein from free dye in the determination of labeling efficiency or in TIRF microscopy. We found exceptional difficulty in our initial attempts to label Get1 and Get2 with Atto647N-maleimide, an insoluble, hydrophobic fluorophpore. The free dye of this fluorophpore proved impossible to removed, even under the harshest washing on Ni-NTA columns. Atto647N labeling efficiency was also lower than with soluble fluorophores. It is possible that this fluorophore became sequestered in detergent micelles and was nonspecifically incorporated into the transmembrane domains (TMD) of the protein. Since the structure and solubility of many commercially available fluorophores are not published by the seller, this presents a broad challenge to the selection of fluorophores, especially for the labeling of membrane proteins.

Labeling Efficiency

Trace amounts of free dye can often be removed by downstream manipulations and reconstitutions of the labeled protein. The use of Bio-Beads in detergent removal during proteoliposome and nanodisc reconstitutions is also an effective way to remove trace amounts of free, fluorescent dye. However, relying only on the downstream removal of free dye is inadvisable, because it is important to accurately determine the labeling efficiency of each

protein after labeling, and the presence of free dye will artificially inflate the labeling efficiency. This labeling efficiency can often not be accurately determined after reconstitution.

Low labeling efficiencies reduce the likelihood of a multimeric complex containing multiple labeled subunits. The greater the number of subunits, the greater the need for high labeling efficiencies to precisely determine the stoichiometry of the complex. If the photobleaching analysis is otherwise well performed, low labeling-efficiencies such as 40-50% can be effective in distinguishing monomers from dimers. However, distinguishing higher order complexes such as pentamers and hexamers generally requires labeling efficiencies >70% to get a statistically significant difference in their distributions.

The best way to determine labeling efficiencies is via UV/VIS spectroscopy, most commonly using a nanodrop spectrophotometer. Using the respective absorbance of the protein and its conjugate, the molar ratio of the two can be determined. It is critical that no UV-absorbing contaminates or free dye is present in the sample to effect this measurement. It is also critical that there is no significant presence of labeled impurities, so SDS-PAGED imaged by fluorescence is important to identifying such contaminants. The labeling efficiency is usually be used to fit a binomial distribution to the final photobleaching data, so is an important measurement. This distribution is even more critical when measuring the proportions of heterogeneous stoichiometries. On the other hand, a stoichiometrically homogeneous complex that is efficiently labeled does not require a precise determination of labeling efficiency, since the highest number of steps measured in a sample will constitute the soichiometry of that complex. The shape of the distribution, which is a function of the precise labeling efficiency, is not critical if the stoichiometry is consistent within the sample. With the case of Get1/2 complexes in proteoliposomes, higher-order complexes were the result of random associations,

and were therefore heterogeneous in size. That required an accurate assessment of labeling efficiency. Fortunately, Get1 and Get2 were able to be labeled in detergent with a large assortment of fluorophores and labeling efficiencies of 65-70%, sufficient for accurate photobleaching calculations.

TIRF Microscopy

Simple glass flow cells were constructed for use in TIRF microscopy, and three methods of attaching the reconstituted Get1/2 complexes in proteoliposomes or nanodiscs were used. The first, simplest method simply involved flowing a highly diluted sample through a clean flow cell, and allowing the fluorescent complexes to adhere to the glass coverslip. This method gives the smallest amount of background fluorescence since there is the least possible chance for contamination. Liposomes readily adhere to clean glass, so this method was used for photobleaching Get1/2 in proteoliposomes. Unfortunately, this method denatures protein complexes on the glass surface, and is therefore not appropriate for structural studies like single-molecule FRET. For these experiments, Get1/2 nanodiscs were attached to the coverslip via biotin/neutravidin linker. Nanodiscs were easily biotinylated by reacting the membrane scaffold protein with NHS-PEG_4-Biotin before reconstitutions. Coverslips were functionalized with neutravidin one of two ways. The first method covalently reacts PEG-silane and Biotin-PEG-silane to freshly cleaned glass, followed by extensive rinsing, flow cell construction, and incubation with neutravidin. This method was effective, but produced a high degree of fluorescent contamination observed in the Cy3 channel. The blocking of the glass with PEG was also incomplete, showing a small amount of recruitment of fluorescent nanodiscs to the glass surface even when no neutravidin was used. The best method used in my hands required no

covalent modification of the glass coverslips. Flow cell incubation with neutravidin followed by high concentrations of β-casein in all successive buffers was extremely effective at blocking nonspecific recruitment of fluorescent nanodiscs and contained very little fluorescent contamination. This method was therefore used for all reported single-molecule FRET experiments in nanodiscs.

One of the biggest challenges in recording clear TIRF videos is achieving good focus. Drift during a video can be an issue, especially during the early analysis of a slide as temperature is still reaching equilibrium. Sealing of the flow cell with epoxy limited this drift. The focus plane is also likely to change at different locations along the coverslip. It's critical not to change the focus during the recording of a video so as not to introduce step-wise changes in fluorescence that may be interpreted later as photobleaching steps. It's critical that the video be in focus from the first frame, however, it is not advisable to excite the field of view to achieve proper focus before recording the video. Even a single frame excites the field of view with the laser, photobleaching a small amount of the sample, and reducing the effective labeling efficiency. The first laser excitation of the field of view must be the first frame of the video. The best way to achieve this is to bring a field of view into focus by taking single images with reduced laser exposure. Then, with the laser off, bring the objective into an adjacent, but unexposed field of view to begin recording the video. If this field of view is not in perfect focus, discard the video. We found that videos recorded within an hour of making the slide was consistent and reproducible, though longer times can produce slightly lower effective labeling efficiencies via latent exposure to the excitation laser, even if not in the recorded field of view.

The next challenge in recording TIRF videos for photobleaching analysis is finding the proper exposure time and laser power for adequate signal to noise ratio. Higher power laser and

longer exposure times will produce clearer fluorescence traces, but too few frames with which to accurately count steps. It is also critical that the laser power be strong enough that all fluorescent complexes photobleach by the end of the video, or at least at some time during the video so as to achieve a baseline. It's best to record as long of a video as is practically possible, so that no photobleaching steps go unobserved.

One important way to improve signal to noise in TIRF microscopy is the use of triplet-state quenchers. Buffers that contained 2 mM trolox proved to be the most effective, though partial oxidation of the trolox before use was critical, as has been previously reported[1]. Insufficient oxidation leads to a strong reducing environment that impairs the fluorescence lifetime. Excessive oxidation leads to fluorescent impurities. Preparation of trolox the night before, or a few minutes under a UV lamp were sufficient to oxidize trolox for use in photobleaching experiments. Trolox was so effective that we had a difficult time photobleaching samples labeled with Atto655, even at full laser power.

The use of the glycose-oxidase/catylase oxygen-scavenging system (GOC) was effective in increasing fluorescence lifetimes, but the increase in brief-dark state 'blinking' was so dramatic that too much noise was introduced into our measurement and it was not used for photobleaching or single-molecule FRET experiments.

Data Analysis

The first task in analyzing these TIRF videos is identifying regions of interest (ROIs), which are used to record time traces of fluorescence intensities from single fluorescent complexes. The mere intensity of fluorescent complexes is insufficient to filter out background noise. Therefore, the first video frame was filtered with a Laplacian of Gaussian filter (Julia),

with a scale parameter of 150nm. This filter was used to find not only intensity, but shape consistent with point sources of fluorescence. Peaks were identified by thresholding and non-maximum suppression over a 360 nm^2 area.

It is key to find as many plausible regions of interest as possible, so as not to exclude relevant data and bias the distributions. We applied only one additional filter, excluding ROIs that were within 540nm of each other or within 540 nm of the image edge. This prevents fluorescence from bleeding into adjacent ROIs, which can give the appearance of additional photobleaching steps. Merely filtering these ROIs based on their location does not bias the step distributions.

Step Counting

Determining the number of photobleaching steps in a fluorescence trace is one of the biggest challenges of this analysis. We analyzed fluorescence traces in R, and worked to decide between two common methods of counting steps: computationally or by hand.

Computationally finding "steps" was straightforward, applying the changepoint package to a trace finds significant changes in the average intensity of fluorescence over time. Since photobleaching is often temporary, we added an additional factor that took into account the direction of the photobleaching step. Down-steps were bleaches, and up-steps were recoveries. The minimum, stable intensity is always the baseline.

Unfortunately, there are many more aspects of step-patterns in noisy, single-molecule fluorescence traces than can be practically programmed into R. The size of the step varies with the intensity of the trace and location in the field of view. Changepoint functions do not consider step size, even though they should be relatively consistent. In reality, step sizes are not perfectly

consistent, even in the cleanest of traces. Usually the first few steps are larger than the last few. Noise varies widely, and the judgment of what is noise or aggregation, and what is a legitimate step, requires the eye of an experienced user. We dedicated a great deal of time and effort experimenting with many parameters such as quantifying noise and setting parameters for considering the shape of legitimate photobleaching steps. However, there always remained photobleaching steps that our code was clearly miscounting when checked by eye. For these reasons, most photobleaching steps are counted by hand.

Our brains are exquisitely good at recognizing patterns. After analyzing thousands of photobleaching steps, a user is well trained to recognize the various complicated behaviors of specific fluorophores, even specific to the software and instruments used. However, the major problem with counting steps by hand is subjective bias. Presumably, the user has an expected distribution of photobleaching steps for whatever dataset they are analyzing. A non-trivial number of traces can be interpreted with more or fewer steps depending on subjective criteria. Even the most virtuous user cannot eliminate subconscious bias. The data that we collected was subject to such potentially biased interpretations. We found that our data was not exceptionally prone to such bias because the number of traces that could be interpreted multiple ways was rarely significant enough to sway the interpretation of the overall stoichiometry. However, when comparing heterogeneous populations of different stoichiometries, this bias needs to be eliminated.

Our breakthrough in solving this problem was to combine the best of both strategies. Steps were counted by hand, but displayed in a way that prevented the user from knowing the sample of origin. We recorded photobleaching videos from many samples of various stoichiometries, as well as monomeric control samples such as free dye and labeled protein in

SDS. ROIs were isolated and fluorescence traces from all samples were combined into a single dataset. From this dataset, R displayed random traces for the user to determine step counts. After analyzing the entire combined dataset, the step distributions of each sample were reported. Due to the nature of the samples, the user expects a broad distribution of step counts, but no bias could sway what we referred to as this "blind analysis".

Quality Control and Error Correction

If a sample can be assumed to be of homogeneous stoichiometry, the analysis of the step-count distribution is straightforward. The data can be fit to a Poisson distribution that accounts for the known labeling efficiency, or the labeling efficiency can be left as a variable. The most important determination is the maximum step-size. Theoretically, a 6-step trace can never be observed for a pentamer, which should produce a significant population of 5-step traces. So, the maximum population of step-counts is the stoichiometry of the protein. In reality, there are usually a few random traces with more steps than the maximum stoichiometry of the protein, but this population should be negligible if the labeling efficiency is high enough. The more subunits in a complex, the greater the need for high labeling efficiencies.

To determine the distribution of a heterogeneous population of protein stoichiometries from the distribution of photobleaching steps, an accurate labeling efficiency, as well as some correction factors, must be applied. Due to the high level of noise, contaminants, and rapid blinking of fluorophores, it is far more likely to observe false steps than to ignore legitimate photobleaching steps. We even observed a significant number of apparent two-step photobleaching traces in an analysis of free dye, for which only a single step is expected. The monomeric controls in our blind analysis were key to quantifying this rate of false step counting.

Non-specific labeling of protein can also produce higher step-counts. Therefore the ratio of step counts with one too many observed steps (whether due to miscounting, photophysics, or nonspecific labeling) was determined from the monomeric control. This ratio was determined for each blind analysis, and was usually 5-10%. This ratio was first applied to the experimental data, used to subtract mis-counted extra steps in proportion to the population of one-fewer step counts. This analysis changed the overall data very little. Next, a Poisson distribution was fit to the histogram to account for labeling efficiency. The parameters of this Poisson distribution were then used to generate a histogram of protein stoichiometries that includes the protein without fluorescent labels. This histogram can be further corrected, as was key in our experiments, for the number of protein molecules in each population. Monomeric complexes contain one protein, but dimers contain twice as much protein, and so on. Therefore, the population of each stoichiometry was multiplied times the number of subunits in that population to give a distribution of how much protein existed in each oligomeric state.

To test that our step-counting analysis was accurate and consistent, we adapted a method used by the lab of Taekjip Ha[2] to compare the initial intensity of ROIs with the number of counted steps. Fluorescence intensity changes widely over the TIRF field of view, so the intensity of an ROI is not an accurate method of determining the number of fluorophores in that complex. However, the average fluorescence intensity of ROIs containing two fluorophores should be twice the average intensity of single fluorophores. Therefore, the initial intensity of one-step and two-step traces were plotted respectively as kernel density functions, and indeed, the two-step traces were an average of twice the intensity of one-step traces. The distribution of intensities also broadens with greater oligomerization, so only comparing the intensities of 1, 2, and sometimes 3-step traces is practical using this method. This analysis was improved by also

plotting the distribution of median step size of multi-step traces, which, as it should be, was remarkably consistent with the intensity of one-step traces. Finally, we also plotted the intensities of discarded data, traces that were too noisy to count. These traces were shown to be predominantly multimeric, which was to be expected since they were primarily observed in samples of higher order complexes. These checks confirm accurate and consistent analysis of photobleaching step counts.

Conclusions

With controls, diverse samples, unbiased analysis, and an experienced user, this analysis proved to be a robust method for determining membrane protein stoichiometry. However, the technical challenges of this technique require a large investment of time and resources to produce reliable data and analyses. Consideration of the many ways in which photobleaching analysis can be misleading must be adapted to the specific needs of each system. This analysis will greatly depend on the size of the oligomeric complex, the specific TIRF microscope, fluorophores, labeling conditions, TIRF sample environment, and controls samples. Care must be taken when casually assessing stoichiometry using this method.

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CHAPTER 5

TAIL-ANCHORED PROTEINS IN ARABADOPSIS

This chapter describes the collaboration with the Howell Lab at Iowa State to identify Arabadopsis tail-anchored protein SYP72, which was published: Srivastava, R., Zalisko, B. E., Keenan, R. J., & Howell, S. H. (2017). The GET System Inserts the Tail-Anchored Protein, SYP72, into Endoplasmic Reticulum Membranes. Plant Physiology, 173(2), 1137–1145.

In collaboration with Stephen Howell and Renu Srivastava at Iowa State University, we identified Arabadopsis protein SYP72 as a tail-anchored protein. For my part, I used yeast Get1/2 and Get3 to show that SYP72 is a potential substrate for the GET pathway in *Arabadopsis*, which has analogous GET components.

First, we showed that SYP72 binds to yeast Get3. Using *in vitro* synthesis of SYP72 containing a twin-strep tag, we selectively pulled down Get3 along with SYP72 (Fig. 10). This complex was then used in a protease protection assay with Get1/2 proteoliposomes (Fig. 11). A protected fragment of the [S35]-SYP72 was only observed when incubated with active Get1/2 proteoliposomes, including yeast rough microsomes and recombinant Get1/2 in proteoliposomes. No insertion was observed in liposomes alone or Get1/2 with disabling Get1-R73E and Get2-R17E mutants. These binding and insertion assays were performed as described in chapter 2.

Figures (Chapter 5 Appendix)

Reprinted from Srivastava, R., Zalisko, B. E., Keenan, R. J., & Howell, S. H. (2017). The GET System Inserts the Tail-Anchored Protein, SYP72, into Endoplasmic Reticulum Membranes. Plant Physiology, 173(2), 1137–1145.

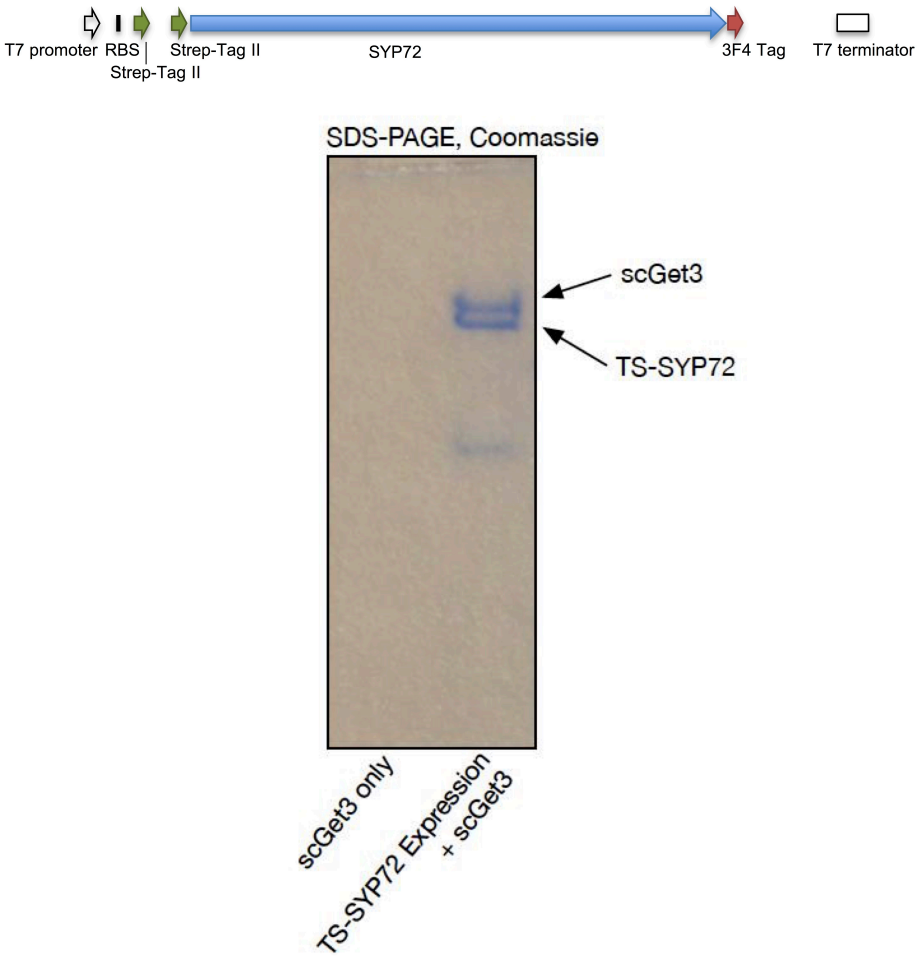

Figure 10. Synthesis of the components used in the proteinase K protection assays. *In vitro* expression and Steptactin purification: Twin-Strep (TS) tagged SYP72-3F4 was expressed in a 250 mL PURExpress reaction in the presence of [^{35}S]-methionine and yeast Get3 (scGet3). This mixture was subjected to purification via Steptactin resin in parallel with a sc-Get3-only control to show that scGet3 was only pulled down by forming a complex with TS-SYP72-3F4

Reprinted from Srivastava, R., Zalisko, B. E., Keenan, R. J., & Howell, S. H. (2017). The GET System Inserts the Tail-Anchored Protein, SYP72, into Endoplasmic Reticulum Membranes. Plant Physiology, 173(2), 1137–1145.

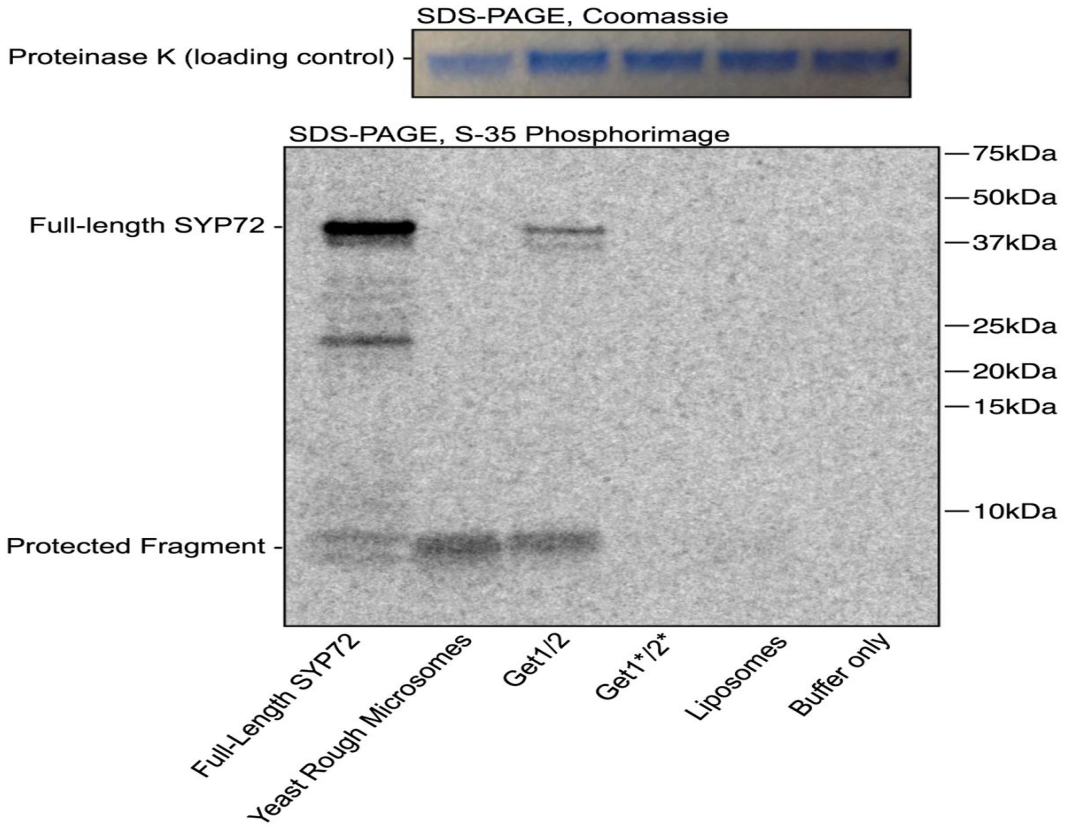

Figure 11. Get3 Inserts SYP72 into proteoliposomes *in vitro*. A proteinase K protection assay was used to demonstrate that yeast Get1/2 can insert Arabidopsis SYP72 into proteoliposomes via yeast Get3. The purified Get3-SYP72 complex was incubated with yeast rough microsomes or proteoliposomes containing the Get1/2 insertase complex. Get1*/2* point mutants that disrupt binding to Get3, empty liposomes, and buffer serve as negative controls. After insertion, SYP72 substrate is digested with proteinase K. Digestion of properly inserted SYP72 results in a C-terminal protected fragment, indicating insertion and correct orientation. Full-length SYP72 contains 4 times as many ^{35}S-Met residues as the protected fragment, corresponding to the difference in band intensity.

Appendix: Laboratory Protocols

This chapter contains experimental protocols could be useful for future experiments relative to this project and are intended for practical use in the laboratory.

Protocol for Expressing and Purifying His-Get1 or His-Get2

Expression:

- Transform Ros2(DE3)pLysS cells with His-Get1 or His-Get2 pET28 plasmids
- Plate on LB/Kan/Chl agar plates overnight at 37°C (14-18hr)
- Use a fresh, single colony to inoculate a 3mL TB/Kan/Chl preculture. Shake at 37°C until OD = 0.5-1
- Add 1mL of preculture to 500mL autoinduction media:
    - 465mL TB + Kan/Chl
    - 25 mL 20x NPS (0.5M ammonium sulfate, 1M potassium dihydrogen phosphate, 1M disodium phosphate)
    - 10 mL 50X 5052 (25%v/v glycerol, 10%w/v α-lactose, 2.5%w/v glucose)
    - 0.5mL 1M magnesium sulfate
- Shake at 250rpm at 37°C for 18-20hr in a 2.8L glass baffled flask
- Harvest cells in a JLA-8.1 rotor at 5,000 rpm for 15 minutes
- Transfer pellet to 50mL falcon tube and store at -80° C. (Typically 4-6g)

Purification:

- 'Lysis buffer': 50mM Hepes, pH 8.0, 200mM NaCl, 5%v/v glycerol, 10mM imidazole
- Resuspend in 40-80mL Lysis buffer + 5mM BME, 1mM PMSF, 30µg/mL DNase, 2mM $MgAc_2$
- Homogenize 5-10 times, microfluidize 2-3 times
- Spin 40min at 35,000rpm in Ti45 rotor, discard supernatant
- Gently resolubilize pellet with brush in 50mL lysis buffer + BME + 1%w/v DDM
- Incubate on cold room wheel for at least 2hr or overnight
- Spin 40min at 35,000rpm in Ti45 rotor, discard supernatant
- Apply supernatant to 3mL Ni-NTA resin, incubate 30-60min on cold room wheel
- Collect FT, wash with 20 x bed volume of lysis buffer containing 20mM imidazole, 1mM TCEP, and 0.1%w/v detergent (UM for Get2, FC12 for Get1)
- Elute with 5 x bed volume of above buffer containing 200mM imidazole
- Concentrate and purify via gel filtration in 50mM Hepes, pH 7.5-8, 200mM NaCl, 5%v/v glycerol, 1mM TCEP (if cysteine mutant)

Construct	Molecular Weight (kDa)	Extinction Coefficient ($mM^{-1}cm^{-1}$)
Get1-His	29.82	55.76
Get1-	27.74	54.48
Get2-His	33.90	42.40
Get2-	31.49	40.91

Expression and Purification of His-Get1 and His-Get2 (Continued)

His-tag removal with TEV

- Note: His tag removal is not necessary for most experiments
- TEV is not active in FC12. His-Get1 must be diluted 10x into UM buffer
- Add 1 TEV aliquot per 10mg of Get1 or Get2 + 1mM EDTA and 1mM DTT
- Monitor cleavage by SDS-PAGE, which is usually complete after ~4hr at RT
- Perform subtraction with Ni-NTA and purify using gel filtration

Things to keep in mind…

- The most reproducible results are obtained with single colonies from a fresh plate, but LB plates can be stored at 4° C for up to 1-2 weeks. Multiple colonies can be used to speed time to the necessary preculture OD, but be sure that the cells are actually growing for at least an hour before inoculation. This is especially important for the auto-induction media.
- Two His-Get1 constructs were constructed. The first had too short of a linker between Get1 and the N-terminal his-tag for efficient TEV cleavage. A second construct was made by Brittney (Manvilla) McClymonds that added 5 residues between Get1 and the TEV site and allow for efficient cleavage. This construct is referred to as "Get1-5H" or "Get1-5-link-his". Unless indicated otherwise, this second construct is always used, but there are many derivatives of the former construct. Be careful of which one you use.
- Autoclave TB and filter the autoinducation media stocks with 0.22 μm filter
- Get1 and Get2 can be resolubilized in DDM or LDAO. If done overnight, do not use LDAO. LDAO is hasher, so 2 hours is sufficient, and longer incubations will decrease yield. Get1 is mildly unstable in DDM and will crash out at higher concentrations. Spiking the resolubilization with a bit of UM will improve yield and stability, though it adds cost and generally isn't necessary since Get1 expression is so high. I generally equilibrate the Ni-NTA resin in UM wash buffer so that whatever DDM-soluble material is in the supernatant after resolubilization is exposed to a little UM right away.
- Do not exchange from LDAO directly into UM. For whatever reason, this causes the protein to crash out. If you must, first exchange into FC12 followed by UM. This can all be done on the same Ni-NTA resin.
- Get1 is noticeably brown above concentrations of 1mg/mL independent of reducing or chelating agents. This is likely due to light scattering due to unfolding or aggregation, though it still shows a monodisperse, single peak on gel filtration.
- I tend to err on the side of adding reducing agents like BME or DTT to most buffers and bubbling nitrogen through my buffers, even when not purifying cysteine mutants. This prevents methionine oxidation, though it's generally not needed. If you bubble nitrogen though buffers, be sure to do so before adding detergent or you will make a bubbly mess.
- Get2 is stable in UM and can be concentrated as high as 5mg/mL with a 50kDa cutoff
- Get1 is stable in FC12, a harsh detergent, and can be concentrated as high as 10mg/mL with a 30kDa cutoff. Get1 tends to aggregate slightly in UM and precipitate in DDM.
- When forming complexes with Get1 and Get2 in UM, diluted the FC12 Get1 stock into Get2 in UM, so that as Get1 exchanges into the UM, it can pair wit Get2 to prevent aggregation.

Expression and Purification of Get2-1sc

Expression:

- Transform and plate LOBSTR-Bl21(DE3)-RIL cells with Get2-1sc pET pET29 plasmid
- Use single colony to inoculate 50mL TB/Kan/Chl preculture
- When OD = 0.5-1.5, transfer 9mL preculture to each of 1L pre-warmed cultures of specially-prepared TB, each in 2.8L non-baffled nalgene flask. (2-6L at a time)
    - Autoclave 50g/L Fisher LB + 0.5%v/v glycerol
    - When cool, add 10x TB salts (170mM $KHPO_4$, 720mM K_2PO_4) + Kan/Chl
- Shake at 250rpm at 37 °C until OD = 0.5
- Transfer to 17 °C shaker at 180rpm for 1hr
- Induce with 0.4 mM IPTG and grow for 17-20hr
- Harvest cells using JLA8.1 rotor at 4,000 rpm for 20min
- Freeze and store pellets in 50mL falcon tubes at -80 °C (5 g pellet per L culture)

Purification:

- Lysis buffer: 50mM Hepes, pH – 8, 500mM NaCl, 5%v/v glycerol, 10mM imidazole
- Resuspend in Lysis buffer (5-10mL/g pellet) + 5mM BME, 1mM PMSF, 30µg/mL DNase, 2mM $MgAc_2$
- Homogenize, microfluidize 3 times
- Remove unlysed cells with slow spin in 50mL falcon tubes (20 min @ 10,000 g)
- Spin supernatant 1hr at 40,000 rpm in Ti45 to pellet membranes
- Resolubilize membranes in Lysis buffer + BME + 1%w/v DDM (~10mL per L culture) with a paintbrush and gently incubate on a wheel at 4 °C for 2 hr.
- Repeat Ti45 spin and incubate supernatant to 1-2mL Ni-NTA for 1hr on cold wheel
- Wash Ni-NTA with 15 x bed volume lysis buffer + BME + 0.05%w/v DDM
- Wash Ni-NTA with 15 x bed volume wash buffer (50mM Hepes, pH 8, 200mM NaCl, 5%v/v glycerol, 30mM imidazole, 1mM TCEP)
- Elute with 5 x bed volume wash buffer + 200mM imidazole
- Add 0.1%w/v LDAO and 1mM EDTA to the elution
- Concentrate with 50 kDa Amicon
- Purify by gel filtration in 50mM Hepes, pH 7.5, 200mM NaCl, 5%v/v glycerol, 0.1%w/v LDAO, 1mM TCEP
- Yields 1-3mg Get2-1sc per liter of culture

Get2-1sc Molecular Weight: 65.14 kDa
Get2-1sc Extinction Coefficient: 96.83 $mM^{-1}cm^{-1}$

Notes:

- Get2-1sc is moderately stable in DDM, but will aggregate when concentrated
- Get2-1sc is slightly more stable in UM
- Get2-1sc is highly stable in LDAO, the detergent used for maleimide labeling

Maleimide Labeling of Membrane Proteins

Notes:

- Every protein is different, but these conditions work best for various cysteine mutants of Get1, Get2, SUMO-Sec22-opsin, and CorA
- Protein concentration should be 30-200 μM. Try to use minimal detergent (~2x cmc)
- Use 1-5 equivalents of label. The more concentrated the protein and the more labile the cysteine, the fewer equivalents of label are needed.
- All buffers must be free of all traces of thiols (BME, DTT), imidazole, and Tris
- Use a pH of 7.5. Lower pH will reduce labeling efficiency. Higher pH will increase non-specific labeling of lysine residues and increase hydrolysis of maleimide.
- Fluorophores or other labels should be hydrophilic and soluble. Excess hydrophobic labels are very difficult to remove from membrane proteins. Cy3, Cy5, Biotin, Atto488, Atto532, and Atto655 are all soluble. Do not use hydrophobic Atto647N with membrane proteins
- Labeling on ice is an effective way to minimize non-specific labeling, which tends to be about 5%
- Use 0.5-1mM TCEP during labeling, but TCEP can damage fluorophores over time, so switch to 1mM DTT after labeling
- According to commercial protocols, solid label is typically first dissolved in DMSO or DMF (Do NOT use DMF), then added to protein. However, I prefer to dissolve soluble labels in buffer, avoiding DMSO, which destabilizes Get1 and Get2. However, this label/buffer stock must be prepared immediately before use and cannot be stored.

Preparation of Maleimide-Label Stock:

- Using a pipette tip, transfer a bit of the solid dye label into 50μL buffer (10mM Hepes, pH 7) and mix thoroughly.
- Dilute 1μL of this stock into water and determine the concentration of the dye stock using a spectrophotometer. If needed, add more solid dye label to the stock and check the concentration again.

Labeling method 1:

- On ice, add 1 equivalent of label to the protein stock
- Mix and incubate on ice overnight
- Add 2-3 additional equivalents of label and incubate on ice 2hr
- Quench with 5mM BME

Labeling method 2:

- On ice, add 2 equivalent of label to the protein stock
- After 1hr, add an additional equivalent of label and mix
- After 1hr, add an additional equivalent of label and mix
- After 1hr, add an additional equivalent of label and mix
- After 2hr, quench with 5mM BME

Maleimide Labeling Continued

Methods to Remove Excess Label

Gel filtration: Optimal, confirming the native conformation of the protein after labeling. Be sure that your dye is quenched and protein is soluble so that you don't ruin the column

PD10: Quick and dirty and can be done by eye, visualizing separation between labeled protein and free label. However, separation is rarely perfect, so take care to ensure that no free dye remains in your sample. It's a good idea to remove most of the free dye by concentrating and diluting the sample first.

Affinity purification: If free dye is difficult to remove, re-bind the protein to an affinity resin like Ni-NTA and wash thoroughly. This method is the most tedious and shouldn't be necessary with soluble labels.

Bio-Beads: Bio-Beads are a good method to remove free labels from soluble proteins, however they also remove detergent, so cannot be used directly with membrane proteins. However, if the labeled protein is immediately reconstituted into nanodiscs, amphipols, etc, the free dye will be removed with the detergent. This is a risky strategy since the labeling efficiency cannot be calculated.

Determining labeling efficiency:

- Confirm with SDS-PAGE that no free label remains in the sample
    - Run alongside free label, and don't run the label off the gel. Use 50%v/v glycerol + 1%w/v SDS instead of loading dye. Image using Chemidoc fluorescence.
- Determine the labeling efficiency by accurately determining the concentration of protein and label using a nanodrop. Record A280 and max absorbance (A max) of the dye
- Calculate protein concentration using a correction factor "CF" which subtracts the proportion of 280nm absorbance that comes from the dye label
- >50% labeling efficiency is sufficient for many experiments. >70% is preferred and typical of fully optimized labeling.

$$[\text{label}] = \frac{\text{A max}}{(\text{path length})*(\text{extinction coefficient})}$$

$$[\text{protein}] = \frac{\text{A280} - (\text{CF}*\text{A max})}{(\text{path length})*(\text{extinction coefficient})}$$

$$\text{Labeling Efficiency} = \frac{[\text{label}]}{[\text{protein}]}$$

Liposome Preparation via Extrusion

- In a glass vial, mix lipid in chloroform stocks (25mg total lipid) with solid DTT (1-10mM final) at RT. For Get1/2, 80%w/w Egg-PC + 20%w/w POPE is used to mimic yeast ER membranes
- Remove the chloroform under a nitrogen stream
- Remove trace chloroform on house vacuum for 15-30min
- Remove remaining chloroform on high vacuum (<1 torr) at RT overnight. To ensure solvent removal, weigh the vial every 30min for the first few hours. The loss will eventually be < 1mg, so be careful not to get the vial dirty. Handle with gloves.
- Add 1.25mL buffer (50mM Hepes-KOH, pH 7.5, 15%v/v glycerol, 1mM DTT) to the lipid film and vortex thoroughly. This should produce a homogeneous, milky suspension
- Cap the vial and rotate on a wheel at room temperature for 2-4hr
- Transfer the mixture to an Epindorff tube
- Flash freeze and thaw three times
- Place the extruder on the hot plate, heated above the respective lipid transition temperature (~65 °C for PC/PE).
- Assemble the extruder (See Avanti website for instructions)
    - Soak (1-2 min) polycarbonate supports and membrane in water
    - Place supports on respective Teflon plug in the center of the o-ring
    - Placing the membrane is the tricky part. It must lay flat (not puckered) across the o-ring. Use the surface tension of the membrane against the steel to drag the membrane onto the Teflon plug without folding.

Filter Supports
Avanti #610014

Membrane
Avanti #800309

- Load the lipid solution into a syringe and wait a few minutes to equilibrate temperature
- Pass the lipid 25 times through the extruder, taking extra care to move slowly for the first few passes.
    - Make sure that the syringe hub is tight because high temperatures will loosen it and cause leaks
    - Pass the lipid through the extruder an odd number of times so that the final mixture is in the opposite syringe
- Aliquot and flash freeze. Liposomes are good for a few months. Do not re-freeze.
- Liposome sizes can be checked using DLS

Get1/2 Proteoliposome Reconstitution

- Reconstitution Buffer: 50mM Hepes-KOH, pH 7.5, 150 mM KAc, 7 mM $MgAc_2$, 250 mM sucrose, 1mM DTT, 0.225%w/v DBC
- Dilute Get1/2 at least 10x into Reconstitution Buffer to a concentration of 1 μM
- After 15min on ice, further dilute to 90 μL in a PCR tube to the desired concentration. (80-320 nM Get1/2 is typical)
- Immediately add 10μL liposome stock and mix well with pipette, but avoiding bubbles
- After 15min on ice, add 25-30mg Bio-Beads and incubate on the cold room wheel overnight
- Remove reconstitution from Bio-Beads with pipette
- 10min spin at 20,000 g's might remove some aggregate, but there shouldn't be any
- Get1/2 concentration can be evaluated using SDS-PAGE and standards. 50% recovery is typical and can often be assumed.

Notes:

- The reconstitution can also be done with 500 mM KAc or without sucrose
- Potassium causes SDS to crash out, so high concentrations of KAc make it difficult to run SDS PAGE
- Proteoliposomes reconstituted in the presence of sucrose can be diluted 5x with water and pelleted by spinning at 75,000 g's for 1hr, then resuspended by pipette in 20μL Reconstitution Buffer (without DBC). This allows any remaining DBC to be removed and to concentrate the sample. However, it is not necessary for effective insertion assays, and can compromise the proteoliposomes. This technique also requires the use of fluorescent lipids to see the pellet, which is not compatible with experiments requiring fluorescently labeled protein.

Proteinase Protection Insertion Assay

Targeting Complex Preparation:

- Express TwinStrep-Sec61β using an *in vitro* PURExpress reaction in the presence of 20 μM Get3 dimers.
- 150 μL reaction -> 2 hr at 37 degrees C
    - 60 μL solution A + 45 μL solution B
    - 8μL RNase inhibitor
    - 20 μM Get3 dimers – Use concentrated stocks (>100μM) to minimally affect the PURExpress buffer conditions
    - 10 μL S-35 Methionine
    - 750ng plasmid
- Incubate with 25 μL equilibrated streptavidin-agarose
    - Wash buffer: 50 mM Hepes-KOH, pH 7.5, 150 mM KAc, 7 mM $MgAc_2$, 1 mM DTT, 10%v/v glycerol
- Spin at 100 g's in filter tube to collect flow through without drying the resin
- Wash three times with 300 μL wash buffer
- Elute by incubating resin 20 min with 50μL wash buffer + 10 mM biotin
- Collect elution and repeat
- Aliquot and store at -80 °C. Determine concentration using SDS-PAGE and comparing to Get3 standards. Concentration should be 0.5-1 μM

Insertion Assay:

- Dilute proteoliposomes in Activity Buffer to a Get1/2 concentration of 32 nM
    - Activity Buffer: 50 mM Hepes-KOH, 150 mM KAc, 7 mM $MgAc_2$, 1 mM DTT, 4 mM ATP (2 mM ATP final)
- For each reaction, aliquot 5 μL this proteoliposome dilution into a PCR tube.
- To initiate the reaction, add 5 μL of targeting complex (diluted to 2x desired concentration) and incubate at 32 °C. Concentration of targeting complex can be adjusted, but there should be a significant excess of targeting complex
- After 30 min at 32 °C, place tubes on ice
- After 2 min on ice, add 1μL 25 mg/mL Proteinase K
- Successively mix with gentle vortexing and quick spins in a microfuge, careful to keep the tubes cold.
- After 2 hr on ice, add a pipette tip (~0.2μL) of 500 mM PMSF in DMSO to each reaction
- In new 0.6mL tubes, heat 10 μL aliquots of 2x loading dye at 95 degrees for 5-10 min
    - 2x loading dye: A 2:1:1 ratio of 4x loading dye, 50%v/v glycerol, and 1% SDS
- Reverse quench each reaction by quickly adding 10 μL of the cold insertion reaction to a tube of the hot, 2X loading dye. Mix quickly by pipette. Continue heating for 10min.
- The Potassium will precipitate in the cold, so store samples at ~40°C and vortex thoroughly before loading. Run 5 μL of the reaction on a 12% Tris Tricine gel, careful not to run the dye front off the gel. Use cold running buffers. Freeze SDS-PAGE samples to prevent further PK digestion.
- Stain the gel so the PK band can serve as a loading control
- Dry the gel and expose for the S-35 phosphorimager

Nanodisc Reconstitutions: Membrane Scaffold Protein (MSP)
(MSP1D1, MSP1E3D1, MSP2N2)

Express, purify, and remove the his tag using the protocol from
Alvarez, et al. (2010). JACS, *132*(28), 9513

Notes:

- Use at least 10mL Ni-NTA per 500mL culture, which should yield 100-200mg
- After Ni-NTA elution, add 5mM DTT and 2mM EDTA. Then, add one TEV aliquot per 20-50mg of MSP. Dialyze this mixture overnight at 4 °C in 50mM Hepes, pH 8, 200mM NaCl to remove DTT, EDTA, and imidazole
- During the subtractive step, collect only the Ni-NTA flow through and 2 x bed volume of wash with buffer + 10-20mM imidazole. Trace amounts of MSP-his should be minimized because nanodisc reconstitutions use large excesses of MSP, and MSP-his is often recovered during these reconstitutions. A second subtraction with 0.5-1mL new Ni-NTA and no washing is appropriate if SDS-PAGE reveals incomplete digestion and subtraction.
- MSP1E3D1 dimerizes in cold temperatures, but is monomeric at RT. Do the subtraction at RT to recover as much MSP as possible. MSP1D1 is always monomeric, and MSP2N2 is always a mixture of dimers and monomers.
- Concentrate MSP as much as possible using an Amicon. (>150μM MSP1D1 and >80μM MSPE3D1)
- Determining the concentration of MSP can be challenging, as a BSA-standard Bradford often gives an artificially high concentration. Use A280 and Lipid:MSP Ratio Optimization to evaluate MSP concentration.

Construct	Extinction Coefficient ($mM^{-1}cm^{-1}$)
MSP1D1-His	21.43
MSP1D1	18.45
MSP1E3D1-His	29.91
MSP1E3D1	26.93
MSP2N2-His	39.88
MSP2N2	36.9

NHS-labeling of MSP:

- NHS-biotinylation of MSP is a useful handle for nanodisc immobilization on glass slides and streptavidin resins.
- After the subtractive step, dialyze against 50mM Hepes, **pH 8.5**, 200mM NaCl
- Recover this purified MSP and accurately determine the concentration. The UV absorbance will change after labeling, so it is important to do so before adding label
- Add 4 equivalents of fresh NHS-PEG_4-biotin (or other label) and incubate 1-2hr at RT
- Quench with 20mM Tris-HCl to quench the NHS and adjust the pH
    - Excess NHS label does not need to be removed because they will be removed during the nanodisc reconstitution
        - Aliquot and store at -80 °C. MSP can be freeze-thawed several times.

Nanodisc Reconstitutions: Mixed Micelles

Notes:

- This protocol will produce 10mM lipid solutions dissolved in a minimal excess of detergent
- Scale: Though initial attempts should be done at small scale to save resources, mixed micelles are best prepared at scales of 20-40mL final volume to minimize variation

Protocol:

- In a glass vial, mix lipid in chloroform stocks with solid DTT (1-10mM final) at RT. For Get1/2, 80%w/w Egg-PC + 20%w/w POPE is used to mimic yeast ER membranes
- Remove the chloroform under a nitrogen stream
- Remove trace chloroform on house vacuum for 15-30min
- Remove remaining chloroform on high vacuum (<1 torr) at RT overnight. To ensure solvent removal, weigh the vial at 30min intervals for the first few hours.
- Add buffer (50mM Hepes, pH 7.5, 200mM NaCl, 1mM DTT) to the lipid film at 80% of the final volume. Vortex thoroughly to give a homogeneous, milky suspension
- Bath sonicate this suspension for 30min alternated with vortexing. Make sure the mixture and sonication bath doesn't get too hot. Repeat until the mixture becomes translucent, though not more than three rounds total.
- Add 1.5 equivalents of the desired detergent and sonicate 30min, followed by vortexing. Add successive 0.2 equivalents and repeat sonication/vortex until solution is completely clear.
    - Note: The number of equivalents depends on the detergent used. Many detergents require no more than 2 equivalents. DDM and UM require at least 3 equivalents. Do not add more detergent than is necessary
    - Once completely resolubilized, a hard spin on the tabletop centrifuge (20krcf) should produce no pellet
- Once clear, add buffer to final volume with 10mM lipid. Record the concentration of detergent.
- Aliquot and store at -80 °C. Aliquots can be freeze-thawed several times.

Nanodisc Reconstitutions: Bio-Beads

Polystyrene Bio-Beads must always be "activated", washed with methanol before use, so that they polystyrene surface is free to adsorb detergent

- Add 10-30g of polystyrene Bio-Beads (Bio-Rad) to a 50mL falcon tube
- Fill the tube with methanol, cap, and agitate for a few minutes
- Allow the beads to settle, and remove the methanol using a 25mL pipette or glass pipette and aspirator
- Repeat this methanol wash
- Repeat this wash 10 times with water to remove all methanol
- Store at 4 °C in water with 0.01%w/v azide.
- Repeat this wash with beads stored for more than a few months

Nanodisc Reconstitutions: Lipid to MSP Ratio Optimization

- Reconstitute nanodiscs at different lipid: MSP ratios. Use the same volume/concentration of mixed micelles, but vary the amount of MSP and additional buffer. Vary lipid:MSP ratios at intervals of 5-10
    - Make sure that the range is wide enough to include free MSP and a ratio that is far too high.
    - For example, for MSP1D1, reconstitute ratios of 0, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 90, 100, 120, 140
- 100µL reconstitutions are sufficient, best done in a PCR strip. Perform the reconstitutions exactly as if there was a target present (see below), but with only MSP and mixed micelles.
- After overnight incubation with Bio-Beads, load the supernatant directly to size exclusion. Observe the elutions of free MSP, empty nanodiscs, and aggregates in the void. High lipid:protein ratios should produce a significant void peak. Low ratios will contain mostly free MSP as well as empty nanodiscs. Keep in mind that lipid does not absorb UV, so the void peak is, in reality, much larger than it appears
- For future reconstitutions, use the highest ratio that contains no aggregate peak but minimizes free MSP. Err on the side of a lower lipid:MSP ratio.

Nanodisc Reconstitutions: Target Reconstitutions

Calculations:

- First, determine your scale. In general, 1-10nmol of target (membrane protein to be reconstituted) is reconstituted at a time. To produce an adequate UV signal on gel filtration, 2-4 nmol of target is required.
- The target should contain a his-tag or other orthogonal tag. If the target contains multiples components, the tagged component should be the limiting reagent.
- The ratio of MSP to target determines the ratio of nanodiscs to target. Higher ratios will produce many more empty nanodiscs and require more materials, but produce a greater proportion of nanodiscs with a single target. Lower ratios generally produce lower yields of target reconstitution. Keep in mind, there are 2 MSP's per nanodisc, and not all MSP's will produce nanodiscs. MSP:target ratios of 10-50 are typical.
- The lipid:MSP ratio should be empirically determined using the Lipid:MSP Ratio Optimization. Choose a ratio that gives **zero** aggregate, but minimizes free MSP. Err on the side of lower ratios (excess MSP).
- The final volume of the reconstitution mixture depends on the amount of mixed micelles used. The final detergent concentration should be 2-10mM and the final lipid concentration must be >1mM, but >2mM is best. Calculate the volume of additional reconstitution buffer (50mM Hepes, pH 8, 200mM NaCl, 1mM DTT) to reach this final volume
- Use reconstitution volumes that fill the entire tube after adding Bio-Beads. Free space will cause frothing during mixing that can lower yield.

Other notes:

- Nanodisc reconstitutions cannot tolerate glycerol. Residual glycerol from protein stocks must be diluted to a final concentration <1%.
- 10%w/v sucrose during the reconstitution is fine.
- Salt concentrations of 150-500mM KAc and 100-500mM NaCl can all be tolerated. This can be adjusted to suit the target.

Procedure:

- All samples should be cooled on ice at all times
- Dilute the target into minimal buffer with minimal detergent.
    - The concentrated Get1 stock in FC12 is added to Get2 diluted in 0.1%w/v UM as a means to form the Get1/2 complex and dilute away the FC12
- Dilute the mixed micelles into the calculated volume of additional reconstitution buffer and place on ice. Avoid bubbles.
- Add the target dilution to the mixed micelle dilution and mix gently. Avoid bubbles.
- After 10min on ice, add the MSP and mix well, but gently, with pipette. Avoid bubbles.
- Incubate on ice for 20min to 3hr. Longer times can increase final yield, especially with low MSP:target ratios.
- After incubation on ice, add Bio-Beads at 10-20%w/v of the total reconstitution volume.
- Slowly rotate on wheel overnight at 4 °C

Recovery:

- After overnight incubation with Bio-Beads, most of the detergent has been removed leaving free MSP, empty nanodiscs, nanodiscs containing target, and various degrees of aggregated MSP, lipid, and target. Do not use detergent in any future buffers.
- Remove the reconstitution mixture from the Bio-Beads using a pipette
- Optional: to remove any aggregate, spin 30min at 150,000 g's. Efficient reconstitutions should produce **no** visible pellet.

Affinity purification:

- This is used to remove free MSP, empty nanodiscs, and any remaining detergent
- Use 20-50 µL of Ni-NTA per nmol of target. More Ni-NTA may be required for larger nanodiscs. Incubate 1hr with Ni-NTA on the wheel at 4 °C
- Wash Ni-NTA with 20 x bed volume of buffer + 20mM imidazole
- Elute with minimal buffer + 300mM imidazole
- Avoid concentrating nanodiscs with Amicons, as this can cause aggregation. Therefore, best results are obtained using <250µL Ni-NTA and carefully taking the peak, 500µL elution to load directly onto gel filtration. This can often be done by eye, observing the elution front in the Ni-NTA resin.

Size Exclusion Chromatography:

- Purify and analyze the elution using a Superose 6 10/300 column in 50mM Hepes, pH 7.5, 200mM NaCl, 1mM DTT
- Peak fractions can then be used or dialyzed overnight at 4 °C against buffer + 10%w/v sucrose. This concentrates the fractions 2x and serves as a cryoprotectant.
- Get1/2 nanodiscs can be flash frozen, stored at -80 °C, and repeatedly freeze thawed. However, they should not be used more than 2 weeks after reconstitution.
- Concentration is best determined via SDS-PAGE compared to standards of the target. Efficient, optimized reconstitutions can often be quantitative, though <10% recovery can still be utilized.

Quality Control:

1. Lipid:MSP Ratio Optimization profiles, using these same stocks of MSP, mixed micelles, and Bio-Beads as the reconstitution, should be obtained before moving on to target reconstitutions.
2. After Biobead incubation, you should not observe a cloudy reconstitution or pellet
3. The final gel filtration profile should not contain large aggregates or a void peak
4. The monodispersity of nanodiscs can be further evaluated using negative-stain EM