Analysis of the architecture of the heterodimeric Get1-Get2 insertase and the inhibition of tail-anchored protein biogenesis by Retro-2

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Accessibility
Analysis of the architecture of the heterodimeric Get1-Get2 insertase
and the inhibition of tail-anchored protein biogenesis by Retro-2

A dissertation presented
by
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to
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in partial fulfillment of the requirements
for the degree of
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Analysis of the architecture of the heterodimeric Get1-Get2 insertase and the inhibition of tail-anchored protein biogenesis by Retro-2

Abstract

Tail-anchored (TA) proteins, including SNAREs involved in vesicle transport, are post-translationally targeted to the endoplasmic reticulum membrane by the guided entry of tail-anchored proteins (GET) pathway in yeast and the transmembrane domain (TMD) recognition complex (TRC) pathway in mammals. The conserved targeting factor Get3 (in yeast; ASNA1 in mammals) binds the carboxyl-terminal TMD of TA proteins and forms a targeting complex, which engages its integral heterodimeric receptor at the ER membrane for TMD release and insertion into the lipid bilayer.

I first addressed the structure and stoichiometry of the yeast Get1-Get2 complex. In vivo site-specific crosslinking and accessibility assays revealed an aqueous interface between the transmembrane regions of Get1 and Get2. To distinguish between competing models for how the Get1-Get2 heterodimer interacts with the Get3-TA targeting complex and mediates TMD insertion, I reconstituted a single-chain version of Get1-Get2 complex into proteoliposomes and nanodiscs. In vitro assays, alongside fluorescence tools, showed a single copy of the Get1-Get2 heterodimer is sufficient to recruit the Get3-TA targeting complex and insert the TMD into the ER membrane.

Next, I investigated the mechanism of action of the small molecule Retro-2, which protects cells from toxins and pathogens that exploit vesicular trafficking. Genetic profiling analysis revealed that Retro-2 treatment resembles inhibition of the TRC pathway. Using dual-
colour self-cleaving fluorescent reporters, I showed the compound inhibited TA protein biogenesis in mammalian cells. *In vitro* assays further revealed the mechanism of action in which Retro-2 blocks the transfer of newly-synthesized TA proteins from the upstream chaperone SGTA to ASNA1. These data propose a model in which Retro-2 directly inhibits ASNA1-mediated TA protein targeting, leading to the depletion of the SNAREs that mediate vesicular transport, and thus ultimately preventing the entry or replication of various pathogens.
# Table of contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title page</td>
<td>i</td>
</tr>
<tr>
<td>Copyright</td>
<td>ii</td>
</tr>
<tr>
<td>Abstract</td>
<td>iii</td>
</tr>
<tr>
<td>Table of contents</td>
<td>v</td>
</tr>
<tr>
<td>List of figures and tables</td>
<td>vi</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>viii</td>
</tr>
<tr>
<td>Chapter 1: Overview of tail-anchored protein biogenesis</td>
<td>1</td>
</tr>
<tr>
<td>Chapter 2: Characterization of the structure and function of the Get1-Get2 heterodimer</td>
<td>8</td>
</tr>
<tr>
<td>Chapter 3: Tail-anchored insertion by a single Get1/2 heterodimer</td>
<td>30</td>
</tr>
<tr>
<td>Chapter 4: Overview of the cytoprotectant small molecule Retro-2</td>
<td>60</td>
</tr>
<tr>
<td>Chapter 5: Retro-2 protects cells from ricin toxicity by inhibiting ASNA1-mediated ER targeting and insertion of tail-anchored proteins</td>
<td>68</td>
</tr>
<tr>
<td>Chapter 6: Evidence that Retro-1 has the same mechanism of action as Retro-2</td>
<td>103</td>
</tr>
<tr>
<td>Chapter 7: Future directions</td>
<td>107</td>
</tr>
<tr>
<td>Appendix 1: Supplemental figures for Chapter 3</td>
<td>112</td>
</tr>
<tr>
<td>Appendix 2: Supplemental figures for Chapter 5</td>
<td>120</td>
</tr>
</tbody>
</table>
## List of figures and tables

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Schematic of TA protein handoff to conserved targeting factor Get3/ASNA1</td>
<td>2</td>
</tr>
<tr>
<td>1.2</td>
<td>Representative structures showing the distinct conformations of Get3</td>
<td>3</td>
</tr>
<tr>
<td>1.3</td>
<td>The membrane-associated steps of TA protein biogenesis</td>
<td>4</td>
</tr>
<tr>
<td>2.1</td>
<td>Site specific crosslinking reveal the interactions between the transmembrane domains of Get2 and Get1</td>
<td>9</td>
</tr>
<tr>
<td>2.1: Continued</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>2.2</td>
<td>Cysteine accessibility assay reveal the local aqueously-exposed environment of the Get1/2 transmembrane region</td>
<td>12</td>
</tr>
<tr>
<td>2.3</td>
<td>Schematics for the models of Get1/2-mediated tail-anchored protein insertion</td>
<td>13</td>
</tr>
<tr>
<td>2.4</td>
<td>Purification and validation of recombinant Get2-1sc</td>
<td>14</td>
</tr>
<tr>
<td>2.5</td>
<td>TA protein insertion into liposomes by a single Get2-1sc heterodimer</td>
<td>15</td>
</tr>
<tr>
<td>2.6</td>
<td>Generation and characterization of Get2-1sc nanodiscs.</td>
<td>16</td>
</tr>
<tr>
<td>3.1</td>
<td>Alternative models for the Get1/2 architecture</td>
<td>32</td>
</tr>
<tr>
<td>3.2</td>
<td>Bulk FRET measurements of Get3 binding to Get1/2 complexes in proteoliposomes</td>
<td>34</td>
</tr>
<tr>
<td>3.3</td>
<td>Single Get1/2 heterodimers mediate TA protein insertion</td>
<td>37</td>
</tr>
<tr>
<td>3.4</td>
<td>Get1 and Get2 bind on opposite sides of Get3 in its post-hydrolysis states</td>
<td>40</td>
</tr>
<tr>
<td>Table 3.1</td>
<td>Labeling efficiencies for Get1, Get2 and Get2-1sc</td>
<td>46</td>
</tr>
<tr>
<td>4.1</td>
<td>The chemical structure of the bioactive form of Retro-2</td>
<td>60</td>
</tr>
<tr>
<td>5.1</td>
<td>Single and paired-gene CRISPRi screens implicate TRC pathway inhibition as the MOA of Retro-2</td>
<td>72</td>
</tr>
<tr>
<td>5.1: Continued</td>
<td></td>
<td>73</td>
</tr>
<tr>
<td>5.2</td>
<td>Retro-2 diverts newly-synthesized TA proteins from ER targeting to degradation</td>
<td>75</td>
</tr>
<tr>
<td>5.2: Continued</td>
<td></td>
<td>76</td>
</tr>
<tr>
<td>5.3</td>
<td>ASNA1 knockdown and Retro-2 treatment both decrease the abundance of Golgi-localized STX5, an ASNA1 substrate.</td>
<td>77</td>
</tr>
<tr>
<td>5.4</td>
<td>Isolation and characterization of A149V ASNA1, a Retro-2-resistance allele</td>
<td>79</td>
</tr>
</tbody>
</table>
Figure 5.4: Continued

Figure 5.5: DHQZ36.1 blocks substrate transfer from SGTA to ASNA1 *in vitro* 82

Figure 5.5: Continued 83

Figure 6.1: ASNA1 knockout and Retro-1 treatment destabilize a TA protein reporter for ER targeting and insertion 104

Figure 6.2: Retro-1 blocks substrate transfer from SGTA to ASNA1 *in vitro* 105

Figure 6.2: Continued 106

Figure 7.1: Summary schematic of the mechanism of action of Retro-2 107

Figure 7.1: Continued 108

Figure 7.2: Chemical structure of an example Retro-2 photoaffinity probe 110

Figure S3.1: Preparation of recombinant Get1/2 constructs 113

Figure S3.2: Additional details for the bulk FRET experiments 114

Figure S3.2: Continued 115

Figure S3.3: Single-molecule photobleaching data analysis 116

Figure S3.3: Continued 117

Figure S3.4: Reconstitution and functional analysis of heterodimeric Get1/2 nanodiscs 118

Figure S3.4: Continued 119

Figure S5.1: Genetic profile analysis of ricin phenotypes for Retro-2 and candidate genes 121

Figure S5.1: Continued 122

Figure S5.2: ASNA1 knockout and Retro-2/DHQZ36.1 treatment destabilize the TA reporter 123

Figure S5.3: Effect of ASNA1 knockdown or Retro-2 and DHQZ36.1 in HeLa cells 124

Figure S5.4: Replication and validation of CRISPR-X screen 125

Figure S5.4: Continued 126

Figure S5.5: *In vitro* analysis of DHQZ36.1’s effect on ER targeting 127

Figure S5.5: Continued 128
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Chapter 1: Overview of tail-anchored protein biogenesis

Eukaryotic cells use sophisticated molecular machinery to accurately target proteins to their intracellular membrane compartments. This is especially evident for tail-anchored (TA) proteins, a class of membrane proteins with a single transmembrane domain (TMD) located near the carboxyl (C)-terminus that serves as both the targeting signal and a membrane anchor for the N-terminal cytosolic domain (Kutay et al., 1993, Beilharz et al., 2003). Many of these proteins play essential roles in cell biology, including SNAREs in vesicular transport. Due to its C-terminal location, the TA protein targeting signal only becomes accessible for targeting after it emerges from the ribosome exit tunnel following completion of protein synthesis.

Many newly-synthesized TA proteins are post-translationally targeted to the endoplasmic reticulum (ER) membrane for insertion by the guided-entry of TA proteins (GET) pathway in yeast or the TMD recognition complex (TRC) pathway in mammals (Stefanovic et al., 2007, Schuldiner et al., 2008, Hegde and Keenan, 2011, Denic et al., 2013, Mateja and Keenan, 2018). During the first step in the GET pathway, the chaperone Sgt2 (SGTA in mammals) recognizes the substrate TMD sequence (Wang et al., 2010). Notably, this initial binding event sorts ER-destined TA proteins from those destined for the mitochondrial outer membrane (Wang et al., 2010). In the ensuing pre-targeting steps, The Get5-Get4 scaffolding complex facilitates transfer of substrates bound to Sgt2 to the conserved ER membrane targeting factor Get3 (ASNA1 in mammals) (Wang et al., 2010, Gristick et al., 2014) (Figure 1.1A). In mammalian cells, the UBL4A-BAG6-GET4 complex performs an analogous scaffolding function to bridge SGTA with ASNA1 during substrate hand-off (Mariappan et al., 2010, Mock et al., 2015, Shao et al., 2017). TA proteins that are inefficiently transferred to ASNA1 are instead channeled via direct binding to BAG6 to an associated quality control module that includes the E3 ligase RNF126 for TA protein ubiquitination and subsequent degradation by the proteasome.
Yeast appear to lack a BAG6 homolog and its associated TA protein degradation mechanism.

**Figure 1.1: Schematic of TA protein handoff to conserved targeting factor Get3/ASNA1.**

(A) In yeast, the newly synthesized TA protein is specifically recognized and bound by Sgl2. Transfer of the TA protein to Get3 is mediated by the Get5-Get4 scaffold complex. (B) In mammalian cells, the TA protein is triaged between ER targeting and degradation. Transfer of the TA protein from SGTA to ASNA1 is mediated by the UBL4A-BAG6-GET4 scaffold complex. Delayed transfer to ASNA1 leads to capture by BAG6 and subsequent ubiquitination by RNF126 and proteasomal degradation.

Get3 is an obligate homodimer associated together by a zinc ion, comprising a Rossmann folded dimer core with two interfacial ATPase active sites that is coupled to the helix-rich subdomain on each subunit, which forms one-half of a composite TMD-binding site (Mateja et al., 2009, Hu et al., 2009) (Figure 1.2). Get3 assumes distinct conformational changes coupled to nucleotide hydrolysis to coordinate TA protein binding and release during different steps in the pathway (Mateja et al., 2009, Hu et al., 2009, Rome et al., 2014) (Figure 1.2). In the “closed” conformation, the two Get3 subunits form a composite hydrophobic groove that cradles the substrate’s TMD (Mateja et al., 2009, Hu et al., 2009, Mateja et al., 2015). A dynamic “lid” shields the TMD from the aqueous cytosol and prevents illicit interactions with off-pathway chaperones (Mateja et al., 2015, Chio et al., 2019). In the “open” conformation, the two subunits are rotated away from each other causing disruption of the TMD binding interface (Mateja et al.,...
2009, Hu et al., 2009). Under oxidative stress, Get3 may also form tetramers and act as an ATP-independent chaperone (Voth et al., 2014).

**Figure 1.2**: Representative structures showing the distinct conformations of Get3. The homodimer Get3 (pale green, green) undergoes conformational changes during TA protein ER targeting. In the “closed” conformation, Get3 has an extensive dimer interface that can bind TMDs (PDB ID: 2WOJ). In the “open” conformation, the subunits are splayed apart (PDB ID: 3H84).

ATP binding enables Get3 to engage Get4 and receive the TA protein from Sgt2 (Rome et al., 2013, Gristick et al., 2014, Chio et al., 2017). TMD binding stimulates Get3 ATP hydrolysis, which is necessary for the targeting complex to ultimately release its cargo at the membrane (Rome et al., 2013, Rome et al., 2014). The Get3-TA protein targeting complex is recruited to the ER membrane by the Get1-Get2 (Get1/2) (WRB-CAMLG in mammalian cells) heterodimeric integral membrane receptor, which induces TA protein release from Get3 (Wang et al., 2011, Vilardi et al., 2011, Yamamoto and Sakisaka, 2012, Vilardi et al., 2014) (Figure 1.3). Specifically, the cytosolic domain of Get2 tethers the Get3-TA protein complex into proximity with the cytosolic domain of Get1. The cytosolic domain of Get1 initially binds to Get3 in the ADP-bound “closed” state but then forces conversion to the “open” state, thereby displacing the spent nucleotide and disrupting the TMD binding site (Mariappan et al., 2011, Stefer et al., 2011, Wang et al., 2011, Kubota et al., 2012) (Figure 1.3). ATP displaces Get3 from Get1/2, allowing the recycling of the empty targeting factor back into the cytosol (Wang et
The Get1/2 receptor has an additional insertase function. An intact Get1/2 transmembrane region, consisting of 6 transmembrane segments that interact with each other, is required for the efficient release of the TA protein from Get3 and directly contacts the released TA protein (Wang et al., 2014, Vilardi et al., 2014). This suggests the presence of a docking site embedded in the lipid bilayer that may provide a path from cytosol to membrane integration for the TA protein (Wang et al., 2014, Anghel et al., 2017). Beyond this mechanistic framework, many details about TMD insertion into the lipid bilayer remain unknown and efforts to determine the full-length structure of Get1/2 have been unsuccessful.

**Figure 1.3: The membrane-associated steps of TA protein biogenesis.** Top: Shown are the structures of N-terminus of Get2 (blue) bound to “closed” nucleotide-bound Get3 (pale green, green) (PDB ID: 3ZS9) and the structure of the cytosolic domain of Get1 (teal) bound to nucleotide-free “open” Get3 (PDB ID: 3ZS8). Bottom: Summary schematic for the interactions between TA protein targeting complex and the heterodimeric ER receptor Get1-Get2 (WRB-CAML in mammals). The cytosolic N-terminus of Get2 recruits the Get3-TA protein complex to the ER membrane, which then allows the cytosolic domain of Get1 to physically disrupt the TMD-binding site on Get3. The Get1/2 transmembrane segments capture the TA protein from Get3, enabling the efficient release of the TA protein from Get3 and the subsequent membrane insertion.
References


Chapter 2: Characterization of the structure and function of the Get1-Get2 heterodimer

The mechanism of how the Get1-Get2 (Get1/2) heterodimeric receptor facilitate the insertion of tail-anchored (TA) proteins into the lipid bilayer remains undefined. One challenge is the lack of structural information about the transmembrane (TM) segments of these two proteins. I employed an in vivo site-specific crosslinking strategy to map interactions between the TMDs of Get1 and Get2 in S. cerevisiae yeast cells. I first screened a library of Get1-Get2 cysteine double mutants for crosslinking with bismaleimidoethane (BMH), a sulfhydryl-specific crosslinker, and analyzed the adducts by western blotting. For specific Get1/2 cysteine double mutants, one high molecular weight crosslinking BMH-dependent product was observed. The product consistently had the expected size for a Get1-FLAG/2 heterodimer, suggesting that its source was crosslinking between Get 1 and Get2; crosslinking between Get1 and non-specific proteins is thus unlikely to be source of this product.

BMH-dependent crosslinking was observed in 11 out of 177 Get1/2 cysteine double mutants (Figure 2.1A). In particular, most crosslinking occurred between the third TM segment of Get2 (positions 266 and 272) and all three TM segments of Get1 (positions 15, 106, 108,193, and 186) (Figure 2.1B,C). The third TM segment of Get2 also crosslinked to positions 142, 143, and 144 of Get1 found in the sole predicted luminal loop region of Get1 (Figure 2.1D). Notably, many of these TM cysteine positions of Get1 (positions 15, 106, 108, and 193) and Get2 (position 266) also crosslinked to TA substrate (Wang et al. 2014). I found the third TM segment of Get1 crosslinked to multiple positions on the TMD of Get2. To resolve distances between these Get1/2 TM positions, we used BMH and bismaleimidoethane (BMOE), two crosslinkers with the same reactivity but different spacer lengths. The shorter linker of BMOE (~8 Å compared to BMH’s ~13 Å linker) spatially restricts the formation of crosslinked adducts, allowing for better resolution of the Get1/2 interactions. We found only one pair of positions, Get1 N193C and Get2 S266C, crosslinked as efficiently with BMOE as with BMH (Figure 2.1E).
**Figure 2.1: Site specific crosslinking reveal the interactions between the transmembrane domains of Get2 and Get1.** (A) Schematic of Get2 and Get1 summarizing the pairwise sets of Get2 and Get1 cysteine alleles that form Get2×Get1 adducts upon BMH treatment. (B, C, D) Yeast cells expressing the indicated pairs of single-cysteine alleles of Get1-FLAG and Get2 were harvested and treated with 0.250 mM bismaleimidohexane (BMH), a sulfhydryl-specific crosslinker. Samples were analyzed by SDS-PAGE and immunoblotting (IB). Hexokinase (Hxk) served as the loading control. (E) Yeast cells expressing the indicated pairs of single-cysteine alleles of Get1-FLAG and Get2 were harvested and treated with 0.250 mM bismaleimidohexane (BMH) or 0.25 mM bismaleimidoethane (BMOE) sulfhydryl-crosslinkers. BMH has a spacer arm of 13.0 Å, while BMOE has a spacer arm of 8.0 Å. Samples were analyzed by SDS-PAGE and immunoblotting (IB). Hexokinase (Hxk) served as the loading control.
Figure 2.1: (Continued)
The chemistry of cysteine-maleimide coupling suggests that these positions are exposed at least temporarily to an aqueous environment, and are within close proximity of each other. I probed the local environment of the TMD of Get1/2 by monitoring the accessibility of single cysteine alleles to N-ethylmaleimide (NEM), which efficiently alkylates cysteine thiols exposed to an aqueous environment. Following alkylation, the membranes were solubilized, allowing the remaining thiols to react with a high molecular weight PEG-maleimide (Mackinnon et al., 2014). By comparing the PEG-reacted species in NEM-treated or mock-treated cells, I can determine the accessibility of the cysteine to the aqueous cytosol. This accessibility assay revealed that the aqueous interface between the TMDs of Get1 and Get2, and between Get1 and TA substrate (Figure 2.2A-C). This observation is consistent with recent bioinformatic evidence showing Get1 is part of the Oxa1 superfamily of proteins, which includes bacterial YidC and archeal Ylp1, whose structures show a lipid-exposed hydrophilic groove that also contact substrates (Anghel et al., 2017, Borowska et al., 2015, Kumazaki et al., 2014).

Mutations to the Get1/2 TM region result in the loss of function of the single-chain version of the Get1/2 heterodimer (Get2-1sc) (Wang et al. 2014). I analyzed how a TM swap of the third TM segment of Get1 of the linked Get2-1sc (Get2-1^{TM3sc}) changed the local environment of the first TM segment of Get1, which is part of the putative TA protein docking site embedded within the lipid bilayer (Wang et al. 2014). I found there was increased accessibility to position 18 and decreased accessibility to position 15 (Figure 2.2D). These changes to the aqueous Get1/2-TA substrate interface may be sufficient to explain the inability of the TM swap Get2-1sc mutants to release TA proteins from Get3 (Wang et al. 2014). Site-specific mutants or chemical modifications that alter the Get1/2 TMD docking site will enable further characterization of the role of Get1/2’s aqueously-exposed TMD in TA protein insertion.
Figure 2.2: Cysteine accessibility assay reveal the local aqueously-exposed environment of the Get1/2 transmembrane region. (A) Yeast cells expressing the indicated single cysteine alleles of Get1FLAG in the first TM segment were treated with 0.2 mM N-ethylmaleimide (NEM) or mock-treated. The cells were quenched, harvested and lysed by digestion with lyticase and bead beating. The ER membranes were collected and treated with 16mM 5 kDa PEG-maleimide. Samples were analyzed by SDS-PAGE and immunoblotting (IB). Cysteine accessibility was calculated by comparing the fraction of Get1FLAG that reacted with the 5 kDa PEG-maleimide (Get1FLAG-PEG) in the corresponding pairs of NEM-treated and mock-treated cells. (B) Yeast cells expressing the indicated single cysteine alleles of Get1FLAG in the TMDs and luminal loop were treated with NEM and 5 kDa PEG-maleimide as above. (C) Yeast cells expressing the single cysteine alleles of Get2 were treated with NEM and 5 kDa PEG-maleimide as above. (D) Yeast cells expressing the single cysteine alleles of Get2-1TM3FLAGsc in the first TM segment of Get1 were treated with NEM and 5 kDa PEG-maleimide as above.
Upon examination of the Get1 and Get2 single cysteine mutants, Get1 positions that led to Get1/2 crosslinking products formed a similar high molecular weight BMH-dependent crosslinking product despite the absence of a cysteine in Get2 (Figure 2.1B-D). Site-specific crosslinking between Get1 is likely the source of this product, as it has the expected size for a Get1-FLAG homodimer. These observations are consistent with structural studies, which have shown symmetric (2:2) Get3 complexes with the individual cytosolic domains of Get1 and Get2 (Mariappan et al., 2011, Stefer et al., 2011). This hypothesizes that Get3 bridges two copies of the Get1/2 heterodimer and Get1/2 functions as a heterotetramer (a dimer of the Get1/2 heterodimer) to mediate insertion of TA proteins (Figure 2.3A). In contrast, a single copy of the Get1/2 heterodimer may be sufficient to capture the TA protein from Get3 and release the TMD into the membrane despite the possibility of higher order Get1/2 structures in the ER membrane (Figure 2.3B).

**Figure 2.3:** Schematics for the models of Get1/2-mediated tail-anchored protein insertion. (A) Schematic of the Get1 (green)-Get2 (blue) heterotetramer model of TA protein insertion. Get3 may bridge two copies of the Get1/2 heterodimer, which then form a composite channel for the TA protein. (B) Schematic of the Get1/2 heterodimer model of TA protein insertion. A single copy of the Get1/2 heterodimer transiently captures the TA protein in a lipid-exposed hydrophilic docking site before releasing the TMD into the membrane.

To determine the minimal number of Get1/2 heterodimers needed for TA protein insertion, I first purified a His-tagged version of Get2-1sc from *E.coli* by metal-affinity chromatography and gel filtration chromatography (Figure 2.4A). The recombinant protein was then validated by observing binding to Get3 in detergent-containing buffer by monitoring complex formation using size-exclusion chromatography (Figure 2.4B). I then reconstituted
Get2-1sc into liposomes, incubated the membranes with affinity-purified Get3 in complex with a radiolabeled model TA substrate with the TMD of Sec22 (Get3~Sec22_{TMD}), and monitored the insertion of Sec22_{TMD}. I observed the presence of a protected fragment after protease treatment that is diagnostic of insertion into the lipid bilayer (Figure 2.4C).

**Figure 2.4: Purification and validation of recombinant Get2-1sc.** (A) Left: Schematic for the purification of His-tagged Get2-1sc from *E. coli*. Top right: His-tagged Get2-1sc was expressed in *E. coli*, solubilized membranes in detergent and purified Get2-1sc by metal-affinity chromatography. Representative samples were analyzed by SDS-PAGE and visualized by Coomassie staining. Bottom right: Ni-affinity purified Get2-1sc was further purified by size-exclusion chromatography. The corresponding fractions were analyzed by SDS-PAGE and visualized by Coomassie staining. See this chapter’s experimental procedures for more details. (B) Get2-1sc and Get 3 (5.5 µM each) were incubated together for 40 minutes in size-exclusion (SEC) buffer (50 mM Hepes- NaOH, 150 mM NaCl, 10% glycerol, 0.1% LDAO) and then analyzed by gel filtration chromatography. The corresponding fractions were analyzed by SDS-PAGE and visualized by SYPRO Ruby staining. (C) Affinity-purified radiolabelled Get3~Sec22_{TMD} complexes were incubated at room temperature for 30 minutes with the indicated membranes and the treated with 5 µg Proteinase K for 1 hour. Samples were analyzed by SDS-PAGE and autoradiography. ER-derived microsomes from Δget3 yeast cells served as the positive control, while empty liposomes served as the negative control.
I generated proteoliposomes which would contain at maximum a single copy of Get2-1sc. I compared the insertion efficiency of radiolabelled Sec22\textsubscript{TMD} of these “dimer” proteoliposomes with “oligomer” proteoliposomes where multiple copies were available (Figure 2.5). When total Get2-1sc amounts were normalized, both types of proteoliposomes were able to insert TA proteins with the same efficiency, while the negative control, a cytosolic domain mutant of Get2-1sc (Get2\textsubscript{CDm}-1\textsubscript{CDm}sc) that cannot bind Get3, did not.

Figure 2.5: TA protein insertion into liposomes by a single Get2-1sc heterodimer. Affinity-purified radiolabelled Get3–Sec22\textsubscript{TMD} complexes were incubated at room temperature for 30 minutes with the indicated membranes containing ~4 ng of Get2-1sc and then treated with 5 µg Proteinase K for 1 hour. Samples were analyzed by SDS-PAGE and autoradiography. A mutant Get2-1sc with point mutations (Get1 N72A, R73A and Get2 R14E, E15R, R16E, R17E) that abolish Get3 binding to each cytosolic domain (Get2\textsubscript{CDm}-1\textsubscript{CDm}sc) served as the negative control. Get2-1sc and Get2\textsubscript{CDm}-1\textsubscript{CDm}sc were reconstituted into liposomes such that for the “dimer” prep, there is a maximum of a single copy of the heterodimer per liposome, while the for the “oligomer” prep, multiple heterodimers per liposome are possible. For the “dimer” prep, 16 nM Get2-1sc was reconstituted into 100-nm liposomes. For the “oligomer” prep, 160 nM Get2-1schis was reconstituted into 200-nm liposomes.

I then reconstituted Get2-1sc into nanodiscs, a model lipid bilayer surrounded by scaffold proteins which wrap around the lipids to form a defined disc (Ritchie et al., 2009). The oligomerization state of Get2-1sc could be controlled by varying the stoichiometry of Get2-1sc to scaffold proteins to lipids. Nanodiscs self-assemble from lipids, scaffold proteins and membrane proteins upon detergent removal. Nanodiscs containing a single copy of Get2-1sc were isolated by size-exclusion chromatography (Figure 2.6A). I then characterized the ability of the Get2-1sc nanodiscs to release a model TA protein, Sec22, from Get3 using the amine-reactive crosslinker, disuccinimidyl suberate (DSS); failure to release TA substrate from Get3 leads to a
Get3×Sec22 adduct. Get2-1sc nanodiscs released Sec22 from Get3, resulting in the loss of the crosslink product (Figure 2.6B).

Figure 2.6: Generation and characterization of Get2-1sc nanodiscs. (A) Top: Schematic for the reconstitution of Get2-1sc into nanodiscs. Purified Get2-1sc, MSP1D1 scaffold proteins and lipids were mixed together in detergent-containing buffer. Upon detergent removal with biobeads, nanodiscs formed by self-assembly. Bottom: Representative separation of empty nanodiscs from Get2-1sc nanodiscs by gel-filtration chromatography. Here, 2 nmol of Get2-1sc was mixed with MSP1D1 and lipids at a molar ratio of 1:4:200. The corresponding fractions were analyzed by SDS-PAGE and visualized by SYPRO Ruby staining. (B) Affinity-purified radiolabelled Get3~Sec22 complexes were incubated at room temperature for 15 or 30 minutes with Get2-1sc and Get2<sub>CDm</sub>-1<sub>CDm</sub>sc nanodiscs (containing ~0.026 µg of Get2-1sc each) and then further incubated with disuccinimidyl suberate (DSS) crosslinker for an additional 30 minutes. Samples were analyzed by SDS-PAGE and visualized by autoradiography. ER-derived microsomes from Δget3 yeast cells served as the positive control; release of Sec22<sub>TMD</sub> from Get3 and subsequent insertion into the microsomes led to the glycosylation of an opsin epitope at the C-terminus of Sec22 (gSec22). For the negative control, no membranes were added to the reaction. (C) Affinity-purified radiolabelled Get3~Sec22<sub>S192C</sub> complexes were incubated at room temperature for 10 minutes with S-protein and then further incubated for the indicated times in the presence of Get2-1Q<sub>19C</sub>sc or Get2<sub>CDm</sub>-1<sub>CDm</sub>Q<sub>19C</sub>Sc nanodiscs. Samples were incubated with 0.2 mM BMH on ice for 1 hour, quenched, analyzed by SDS-PAGE and visualized by autoradiography.

To exclude the possibility that nanodisc lipids are able to drive substrate release by forming peripheral membrane interactions with the TMD, I adapted a strategy to capture the
transient docking event by crosslinking single-cysteine TMD mutants of Get1 and Sec22 (Get2-1Q19Csc and Sec22S129Cs, respectively) (Wang et al. 2014). Moreover, previous work demonstrated that Sec22 with a C-terminal S-tag (Sec22s) can be blocked en route to insertion by attaching a S-protein, which stabilizes the Get1/2-TA protein-docked intermediate (Wang et al. 2014). While I observed crosslinking between Get2-1Q19Csc and Sec22S129Cs that was dependent on the functional cytosolic domains of Get2-1sc and was also stabilized by S-protein, Get2-1Q19Csc x Sec22S129Cs adduct did not decrease in the absence of the S-protein over time as expected (Figure 2.6C). This may be due to the confined space within the nanodisc to accept multiple new transmembrane segments. While monitoring TA protein insertion into nanodiscs is still a challenge, one possible approach is to use an environment-sensitive probe that changes fluorescence to detect TA protein docking or Get2-1sc dynamics (Kedrov et al., 2016). Nonetheless, Get2-1sc reconstituted into proteoliposomes and nanodiscs show a single Get1/2 heterodimer is sufficient to insert TA proteins.

In the future, Get2-1sc nanodiscs coupled with cryo-electron microscopy and single molecule fluorescence analyses can be potentially used to establish a more detailed structural and mechanistic description of the Get1/2 insertase.

Experimental Procedures

S. cerevisiae strain construction

The strains used were previously described in Wang et al., 2014.

Antibodies

Mouse monoclonal ANTI-FLAG M2 antibody (α-FLAG) was obtained from Sigma-Aldrich. α-Hexokinase (α-Hxk) was obtained from United States Biological. α-Get2 antibody was a gift from Ramanujan Hegde. Secondary antibodies were goat α-mouse-IgG-HRP (Biorad), goat α-rabbit-IgG-HRP (Biorad), goat α-mouse-IgG-Cy5 (Thermo Fisher Scientific) and goat α-rabbit-IgG-Cy3 (Thermo Fisher Scientific).
Vectors

pBB343 MSP1D1 was a gift from Brianna Burton. Templates for model TA protein substrates, Sec22, Sec22S192Cs and SumoTMDV5 (Sec22TMD), were previously described (Schuldiner et al., 2008, Wang et al., 2011, Wang et al., 2014). pETDUET Get4-His, Get5 and pET29b Get3-FLAG were previously described (Wang et al., 2010). Get2-1sc and Get2cdm-1cdmsc were PCR amplified from pRS316 Get2-1scFLAG and pRS316 Get2(RC)-1(NR)scFLAG, respectively (Wang et al., 2014) and subcloned into Ndel/KpnI site of pET29b vector to yield pET29b Get2-1sc-His and pET29b Get2cdm-1cdmsc-His. Get2cdm-1cdmsc has the point mutations Get1 N72A, R73A and Get2 R14E, E15R, R16E, R17E that prevent binding to Get3 (Wang et al., 2011). The Q19C point mutation was introduced using QuikChange site-directed mutagenesis with PfuTurbo DNA polymerase (Aligent).

In vivo crosslinking screen

BY4741 yeast cells with carrying the pairwise library of GET1cys/GET2cys alleles were grown in 96-well plate in YPD at 30°C for 4.5 hours with shaking. The plates were spun down at 1600 rpm for 3 minutes and washed with 200 µL phosphate buffered saline (PBS; 10 mM Na2HPO4, pH 7.4, 1.8 mM KH2PO4, 2.7 mM KCl, 137mM NaCl). The cells were resuspended with 200 µL per well of PBS supplemented with 0.250 mM of bismaleimidohexane (BMH) (Thermo Fisher Scientific), and incubated on ice for 1 hour. Cells were pelleted by centrifugation at 1600 rpm for 3 minutes and the reaction quenched with 20 µL per well 2× protein sample buffer supplemented with 50 mM DTT and protease inhibitor and heated at 65°C for 10 minutes. The supernatant was analyzed by SDS-PAGE and visualized by immunoblotting with α-FLAG (1:2000) and α-Hxk (1:10000).

In vivo site-specific cysteine-cysteine crosslinking

Yeast cells with the indicated GET1cys/GET2cys alleles were grown in YPD at 30°C to mid-log phase (OD600=0.67). For each sample, 0.5 OD600 units of yeast cells were harvested by
centrifugation and washed with PBS. The cells were resuspended with 0.75 mL of PBS supplemented with 0.250 mM of the indicated sulfhydryl-crosslinker, either bismaleimidohexane (BMH) or bismaleimidoethane (BMOE) (Thermo Fisher Scientific). Crosslinking reactions were incubated at 4˚C for 1 hour with rotation. Cells were pelleted by centrifugation and lysed with 50µL 2× protein sample buffer and heated at 65˚C for 10 minutes. The supernatant was analyzed by SDS-PAGE and visualized by immunoblotting with α-FLAG (1:2000) and α-HXK (1:10000).

In vivo NEM accessibility assay

For each strain of yeast cells with the indicated single-cysteine alleles of Get1-FLAG, two samples of 10 OD<sub>600</sub> units of cells grown in YPD at 30˚C to mid-log (OD<sub>600</sub>=0.67) were harvested by centrifugation and washed with PBS. One sample was resuspended with 3 mL of ice-cold PBS supplemented with 0.2 mM N-ethylmaleimide (NEM) (Sigma-Aldrich). The other sample was resuspended with PBS and mock-treated with DMSO. The samples were incubated at 4˚C for 1 hour with rotation, followed by quenching with 5 mM dithiothreitol (DTT) for 10 minutes on ice. The cells were pelleted and resuspended with 50 µL 100 mM Tris-HCl, pH 9.5, 10 mM DTT solution and incubated at room temperature for 10 minutes. The cells were pelleted and gently resuspended with 60 µL lyticase buffer (10mM Tris pH 7.9, 0.7M sorbitol, 0.75× yeast extract-peptone, 0.5% dextrose), followed by 15 µL of homemade lyticase (in 50 mM sodium citrate, pH 5.8). The cells were digested for 40 minutes at 30˚C with rotation. 100 µL of the digested cell suspension was applied to the top of 100 µL ice-cold Cushion 1 (20 mM Hepes, pH 7.4, 0.8 M sucrose, 1.5% Ficoll 400) and spun at 3000 rpm for 10 minutes at 4˚C. The supernatant was removed and the spheroplasts were mixed with 250 µL lysis buffer (20 mM Hepes, pH 7.4, 0.1 M sorbitol, 50 mM KOAc, 2 mM EDTA, 1 mM DTT, 1 mM PMSF) and 100 µL of 0.5 mm glass beads, and lysed by bead beating. The samples were spun at 1000 g for 10 minutes at 4˚C and the supernatant was collected and spun again at 13000 g for 15 minutes.
The supernatant was removed and the remaining microsomes were solubilized with 10 µL of 50 mM Hepes, pH 7.5, 1% SDS, 0.25 mM TCEP. The microsomes were treated with 10µL of 16 mM 5 kDa PEG-maleimide in 50 mM Hepes, pH 7.5 at 30˚C for 1 hour with rotation. The reactions were quenched with 20 mM DTT for 20 minutes at room temperature, followed by 2× protein sample buffer (10 µL of sample buffer/ 5 µL of sample). The samples were analyzed by SDS-PAGE, immunoblotting, and image analysis in ImageJ. Cysteine accessibility was calculated by comparing the fraction of Get1-FLAG that reacted with the 5 kDa PEG-maleimide (Get1FLAG-PEG), which migrates slower with a higher molecular weight, in NEM-treated and mock-treated cells.

Recombinant protein purification

Get3-FLAG

His-tagged Get3-FLAG was purified from BL21-CodonPlus (DE3)-RIPL competent E. coli cells as previously described (Wang et al. 2010). In brief, cells were transformed with the pET29b expression plasmid and grown in LB media at 37˚C under kanamycin selection. Cells were grown to A_{600} = 0.4 and expression was induced with 0.4 mM IPTG for 4 hours. Cells were then harvested by centrifugation, washed in 20 mM Tris, pH 7.5, 150 mM NaCl, then stored at -80˚C until lysis.

Cells were resuspended in Tris lysis buffer (50 mM Tris, pH 7.5, 300 mM NaCl, 2% glycerol, 2 mM beta mercaptoethanol (BME)) supplemented with cOmplete Protease Inhibitor Cocktail (Roche) and phenylmethane sulfonyl fluoride (PMSF). Cells were lysed by high pressure homogenization by two passes through an EmulsiFlex®-C3 (Avestin, Inc.). The cell lysate was clarified by centrifugation for 1 hour at 30000 g at 4˚C and bound to a Ni-NTA resin column by gravity flow.

Columns were washed first with a high salt wash buffer (50 mM Tris, pH 7.5, 500 mM NaCl, 2% glycerol, 2 mM BME), followed by 10 mM imidazole wash buffer (50 mM Tris, pH 7.5,
300 mM NaCl, 2% glycerol, 2 mM BME, 10 mM imidazole), and 50 mM imidazole wash buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 2% glycerol, 2 mM BME, 50 mM imidazole). Get3 was eluted with 50 mM Tris, pH7.5, 150 mM NaCl, 2% glycerol, 2 mM BME, 250 mM imidazole. Protein was desalted with Econo-Pac® 10DG Desalting Prepacked Gravity Flow Columns (Bio-Rad) into 50 mM Tris pH 7.5, 50 mM NaCl, 2 mM BME and 2% glycerol.

Get3 was further purified by ion-exchange (IEX) chromatography using a Resource Q column using IEX buffer A (50 mM Tris, pH 7.5, 2% glycerol, 2 mM BME) and IEX buffer B (50 mM Tris, pH 7.5, 2% glycerol, 2 mM BME, 1 M NaCl).

Get4-Get5 complex

His-tagged Get4 was purified in complex with Get5 from BL21-CodonPlus (DE3)-RIPL competent E. coli cells as previously described (Wang et al. 2010). Cells were transformed with the pETDUET expression plasmid and grown in 2×LB with phosphate salts (17 mM KH₂PO₄, 72 mM K₂HPO₄) at 37°C under ampicillin selection. Cells were grown to A₆₀₀=0.5 then cooled at 17°C. Expression was induced with 0.4 mM of IPTG overnight at 17°C. Cells were then harvested by centrifugation, washed in 20 mM Tris, pH 7.5, 150 mM NaCl, then stored at -80°C until lysis.

Cells were resuspended in Tris lysis buffer supplemented with cOmplete Protease Inhibitor Cocktail and phenylmethane sulfonyl fluoride (PMSF). Cells were lysed by high pressure homogenization by two passes through an EmulsiFlex®-C3 (Avestin, Inc.). The cell lysate was clarified by centrifugation for 1 hour at 30000 g at 4°C and bound to a Ni-NTA resin column by gravity flow. Columns were washed first with a high salt wash buffer, followed by 10 mM imidazole wash, and 50 mM imidazole wash buffers. Get3 was eluted with 50 mM Tris, pH7.5, 150 mM NaCl, 2% glycerol, 2 mM BME, 250 mM imidazole. Protein was desalted with Econo-Pac® 10DG Desalting Prepacked Gravity Flow Columns (Bio-Rad) into 50 mM Tris 7.5, 50 mM NaCl, 2 mM BME and 2% glycerol.
Get4/5 complex was further purified by ion-exchange chromatography using a Resource Q column using IEX buffer A (50 mM Tris, pH 7.5, 2% glycerol, 2 mM BME) and IEX buffer B (50 mM Tris, pH 7.5, 2% glycerol, 2 mM BME, 1 M NaCl). Protein was desalted with Econo-Pac® 10DG Desalting Prepacked Gravity Flow Columns (Bio-Rad) into 50 mM Hepes, pH 7.5, 150 mM NaCl, 2 mM BME and 10% glycerol, concentrated with a 10k MWCO Amicon concentrator and stored at -80°C until use.

**Get2-1sc and Get2\textsuperscript{CDm-1CDm sc}**

His-tagged Get2-1sc and Get2\textsuperscript{CDm-1CDm sc} were purified from BL21-CodonPlus (DE3)-RIPL competent *E. coli* cells. Cells were transformed with the pET29b expression plasmid and grown in 2× LB with phosphate salts (17 mM KH\textsubscript{2}PO\textsubscript{4}, 72 mM K\textsubscript{2}HPO\textsubscript{4}) at 37°C under kanamycin selection. Cells were grown to A\textsubscript{600}=0.5 then cooled at 17°C. Expression was induced with 0.4 mM of IPTG overnight at 17°C. Cells were then harvested by centrifugation, washed in 20 mM Tris, pH 7.5, 150 mM NaCl, then stored at -80°C until lysis.

Cells were resuspended in lysis buffer (50 mM Hepes pH 8.0, 500 mM NaCl, 10 mM imidazole, 10% glycerol) with cOmplete Protease Inhibitor Cocktail (PIC) and phenylmethane sulfonyl fluoride (PMSF). Cells were lysed by Cells were lysed by high pressure homogenization by two passes through an EmulsiFlex®-C3 (Avestin, Inc.). The cell lysate was clarified by centrifugation 12000 g for 20 minutes using the JA 25.50 at 4°C. The membrane pellet was collected at 40000 rpm for 1 hour using the Ti45 and then solubilized in lysis buffer with 1% n-Dodecyl β-D-maltoside (DDM) at 4°C. The solubilized membranes were cleared for 1 hour at 30000 g at 4°C using the JA25.50 and then bound to Ni-NTA resin column (equilibrated in lysis buffer with 0.02% DDM) by gravity flow. The column was washed equilibration buffer, then 50 mM Hepes, pH 8, 25 mM imidazole, 300 mM NaCl, 10% glycerol, 0.02% DDM, and 50 mM Hepes, pH 8, 40 mM imidazole, 150 mM NaCl, 10% glycerol, 0.02% DDM. The proteins were eluted with 50 mM Hepes, pH 8, 200 mM imidazole, 150 mM NaCl, 10% glycerol, 0.0.2% DDM. The proteins were concentrated to ~ 1 mg/mL with a 50k MWCO Amicon concentrator. Proteins
were further purified by gel-filtration using a Superdex 200 10/300 GL column in size-exclusion chromatography (SEC) buffer (50 mM Hepes, pH 8, 150 mM NaCl, 2% glycerol, 0.02% DDM). The pooled fractions were concentrated to ~1 mg/mL.

To analyze the interaction between Get2-1sc and Get3 by gel filtration chromatography, Get2-1schis and Get3his were incubated at 5.5 µM each in 100 µL of size-exclusion (SEC) buffer (50 mM Hepes-NaOH, 150 mM NaCl, 10% glycerol, 0.1% LDAO) for 40 minutes at 4°C with agitation. Samples were diluted to a total volume of 200 µL upon addition of 100 µL of SEC buffer and analyzed by Superdex 200 10/300 GL. The fractions were analyzed by SDS-PAGE and visualized by SYPRO Ruby staining (Invitrogen).

**MSP1D1**

His-tagged MSP was purified from BL21-CodonPlus (DE3)-RIPL competent *E. coli* cells as previously described (Ritchie et al., 2009). Cells were transformed with the pET15 His6-TEV-MSP1D1 expression plasmid and grown in in 2×LB with phosphate salts at 37°C under ampicillin selection. Cells were grown to A<sub>600</sub> = 0.6-0.8 and expression was induced with 1 mM IPTG for 4 hours. Cells were then harvested by centrifugation, washed in 20 mM Tris, pH 7.5, 150 mM NaCl, then stored at -80°C until lysis.

Cells were resuspended in lysis buffer (50 mM Tris, pH8, 500 mM NaCl, 1% TritonX-100, 1 mM EDTA) supplemented with PIC and PSMF. Cells were lysed by high pressure homogenization by two passes through an EmulsiFlex®-C3 or sonication. The cell lysate was treated with 5 mM MgCl<sub>2</sub> and 100 U benzonase and then clarified at 16000 rpm for 45 minutes using the JA 25.50. The supernatant was bound to a Ni-NTA resin column (equilibrated 50 mM Tris pH 8, 500 mM NaCl, 1% Triton-X-100) by gravity flow. The column was washed with wash buffer (50 mM Tris pH 8, 500 mM NaCl) supplemented with 1% TX-100, then wash buffer with 50 mM sodium cholate, followed by detergent-free wash buffer, then wash buffer with 20mM imidazole. The proteins were eluted with 50 mM Tris, pH 8, 500 mM NaCl, 500 mM imidazole. The proteins were then dialyzed into 50 mM Tris, pH 8, 20 mM NaCl, 1 mM EDTA, 2 mM DTT
and cleaved with TEV protease overnight at 4°C. The buffer was exchanged into 20 mM Tris, pH 7.5, 500 mM NaCl, using Econo-Pac® 10DG Desalting Prepacked Gravity Flow Columns. The protease and tags were removed from purified MSP1D1 by passing the protein through a Ni-NTA column. The flow through was collected, exchanged into 20 mM Tris, pH 7.5, 100 mM NaCl, 0.5 mM EDTA, concentrated to 1 mM and stored at -80°C.

Isolation of ER-derived microsomes from yeast cells

ER-derived microsomes were prepared as previously described (Schuldiner et al., 2008). Yeast cells were grown in YPD at 30°C to ~2 OD overnight. Cells were harvested by centrifugation and washed with water. Cells were resuspended with 10 mM DTT, 100 mM Tris-HCl, pH 9.4 (15 mL per litre of cells) and nutated at room temperature for 10 minutes. Cells were pelleted at 3000 g for 3 minutes and the supernatant removed. Cells were resuspended in 12 mL of lyticase buffer (10mM Tris pH 7.9, 0.7M sorbitol, 0.75× yeast extract-peptone, 0.5% dextrose) and 3 mL of homemade lyticase (per 1 litre of cells) and incubated at 30°C for ~1 hour. The cell suspension overlaid on top of 15 mL of ice-cold Cushion 1 (20 mM Hepes, pH 7.4, 0.8 M sucrose, 1.5% Ficoll 400) and the spheroplasts were collected by centrifugation for 25 minutes at 4°C at 3000 g in a swing bucket rotor. The spheroplasts were resuspended in 20 mL of ice-cold lysis buffer (20 mM Hepes, pH 7.4, 0.1 M sorbitol, 50 mM KOAc, 2 mM EDTA, 1 mM DTT, 1 mM PMSF) and lysed by douncing on ice (5 times per round, for a total of 5 rounds). 15 mL of lysed cells were overlaid onto 15 mL of Cushion 2 (1M sucrose, 50 mM KAcO, 20 mM Hepes, pH 7.4) and centrifuged for 10 minutes at 4°C at 6750 g using the JLA 16.250. The supernatant was carefully collected and centrifuged at 16700 rpm for 25 minutes in 70 Ti rotor. The pellet was then resuspended in 1 mL of Buffer 88 (20 mM Hepes, pH 6.8, 150 mM KAcO, 250 mM sorbitol, 5 mM MgAcO), and transferred into a 1.5 mL tube. The microsomes were spun again at 4°C for 10 minutes on a table top centrifuge and the supernatant was removed. The A_{280} of the microsomes were normalized to 80.

Preparation of nanodiscs
The reconstitution of Get2-1sc into nanodiscs is based on the procedure described in Ritchie et al., 2009. Nanodiscs were made with a mix of L-\(\alpha\)-phosphatidylcholine (PC), 16:0-18:1 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (PE) lipids. Lipids (molar ratio of 4 PC: 1 16:0-18:1 PE) were mixed as chloroform stocks, and dried under vacuum. Lipids were solubilized in cholate buffer (50 mM HEPES, pH 7.4, 150 mM KOAc, 7 mM Mg(OAc)\(_2\), 1 mM DTT, 50 mM sodium cholate, 0.25% DDM) to 10 mg/mL by sonication and pipetting. To reconstitute Get2-1schis into nanodiscs, 2 nmol (130 \(\mu\)g) of Get2-1schis was mixed with MSP1D1 and lipids at a molar ratio of 1:4: 200 or 1: 10: 500 in reconstitution buffer (50 mM HEPES, pH 7.4, 150 mM KOAc, 7 mM Mg(OAc)\(_2\), 1 mM DTT, 0.1% DDM) to a final volume of 400\(\mu\)L. Empty nanodiscs were prepared as a control by mixing 8 nmol of MSP1D1 and lipids at a molar ratio of 1:65. After the protein-lipid mixes were incubated on ice for 1 hour, 400\(\mu\)L of a 50% Biobeads™ SM-2 adsorbent media slurry in 50 mM HEPES, pH 7.4, 150 mM KOAc, 7mM Mg(OAc)\(_2\) buffer were added and detergent was removed overnight at 4˚C with overhead mixing. Biobeads were removed by puncturing the bottom of the tube a 25 gauge needle, spinning at 1000 g for 1 minute and collecting the nanodiscs in a 15-mL tube. 500\(\mu\)L of the nanodisc mix were analyzed by gel filtration using a Superdex 200 10/300 GL column in SEC buffer (50 mM HEPES, pH 7.4, 150mM KOAc, 7mM Mg(OAc)\(_2\), 1 mM DTT, 10% glycerol).

Preparation of liposomes

Liposomes were prepared as described in Mariappan et al., 2011. Liposomes usually consist of a molar ratio of 8 L-\(\alpha\)-phosphatidylcholine (PC) : 1.9 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (16:0-18:1 PE) : 0.1 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (rhodamine-PE) (Avanti Polar Lipids, Inc.). Lipids were mixed as chloroform stocks, adjusted to 10 mM DTT and then dried under vacuum for 12 hours. Lipids were resuspended in lipid buffer (50 mM Hepes, pH 7.4, 15% glycerol) to 20 mg/mL for 6 hours at room temperature by overhead mixing with
intermittent vortexing. The lipid suspension was then freeze-thawed 3 times in liquid nitrogen and 37°C, followed by extrusion at 65°C through 100-nm or 200-nm polycarbonate membranes 11 times using a mini-extruder (Avanti Polar Lipids, Inc.). Liposomes were stored at -80°C.

*Preparation of Get2-1sc proteoliposomes*

Proteoliposomes were prepared as described in Mariappan et al., 2011. Purified Get2-1sc and 10µL of 20 mg/mL liposomes were mixed together in 100 µL of reconstitution buffer (50 mM Hepes-KOH, pH 7.4, 500 mM KOAc, 5 mM Mg(OAc)₂, 250 mM sucrose, 1 mM DTT, 0.25% Deoxy Big CHAP). ~30 mg of biobeads were added the protein-liposome mix and incubated overhead mixing for 12 hours at 4°C. The proteoliposomes were separated from biobeads by puncturing the tube, spinning and collecting the fluid phase. The proteoliposomes were diluted with 5 volumes of ice-cold water and then sedimented by centrifugation at 75000 rpm for 30 minutes at 4°C using a TLA 100.3. The supernatant is removed and the proteoliposomes were resuspended in membrane buffer (50 mM Hepes, pH 7.4, 100 mM KOAc, 5 mM Mg(OAc)₂, 1 mM DTT).

*In vitro transcription*

Templates for transcription were generated by PCR using a 5’ primer containing the T7 promoter that anneals to the first ~30 bp of the open reading frame and a 3’ primer containing the stop codon and polyA tail that anneals to the last ~30 bp of the open reading frame. PCR templates for transcription were used to make mRNAs by incubation with components of mMESSAGE mMACHINE® T7 transcription kit (Thermo Fisher Scientific) at 37°C for 2 hours. The mRNA was purified by acid phenol-chloroform extraction before being in vitro translated. Alternatively, Transcription reactions were carried out using T7 polymerase at 37°C for 2 hours and used directly for *in vitro* translation.

*Generation of Get3-TA protein complexes*

Unpurified mRNA was *in vitro* translated in the presence of ³⁵S-methionine in
prepared rabbit reticulate lysate (Stefanovic and Hegde, 2007) supplemented with 160 ng/µL of recombinant Get3-FLAG and 80 ng/µL of recombinant Get4/5 for 1.5 hours at 30˚C. The translation reaction was stopped with 0.6 mM cycloheximide, then diluted two-fold with Buffer A. Get3-FLAG~TA complexes were affinity-purified with α-FLAG M2 affinity gel (10 µL resin per 10 µL translation reaction) (Sigma Aldrich) for 1 hour at 4˚C with agitation and eluted with 0.5 mg/mL FLAG peptide (Sigma Aldrich).

Alternatively, purified mRNA was *in vitro* translated in the presence of 35S-methionine, 32 ng/µL Get3-FLAG and 64 ng/µL Get4/5 in prepared wildtype yeast extract (Schuldiner et al., 2008) at room temperature for 30 minutes and then stopped with 1 mM cycloheximide. Get3-FLAG~TA complexes were affinity-purified with α-FLAG resin (10 µL resin per 10 µL translation reaction) for 1 hour at 4˚C with agitation and eluted with 0.5 mg/mL FLAG peptide.

**Insertion assay**

Get3-FLAG~TA complexes were incubated at room temperature in the presence of indicated membrane in insertion buffer (PBS supplemented with 22 mM Hepes, pH 7.4, 120 mM KOAc, 15 mM Mg(OAc)$_2$, 0.75 mM rATP, 0.0250 mM creatine phosphate, 1.7 mM DTT) and 3 mg/mL of creatine kinase. Insertion reactions were stopped by placing the samples on ice.

For protease protection assays, the samples were incubated with 5 µg of proteinase K on ice for 1 hour. Digestion was stopped by adding PMSF and incubated on ice for an additional 5 minutes. The samples were transferred to boiling 2× sample buffer and boiled for 2 minutes. Samples were analyzed by SDS-PAGE and autoradiography.

**Get3 substrate release assay**

Get3-FLAG~TA complexes were incubated at room temperature in the presence of indicated membrane in insertion buffer. The samples were then incubated with 0.55 mM disuccinimidyl suberate (DSS) for an additional 30 minutes. The samples were quenched with protein sample buffer and analyzed by SDS-PAGE and autoradiography.

**Docking assay**
2 µL of Get3-FLAG~TA complexes were incubated with 0.4 µL of S-protein HRP conjugate (EMD Millipore) for 10 minutes in insertion buffer at room temperature and further incubated in the presence of the indicated membranes. The samples were then incubated with 0.2 mM BMH for 1 hour on ice. The samples were quenched with protein sample buffer and analyzed by SDS-PAGE and autoradiography.

References


Summary

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

Introduction

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

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

Despite these mechanistic insights, how full-length Get1 and Get2 function together to coordinate events at the membrane remains unclear. Although Get1/2 complexes can be isolated with Get3 from yeast rough microsomes (Auld et al., 2006, Jonikas et al., 2009), the quaternary structure of the Get1/2/3 complex is undefined. Crystal structures of the cytosolic Get1 or Get2 fragments bound symmetrically to different nucleotide states of homodimeric Get3 have led to closely related models involving a heterotetrameric Get1/2 assembly of two Get1 and two Get2 subunits (Figure 3.1A) (Mariappan et al., 2011, Stefer et al., 2011); such an assembly might exist constitutively, or it might form dynamically in the presence of Get3 (Figure 3.1B). Simpler models are also plausible, including a heterodimeric Get1/2 assembly with only one copy of each subunit (Figure 3.1C). Defining the oligomeric state of the functional Get1/2 complex is critical for understanding its molecular mechanism. For example, the number of subunits present in the functional complex likely dictates whether the Get1 and Get2 cytosolic domains bind competitively or simultaneously to the same or opposite sides of the Get3 homodimer at various stages along the pathway (Figure 3.1) (Mariappan et al., 2011, Stefer et al., 2011). Likewise, the number of subunits has important implications for how the Get1/2
TMDs guide TA substrates into the bilayer (Wang et al., 2014) and whether conformational changes in Get3 can be coupled to these TMDs during insertion (Denic et al., 2013, Hegde and Keenan, 2011, Stefer et al., 2011). Thus, a rigorous description of how Get1/2 coordinates key steps at the membrane requires knowledge of its quaternary structure. Here we show that the minimal functional unit of the full-length Get1/2 complex is a heterodimer, which drives the insertion of TA proteins by binding to opposite sides of the Get3 homodimer.

**Figure 3.1: Alternative models for the Get1/2 architecture.** (A) In static heterotetramer models, based on structural and biochemical studies (Mariappan et al., 2011, Stefer et al., 2011), Get1/2 exists as a constitutive complex comprising two copies of each subunit. Accordingly, the Get3-TA protein complex is captured by two copies of Get2, which bind on opposite sides of the symmetric Get3 dimer. Subsequently, the ADP or apo form of the Get3-TA complex is handed off to Get1, which displaces each Get2 subunit such that two Get1 subunits bind on opposite sides of Get3. Alternatively, Get1 only partially displaces Get2, such that two Get1 and two Get2 subunits are bound to Get3. All heterotetramer models predict that two copies of Get1 and/or Get2 bind simultaneously to Get3 at different steps in the pathway. In such models, conformational changes in Get3 could be coupled to the transmembrane domains of Get1/2 by rigid interactions mediated by the coiled coil (not shown). (B) In a dynamic model, Get3 binding drives the transient assembly of two heterodimeric Get1/2 complexes into a single heterotetrameric complex. (C) In a static heterodimer model, the Get3-TA complex is initially captured by a single copy of Get2 bound to one side of the Get3 dimer; Get1 then engages the ADP-bound or apo form of the Get3-TA complex by partially displacing Get2 or by binding to the opposite side of the Get3 dimer.

**Results**

To gain insight into the organization of the Get1/2 complex, we developed a bulk fluorescence resonance energy transfer (FRET) assay in proteoliposomes that reports on changes in the proximity of Get1 and/or Get2 subunits upon binding to Get3. We introduced single cysteines at membrane-proximal (Get1-A95C; Get2-E220C) or cytosolic (Get1-S77C; Get2-S28C) positions within Get1 and Get2 (Figure 3.2A). After purification (Figure S3.1), the individual subunits were labeled with FRET donor (Cy3) or acceptor (Cy5) fluorophores.
Cytosolically or membrane-proximal-labeled Get1 and Get2 subunits were then reconstituted into proteoliposomes in different donor-acceptor combinations: Get1\textsuperscript{Cy3}-Get1\textsuperscript{Cy5}-Get2, Get1\textsuperscript{Cy3}-Get2\textsuperscript{Cy5} (membrane proximal), Get1\textsuperscript{Cy5}-Get2\textsuperscript{Cy3} (cytosolic), and Get1-Get2\textsuperscript{Cy3}-Get2\textsuperscript{Cy5}. These proteoliposomes are reconstituted at a high protein-to-lipid ratio (12:10,000) such that they contain multiple copies of Get1/2. This allows for an unbiased analysis of Get3 binding to different oligomeric states of Get1/2. After verifying the insertion activity of the different proteoliposomes (Figure S3.2a), we monitored changes in FRET as a function of binding to different nucleotide states of Get3.

We first explored whether Get3 binding drives Get1/2 toward a higher oligomeric state, as would be expected in the dynamic model (Figure 3.1B). When proteoliposomes containing different combinations of membrane-proximal labels were incubated with Get3, we observed no significant FRET increase, regardless of which subunits were labeled or the nucleotide state of Get3 (Figure 3.2B; Figure S3.2b). Thus, consistent with static models, Get3 binding does not drive assembly of a higher-order oligomer of Get1/2.

Next, we used proteoliposomes containing different combinations of cytosolically labeled Get1 and Get2 to explore how they engage Get3. The cytosolic coiled coil of Get1 and the long (150-residue) unstructured N terminus of Get2 do not interact with each other but instead bind to overlapping sites present on each of two sides of the Get3 homodimer (Mariappan et al., 2011, Stefer et al., 2011). These cytosolic regions are expected to FRET most efficiently when brought into proximity by simultaneous binding to Get3. Studies with the isolated cytosolic fragments show that the Get2-Get3 interaction is insensitive to nucleotide but that the Get1-Get3 interaction is weakened by ADP and completely disrupted by ATP (Mariappan et al., 2011, Rome et al., 2014, Stefer et al., 2011, Wang et al., 2011).
Figure 3.2: Bulk FRET measurements of Get3 binding to Get1/2 complexes in proteoliposomes. (A) Get1 and Get2 subunits were labeled with FRET donor Cy3 (green) or FRET acceptor Cy5 (red) fluorophores at membrane-proximal or cytosolic positions and then reconstituted into proteoliposomes in different donor-acceptor combinations. (B) Proteoliposomes were reconstituted at 12 Get1/2 molecules per 10,000 lipids (“High Get1/2-to-lipid”). The histogram shows the FRET change after addition of 50 nM Get3 (dark gray), Get3 + ADP (gray), or an ATPase-deficient Get3-D57N mutant + ATP (white) to each of the six proteoliposomes (10 nM Get1/2). A significant Get3-dependent FRET increase is only observed when the donor-acceptor pairs are located on the cytosolic positions of Get1 and Get2 subunits. All samples show a non-specific FRET component in the absence of Get3 due to coreconstitution of multiple donors and acceptors in the same proteoliposomes (Figure S3.2b); we interpret a Get3-dependent FRET decrease as an increase in the average distance between labeled Get1/2 heterodimers due to steric hindrance caused by Get3 binding. This decreased FRET is eliminated in proteoliposomes reconstituted at lower protein-to-lipid ratios (D and Figure S3.2b). (C) At left, FRET-based titration of cytosolically labeled Get1<sup>Cy5</sup>-Get2<sup>Cy3</sup> proteoliposomes (10 nM Get1/2) with Get3-D57N. At right, disruption of the Get1-Get2-Get3 interaction in the same proteoliposomes, monitored by the change in FRET upon titration with ATP. (D) As in (B), but after reconstitution at lower Get1/2-to-lipid ratios (1.2 Get1/2 molecules per 10,000 lipids; “Low Get1/2-to-lipid”).
When Get3 was incubated with cytosolically labeled Get1\textsuperscript{Cy3}-Get2\textsuperscript{Cy5} proteoliposomes, no significant FRET increase was observed, regardless of the nucleotide state (Figure 3.2B; Figure S3.2b). Likewise, no significant FRET increase was observed when ATP-bound Get3 was incubated with Get1\textsuperscript{Cy5}-Get2\textsuperscript{Cy3} proteoliposomes. However, when ADP-bound or nucleotide-free Get3 was incubated with Get1\textsuperscript{Cy5}-Get2\textsuperscript{Cy3} proteoliposomes we observed a strong FRET increase (Figure 3.2B; Figure S3.2b). When cytosolically labeled Get1\textsuperscript{Cy5}-Get2\textsuperscript{Cy3} proteoliposomes (10 nM) were titrated with Get3, we observed a linear FRET increase that became saturated at one equivalent of Get3 (10 nM homodimer) (Figure 3.1C) and could be reversed by ATP (Figure 3.2C). These data are consistent with Get3 binding with sub-nanomolar affinity to a single Get1/2 heterodimer.

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

To recapitulate the result with single Get1/2 heterodimers per liposome, we repeated the bulk FRET experiment with all donor-acceptor combinations reconstituted at a lower protein-to-lipid ratio (1.2:10,000). Consistent with the presence of ~1 Get1/2 heterodimer per liposome, we observed negligible FRET in Get1\textsuperscript{Cy3}-Get1\textsuperscript{Cy5}-Get2 and Get1-Get2\textsuperscript{Cy3}-Get2\textsuperscript{Cy5} proteoliposomes but clear FRET for both cytosolically labeled Get1\textsuperscript{Cy5}-Get2\textsuperscript{Cy3} and membrane-proximal Get1\textsuperscript{Cy3}-Get2\textsuperscript{Cy5} proteoliposomes (Figure S3.2b). Get3 binding did not significantly change FRET between membrane-proximal-labeled Get1\textsuperscript{Cy3}-Get2\textsuperscript{Cy5} (Figure 3.2D), indicating that the Get3-dependent FRET decreases observed at higher protein-to-lipid ratios (Figure 3.2C) result from the presence of multiple copies of labeled Get1/2 in the same proteoliposome. However, as with
the higher protein-to-lipid ratio reconstitutions, the addition of ADP-bound or nucleotide-free Get3 increased FRET between cytosolically labeled Get1<sup>Cy5</sup>-Get2<sup>Cy3</sup> (Figure 3.2D). While these bulk FRET data are consistent with a model in which Get3 binds to a heterodimeric Get1/2 complex comprising a single copy of each subunit, they do not formally exclude the possibility that Get1/2 functions as a heterotetrameric or higher-order complex.

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

To prevent dissociation of the Get1 and Get2 subunits during reconstitution, we took advantage of an engineered single-chain Get1/2 (Get2-1sc) construct shown previously to be functional in yeast (Wang et al., 2014). We expressed and purified Get2-1sc from <i>E. coli</i> and verified its activity <i>in vitro</i>. Like native Get1/2, Get2-1sc, but not its variants containing inactivating mutations in the cytosolic fragments of Get1 (R73E) or Get2 (R17E), is functional for TA protein insertion in proteoliposomes (Figure 3.3A).
Figure 3.3: Single Get1/2 heterodimers mediate TA protein insertion. (A) Yeast rough microsomes (yRMs) or the indicated proteoliposomes were tested for insertion of radiolabeled TA protein, Sec61β (FL), by a proteinase K protection assay. The appearance of a protected fragment (PF), which is diagnostic for insertion, was quantified by SDS-PAGE and autoradiography. Co-reconstituted Get1 and Get2 subunits (Get1/2) show equivalent specific activity to Get2-1sc proteoliposomes. Mixtures of Get1-only and Get2-only proteoliposomes (Get1, Get2) and single-chain constructs containing inactivating point mutations in either the Get1 (Get2-1sc; R73E in Get1) or the Get2 (Get2-1sc; R17E in Get2) cytosolic domains show no activity. The four Get2-1sc-Cy5 samples correspond to the four protein-to-lipid ratio reconstitutions in (B), at increasing protein-to-lipid ratios. All reconstitutions were diluted with empty liposomes to a final concentration of 10 nM Get1/2. The normalized insertion activity is independent of the number of Get2-1sc-Cy5 molecules in each proteoliposome. Coomassie-stained proteinase K (PK) was used as a loading control. Before performing the insertion assay, proper Get2-1sc-Cy5 normalization was confirmed by Cy5-imaged SDS-PAGE. For completeness, the concentration of Get2-1sc-Cy5 samples was also compared with unlabeled Get1/2 samples by stain-free SDS-PAGE; no sample was run for the most dilute Get2-1sc-Cy5 sample (marked with an asterisk), because of high lipid content, low protein concentration, and the large amount of sample required for stain-free detection. (B) Single-molecule photobleaching analysis of proteoliposomes from (A), reconstituted with Cy5-labeled single-chain Get1/2 (Get2-1sc-Cy5) at different protein-to-lipid ratios. The number of photobleaching steps per labeled proteoliposome is shown in red; x represents discarded traces. SDS-solubilized Get2-1sc-Cy5 serves as a monomeric control. The inset (black) shows the calculated proportion of Get2-1sc-Cy5 found in different oligomeric states, as described in this chapter’s Experimental Procedures. (C) In parallel with the assay shown in (A), Get2-1sc-Cy5 proteoliposomes (reconstituted at a ratio of 12 proteins per 10,000 lipids) were diluted to the indicated final concentrations, and TA protein insertion was quantified by autoradiography. This control experiment demonstrates that the assay is linear up to a total Get2-1sc-Cy5 concentration of ~10 nM and is not limited by the active targeting complex.
Next, we purified and fluorescently labeled a Get2-1sc construct harboring the Get1-S77C mutation (Figure S3.1). 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 total internal reflection fluorescence (TIRF) microscopy (Figure 3.3B; Figures S3.3a–S3.3d). At the lowest protein-to-lipid ratio tested, more than 80% of Get2-1sc-Cy5 was incorporated into liposomes containing only one Get2-1sc-Cy5 molecule. At the highest protein-to-lipid ratios, we observed a corresponding increase in the number of molecules per liposome, with as much as 80% of Get2-1sc-Cy5 incorporated into liposomes containing two or more Get2-1sc-Cy5 molecules. Thus, proteoliposomes reconstituted at the highest protein-to-lipid ratio contain about four times as many Get2-1sc-Cy5 molecules—that could, in principle, oligomerize—than proteoliposomes reconstituted at the lowest protein-to-lipid ratio.

To measure the specific insertion activity of the different proteoliposome reconstitutions, we normalized the total Get2-1sc-Cy5 concentration in each sample by adding the appropriate volume of empty liposomes. If oligomerized Get2-1sc-Cy5 is required for insertion, photobleaching analysis predicts that proteoliposomes reconstituted at the lowest protein-to-lipid ratio should show at least 4-fold-lower insertion activity than Get2-1sc-Cy5 reconstituted at the highest protein-to-lipid ratio. We found that the specific insertion activity was essentially independent of the number of copies of Get2-1sc-Cy5 per liposome; robust insertion was observed in proteoliposomes containing the largest proportion of single Get2-1sc-Cy5 molecules (Figures 3.3B and 3.3C; Figures S3.3e–S3.3i). Thus, we conclude that a single Get1/2 heterodimer is minimally required for TA protein insertion.

Finally, we sought structural information on how the two subunits of Get1/2 simultaneously contact Get3. Previous nuclear magnetic resonance (NMR) studies showed that isolated cytosolic fragments of Get1 and Get2 can bind simultaneously to the same side of the
Get3 dimer (Figures 3.1C and 3.4A) (Stefer et al., 2011). In this configuration, the two subunits are close, with Get1 displacing the second helix of Get2 (Figure 3.4A; Figure S3.4e). Because Get1 and Get2 are bound to the same subunit of Get3, structural modeling predicts that their relative position will remain fixed irrespective of the Get3 conformational state (Figure 3.4A). Alternatively, Get1 and Get2 might bind on opposite sides of the Get3 homodimer. In this case, the two subunits should be farther apart. Moreover, because Get1 and Get2 are bound to different subunits of Get3, the distance between them is expected to increase as the Get3 dimer changes conformation from its open to a semi-open state in response to ADP binding (Figure 3.4A) (Kubota et al., 2012, Mariappan et al., 2011, Stefer et al., 2011).

To distinguish between these two possibilities, we used single-molecule FRET as a qualitative measure of the distance between Get1 and Get2 bound to different conformational states of Get3. Here we reconstituted cytosolically labeled Get1\textsuperscript{Cy5}-Get2\textsuperscript{Cy3} into nanodiscs, yielding a more homogeneous population of single Get1/2 heterodimers (Figure S3.4) and providing a cleaner system for use in TIRF flow cells. After verifying the heterodimeric Get1/2 composition of the nanodiscs by single-molecule photobleaching, we showed that they are functional for TA protein release from Get3 (Figures S3.4c and S3.4d).

Next, we measured the distribution of FRET efficiencies between the Get1 and the Get2 cytosolic domains bound to different nucleotide states of Get3 and fit each dataset to two Gaussian distributions (Figure 3.4B). In the absence of Get3, we observed a broad distribution of low FRET states corresponding to long inter-dye distances, as expected for non-interacting cytosolic domains. Similarly, in the presence of ATP-bound Get3, we observed a broad distribution of low FRET states, consistent with the inability of Get1 to interact with ATP-bound Get3. However, when ADP-bound Get3 was added, an intermediate FRET state was observed at 53% ± 4%, and this shifted to a higher FRET state of 71% ± 2% in the presence of nucleotide-free Get3.
Figure 3.4: Get1 and Get2 bind on opposite sides of Get3 in its post-hydrolysis states. (A) Models of Get1 (magenta) and Get2 (yellow) bound to the same or opposite sides of the nucleotide-free (open; PDB: 3ZS8) and ADP-bound (semi-open; PDB: 3VLC) conformations of the Get3 homodimer (blue, green). The models are aligned on one Get3 subunit (blue) to highlight the pseudo-rigid-body swivel (gray arrows) of the other subunit (green) that accompanies ADP binding. When bound to opposite sides of Get3, Get1 and Get2 move apart in response to ADP binding; thus, the distance between donor and acceptor fluorophores on Get1-S77C-Cy5 (red) and Get2-S28C-Cy3 (green) is expected to increase when ADP is added. In contrast, when bound to the same side of Get3, the relative positions of Get1 and Get2 are fixed, because the primary contacts are made to the same subunit (blue). Thus, the distance between fluorophores is expected to remain constant as Get3 changes conformation in response to ADP binding. (B) Distributions of single-molecule FRET efficiencies (gray histograms) in nanodiscs containing cytosolically labeled Get1-S77C-Cy5 and Get2-S28C-Cy3 heterodimers during incubation with different nucleotide states of Get3. The solid black curves are the sums of the individual Gaussian functions (red lines) used to fit the raw data. In contrast with the constitutively high FRET efficiencies (>99%) predicted for same-side binding (Figure S3.4e), intermediate FRET efficiencies are observed in the nucleotide-free and ADP-bound states. Moreover, the FRET efficiency of the ADP-bound state (53% ± 4%) is less than in the nucleotide-free state (71% ± 2%), as expected for an increase in the distance between fluorophores as Get3 changes conformation from the semi-open to an open state. (C) Model of the conformation-specific interactions between Get3 and the heterodimeric Get1/2 complex.
The magnitude of the observed FRET efficiencies is inconsistent with simultaneous Get1 and Get2 binding on the same side of Get3, which would be expected to give rise to high FRET (∼99%) in the nucleotide-free and ADP-bound Get3 samples. Moreover, the observed shift from intermediate FRET in the ADP-bound state to higher FRET in the nucleotide-free state is consistent with the expected changes if Get1 and Get2 bind on opposite sides of the Get3 homodimer. Given the Förster distance of the Cy3 and Cy5 FRET pair (60 Å) (Murphy et al., 2004) and assuming that the fluorophores freely rotate at the labeling site (i.e., \( \kappa^2 = 2/3 \)), the relative change in distance between fluorophores in the nucleotide-free and ADP-bound states (∼7 Å) is in qualitative agreement with the expected distance change based on structural modeling (∼10 Å) (Figure 3.4A; Figure S3.4e). These data are most consistent with a model in which Get1 and Get2 bind simultaneously to opposite sides of the Get3 homodimer.

Discussion

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

The bulk and single-molecule FRET studies described here were performed in the absence of TA protein, because we lack an experimental means to trap a post-hydrolysis Get3-TA targeting complex that does not release TA protein to Get1/2. Nevertheless, we qualitatively extend our conclusions regarding the architecture of the Get1/2/3 complex to the targeting complex, because previous structural and biophysical analyses have shown that TA protein binding stabilizes closed conformations resembling those sampled by ATP- and ADP-bound
Get3 (Mateja et al., 2009, Mateja et al., 2015). Thus, we propose a simplified model for how docking, TA protein release and insertion, and Get3 recycling are coordinated by a Get1/2 heterodimer (Figure 3.4C).

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

**Experimental Procedures**

*DNA constructs*

Full-length Get1, Get2, and Get3 were subcloned into a pET28 derivative containing a tobacco etch virus (TEV) cleavage site between an N-terminal 6xHis tag and the polylinker, essentially as described previously (Mariappan et al., 2011; Mateja et al., 2009). A single-chain Get2-Get1 construct described previously (Wang et al., 2014) was modified for bacterial expression by fusing Get1 to Get2 with a 27-residue linker (LGAGGSEGGENLYFQSGEGGTSGATS), 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 DHFR 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, purification, and labeling**

Get3 was expressed and purified as described previously (Mateja et al., 2009). Get1 and Get2 were individually expressed in E. coli Ros2(DE3)/pLysS (Novagen) cells. All growth media was supplemented with 50 μg/mL kanamycin (TCI) and 34 μg/mL chloramphenicol (EMD). Fresh, single colonies from LB/agar plates were grown in 3 mL TB (Fisher) precultures until OD$_{600}$=0.5-1, and then 1 mL of this preculture was used to inoculate 500 mL of prewarmed, homemade TB autoinduction medium (Studier, 2005) in a 2.8 L baffled glass flask. After 18 hr at 37°C and 250 rpm, cells were harvested in a JLA-8.1 rotor at 6,000 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 MgAc2). 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 hours, the suspension was spun for 40 minutes in a Ti45 rotor at 35,000 rpm at 4°C. The detergent-soluble supernatant was batch purified by gently incubating with a 3 mL bed volume of Ni-NTA resin (Qiagen) at 4°C for 40 minutes. After removing the flow-through, the Ni-NTA resin was exchanged into a new detergent by washing with 20 column volumes of Buffer A supplemented with 20 mM imidazole and 0.1% n-Undecyl-β-D-Maltopyranoside (UM, Anatrace) for Get2 or 0.1% Fos-Choline-12 (FC12, Anatrace) for Get1. The protein was eluted from the resin with 5 column volumes of the same buffer supplemented
with 200 mM imidazole. For cysteine-containing mutants, wash and elution buffers were supplemented with 1 mM TCEP. After elution, 1 mM EDTA was added, and the elution was concentrated in an Amicon centrifugal filter (Millipore) (30 kDa MWCO for Get1 in FC12, and 50 kDa MWCO for Get2 in UM). Concentrated protein was further purified by gel filtration using a Superdex 200 10/300 column (GE Healthcare) equilibrated in 50 mM Hepes-KOH, pH 7.5, 200 mM NaCl, 5% glycerol, 0.1% detergent, and 1 mM TCEP. Peak fractions were concentrated to 50-100 µM for Get1, and 20-50 µM for Get2. Aliquots were flash frozen in liquid nitrogen and stored at -80°C. Typical yields were between 5 and 20 mg of purified protein per liter of culture.

Single-chain Get2-Get1 (Get2-1sc) was expressed in LOBSTR-BL21(DE3)-RIL cells, a gift from Thomas U. Schwartz (MIT) (Andersen et al., 2013). All growth media was supplemented with 50 µg/mL kanamycin and 34 µg/mL chloramphenicol. A single colony was transferred to a 50 mL TB preculture shaking at 250 rpm at 37°C. The TB for Get2-1sc 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 OD600=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 OD600=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 hours 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 Mg(OAc)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 hours. This suspension was spun for 1 hour 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.

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.

**Table 3.1: Labeling efficiencies for Get1, Get2 and Get2-1sc**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Cysteine Mutation</th>
<th>Cy5 Labeling Efficiency</th>
<th>Cy3 Labeling Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Get2-1sc</td>
<td>S77C (on Get1)</td>
<td>80%</td>
<td>N/A</td>
</tr>
<tr>
<td>Get1</td>
<td>S77C</td>
<td>70%</td>
<td>71%</td>
</tr>
<tr>
<td>Get1</td>
<td>A95C</td>
<td>49%</td>
<td>46%</td>
</tr>
<tr>
<td>Get2</td>
<td>S28C</td>
<td>67%</td>
<td>66%</td>
</tr>
<tr>
<td>Get2</td>
<td>E220C</td>
<td>59%</td>
<td>71%</td>
</tr>
</tbody>
</table>

*Get1/2 pull-down in different detergents*

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

*Multi-angle laser light scattering*

The absolute molecular mass of the Get1/2 complex in UM was determined by static multi-angle laser light scattering (MALLS). 10 nmol of His-Get1 and His-Get2, purified in FC12 and UM respectively, was diluted into 3 mL UM buffer (50 mM Hepes, pH 8, 200 mM NaCl, 0.1% UM), diluting the FC12 left from the His-Get1 stock 30x. The sample was concentrated to 200 µL in a 50 kDa MWCO Amicon, and then diluted again with UM buffer to a final volume of 3 mL. After concentrating to 100 µL, the sample was loaded into a Superdex 200 10/300 column equilibrated with UM buffer. The column was coupled to an online UV detector (UPC-900, GE Healthcare), static light scattering detector (Dawn HELEOS II, Wyatt Technology), and a
refractive index detector (Optilab rEX, Wyatt Technology). Complex mass and protein conjugate analysis was calculated using ASTRA software (Wyatt Technology). ExPasy was used to calculate extinction coefficients; dn/dc for UM (0.1506 mL/g) is from Anatrace, and the dn/dc for Get1/2 (0.1872) was calculated based on its sequence (Zhao et al., 2011).

Proteoliposome reconstitutions

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 were prepared by diluting protein to 1 µM in reconstitution buffer (50 mM Hepes-KOH, pH 7.5, 150 mM KOAc, 10% sucrose, 0.01% NaN₃, 1 mM DTT, 0.225% DBC) and incubating on ice for 30 minutes. This protein mixture was further diluted to the desired concentration into 90 µL of cold reconstitution buffer in a 0.2 mL PCR tube. 10 µL of liposomes (20 mg/mL) was immediately added and gently mixed by pipette. After 15 minutes on ice, 25-30 mg of activated biobeads (Bio-Rad) were added, followed by a very brief spin on a tabletop centrifuge. After gently revolving on a wheel at 4°C overnight, the supernatant was removed from the biobeads by pipette and centrifuged at 20,000 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 hours 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 x 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 KOAc, 1 mM
DTT buffer and incubated in the 15 μL flow cells for 3 minutes followed by a 50 μL wash with buffer containing 10 mM DTT and 2 mM partially oxidized trolox as triplet state quenchers, as previously described (Cordes et al., 2009). Samples were imaged using Total Internal Reflection Fluorescence (TIRF) microscopy, and 500 frame videos were recorded using a 200 ms exposure.

Fluorescent spots were selected from the first video frame by applying a Laplacian of Gaussian filter with a scale parameter of 150 nm. Peaks were identified by thresholding and non-maximum suppression over a 360 nm² 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 Figure 3.3B and Figure S3.3d (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 (Figure S3.3b) to confirm that steps were accurately counted, similar to previously reported photobleaching controls (Jain et al., 2014).

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

Yeast microsomes

Yeast microsomes were prepared essentially as described previously (Mariappan et al., 2011). A Get3 knock-out strain of S. cerevisiae (Open Biosystems) was grown at 32°C in YPD supplemented with 15 µg/mL Kanamycin to a final OD600= 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 Hapes-KOH, pH 7.5, 100 mM KOAc, 2 mM Mg(OAc)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 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 35S-Methionine and 25 μM purified Get3. After 2.5 hours 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 KOAc, 7 mM Mg(OAc)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 OD280=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 3.2B). 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 3.2B).

The normalized proteoliposomes were then diluted 2× with insertion buffer (50 mM Hepes-KOH, pH 7.5, 150 mM KOAc, 7 mM Mg(OAc)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
2× loading dye (2:1:1 4× 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 6×His-tagged construct of membrane
scaffold protein (MSP) was purchased from Addgene (plasmid 20066). MSP was expressed,
purified, and TEV cleaved as described previously (Alvarez et al., 2010). Cleaved protein was
dialyzed against buffer (50 mM Hepes, pH 8.5, 150 mM NaCl) and then biotinylated using 4
equivalents of NHS-PEG4-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 hour in a room temperature bath until the
suspension appears homogeneous, a translucent milky white. Next, two equivalents of UM
detergent were added followed by 30 minutes of sonication at room temperature. Successively,
0.2 eq of UM was added followed by 10 minutes of sonication until the solution was completely
clear. This solution was diluted with buffer to a final lipid concentration of 10 μM and stored in
aliquots at -80°C. The concentration of UM in this stock is typically 25-30 mM.

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

Next, biobeads, in an amount weighing 10% of the total reconstitution volume, were added to the reconstitution. The mixture was gently revolved overnight on a wheel at 4°C. After removing the biobeads by pipette the supernatant was spun at 20,000 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 Heps, 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 Heps-KOH, pH 7.5, 200 mM NaCl, 1 mM DTT) and shows a single peak that contains His-Get1, Get2, and MSP in a ratio of 1:1:2 (quantified by stain-free SDS-PAGE and ImageJ). Nanodiscs were used immediately after reconstitution or flash frozen in aliquots after dialysis against buffer containing 10% sucrose.

TA substrate release assay

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

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

The substrate release assay was performed as previously described (Wang et al., 2014). In brief, 2 μL of affinity-purified Get3-FLAG-Sec22-opsin targeting complex was mixed with 30 nM Get1/2 nanodiscs or microsomes and incubated at room temperature for 30 minutes. Samples were then incubated with 0.5 mM disuccinimidyl suberate (Pierce) at room temperature for an additional 30 minutes. As a positive control for Sec22-opsin release for Get3-FLAG, 2 μL of GET1E166C-FLAG microsomes (OD280 = 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 efficiency, $E$, was determined as described previously (Roy et al., 2008), to be $1.5 \pm 0.09$, 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 = $1/(1+Y^*(A/D))$.

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

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

Statistical methods

Errors are reported ± SEM. The bulk FRET experiments in Figure 3.2 and Figure S3.2 combine data from three independent proteoliposome reconstitutions for each Get1/2 fluorophore combination. The single-molecule photobleaching and insertion data in Figure 3.3
are from the same reconstitution, one Get2-1sc-Cy5 proteoliposome reconstitution for each condition, and are representative of multiple experiments (>3). Figure S3.3 shows variance of the insertion assay by splitting each reconstitution into triplicates. The single-molecule FRET distributions in Figure 3.4 were fit to two Gaussian curves using the mle package in R.

**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.

**Author Contributions**

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

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**References**


Chapter 4: Overview of the cytoprotectant small molecule Retro-2

Eukaryotic cells use vesicle transport to exchange information and material with their surroundings. This normally beneficial process can also lead to pathogenesis by many toxins and pathogens that take advantage of it to either gain entry into the cytoplasm or to replicate. First, identified from a high throughput screen for inhibitors of ricin cytotoxicity, Retro-2 and its analogs (referred here collectively as Retro-2) have emerged as compounds that confer broad cytoprotection against a variety of cellular threats dependent on vesicle transport (Stechmann et al., 2010, Gupta et al., 2017) (Figure 4.1).

Figure 4.1: The chemical structure of the bioactive form of Retro-2.

Retro-2 was originally identified due to its ability to protect cells from the plant toxin ricin and bacterial Shiga (as well as Shiga-like) and cholera toxins (Stechmann et al., 2010). These toxins bind to the cell surface, are endocytosed, and routed by retrograde vesicles to the endoplasmic reticulum (ER) via the early endosome and trans-Golgi network (TGN) (Mallard et al., 1998, Spooner et al., 2006). Once in the ER, they retro-translocate into the cytosol, where they exert their action on their targets. The retrograde route for Shiga and cholera toxins is dependent on the Syntaxin 5 (STX5)/GS28/Ykt6/GS15 and the Syntaxin 16 (STX16)/Syntaxin 6 (STX6)/Vit1a/VAMP4 SNARE complexes, and the GTPase Arl1 and its effector Golgin-97 (Lu et al., 2004, Amessou et al., 2007). Ricin also traverses through the early endosome to reach the Golgi on the STX16-dependent pathway (Iversen et al., 2001, Moreau et al., 2011). For all these toxins, pre-treatment with Retro-2 blocks retrograde transport to the TGN, trapping the toxins in the endosome (Stechmann et al., 2010, Noel et al., 2013).
Retro-2 also interferes with the infection of non-enveloped viruses. The cellular entry of papillomaviruses (such as HPV16) depends on endocytosis and a retromer-mediated path from early endosomes to the TGN before further transport to the ER (Day et al., 2003, Lipovsky et al., 2013, Zhang et al., 2014) In contrast, polyomaviruses (such as JCPyV, BKPyV, and simian virus 40) also enter cells by endocytosis, but traffic from the early endosome to the ER using a retromer-independent route that bypasses the TGN (Norkin and Kuksin, 2005, Maginnis et al., 2015). Despite taking two different retrograde trafficking pathways, the transport of both papillomaviruses and polyomaviruses to the ER is inhibited by Retro-2 (Nelson et al., 2013, Carney et al., 2014, Maru et al., 2017). Moreover, adeno-associated virus (AAV) capsids traffic from the early endosome to the Golgi apparatus, where they escape into the cytoplasm (Bantel-Schaal et al., 2002). This retrograde route is sensitive to STX5 knockdown, but independent of the retromer and the SNAREs STX16 and STX6 (Nonnenmacher et al., 2015). Treatment with Retro-2 disrupts AAV transport to the TGN and decreases transduction (Nonnenmacher et al., 2015).

Retro-2 has also been shown to inhibit infection with enveloped viruses. Retro-2 has an antiviral effect against Herpes simplex virus 2 (HSV-2), possibly by interfering with capsid membrane envelopment and tegument protein (a component of the space between viral capsid and envelope) acquisition which occurs at the TGN (Henaff et al., 2012, Dai et al., 2018). Furthermore, Retro-2 inhibits infection with human cytomegalovirus (HCV) by interfering with the assembly of the cytoplasmic viral assembly compartment (cVAC). During a HCV infection, the endosomal system and the Golgi are extensively reorganized into the cVAC, where newly-synthesized capsids undergo tegumentation and envelopment. This reorganization is dependent on the host cell’s Rab GTPases and SNARE proteins (Lučin et al., 2018, Henaff et al., 2012). Retro-2 decreases STX5 levels and leads to the loss of the STX5 at the cVAC (Cruz et al., 2017). This results in the altered formation of the cVAC and inhibits the formation of infectious virions.
Additionally, Retro-2 inhibited the spread of poxviruses (such as vaccinia (VACV) and monkeypox viruses). Poxviruses depend on a variety of retrograde transport factors, such as the GARP complex and STX6, for viral spread; when these factors are depleted, the viral particles are not wrapped with membranes and are consequently retained within the host cell (Sivan et al., 2016). Likewise, Retro-2 prevents membrane wrapping in vaccinia virus, leading to retention of viral particles within the host cell (Sivan et al., 2016, Harrison et al., 2016). Additionally Retro-2 was effective against RNA viruses, inhibiting progeny release of Enterovirus 71, and blocking cellular entry of certain filoviruses (Dai et al., 2017, Shtanko et al. 2018).

Retro-2 is also effective against intracellular pathogens. Leishmania parasites reside in membrane-bound compartments called Leishmania parasitophorous vacuoles (LPVs), which are derived from endocytic/secretory vesicles (Russell et al., 1992, Canton et al., 2012). Treatment with Retro-2 blocks Leishmania infection by preventing the LPV from maturing into its fully distended size (Canton and Kima, 2012, Craig et al., 2017). Surprisingly, Retro-2 also directly blocks Leishmania parasite replication in the absence of host cells. (Canton and Kima, 2012). Similarly, Simkania negevensis is an obligate intracellular gram-negative bacterium, related to pathogenic Chlamydia, that dwells within a host-derived membrane-bound compartment called an inclusion to avoid lysosomal degradation while it replicates (Fields and Hackstadt, 2002, Herweg et al., 2016). The parasite acquire lipids for growth by interception of Golgi-derived sphingomyelin-rich vesicles (Hackstadt et al., 1995). Retro-2 interfered with inclusion formation and lipid acquisition, leading to reduced progeny formation (Herweg et al., 2016).

The anti-toxic and anti-viral in vitro effects of Retro-2 extend to whole organisms. Retro-2 can be tolerated in high doses and was shown to rescue mice from lethal doses of ricin and protecting mice from infection with Shiga toxin-producing E. coli O104:H4, polyomavirus, VACV, and enterovirus 71 (Stechmann et al., 2010, Secher et al., 2015, Maru et al., 2017, Harrison et
The potential therapeutic applications of Retro-2, therefore, drive an impetus to ascertain the cellular target of the small molecule.

While the mechanism of action of Retro-2 is not well-understood, one notable phenotype of Retro-2 treatment is the change in localization of STX5 and less acutely STX6 (Stechmann et al., 2010, Gupta et al., 2017). STX5 is a target-SNARE found as a part of the STX5/GS27/Sec22/Bet1 complex at the cis-Golgi-ER interface, where it regulates the early secretory pathway by mediating ER to Golgi transport and the assembly of pre-Golgi intermediates (Dascher et al., 1994, Hay et al., 1997, Rowe et al., 1998) STX5 is also a component of the STX5/GS28/Ykt6/GS15 complex on Golgi membranes that enables retrograde transport from the early/recycling endosomes to the TGN (Zhang and Hong, 2001, Tai et al., 2004, Bonifacino and Rojas, 2006, Amessou et al., 2007). Additionally, STX5 interacts with both NSF- and p97-mediated membrane fusion pathways that assemble the stacked cisternae of the Golgi apparatus (Rabouille et al., 1998, Uchiyama and Kondo, 2005, Meyer, 2005). Consequently, knockdown of STX5 leads to a variety of phenotypes, including disruption of Golgi morphology and inhibition of retrograde transport (Suga et al., 2005, Amessou et al., 2007). Loss of STX5 function may account for some of Retro-2’s anti-toxin and anti-viral effects but the compound’s remarkable efficacy against such a broad range of other pathogens implies that STX5 is just one effector branch of its MOA rather than its sole, direct target. Determining the actual cellular target of Retro-2 remains an important future goal that should facilitate ongoing pharmaceutical efforts to develop this compound into a drug.

References


Noel, R., Gupta, N., Pons, V., Goudet, A., Garcia-Ca
dihydroquinazolinone derivatives of Retro-2 with enhanced efficacy against Shiga toxin. J. Med. Chem. 56, 3404-3413.


Chapter 5: Retro-2 protects cells from ricin toxicity by inhibiting ASNA1-mediated ER targeting and insertion of tail-anchored proteins

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Summary

The small molecule Retro-2 protects cells from ricin through a poorly-defined mechanism of action (MOA), which involves halting retrograde vesicle transport of endocytosed ricin toxin to the endoplasmic reticulum (ER). We discover by CRIPSRi genetic interaction analysis that Retro-2 cell treatment resembles genetic disruption of the transmembrane domain recognition complex (TRC) pathway, which mediates post-translational ER targeting and insertion of tail-anchored proteins, including SNAREs required for retrograde vesicle transport. Cell-based and in vitro assays reveal a MOA in which Retro-2 blocks the delivery of newly-synthesized tail-anchored proteins to the ER targeting factor ASNA1 (TRC40). A point mutant of ASNA1 identified using a CRISPR-mediated mutagenesis screen abolishes both the cytoprotective effect of Retro-2 against ricin and its inhibitory effect on ASNA1-mediated ER targeting. Together, our work argues that Retro-2 blocks retrograde trafficking of ricin by indirectly disrupting vesicular transport machinery, describes a general CRISPR strategy for predicting the MOA of small molecules, and paves the way for drugging the TRC pathway to treat broad classes of viruses known to be inhibited by Retro-2.

Introduction

Eukaryotic cells destroy many endocytosed pathogens by targeting them to the lysosome. The plant toxin ricin, however, evades this fate by being sorted into early endosome-derived vesicles destined for fusion with the trans-Golgi network (TGN). Following further
retrograde transport from the Golgi to the endoplasmic reticulum (ER), ricin retrotranslocates into the cytosol where it catalytically disables ribosomes, halting translation and killing the cell (Bassik et al., 2013; Crispin et al., 2009; Johannes and Popoff, 2008; Lord et al., 2003; Sandvig and van Deurs, 2005). Recently, a high-throughput screen of small molecules identified Retro-2 as an inhibitor of ricin and Shiga-like toxins that halts their retrograde progression to the ER by trapping them in the early endosome (Stechmann et al., 2010). Remarkably, Retro-2 was able to rescue mice from lethal doses of ricin and was tolerated at high doses. Subsequent studies of the bioactive cyclic derivative of Retro-2 (hereafter Retro-2) (Nelson et al., 2013; Park et al., 2012) and its analogs have also revealed the ability of these compounds to interfere with infection of papillomaviruses (such as HPV16) (Lipovsky et al., 2013) and polyomaviruses (such as JCPyV, BKPyV, and simian virus 40) (Nelson et al., 2013), which are known to rely on retrograde transport during infection.

While the mechanism of action (MOA) of Retro-2 is not well understood, Retro-2 has been shown to affect the localization of Syntaxin 5 (STX5) and less acutely Syntaxin 6 (Stechmann et al., 2010). STX5 – a target-SNARE – is required for fusion of retrograde vesicles with the TGN, and knockdown of its expression is sufficient to trap ricin in the early endosome (Amessou et al., 2007; Bennett et al., 1993; Dascher and Balch, 1996; Norlin et al., 2016; Suga et al., 2005). Most SNAREs like STX5 are tail-anchored (TA) proteins – a class of membrane proteins with a single, C-terminal transmembrane domain (TMD) that are post-translationally targeted to the ER for insertion by the transmembrane domain recognition complex (TRC) pathway (Cho et al., 2018; Denic, 2012; Hegde and Keenan, 2011). Notably, our previous genome-wide CRISPR/Cas9 deletions screens have identified STX5 and its ER targeting factor, ASNA1 (also known as TRC40) among the top hits that confer resistance to ricin (Morgens et al., 2017).

A variety of genetic approaches have been used in mammalian cells to successfully define the MOA of novel small molecules with therapeutic potential (Acosta-Alvear et al., 2015;
Deans et al., 2016; Jost and Weissman, 2017; Matheny et al., 2013; Pabon et al., 2018; Sidrauski et al., 2015). In yeast, novel small molecules have been studied by measuring the extent to which the small molecule phenotypes are modified by the presence of a defined, single mutation across the yeast genome. The resulting compound’s genetic profile is analogous to a gene’s genetic profile, obtained by measuring a particular gene's mutant phenotype in the presence of many second mutations. Remarkably, the genetic profile of the compound often resembles the genetic profile of the compound’s target, allowing for target identification (Costanzo et al., 2010; Giaever et al., 1999; Hillenmeyer et al., 2008; Hoepfner et al., 2014; Lee et al., 2014; Parsons et al., 2004, 2006; Simpkins et al., 2018; Wildenhain et al., 2015). Here, we developed a conceptually analogous genetic profiling approach in mammalian cells and applied it to Retro-2. Analysis of single and paired-gene CRISPRi screens revealed a robust link between Retro-2 and TA protein biogenesis mediated by the TRC pathway. Flow cytometry and quantitative cell microscopy showed that Retro-2 destabilizes a fluorescent TA protein reporter and induces a decrease in endogenous STX5 abundance at the Golgi. Targeted mutagenesis of the ASNA1 genomic locus using a dCas9-AID*Δ fusion (CRISPR-X) identified a point mutant that suppressed the ability of Retro-2 to protect cells from ricin and to interfere with TA protein biogenesis. Finally, biochemical reconstitution with purified components demonstrated that Retro-2 blocked TA protein delivery to the ER targeting factor ASNA1 (TRC40). Collectively, these findings support a model in which Retro-2 directly inhibits ASNA1, leading to inefficient ER targeting of TRC pathway clients such as STX5, which ultimately prevents retrograde trafficking of ricin and protects the cell.

**Results**

*Genetic profiling reveals that Retro-2 treatment resembles TRC pathway inhibition*

Previously, potential drug targets have been defined in yeast by looking for correlations between the chemical-genetic profile of a drug and that of its target (Costanzo et al., 2010; Giaever et al., 1999; Hillenmeyer et al., 2008; Hoepfner et al., 2014; Lee et al., 2014; Parsons et
al., 2004, 2006; Simpkins et al., 2018; Wildenhain et al., 2015). To obtain a chemical-genetic profile of Retro-2, we used CRISPRi to measure the effect of Retro-2 on the ricin phenotypes of 288 hits from a previous genome-wide shRNA screen in the human leukemia cell line K562 (Bassik et al., 2013). Using established CRISPRi sgRNA designs (Horlbeck et al., 2016), we created a lentiviral library comprising 10 sgRNAs per gene along with 2,000 non-targeting and safe-targeting controls (Morgens et al., 2017), which we installed into K562 cells engineered to express dCas9-KRAB (Gilbert et al., 2014). We then grew infected K562 cells in replicate in the presence of Retro-2 or in the presence of both Retro-2 and ricin (Figure 5.1A). Additional untreated and ricin-only replicates were included as controls. We then used casTLE to compare the enrichment of sgRNAs between conditions (Morgens et al., 2016), measuring the ricin phenotype of each gene knockdown in the absence and presence of Retro-2, as well as the effect of the knockdown on the activity of Retro-2 (Figure S5.1a-c). The ricin phenotypes of 288 gene knockdowns in the presence of Retro-2 yielded a genetic profile of Retro-2, which we compared to profiles of candidate genes, as described below.

To measure genetic profiles for candidate genes, we performed a paired-gene CRISPRi screen (Figure 5.1B). Using our previously established paired-guide platform (Han et al., 2017), we constructed a library containing CRISPRi guides targeting 105 × 105 pairs of genes with three guides per gene. We chose 100 of these genes based on having the strongest effect on the activity of Retro-2 (Figure S5.1c). Since ASNA1 was one of the top genes in this unbiased group, we also included an additional five genes comprising four additional TRC pathway components (GET4, WRB, CAMLG, and SGTA) and STX5, a TRC pathway substrate that has been implicated in the MOA of Retro-2 (Stechmann et al., 2010). Following installation of this library into K562 cells expressing dCas9-KRAB (Gilbert et al., 2014), we grew the pool of infected cells in replicate in the presence or absence of ricin and monitored the phenotype of the double knockdowns by comparing the enrichment of paired guides between conditions using
casTLE (Morgens et al., 2016). For each of the 105 genes included, these double phenotypes represent a genetic profile that we compared to the genetic profile of Retro-2 measured in the single-guide CRISPRi screen (Figure 5.1A,C; Figure S5.1b,c); this allowed us to identify genes whose knockdown most closely resemble Retro-2 treatment.

Figure 5.1: Single and paired-gene CRISPRi screens implicate TRC pathway inhibition as the MOA of Retro-2.
Figure 5.1: (Continued). (A) Schematic of single-gene CRISPRi screen. A 288 gene library with 10 guides per gene targeting previously identified ricin hits and 2000 negative controls was lentivirally infected into a K562 cell line expressing a dCas9-KRAB fusion. The pool was then grown in replicate in the presence of 10µM Retro-2 and presence or absence of 2.5 ng/µL ricin. The ricin phenotypes of the gene knockdowns in the presence of Retro-2 yielded a genetic profile of Retro-2. (B) Schematic of paired-gene CRISPRi screen. A library of 105 × 105 genes with 3 guides per gene and 50 negative controls were lentivirally infected into a K562 cell line expressing a dCas9-KRAB fusion. The pool was then grown in replicate in the presence or absence of ricin. For each of the genes included, the ricin phenotype of the double knockdowns represent a genetic profile. (C) Summary of paired-guide screen results. The genetic profile of each gene in the paired-gene CRISPRi screen i.e. the ricin phenotype of each other gene in that background – was correlated (Pearson) with the genetic profile of Retro-2 i.e. the ricin phenotype of each gene in the presence of Retro-2 as measured in the single-gene CRISPRi screen. The x-axis is the rank of the Pearson correlation coefficient. The y-axis is the negative log10 p-value of the correlation. The top four ranked genes are labeled and highlighted in red. (D) Schematic of the TRC pathway with the candidates from the single and pair-gene CRISPRi screens highlighted in color.

The genes whose genetic profile correlated most strongly with the profile of Retro-2 were WRB, STX5, SGTA, and GET4 (Figure 5.1C; Figure S5.1d-f). Notably, the genetic profiles of ASNA1 and CAMLG were uninformative (and did not correlate with Retro-2) (Figure S5.1f); this is likely because the guides targeting these genes individually had extremely protective ricin phenotypes (independent of the second guide), suggesting either that their phenotypes cannot be modified or the variance is outside the dynamic range of this experiment. Regardless, our analysis revealed that Retro-2 treatment closely resembles genetic perturbation of the TRC pathway (Figure 5.1D). On the basis of this finding, we hypothesized that Retro-2 protects cells against ricin by inhibiting the TRC pathway, interfering with ER targeting and insertion of TA proteins, including the SNARE STX5. This in turn disrupts retrograde transport of ricin to the ER and subsequently protects against toxicity. We proceeded to test three predictions of this working model for the MOA of Retro-2: 1) Retro-2 treatment should disrupt biogenesis of TRC pathway substrates, 2) mutations in the relevant TRC pathway component should confer resistance to Retro-2, and 3) Retro-2 should disrupt a specific step along the TRC pathway.

Retro-2 inhibits targeting of newly-synthesized TRC pathway substrates

To test whether Retro-2 disrupts the biogenesis of TRC pathway substrates, we exploited the molecular triage decision that channels newly-synthesized TA proteins that are not
efficiently delivered to ASNA1 to degradation (Shao et al., 2017). To examine if Retro-2 induces
this form of TA protein instability, we adapted an established approach (Chitwood et al., 2018;
Guna et al., 2018) by constructing a doxycycline (dox)-inducible cassette expressing green
fluorescent protein (GFP) linked by a self-cleaving P2A peptide to a red fluorescent protein
(RFP) fused to a C-terminal SEC61B TMD sequence (GFP-2A-RFP-SEC61B_{TMD}) (Figure 5.2A).
When expressed in cells, the RFP-SEC61B_{TMD} fusion protein will be inserted into the ER
membrane as a TA protein, but failed insertion will result in its degradation and loss of RFP
signal. We then lentivirally delivered GFP-2A-RFP-SEC61B_{TMD} into wildtype and ASNA1
knockout (ASNA1^{KO}) HEK293T cells (Figure S5.2a). As expected, we observed by cytometry
significantly lower RFP signal (relative to GFP) in the ASNA1^{KO} cells following dox treatment,
confirming the dependence of the reporter on the TRC pathway (Figure 5.2B). Strikingly, pre-
treatment of wild-type cells with either Retro-2 or a hyperactive analog of Retro-2, DHQZ36.1
(Craig et al., 2017), resulted in a comparable RFP:GFP ratio decrease that was not further
aggravated by the additional absence of ASNA1 (Figure 5.2B; Figure S5.2b,c). In contrast, we
detected no significant reporter changes when we treated cells with an inactive analog of Retro-
2, DHQZ5 (Carney et al., 2014) (Figure S5.2b) or when the reporter lacked a transmembrane
domain (Figure 5.2B).
**Figure 5.2: Retro-2 diverts newly-synthesized TA proteins from ER targeting to degradation.** (A) Schematic of the dual-colour reporter consisting of a self-cleaving P2A peptide between a GFP and a RFP with a C-terminal TMD. Genetic and chemical perturbations to targeting pathways will promote destabilization of RFP-TATTMD, as TA proteins are diverted for degradation by accessory TRC pathway components (not shown) if they are not efficiently captured by ASNA1. (B) Wildtype and ASNA1KO HEK293T cell lines with indicated reporters were pre-treated with 3 µM DHQZ36.1 for 1 hour prior to induction with dox for approximately 18 hours and FACS analysis. Shown are bar graphs of reporter RFP to GFP ratios with standard deviations derived from three experiments as relative means to their corresponding mock-treated wildtypes.
Figure 5.2: (Continued) (C) Schematic of the post-translational and co-translational ER targeting of RFP-SEC61B\textsubscript{TMD} and RFP-SEC61B\textsubscript{TMD}-BFP, respectively. (D) Cells with indicated genotypes and reporters were treated and analyzed as in part (B).

We tested the prediction that the sensitivity of the TMD to DHQZ36.1 is not solely determined by its amino acid sequence but additionally relies on its positional context within the protein. Previous \textit{in vitro} work with SEC61B showed that recognition by ASNA1 can be abolished by appending a C-terminal sequence, leading to preferential TMD engagement by the signal recognition particle (SRP) on the ribosome (Stefanovic and Hegde, 2007). To switch the ER targeting specificity of SEC61B TMD in cells by a similar approach, we fused the blue fluorescent protein (BFP) to the C-terminus of GFP-RFP-SEC61B\textsubscript{TMD} (GFP-2A-RFP-SEC61B\textsubscript{TMD}-BFP) and confirmed that the resulting co-translational reporter was no longer destabilized in ASNA1\textsuperscript{KO} cells (Figure 5.2C). Consistent with our model, this modification also conferred reporter resistance to DHQZ36.1 in both wildtype and ASNA1\textsuperscript{KO} cells (Figure 5.2D).

To test our model on the endogenous STX5 protein, we measured STX5 levels at the Golgi upon Retro-2 treatment or genetic perturbation of ASNA1. First, we established HeLa cells expressing either shRNAs targeting ASNA1 or scrambled negative control shRNAs and demonstrated that genetic inhibition of the TRC pathway conferred resistance to ricin toxicity that was comparable to that caused by Retro-2 (Figure S5.3a). As expected given the dependence of STX5 on the TRC pathway (Rivera-Monroy et al., 2016) and the effect of Retro-2 on STX5 localization (Stechmann et al., 2010), immunofluorescence and confocal microscopy revealed a reduction in STX5 fluorescence density at the Golgi upon Retro-2-treatment of control cells that was comparable to that observed in untreated ASNA1 knockdown cells (Figure 5.3A,B; Figure S5.3c). Combining Retro-2 treatment with ASNA1 knockdown resulted in a more severe loss of STX5 at the Golgi (Figure 5.3A,B; Figure S5.3c). We also monitored the activity of the hyperactive Retro-2 analog DHQZ36.1 (Craig et al., 2017) and observed that it caused a severe loss of Golgi STX5 similar to Retro-2-treated ASNA1 knockdown cells (Figure 5.3B; Figure S5.3b,c). Taken together, our fluorescent protein stability reporter and cell microscopy
data argue that Retro-2 and DHQZ36.1 disrupt biogenesis of TRC pathway clients leading to their enhanced degradation, which could explain their effect on STX5 abundance at the Golgi.

Figure 5.3: ASNA1 knockdown and Retro-2 treatment both decrease the abundance of Golgi-localized STX5, an ASNA1 substrate. (A) HeLa cells expressing either ASNA1-targeting or scrambled control (shCtrl) shRNAs were treated for 24 hours with DMSO or 10 µM Retro-2 before fixation and staining for STX5, a Golgi marker (GM130), and a nuclear marker (DAPI). Shown are maximal signal projections of z-stacked confocal micrographs taken with a 100× objective and made without contrast or LUT adjustments. Scale bar represents 25 µm and applies to all images. (B) HeLa cells were treated as in part a) but including additional shRNAs and the hyperactive Retro-2 analog DHQZ36.1. Images were collected using a 60× objective and quantitatively analyzed. Shown are box plots of per cell mean STX5 intensity at GM130-marked Golgi for the indicated treatments. Asterisks specify significant differences between treatments as calculated by the MW U test.

CRISPR-X screen identifies point mutants of ASNA1(TRC40) resistant to Retro-2

To test if the TRC pathway is the functional target of Retro-2 in cells, we attempted to identify mutations in ASNA1 that result in resistance to the compound. We noted that ASNA1 knockdown sensitizes cells to growth inhibition by high concentrations of Retro-2 even in the absence of ricin (Figure S5.3a) and that high doses of DHQZ36.1 are toxic to cell growth even in wildtype cells (Figure S5.3b). Along with our previous result that ASNA1 is an essential gene for cell growth in CRISPR/Cas9 screens (Morgens et al., 2017), these observations suggested
that the toxicity of Retro-2 and DHQZ36.1 may be mediated by strong inhibition of ASNA1. We used this toxicity to select for ASNA1 alleles resistant to growth inhibition by DHQZ36.1 by first randomly mutagenizing the ASNA1 coding region with CRISPR-directed diversification. Here, a library of sgRNAs tiling the ASNA1 coding region was lentivirally infected into a K562 cell line stably expressing an N-terminal, dCas9-AID*Δ fusion (CRISPR-X) (Hess et al., 2016), which results in a high frequency of diverse point mutations where it is recruited to a specific genomic locus. We then grew the mutagenized cell population in the presence of toxic doses of DHQZ36.1 for ~4 weeks and deep sequenced the ASNA1 cDNA to identify variant ASNA1 alleles (Figure 5.4A). By this method, we detected a number of distinct ASNA1 mutations which appeared in a moderately high fraction of selected cells relative to the starting populations (Figure 5.4B; Figure S5.4a). The most common allele in both replicates, C460T, results in an alanine to valine coding change (A149V), and we proceeded to test in multiple assays if this mutant represents a bona fide resistance allele.

First, we specifically introduced the A149V mutation into a Cas9-expressing K562 line using CRISPR-mediated homologous recombination to modify the endogenous ASNA1 locus (Figure S5.4b). Confirming our screen results, cells containing this allele were resistant to the toxicity of DHQZ36.1, whereas cells containing a synonymous control mutation (C461T; A149A) were not (Figure S5.4c). More critically, we then tested whether the A149V ASNA1 allele conferred resistance to the protective activity of Retro-2 against ricin. Indeed, the A149V mutation significantly compromised the ability of Retro-2 to protect cells from ricin (Figure 5.4C; Fig. S5.4d) where again the synonymous A149A allele had little effect.

Lastly, we analyzed the effect of the A149V mutation on the ability of Retro-2 to interfere with ER targeting as measured by our two-color GFP-2A-RFP-SEC61B_{TMD} reporter. To this end, we first established that we could complement the reporter phenotype in ASNA1^{KO} HEK293T cells by expressing wildtype ASNA1 but not the ATPase-dead version (ASNA1 D74N), which is unable to engage the membrane-associated steps of the TRC pathway (Mateja et al., 2015;
Norlin et al., 2018; Wang et al., 2011). Notably, Retro-2 was still able to induce reporter destabilization in complemented cells. By contrast, even though expression of the A149V ASNA1 mutant only resulted in partial complementation (Figure 5.4D), it conferred full reporter protection from Retro-2. In sum, our analysis of the A149V ASNA1 mutant demonstrates that the allele is resistant to the ability of DHQZ36.1 to inhibit cell growth, the ability of Retro-2 to protect cells from ricin, and the ability of Retro-2 to disrupt ER targeting of a TA reporter. These data provide further support for our model that Retro-2/DHQZ36.1 interferes with the TRC pathway, possibly by inhibiting ASNA1 directly.

Figure 5.4: Isolation and characterization of A149V ASNA1, a Retro-2-resistance allele.
Figure 5.4: (Continued). (A) Schematic of ASNA1 mutagenesis by CRISPR-X. A 172 sgRNA library tiling the ASNA1 coding region was lentivirally infected in a K562 cell line expressing a dCas9-AID*Δ N-terminal fusion. The pool was then grown in duplicate in the presence of 20 µM DHQZ36.1 for 4 weeks. ASNA1 was amplified separately from cDNA of naive and treated cell populations before being sequenced by Nextera XT. (B) Plot of the frequency of ASNA1 alleles across the gene-body (x-axis) in the initial CRISPR-X population (pre-selection) and in one replicate of the selected population. Allelic fraction was calculated by determining the per-base variant frequency i.e. the number of reads which contain a mutated base at a given position vs the number of reads which contain the wildtype base at that position. Bases which had less than 500\(^x\) read coverage were excluded, resulting in no resolution of the 5’/3’ ends of ASNA1. The top selected mutation in both replicates is highlighted. (C) A149V was installed by homology directed repair in a K562 cell line expressing Cas9. The resulting mutant line was treated with ricin toxin in the presence of 10 µM Retro-2 or DMSO. Live cells were counted using forward/side scatter by cytometry. Shown are bar graphs of the mean with standard error from three technical replicates. Also shown are data for five control lines treated in the same way. See methods for further details. (D) ASNA1\(^{K0}\) HEK293T cells expressing GFP-2A-RFP-SEC61B\(_{\text{TMD}}\) were transiently transfected with indicated BFP-ASNA1 variants or BFP. The resulting transfected cells and untransfected wildtype control were pre-treated with 10 µM Retro-2 for 1 hour prior to induction with dox for 24 hours and FACS analysis. Shown are bar graphs of the means ± standard deviation (3 experiments) of RFP to GFP ratios normalized to untransfected wildtype.

DHQZ36.1 directly inhibits TA protein delivery to ASNA1

As the final test of our MOA model, we investigated whether DHQZ36.1 disrupts TA protein delivery to ASNA1 using an established cell-free assay (Guna et al., 2018; Mariappan et al., 2010). Following in vitro translation in crude rabbit reticulocyte lysate (RRL) of a radiolabeled model TA protein substrate with the TMD of STX5 (STX5\(_{\text{TMD}}\)), we analyzed ASNA1~STX5\(_{\text{TMD}}\) complex formation by size-separation on a sucrose gradient and subsequent chemical crosslinking with bismaleimidohexane (BMH) and ASNA1 immunoprecipitation (Figure 5.5A). DHQZ36.1 caused a small but significant decrease in the formation of the crosslinked ASNA1\(_{\text{x}}\)STX5\(_{\text{TMD}}\) adduct (Figure 5.5B,C; Figure S5.5a). In contrast, DHQZ5, the inactive Retro-2 analog (Figure 5.5B,C; Figure S5.5a). In contrast, DHQZ5, the inactive Retro-2 analog (Carney et al., 2014), behaved like the vehicle control. We also repeated our crosslinking analysis on SEC61B, a classic ASNA1 substrate in vitro, and found DHQZ36.1 treatment also led to decreased ASNA1~SEC61B protein interactions (Figure S5.5b,c).
Formation of ASNA1-TA protein complexes is preceded by TMD capture by SGTA, the most upstream factor in the TRC pathway. Following this pre-targeting step, TA proteins are transferred from SGTA to ASNA1 by a mechanism mediated by the BAG6 complex (comprising BAG6, GET4, and UBL4A) (Shao et al., 2017). We thus hypothesized that DHQZ36.1 interferes with TA protein delivery to ASNA1 from SGTA. This could explain our *in vitro* observation of the accumulation of another crosslinked adduct in the presence of DHQZ36.1 (Figure 5.5B,C; Figure S5.5b,c) that based on its molecular weight and native interaction with BAG6, could result from enhanced substrate crosslinking to GET4 (Mariappan et al., 2010; Shao et al., 2017). To test this hypothesis, we used an established *in vitro* assay with entirely purified components (Shao et al., 2017). Following *in vitro* translation of model TA substrate STX5\textsubscript{TMD} in the presence of recombinant SGTA in the *E. coli* PURE system, we isolated SGTA-bound to STX5\textsubscript{TMD} by sucrose fractionation (Figure S5.5d). These complexes were then briefly incubated in the presence of ASNA1 and a minimal BAG6 scaffold complex (UBL4A/cBAG6/GET4) (Figure 5.5C) (Shao et al., 2017). BMH crosslinking analysis revealed robust formation of ASNA1\texttimes{}STX5\textsubscript{TMD} adducts that was dependent on addition of the scaffold complex (Figure 5.5D). Consistent with our hypothesis, addition of DHQZ36.1 but not its biologically inactive derivative DHQZ5 (Carney et al., 2014) obstructed STX5\textsubscript{TMD}, as well as SEC61B, delivery to ASNA1 under these conditions (Figure 5.5D; Figure S5.5e). Taken together, these data demonstrate that Retro-2/DHQZ36.1 specifically interferes with the substrate hand-off step in the TRC pathway.
Figure 5.5: DHQZ36.1 blocks substrate transfer from SGTA to ASNA1 in vitro. (A) Schematic of the fractionation and crosslinking assay to monitor the cytosolic interactions of in vitro translated TA proteins. After translation in crude rabbit reticulate lysate (RRL), the TA proteins are captured by endogenous factors. (B) In vitro translation of a model TA substrate containing the TMD of STX5 (STX5TMD) in the presence of 35S-methionine and 15 µM DHQZ36.1 in RRL. Completed lysate reactions were subjected to size fractionation by centrifugation in a 5-25% sucrose gradient and their individual fractions chemically crosslinked with 0.250 mM bismaleimidohexane (BMH). Samples were resolved by SDS-PAGE and visualized by autoradiography. Adducts to STX5TMD are denoted with ×. (C) 35S-methionine-labeled model TA substrate STX5TMD, which has a C-terminal V5 epitope, was translated in crude RRL lysate in the presence of 15 µM of the indicated compounds. Translation reactions were subjected to chemical crosslinking (XL) with 0.250 mM BMH. Non-crosslinked samples were directly analyzed by SDS-PAGE. Crosslinked adducts to the TA protein substrate or ASNA1 were analyzed after denaturing immunoprecipitation (IP) with α-V5 or α-ASNA1 antibodies, respectively. For crosslinked products to GET4, samples were subjected to a non-denaturing IP with α-BAG6 antibody, which maintains the BAG6/GET4 interaction. Samples were visualized by autoradiography. Adducts to STX5TMD are denoted with ×. (D) Schematic of the TA protein transfer assay used in part e). Model TA substrate STX5TMD bound to SGTA is scaffolded into proximity with ASNA1 by a minimal BAG6 complex comprising the indicated components (see Shao et al. 2017 for more details). Substrate transfer to ASNA1 is detected by chemical crosslinking (XL) with BMH.
Figure 5.5: (Continued). (E) Purified SGTA bound to STX5\textsubscript{TMD} (SGTA–STX5\textsubscript{TMD}) and indicated recombinant TRC pathway components were incubated in the presence of 15 µM of DHQZ36.1 or DHQZ5 or mock treated (DMSO). Completed reactions were subjected to chemical crosslinking (XL) with 0.250 mM BMH. Crosslinked samples were resolved by SDS-PAGE and visualized by autoradiography (top) and Coomassie blue staining (bottom). Adducts to STX5\textsubscript{TMD} are denoted with ×.

Discussion

Here, we have demonstrated that Retro-2 and its hyperactive analog, DHQZ36.1, target the TRC pathway. Based on similarities between their genetic profiles (Figure 5.1), we hypothesized that Retro-2 acts via inhibition of TRC pathway members. Our subsequent findings provide three supporting lines of evidence for this model. First, DHQZ36.1 inhibits transfer of TA proteins from SGTA to ASNA1 in a biochemically defined system (Figure 5.5). Second, Retro-2 and DHQZ36.1 prevent the insertion of newly-synthesized TA protein reporters (Figure 5.2-3). Third, an ASNA1 mutation prevents the cytoprotective effect of Retro-2/DHQZ36.1 against ricin as well as its ability to destabilize the reporter containing the SEC61B TMD (Figure 5.4).

Our Retro-2 mechanism of action model also provides a satisfying explanation for two previous observations. First, Retro-2 treatment interferes with STX5 localization to the Golgi (Stechmann et al., 2010), which can now be understood as an expected consequence of reduced ER targeting of newly-synthesized STX5. Second, Retro-2 treatment or ASNA1 knockout both reduce insulin content and induces ER stress in isolated mouse islet cells (Norlin et al., 2016). This effect can now be explained by Retro-2 targeting the TRC pathway. Beyond this, determining in greater detail how Retro-2 functions to block TA protein transfer from SGTA to ASNA1 is an important future goal. Our identification of ASNA1 resistance alleles will also facilitate development of in vitro assays for defining any relevant binding interactions with this high priority target candidate.

The ability of Retro-2 to inhibit the TRC pathway and disrupt endogenous STX5 may be sufficient to explain its protective activity against ricin and other toxins and viruses that similarly
rely on retrograde trafficking (Lipovsky et al., 2013; Maru et al., 2017; Nelson et al., 2013). However, retrograde trafficking has not been implicated in the life cycles of many Retro-2-sensitive viruses and endocytic pathogens such as human cytomegalovirus (Cruz et al., 2017), filoviruses such as Marburg and Ebola (Shtanko et al., 2018), poxviruses (Sivan et al., 2016), chlamydia (Herweg et al., 2016), *leishmania* parasites (Craig et al., 2017), and enteroviruses (Dai et al., 2017). In those instances, we speculate that either functions of STX5 beyond retrograde trafficking or other clients of the TRC pathway are responsible for the protective activity of Retro-2. In addition, our work provides impetus to examine the efficacy of Retro-2 against viruses such as Herpes simplex virus 1 (Ott et al., 2016), Epstein-Barr (Horst et al., 2011), and human papillomavirus 6 and 8 (Sullivan and Coscoy, 2010), which are known to depend on the TRC pathway.

Together, our work demonstrates the use of paired-gene CRISPR screening technology in mammalian cells to translate the chemical-genetic signature of Retro-2 into a testable model for its MOA that explains its ability to protect cells against ricin. Additionally, it illustrates a powerful and generalizable approach for model validation by targeted genomic mutagenesis of an MOA candidate using dCas9-targeted AID (CRISPR-X). Together, this combination of technologies charts a new path for direct mechanistic dissection of novel compounds in human cells. Finally, this study finds the first known chemical inhibitor of the TRC pathway, which may enable future work to drug this pathway for therapies against diverse viruses and pathogens.

**Experimental Procedures**

*Cell culture and cell lines*

Cell culture was carried out as previously described (Deans et al., 2016). K562 cells (ATCC) were cultured in RPMI 1640 (Gibco) media and supplemented with 10% FBS (Hyclone), pen-strep (10,000 I.U./mL), and L-glutamine (2 mM). HeLa cells (ATCC) were cultured in DMEM (Gibco) media and supplemented with 10% FBS (Hyclone), pen-strep (10,000 I.U./mL), and L-
glutamine (2 mM). These cell lines were maintained in a controlled humidity incubator at 37° C with 5% CO₂. HEK293T cells were grown in a standard water-jacketed incubator with 5% CO₂ and maintained in DMEM (Gibco) media supplemented with 10% FBS (ATCC) and penicillin-streptomycin (10,000 U/mL) (Thermo Fisher Scientific). All cell lines were passaged less than 25 times passaged by trypsinization with 0.05% Trypsin-EDTA (Life Technologies).

**Chemicals and drugs**

Retro-2, DHQZ5, and DHQZ36.1 were chemically synthesized as previously described (Carney et al., 2014; Craig et al., 2017; Nelson et al., 2013). Stock solutions were prepared in dimethylsulfoxide (DMSO).

**Antibodies**

α-ASNA1, α-SGTA and α-BAG6 antibodies were gifts from Susan Shao. α-GET4 and α-SQSTM1 antibodies were obtained from Abcam. α-V5 antibody was obtained Invitrogen. α-STX5 was obtained from Synaptic Systems (110053), Secondary antibodies were goat α-mouse-IgG-HRP (Biorad), goat α-rabbit-IgG-HRP (Biorad), goat α-mouse-IgG-Cy5 (Thermo Fisher Scientific), goat α-rabbit-IgG-Cy3 (Thermo Fisher Scientific), goat α-rabbit 488 Alexa Fluor (Thermo Fisher Scientific), and goat α-mouse 647 Alex Fluor (Thermo Fisher Scientific).

**Vectors**

LentiCRISPR v2 was a gift from Feng Zhang (Addgene plasmid #52961). pCW57-GFP-2A-MCS was a gift from Adam Karpf (Addgene plasmid #71783). pLV-azurite was a gift from Pantelis Tsoulfas (Addgene plasmid #36086). pET29b was obtained from Novagen. pCS470 tfLC3, pCS625 BFP, psPAX, and pVSV were gifts from Christopher Shoemaker. pGEX-6P1 GST-SGTA was a gift from Robert Keenan. pGEX GST-GET4, pACYC His-cBAG6[1004-1132], pRSETA His-UBL4A, pET28a zebrafish ASNA1-His and PURExpress 3HA-SEC61B were gifts from Susan Shao. The vector for SEC61B-3F4 was a gift from Ramanujan Hegde.

**Single-gene CRISPRi library construction, screening, and analysis**
A sgRNA library targeting 288 genes involved in ricin resistance were chosen from a previously designed CRISPRi library (Horlbeck et al., 2016) along with 2,000 non-targeting and safe-targeting controls from a separate design (Morgens et al., 2017). As previously described (Morgens et al., 2017), sgRNAs were synthesized (Agilent) and transformed into mCherry positive, puromycin resistant, third generation, lentiviral plasmids driven by an mU6 promoter (Addgene pMCB320). Lentivirus was created using these plasmid libraries by PEI transfection into HEK293T cells along with packaging vectors. Virus was then spin infected into dCas9-KRAB expressing K562 cells (Gilbert et al., 2014) at 500x guide coverage and low MOI. Three days post-infection, cells were selected with puromycin (1 µg/mL; Sigma) for an additional three days. Cells were then split into four conditions with two replicates each at 1000x guide coverage. Cells were then pretreated with 10 µM Retro-2 or DMSO for 24 hours and remained in Retro-2 for the entire course of the experiment. Ricin treatment (1.25 ng/mL; Vector Labs) was carried out for 24 hours initially, followed by a three day recovery before being treated again (2.5 ng/mL) for 24 hours. Cells were again allowed to recover for three days before receiving a third dose of ricin at 2.5 ng/µL for 24 hours. Untreated and Retro-2 treated replicates were split each day to maintain cells at 500,000 cells per mL. Four days after the last third ricin pulse and 13 days after the pretreatment, all cells were spun into fresh media and expanded before being spun down for DNA extraction. Genomic DNA was extracted using QIAamp DNA mini kits, guides were amplified as previously described (Deans et al., 2016), and libraries were sequenced on a NextSeq 550. Gene-level effects and scores were then generated using casTLE version 1.0 (Morgens et al., 2016). Scripts are available at https://bitbucket.org/dmorgens/castle.

Paired-gene CRISPRi library construction, screening, and analysis

Three sgRNAs each for 105 genes and 50 safe-targeting sgRNAs (Morgens et al., 2017) were selected, and a paired-guide library was cloned as previously described (Han et al., 2017). Briefly, two libraries of these were synthesized and cloned into distinct mU6 and hU6 containing
plasmids. The two plasmid pools were then combined in an all-by-all cloning step, creating a pool of mU6 and hU6 containing plasmid each containing two guides. This library was then lentivirally installed into a K562 line stably expressing dCas9-KRAB fusions as above (Gilbert et al., 2014). Cells were allowed to recover for three days before selection with puromycin (1 µg/mL; Sigma) for three additional days. Cells were then split into four pools, and treated in duplicate with ricin or left untreated as above. After two weeks and three treatments, cells were frozen down and DNA was extracted using QIAGEN maxi-prep kits. The double-sgRNA vector was amplified from the genome and sequenced on a NextSeq 550 as previously described (Han et al., 2017). Double-gene level effects were obtained using casTLE version 1.0 (Morgens et al., 2016) by first combining counts for reciprocal pairs (i.e. guideA_guideB and guideB_guideA) and the nine such pairs considered as independent measurements with all safe_safe pairs considered as negative controls.

Generation of the vectors for two-color reporters and in vitro translation

RFP was PCR amplified from pCS470 tfLC3 (Shoemaker et al., 2019) and subcloned into the MluI/BamHI sites of pCW57-GFP-2A-MCS to yield pCW57-GFP-2A-RFP. The coding region of SEC61B protein was PCR amplified from the vector for SEC61B-3F4, and subcloned into the BamHI/NdeI sites of a pET29b backbone with C-terminal opsin, V5 and His tags. These vectors served as templates for amplifying the TMD with a C-terminal opsin tag and subcloning into the SgrAI/BamHI sites of pCW57-GFP-2A-RFP. To generate pCW57 GFP-2A-RFP-SEC61B_{TMD}-BFP, BFP was PCR amplified from pLV-azurite and appended via a short GGGS linker to the opsin tag by overlap extension PCR.

The model TA protein substrate with the TMD of STX5 consists of the cytosolic domain of SEC61B, the TMD of STX5 and a C-terminal V5 epitope. To make the vector for SEC61B-STX5_{TMD} for in vitro translation in rabbit reticulocyte lysate, the coding region of the cytosolic domain of SEC61B was appended to the TMD of STX5 by overlap extension PCR and then subcloned into the BamHI/Ndel sites of a pET29b backbone with C-terminal opsin, V5 and His
tags to yield pET29b SEC61B-STX5TMD. To make the vector for in vitro translation of SEC61B-STX5TMD in the PURE system, SEC61B-STX5TMD was PCR amplified from pET29b SEC61B-STX5TMD and subcloned into the Ndel/BamHI sites of the DHFR PURExpress Control Template (New England Biolabs).

Generation of ASNA1KO HEK293T cells

Oligos encoding a guide RNA against ASNA1 (CTGAAGTGGATCTTCGTCGG) were cloned into the BsmBI site of lentiCRISPRv2. HEK293T cells were transfected using lipofectamine 3000. Cells were allowed to recover for 48 hours and then treated with puromycin (2 µg/mL) for 24 hours to select for transfected cells. Puromycin was removed and cells were passaged for another 72 hours. Cells were diluted by limiting dilution into 96 well plates to generated clonal isolates. Colonies were allowed to grow up for ~10 days. Wells were scored for the presence of single colonies and single-colony wells were propagated. Knockout cells were confirmed by immunoblot against ASNA1. Indel formation in the ASNA1KO cells was confirmed by T7 endonuclease assay and standard commercial Sanger sequencing (Eton Bioscience). In brief, genomic DNA (gDNA) was extracted using QuickExtract DNA extraction solution (Lucigen) according to manufacturer’s instructions. The ASNA1 locus was amplified using the primers ASNA1 KO confirm F4 (CTGGAGCCTACACTTAGCAAC) and ASNA1 KO confirm R1 (CGCCCAGTGGTATATCCTAC). The resulting amplicon was purified using a Zymoclean Gel DNA Recovery Kit (Zymo Research) and either hybridized and treated with T7 endonuclease I (New England Biolabs) according to manufacturer’s instruction or cloned into pJET1.2/blunt with the CloneJET PCR Cloning Kit (Thermo Fisher Scientific), and sequenced at Eton Bioscience.

Lentiviral generation

Wildtype cells were grown to 90% confluency in Gibco™ Opti-MEM™ reduced serum media (Thermo Fisher Scientific) supplemented with 5% FBS. Cells were transfected with 1 µg psPAX, 0.25 µg pVSV, 0.75 µg vector using lipofectamine 3000 (Thermo Fisher Scientific) and
incubated for >6 hours, at which time the media was replaced with fresh Opti-MEM™ reduced serum media. The supernatant was harvested twice over two days and pooled together. Cell debris was removed by centrifugation and the lentivirus was stored at -80°C.

**Viral transduction of HEK293T cells**

Cells were grown to 75-90% confluent in a 12-well plate and transduced with lentivirus overnight in DMEM supplemented with 10% FBS and 8 µg/mL polybrene (Sigma) but lacking penicillin/streptomycin. Media was exchanged to remove polybrene. Cells were allowed to grow for an additional 24 hours prior to selection with puromycin (2 µg/mL).

**Flow cytometry analysis**

Cells expressing two-color reporters were grown in a 6-well culture plate and pre-treated with indicated compound or mock-treated with DMSO for 1 hour followed by doxycycline hyclate (final concentration of 250 ng/mL) (Sigma) for approximately 18 hours. Cells were detached, pelleted, and resuspended with ice-cold phosphate buffered saline (10 mM Na₂HPO₄, pH 7.4, 1.8 mM KH₂PO₄, 2.7 mM KCl, 137mM NaCl) (PBS), and analyzed by flow cytometry using a BD LSRII (BD Biosciences). Data were analyzed by FlowJo (FlowJo, LLC) and R. In particular, the dose response curves were analyzed with the drc package in R.

**Automated Microscopy**

shRNAs targeting ASNA1 or scrambled shRNA controls derived from a previous library design (Kampmann et al., 2015) were stably expressed in wildtype HeLa cells using lentivirus. Cells were plated in 24 well plates at 25,000 cells per well and pretreated for 1 hour at the appropriate drug concentration. They were then treated for 24 hours with 2.5 ng/mL ricin (Vector Labs). Ricin media was replaced with fresh media with the appropriate drug concentration along with 50 nM CellTox Green. Cells were then imaged on an Incucyte Zoom for 72 hours, imaging every 4 hours. Confluence was determined from phase images using Incucyte software.

**Confocal microscopy**
HeLa cells stably expressing shASNA1 or scrambled control shRNAs were plated at 50,000 cells per well in glass bottom plates and treated either 10 µM Retro-2 or 3 µM DHQZ36.1 for 24 hours. Cells were fixed in 4% PFA for 10 minutes, permeabilized in 0.3% Triton-X for 10 minutes, and blocked in fresh 3% BSA for >1 hour. Fixed cells were stained with primary antibodies for 1 hour (1:300 STX5-rabbit; 1:250 GM130-mouse) and secondary antibodies for 1 hour (1:2000 anti-mouse-488; 1:2000 anti-rabbit-647). After high-volume Dulbecco’s phosphate-buffered saline (DPBS) (Thermo Fisher Scientific) washes, cells were mounted with VectaShield w/ DAPI. Cells were imaged using an inverted Nikon Eclipse Ti confocal microscope with an oil immersion objective (Plan Apo, numerical aperture (NA)-1.5, 60× or 100×, Nikon), and an Andor Ixon3 EMCCD camera or Andor Zyla sCMOS camera. All images presented are max-projections using FIJI (Schindelin et al., 2012) from 0.2 µm Z-stacks. Image analysis was carried out using csth imaging package and pyto segmenter (Weir et al., 2017).

**CRISPR-X mutagenesis**

172 guides tiling the ASNA1 coding sequence were obtained from CHOP-CHOP (Montague et al., 2014), synthesized (Agilent), and cloned into an mU6 driven guide expression vector. Lentivirus was produced as above and the tiling library was infected into an N-terminal fusion, CRISPR-X (dCas9-XTEN-AID*Δ; unpublished; GTH) expressing K562 at low MOI. This construct is an N-terminal fusion to a nuclease dead version of Cas9 (dCas9) of a hyperactive version of Activation-Induced cytidine Deaminase (AID*) (Wang et al., 2009) with the nuclear exclusion sequence removed (AID*Δ) with the XTEN linker (Schellenberger et al., 2009). Three days post-infection, cells were selected using 1 µg/mL puromycin. Cells were then treated for ~4 weeks with 20 µM DHQZ36.1. RNA was extracted from cells using Qiagen RNeasy mini kits, and an ASNA1-specific primer were used for first-strand synthesis (Roche AMV) and PCR. The isolated ASNA1-cDNA was then sequenced using Nextera XT on a NextSeq 550. Reads were aligned using BWA (Li and Durbin, 2009), and variants were called using a custom script (Hess
et al., 2016). For each base with a minimum of 500\(\times\) read coverage, a percent allele frequency was calculated. Allele frequency from pre-selected cells was measured as above from cells after puromycin selection but before DHQZ36.1 treatment.

**ASNA1 allele installation and validation**

1 µg of 100 basepair ssDNA ultramer template (IDT) containing the C460T mutation and 2 µg a mU6 driven sgRNA targeting ASNA1 were electroporated into Cas9-expressing K562 using the T-175 program with Lonza Nucleofecter 2b. A safe-targeting guide, a template for C461T, a no-template control, and a mock transfection were included as controls. After three days, cells were sorted for guide expression by GFP. After 4 more days, cells were sorted for dark to eliminate random integrants. To monitor resistance to DHQZ36.1 toxicity, cells were treated with 20 µM DHQZ36.1 or DMSO for 4 days and viability was monitored via cytometry. To monitor resistance to Retro-2’s protective activity, cell were pretreated with 10 µM Retro-2 or DMSO for 24 hours, then treated with 3.75 ng/mL ricin (Vector Labs) for 24 hours. The toxin was spun out and viability and cell number were measured after three days via cytometry.

**Generation of the expression vectors of BFP-ASNA1, BFP-ASNA1 A149V, and BFP-ASNA1 D74N**

ASNA1 was PCR amplified from human ASNA1 (Accession: BC002651) cDNA from the Mammalian Gene Collection (Dharmacon) and subcloned into the NdeI/XhoI sites of pET29b. Point mutations were introduced using QuikChange site-directed mutagenesis with Pfu Turbo DNA polymerase (Agilent). To N-terminally tag ASNA1 with BFP, BFP and ASNA1 alleles were PCR amplified from pCS625 BFP and pET29b ASNA1 vectors, respectively, and inserted into the EcoRV/BamHI site of pCS625 BFP by Gibson assembly. Primer sequences were used to introduce a GGGS linker separating ASNA1 from BFP.

**Two-color reporter analysis of A149V BFP-ASNA1**

Wildtype and \(\text{ASNA1}^{\text{KO}}\ GF\text{P-2A-RFP-SEC61B}_{\text{TMD}}\) HEK293T cells were grown in a 6-cm plate and treated with doxycycline hyclate (final concentration of 250 ng/mL) (Sigma) for
approximately 20 hours. Cells were detached, pelleted, and resuspended with ice-cold PBS and bulk sorted using a MoFlo XDP cell sorter (Beckman Coulter) for GFP and RFP positive cells. The latter cells were then grown in a 6-cm plate and transfected with 2 µg of vectors expressing BFP-ASNA1 variants or BFP alone using lipofectamine 3000. After approximately 40 hours, cells were allowed to recover in fresh Gibco™ Opti-MEM™ reduced serum media (Thermo Fisher Scientific) for 1 hour before incubation with Retro-2 (10 µM) or mock-treatment with DMSO for an additional hour followed by treatment with doxycycline hyclate (250 ng/mL; Sigma) for 24 hours. Sorted but non-transfected wildtype control cells were grown and treated in parallel. Cells were detached, pelleted, and resuspended with ice-cold PBS, and analyzed by flow cytometry using a BD LSRII (BD Biosciences). Data were analyzed by FlowJo (FlowJo, LLC) and R.

Recombinant protein purification

GST-tagged SGTA, zebrafish His-tagged ASNA1, GST-tagged GET4, His-tagged cBAG6, and His-tagged UBL4A were purified from BL21-CodonPlus (DE3)-RIPL or BL21 (DE3) competent E. coli cells as previously described (Shao et al. 2017). In brief, cells were transformed with the expression plasmids encoding the protein and were grown in LB media at 37°C under the appropriate antibiotic selection. For GST-tagged SGTA, zebrafish His-tagged ASNA1 and His-tagged UBL4A, cells were grown to A600=0.4-0.6 and expression was induced with 0.1 mM-0.2 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) for 2-3 hours. For GST-tagged GET4 and His-tagged cBAG6, cells were grown to A600=0.7-0.9, cooled and expression was induced with 0.2 mM of IPTG overnight at 16°C. Cells were then harvested.

For ASNA1, cells were resuspended in Tris lysis buffer (50 mM Tris, pH 7.5, 300 mM NaCl, 2% glycerol, 2 mM beta mercaptoethanol (BME)) supplemented with cOmplete™ Protease Inhibitor Cocktail and phenylmethane sulfonyl fluoride (PMSF). Cells were lysed by high pressure homogenization by two passes through an EmulsiFlex®-C3 (Avestin, Inc.). The cell lysate was clarified by centrifugation and bound to a Ni-NTA resin (Thermo Fisher Scientific).
column by gravity flow. Columns were washed and then eluted with 50 mM Tris, pH 7.5, 150 mM NaCl, 2% glycerol, 2 mM BME, 250 mM imidazole. Protein was desalted with Econo-Pac® 10DG Desalting Prepacked Gravity Flow Columns (Bio-Rad) into 50 mM Tris 7.5, 50 mM NaCl, 2 mM BME and 2% glycerol and stored -80˚C until use.

For SGTA, cells were resuspended in phosphate salt lysis buffer (10 mM Na₂HPO₄, pH 7.4, 1.8 mM KH₂PO₄, 2.7 mM KCl, 250 mM NaCl, 10 mM imidazole, 1 mM dithiothreitol (DTT)) and lysed by high pressure homogenization. The cell lysate was clarified by centrifugation and bound to a glutathione-sepharose (Millipore Sigma) column by gravity flow. Columns were washed and then eluted with 50 mM Tris, pH 8, 25 mM reduced glutathione. Peak elutions were determined by SDS-PAGE and Coomassie staining then pooled and dialyzed against 25 mM Hepes, pH 7.4, 150 mM KAcO, 10 mM imidazole, 10% glycerol, 1 mM DTT in the presence of PreScission Protease (Millipore Sigma). The protease and tags were removed from purified SGTA by passing the dialyzed protein through a glutathione-sepharose column. The flow through was collected and stored at -80˚C until use.

For the minimal scaffold complex (UBL4A/cBAG6/GET4), cells were resuspended in phosphate salt lysis buffer and lysed with two passages through the EmulsiFlex®-C3. The cell lysates then were clarified by centrifugation. The cell lysates for GST-GET4 and His-tagged cBAG6 incubated together at 4˚C for 1 hour with shaking and were co-purified together by metal-affinity chromatography through a Ni-NTA resin column. His-tagged UBL4A was also bound to a Ni-NTA resin column by gravity flow. Columns were washed with phosphate salt lysis buffer and eluted with elution buffer (10 mM Na₂HPO₄, pH 7.4, 1.8 mM KH₂PO₄, 2.7 mM KCl, 250 mM NaCl, 250 mM imidazole, 1 mM DTT). Peak elutions were determined by SDS-PAGE and Coomassie staining, and combined for complex formation. Proteins were dialyzed against PBS supplemented with 5% glycerol and 1 mM DTT in the presence of acTEV™ protease (Thermo Fisher Scientific). The dialyzed material was clarified by centrifugation and the supernatant was bound to a glutathione-sepharose column by gravity flow. The complex
was eluted with 50 mM Tris, pH 8, 25 mM reduced glutathione. Peak elutions were pooled together and dialyzed in 25 mM Hepes, pH 7.4, 150 mM KOAc, 10 mM imidazole, 10% glycerol and 1mM DTT in the presence of PreScission Protease. The minimal scaffold complex was cleared of proteases and cleaved tags by subtraction through Ni-NTA and glutathione-sepharose columns. The minimal scaffold complex was stored at -80˚C until use.

*In vitro transcription*

Templates for transcription were generated by PCR using a 5’ primer containing the T7 promoter that anneals to the first ~30 bp of the open reading frame and a 3’ primer containing the stop codon and polyA tail that anneals to the last ~30 bp of the open reading frame. PCR templates for transcription were used to make mRNAs by incubation with components of mMESSAGE mMACHINE® T7 transcription kit (Thermo Fisher Scientific) at 37˚C for 2 hours. The mRNA was purified by acid phenol-chloroform extraction before being *in vitro* translated.

*In vitro translation, fractionation and crosslinking assay*

SEC61B-STX5<sub>TMD</sub>-V5 was synthesized from purified mRNA (5 ng of mRNA/10 µL of translation reaction) with prepared rabbit reticulocyte lysate (RRL) (Stefanovic and Hegde 2007) supplemented with Mg<sup>2+</sup> and spermidine (final concentration of 1.2 mM and 0.4 mM, respectively) in the presence of DMSO or 15 µM DHQZ36.1. Translation was initiated with <sup>35</sup>S-methionine and mRNA and incubated for 30 minutes at 32˚C. For SEC61B-V5, translation was initiated with purified mRNA at 100 ng of mRNA/10 µL of translation reaction.

20 µL of the translation reaction was subjected to size fractionation through a 200-µL 5-25% sucrose gradient in physiological salt buffer (50 mM Hepes pH 7.4, 100 mM KOAc, 2 mM Mg(OAc)<sub>2</sub>) (PSB). Samples were centrifuged for 145 minutes at 4˚C using a TLS-55 rotor (50k rpm) with the slowest acceleration and deceleration settings. Eleven 20 µL fractions were collected. Fractions were incubated with 0.250 mM bismaleimidohexane (BMH) for 1 hour on ice. Reactions were quenched with protein sample buffer and analyzed by SDS-PAGE and autoradiography.
To analyze the crude lysate by fractionation, 20 µL of RRL was directly subjected to size fractionation through a 200 µL 5-25% sucrose gradient in PSB. Samples were centrifuged for 145 minutes at 4˚C using a TLS-55 rotor (50k rpm) with the slowest acceleration and deceleration settings. Eleven 20µL fractions were collected and analyzed by SDS-PAGE and immunoblotted with α-BAG6, α-ASNA1, α-SGTA, and α-GET4.

In vitro translation, crosslinking and immunoprecipitation

Following in vitro translation, reactions were diluted 10-fold in PSB and incubated on ice for 1 hour with 0.250 mM BMH. Crosslinked samples were split and treated with DTT and phenylmethane sulfonfyl fluoride (PMSF). For denaturing immunoprecipitation (IP) of the substrate or ASNA1, samples were solubilized with the addition SDS-containing Tris buffer (final concentration of 1% SDS) and boiled. The samples were then diluted with ice-cold IP buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100) and incubated with α-V5 antibody or α-ASNA1 antibody and Protein G dynabeads or Protein A agarose, respectively, at 4˚C overnight. For native IP of Bag6-associated crosslinked products, samples were diluted with ice-cold IP buffer and incubated with α-BAG6 antibodies and Protein A agarose at 4˚C overnight. The beads were washed three times with IP buffer and then eluted with protein sample buffer. Samples were analyzed by SDS-PAGE and autoradiography.

TA substrate transfer assay

SGTA~SEC61B-STX5TMD (SGTA~STX5TMD) and SGTA~3HA-SEC61B (SGTA~SEC61B) complexes were generated as previously described (Mateja et al., 2015; Shao et al., 2017). Briefly, the PURExpress vectors were directly transcribed and translated with the commercial PURExpress® in vitro protein synthesis kit (New England Biolabs) supplemented with purified SGTA (final concentration of ~14 µM) for 90 minutes at 37˚ C. 50 µL of the translation reaction was diluted to 200 µL with PSB and applied to a 2-mL 5-25% sucrose gradient in PSB. Gradient centrifugation was carried out for 5 hours at 4˚ C using a TLS-55 rotor (55k rpm) with the
slowest acceleration and deceleration settings. Eleven 200µL fractions were collected and fractions 3-5 containing SGTA~TA protein complexes were pooled and stored at -80°C until used.

Substrate transfer reactions were assembled on ice by mixing SGTA~TA protein complexes with recombinant zebrafish ASNA1 and UBL4A/cBAG6 /GET4 scaffold complex (all proteins were present at ~1 µM final concentration) in the presence of 1 mM ATP, 2 mM Mg(OAc)₂, and 15 µM DHQZ36.1 or DHQZ5. Transfer was initiated by warming up the reactions to 32°C for 1 minute before placing them back on ice and incubating them with 0.250 mM BMH for 1 hour. Crosslinking reactions were quenched in protein sample buffer and analyzed by SDS-PAGE on a 10% gel and autoradiography.

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Author Contributions

produced and provided CRISPR-X reagents. N.P. generated the ASNA1 knockout cell line. A.L. and R.B.A. performed additional analysis. All authors read and approved the manuscript.

Declaration of Interests

The authors declare no competing financial interests.

References


Chapter 6: Evidence that Retro-1 has the same mechanism of action as Retro-2

Retro-1 was originally identified in the same high-throughput screen that identified Retro-2 (Stechmann et al., 2010). This pioneering study showed that both Retro compounds share the ability to block retrograde transport of ricin and Shiga-like toxins from endosome to the trans-Golgi network and disrupt the localization of two SNARE proteins, syntaxin 5 (STX5) and syntaxin 6 (STX6) (Stechmann et al., 2010). Retro-2’s relatively greater bioavailability, however, enabled it to rescue mice from lethal doses of ricin without significant side effects (Stechmann et al., 2010). This remarkable result has driven disproportionate amount of follow-up work on Retro-2, leaving Retro-1 largely uncharacterized with two notable exceptions. First, Retro-1 was subsequently shown to interfere with inclusion size, progeny formation and nutrient acquisition of the intracellular bacterium, Simkania negevensis, and inhibit viral spread of vaccinia virus, like Retro-2 (Herweg et al., 2016, Harrison et al., 2016). In a second distinct line of inquiry, high concentrations of Retro-1 were shown to enhance the pharmacological activity of oligonucleotides by aiding their release from Rab7/9 positive late endosomes (Ming et al., 2013, Yang et al., 2016).

I hypothesized that Retro-1 has the same mechanism of action (MOA) as Retro-2. Previously, I engineered a doxycycline (dox)-inducible cassette expressing GFP linked by a self-cleaving P2A peptide to a RFP fused to a C-terminal SEC61B transmembrane domain (TMD) sequence (GFP-2A-RFP-SEC61B_{TMD}). Fluorescence activated cell sorting (FACS) analysis of wildtype HEK293T and ASNA1 knockout (ASNA1^{KO}) cells expressing this reporter for ER targeting and insertion of tail-anchored (TA) proteins revealed a reduced RFP:GFP ratio in the latter. I also found that pre-treatment of wildtype cells with Retro-2 induced a decrease of the RFP:GFP ratio, comparable to the destabilization of the reporter in untreated (or Retro-2 treated) ASNA1^{KO} cells. My interpretation of these data is that interfering with ASNA1-mediated
ER targeting of the TA protein reporter, either by gene deletion or chemical inhibition of ASNA1 by Retro-2, leads to enhanced degradation of the reporter by the proteasome.

To test whether Retro-1 inhibits ASNA1-mediated ER targeting, I first monitored its effects on wildtype HEK293T cells with the GFP-2A-RFP-SEC61B<sub>TMD</sub> reporter. Upon pre-treatment with Retro-1 and dox induction of the reporter, I found that Retro-1 caused a dose-dependent decrease in the RFP:GFP ratio (Figure 6.1A). Notably, this effect was not further aggravated by the additional absence of ASNA1 (Figure 6.1B). A Retro-1 analog without a benzyl-group, hydro-R1, was inactive and did not decrease the RFP:GFP ratio.

Figure 6.1: ASNA1 knockout and Retro-1 treatment destabilize a TA protein reporter for ER targeting and insertion. (A) Wildtype HEK293T cells expressing the GFP-2A-RFP-SEC61B<sub>TMD</sub> reporter were pre-treated with the indicated concentrations of Retro-1 for 1 hour prior to induction with dox for approximately 18 hours and FACS analysis. Shown is the dose-response curve for the reporter RFP to GFP ratios as relative means (3 experiments) to the mock-treated cells. The dose response was modeled using the four parameter logistic regression to determine the half maximal effective concentration (EC50 ± standard error). Error bars for the means represent the standard error calculated from the four parameter logistic regression. (B) Top: Chemical structures of Retro-2, Retro-1, and hydro-R1, an inactive analog of Retro-1. Bottom: Wildtype and ASNA1<sup>KO</sup> HEK293T cell lines with the GFP-2A-RFP-SEC61B<sub>TMD</sub> were pre-treated with the indicated compounds for 1 hour prior to induction with dox for approximately 18 hours and FACS analysis. Shown are bar graphs of reporter RFP to GFP ratios with standard deviations derived from three experiments as relative means to mock-treated wildtype cells.

To further investigated the MOA of Retro-1, I tested whether Retro-1 disrupts TA protein delivery to ASNA1 by translating a radiolabeled model TA protein with the transmembrane
domain of STX5 (STX5\textsubscript{TMD}) in crude rabbit reticulocyte lysate. To monitor the formation of ASNA1\textendash;STX5\textsubscript{TMD} protein targeting complexes, I analyzed STX5\textsubscript{TMD} by size-separation through a sucrose gradient, followed by chemical crosslinking of fractions and SDS-PAGE. Retro-1 caused a decreased in the formation of the crosslinked ASNA1, and a corresponding increase in a lower molecular weight adduct, which could be GET4\texttimes;STX5\textsubscript{TMD} (Figure 6.2A). Next, we directed monitored the transfer of TA protein from SGTA to ASNA1 by first forming SGTA\textendash;STX5\textsubscript{TMD} complexes in a PURE translation system and initiating transfer to recombinant ASNA1 upon addition of a minimal scaffold complex (UBL4A/cBAG6/GET4), which can be detected by chemical crosslinking. In the vehicle-control treated samples, STX5\textsubscript{TMD} efficiently transferred from SGTA to ASNA1 in the presence of the scaffold complex (Figure 6.2B). In the presence of Retro-1, the formation of ASNA1\textendash;STX5\textsubscript{TMD} decreased, revealing TA substrate transfer was inhibited. Overall, these cell-based and \textit{in vitro} results support my hypothesis that Retro-1 interferes with the transfer of newly synthesized tail-anchored proteins from SGTA to ASNA1.

**Figure 6.2: Retro-1 blocks substrate transfer from SGTA to ASNA1 \textit{in vitro}.

![Diagram](image)
Figure 6.2: (Continued). (A) *In vitro* translation of a model tail-anchored substrate consisting of the cytosolic domain of SEC61B and the TMD of STX5 followed by C-terminal opsin and V5 tags (STX5_{TMD}) in the presence of \(^{35}\)S-methionine and 20 \(\mu\)M Retro-1 or 15 \(\mu\)M DHQZ5 in rabbit reticulocyte lysate. Completed lysate reactions were subjected to size fractionation by centrifugation in a 5-25% sucrose gradient and their individual fractions chemically crosslinked with 0.250 mM bismaleimidohexane (BMH). Samples were resolved by SDS-PAGE and visualized by autoradiography. Adducts to STX5_{TMD} are denoted with \(\times\). *Note: The vehicle-control from the translation and fractionation/crosslinking analysis performed on a separate day was included for comparison. (B) *In vitro* translation of \(^{35}\)S-methionine labeled model tail-anchored substrate consisting of the cytosolic domain of SEC61B and the TMD of STX5 followed by C-terminal opsin tag (STX5_{TMD}) in the presence of recombinant SGTA (final concentration of ~14 \(\mu\)M) in the PURE system. SGTA bound to STX5_{TMD} (SGTA~STX5_{TMD}) was purified by size-separation through a 5-25% sucrose gradient. SGTA~ STX5_{TMD} complex and indicated recombinant TRC pathway components were incubated in the presence of 20 \(\mu\)M Retro-1 or mock treated (DMSO). Completed reactions were subjected to chemical crosslinking (XL) with 0.250 mM BMH. Crosslinked samples were resolved by SDS-PAGE and visualized by autoradiography (top) and Coomassie blue staining (bottom). Adducts to STX5_{TMD} are denoted with \(\times\).

References


Chapter 7: Future directions

The small molecules Retro-2 and Retro-1 are lead compounds with broad spectrum activity against a variety of toxins and pathogens that exploit the host cell’s endomembrane system and vesicle transport machinery. To find the cellular target of Retro-2, the ricin phenotype of a pairwise set of candidate genes was measured, and then compared to the chemical-genetic signature of Retro-2. Analysis of the genetic profiles suggested the compound did not inhibit vesicle transport directly, but instead acted upstream on the TRC pathway, which mediates the post-translational targeting and insertion of tail-anchored (TA) proteins to the endoplasmic reticulum (ER) membrane. Here, I show Retro-2, and most likely, Retro-1, block the delivery of newly-synthesized TA protein proteins, such as syntaxin 5 (STX5), to ASNA1 (Figure 7.1). Since most SNARE proteins are tail-anchored, Retro-2 treatment could convey broad spectrum protection against toxins and pathogens by interfering with many SNARE-mediated vesicular transport events in the secretory/endocytic pathway.

Figure 7.1: Summary schematic of the mechanism of action of Retro-2.
Figure 7.1: (Continued). Top: In the absence of Retro-2, SGTA captures newly synthesized TA proteins, such as STX5, which are then transferred to the ER targeting factor ASNA1. TA proteins are subsequently inserted into the ER membrane and travel to their destination membranes. Ricin gains cellular access by endocytosis and STX5-mediated retrograde transport to the ER, where the toxin retrotranslocates into the cytosol and catalytically inactivate ribosomes. Bottom: Retro-2 blocks the transfer of TA proteins from SGTA to ASNA1. This results in the loss of STX5 at the Golgi, disrupting the retrograde transport of ricin to the ER and conveying protection against ricin intoxication. PM: plasma membrane; Endo.: endosome; TGN: trans-Golgi network.

Retro-2 inhibited the transfer of TA substrates with the TMDs of STX5 and SEC61B from SGTA to ASNA1 in vitro. However, an emerging theme in the field is that the TRC pathway has overlapping TA protein clients with additional ER targeting and insertion pathways mediated by certain folding chaperones, the SND factors, and the ER membrane complex (EMC) (Abell et al., 2007, Aviram et al., 2016, Casson et al., 2017; Guna et al., 2018, Rivera-Monroy et al., 2016). Moreover, ASNA1 knockout has been shown to induce ER stress, the unfolded protein response (Norlin et al., 2016), and possibly the heat shock response as with the get mutants in yeast (Brandman et al., 2012), which may cause compensatory changes to the mutant phenotype. The complete set of TA proteins, which are strictly dependent on the TRC pathway in mammalian cells, has yet to be defined; mass spectrometric-based quantitative proteomics, such as stable isotope labeling by amino acids in cell culture, offers the potential to identity these TRC pathway clients and also reveal the adaptive response to the loss of the TRC pathway upon Retro-2 treatment or genetic manipulation.

Nonetheless, until a set of TRC pathway-dependent TA proteins can be established, the use of Retro-2 as a chemical probe to determine the identity of host factors used by pathogens must be re-evaluated. Because previous work had strongly linked Retro-2 treatment to the disruption of STX5 localization and retrograde trafficking inhibition, effectiveness of Retro-2 against a pathogen has been used to infer a role for retrograde transport and STX5 in the life cycle of the pathogen (Gupta et al., 2017). Although STX5 has broad function in maintaining Golgi structure and endomembrane trafficking pathways, the role of other TA proteins that may also be affected by Retro-2 should not be excluded.
I showed Retro-2 blocks the delivery of TA proteins substrates from SGTA to ASNA1. Nevertheless, the TRC pathway component targeted by the compound and the exact mechanism of inhibition still remain to be characterized. The identification of a Retro-2-resistant allele of ASNA1 (A149V) strongly suggests that the target is ASNA1 itself. Ectopic expression of the ASNA1 A149V mutant could partially complement TA reporter destabilization in an ASNA1 knockout cell line, but was not further affected by the small molecule. While the A149V substitution may perturb the binding site for Retro-2, it also confers partial complementation of an ASNA1 knockout phenotype in the absence of Retro-2 suggesting it causes partial loss of ASNA1 function. Residue A149 corresponds to residue M143 of yeast Get3 found on helix α6, which is part of the α-helical domain that forms the hydrophobic groove and is adjacent to the ATPase domain; an aspartate substitution at this position reduced the rate of ATP hydrolysis, but did not affect TA protein binding (Mateja et al. 2009). Whether ASNA1 A149V allosterically causes a similar ATPase defect and whether the mechanism of action of Retro-2 involves modulation of ASNA1’s ATPase activity require further study.

In order to further dissect how Retro-2 functions to inhibit TA protein transfer and to structurally optimize the compound for efficacy, identification of the direct target of Retro-2 should be prioritized. To this end, Retro-2 photoaffinity probes, Retro-2 analogs with photoreactive and reporter functional groups, have been synthesized (Figure 7.2). These probes allow for photoaffinity labeling in complex protein mixtures such as live cells or crude lysate. The photoreactive moiety of the probe compound crosslinks the compound to its binding partner upon UV irradiation; the photocrosslinked proteins can then be visualized by fluorescently labeling the compound’s reporter moiety and identified (MacKinnon and Taunton, 2009, Smith and Collins, 2015). Subsequently, the binding interaction between Retro-2 and its target can be further characterized using purified TRC pathway components using fluorescence polarization or isothermal titration calorimetry.
Figure 7.2: Chemical structure of an example Retro-2 photoaffinity probe. The photoreactive phenyl azide crosslinks to the target protein upon exposure to UV light and the alkyne functional group can be fluorescently labeled using Cu(I)-catalyzed click chemistry.

Another strategy is to determine whether Retro-2 also inhibits the transfer of TA proteins to yeast Get3. Get3 can efficiently acquire TA substrates from Sgt2 in the presence of the Get4-Get5 scaffold complex (Wang et al., 2010, Mateja et al., 2015). The use of yeast components offers several advantages. First, while the basal ATP hydrolysis by ASNA1 is difficult to detect, the ATPase activity of yeast Get3 can be measured by a standard photometric assay in vitro (Mateja et al. 2009). Moreover, the abundant structural information on Get3 - which is entirely lacking for ASNA1 - can expedite the identification of the potential binding site and any effect its occupancy would have on Get3 conformation dynamics.

Identification of Retro-2’s direct target and their binding interactions could elucidate the mechanistic details of how the compound inhibits TA substrate transfer. Additionally, future structural work in tandem with functional analysis with TA reporters will enable more directed optimization of Retro-2 and Retro-1. Optimization of these compounds will have great therapeutic potential, and can further increase their efficacy against a wide variety of toxins and pathogens.

References


Appendix 1: Supplemental figures for Chapter 3
Figure S3.1: Preparation of recombinant Get1/2 constructs. (a-c) Individual His-Get1 and His-Get2 subunits and a single-chain Get2-Get1 fusion protein (Get2-1sc-His) were expressed in E. coli and purified by Ni-NTA affinity chromatography and gel filtration. Fractions from representative purifications were analyzed by SDS-PAGE and Coomassie blue staining; Lys = total lysate, Sol = crude lysate supernatant, Insol = resuspended lysate pellet, Det = detergent solubilized supernatant, FT = flow-through, W = wash, Elu = imidazole elution, MW = markers. After fluorescent labeling the cysteine mutants, the samples were further purified by gel filtration in the indicated detergent-containing buffers. (d) Purified Get1 (in FC12) was added to a 2-fold molar excess of His-Get2 (in UM), diluted at least 10-fold into the indicated detergents, subjected to Ni-NTA pull-down, and the elution fraction was analyzed by stain-free SDS-PAGE. Get1 is only recovered in the presence of His-Get2, and only in mild detergents. In SC, the subunits are not completely soluble, while in harsh detergents including LDAO and FC12, the Get1-Get2 interaction is disrupted. LDAO = 0.1% n-Dodecyl-N,N-Dimethylamine-N-Oxide, UM = 0.1% n-Undecyl-β-D-Maltopyranoside, DDM = 0.1% n-Dodecyl-α-D- Maltopyranoside, Tw20 = 0.1% Tween 20, DBC = 0.1% Deoxy Big Chap, SC = 1% Sodium Cholate. (e) SEC-MALLS analysis of an equimolar mixture of purified His-Get1 and His-Get2 after exchanging into UM. The observed molecular mass of the complex (70 +/- 3 kDa) is consistent with a 1:1 Get1/2 complex (His-Get1 = 30 kDa; His-Get2 = 34 kDa). The observed molecular masses of the protein-detergent complex (‘PDC’) and the UM micelle are 119 +/- 5 kDa and 49 +/- 3 kDa, respectively. All reconstitutions were performed in DBC (proteoliposomes) or UM (nanodiscs).
Figure S3.2: Additional details for the bulk FRET experiments. (a) Proteoliposomes reconstituted with the indicated labeled Get1 and Get2 subunits at a protein-to-lipid of 12:10,000 were tested for insertion of radiolabeled TA protein, Sec61β (FL), by a PK protection assay. The appearance of a protected fragment (PF) is diagnostic of insertion. Full-length, undigested Sec61β (FL) is shown; empty liposomes and buffer only samples were included as negative controls. (b) Absolute FRET efficiencies before and after addition of Get3 for proteoliposomes containing the indicated combinations of membrane-proximal- or cytosolically-labeled Get1 and Get2 subunits, reconstituted at ‘high’ (12:10,000; dark grey) or ‘low’ (1.2:10,000; grey) protein-to-lipid ratios. Reconstitutions were done in triplicate, and are reported ±SEM.
Figure S3.2: (Continued). Proteoliposomes with high protein-to-lipid ratios show a non-specific FRET component (~15-30%) in the absence of Get3 due to the presence of multiple Get1/2 complexes labeled with donor and acceptor fluorophores in the same liposome. We interpret Get3-dependent decreases in this FRET signal as an increase in the average distance between Get1/2 heterodimers arising from steric hindrance caused by the recruitment of Get3 to the membrane (see also Figure 3.2).
Figure S3.3: Single-molecule photobleaching data analysis. (a) Regions of interest (ROIs, yellow circles) were selected using an automated method of fit. ROIs were discarded if within 540 nm (purple circles) of another ROI, or the edge of the image. This filter was used as an unbiased means of preventing additional photobleaching steps from an adjacent ROI. (b) Fluorescence intensity varies by location in the field of view and is therefore not as accurate a means of determining stoichiometry as counting photobleaching steps. However, the average intensity of sorted photobleaching steps can be used to confirm accurate counting (Jain et al., 2014). The initial intensity or step size of traces for all reported photobleaching analyses are shown here as a kernel density function. Single step photobleaching traces (red) have the same average, initial intensity as the median step size of multi-step traces (magenta), and half the average, initial intensity of two-step traces (blue). (c) Representative fluorescent intensities of ROIs are plotted in black; step traces, determined using the ‘changepoint’ package in R, are shown in red. d-i) An independent replicate of the single-molecule photobleaching and quantitative insertion analysis shown in Figure 3.3. Proteoliposome were reconstituted with Cy5-labeled single-chain Get1/2 (‘Get2-1sc-Cy5’) at different protein-to-lipid ratios. The number of photobleaching steps per labeled proteoliposome is shown in red; ‘X’ represents discarded traces. The inset (black) shows the calculated proportion of Get2-1sc-Cy5 found in different oligomeric states, as described in Supplemental Experimental Procedures. (e) Yeast rough microsomes (‘yRM’), Get2-1-sc-Cy5 proteoliposomes from panel (d), or empty liposomes (negative control) were tested for insertion of radiolabeled TA protein, Sec61β (FL), by a proteinase K protection assay, in triplicate. The appearance of a protected fragment (PF), which is diagnostic for insertion, was quantified by SDS-PAGE and autoradiography. Coomassie-stained PK serves as a loading control. (f) All Get2-1sc-Cy5 proteoliposomes were diluted with empty liposomes to a final concentration of 10 nM Get2-1sc-Cy5, and equivalent concentrations were confirmed via Cy5 fluorescence of these dilutions in 1% SDS. (g) Insertion activity quantified from the PF bands in panel (d) and displayed with standard errors of the mean. As shown in Figure 3.3, specific insertion activity does not decrease as proteoliposomes are enriched for single copies of Get2-1sc-Cy5 per liposome. The small decrease in insertion activity seen for the proteoliposomes reconstituted at the highest protein- to-lipid ratios may reflect overcrowding in liposomes containing more than ~4 copies of Get2-1sc-Cy5. (h) As in Figure 3.3c, Get2-1sc-Cy5 proteoliposomes reconstituted at 12 proteins per 10,000 lipids were diluted to the indicated concentration using empty liposomes and subjected to an insertion and protease protection assay, in triplicate. (i) Plot showing insertion activity quantified from the PF bands for each concentration in (h), with standard errors of the mean. This control experiment demonstrates that the assay is linear up to a total Get2-1sc-Cy5 concentration of ~10 nM, and is not limited by active targeting complex (see also Figure 3.3).
Figure S3.3: (Continued)
Figure S3.4: Reconstitution and functional analysis of heterodimeric Get1/2 nanodiscs.
(a) Representative Ni-NTA purification of Get1/2 nanodiscs, analyzed by stain-free SDS-PAGE. (b) SEC of Ni-NTA purified Get1/2 nanodiscs (black); empty nanodiscs (grey) and MSP (light grey) shown for comparison. Peak fractions were analyzed by stain-free SDS-PAGE, which allows for direct protein quantification based on the number of tryptophan residues (His-Get1 [8], Get2 [5] and MSP [3]). ImageJ analysis of band intensities are consistent with a 1:1:2 ratio of Get1:Get2:MSP after purification. (c) Single-molecule photobleaching analysis of labeled Get1/2 nanodiscs. Insets show the proportion of each subunit found in different oligomeric states, as described in the Supplemental Experimental Procedures; this analysis shows that more than 90% of Get1 and Get2 are present in nanodiscs containing only one copy of each subunit, as expected for nanodiscs containing single Get1/2 heterodimers. (d) Get3-TA protein complexes were incubated with yeast rough microsomes (yRMs) or the indicated Get1/2 nanodiscs, and release of radiolabeled TA protein from Get3 was monitored by chemical cross-linking using DSS. Incubation with yRMs results in loss of Get3-TA crosslinks and appearance of glycosylated TA protein via its C-terminal opsin tag. Incubation with labeled Get1/2 nanodiscs (30 nM) also results in loss of Get3-TA crosslinks. In contrast, Get1/2 nanodiscs harboring inactivating mutations (Get2-R17E or Get1-R73E) fail to drive TA release from Get3. (e) An NMR titration of nucleotide-free Get3 (grey surface) bound to the isolated cytosolic Get2 fragment with the isolated cytosolic Get1 coiled coil reveals that the Get2 helix 2 (blue) becomes displaced by Get1 (magenta), while Get2 helix 1 (yellow; residues 4-20) is unperturbed (Stefer et al., 2011); the labeling sites on Get1 (S77C; red) and Get2 (S28C; green) are indicated for reference. If full-length Get1 and Get2 bind simultaneously to the same side of Get3, the donor fluorophore at position S28 of Get2 will be displaced. We estimate the average distance between the displaced Get2-S28C-linked donor and the Get1-S77C-linked acceptor fluorophores by accounting for the fact that residues 21-28 of Get2, but not the helix 1 residues 4-20, become displaced.
Figure S3.4: (Continued). Assuming this segment acts as a wormlike chain with a persistence length of unfolded polypeptide (Schwaiger et al., 2004), the RMS distance between K20 and S28 is ~18 Å (Howard, 2001). Combining this with the observed distance between K20 and S77 (10 Å; dotted line), the expected distance between fluorophores should be 28 Å or less, depending on which direction the displaced chain points. Given the Cy3-Cy5 $R_0 \sim 60$ Å (Murphy et al., 2004), same-side binding of Get1 and Get2 should give rise to a high (>99%) FRET state.
Appendix 2: Supplemental figures for Chapter 5
Figure S5.1: Genetic profile analysis of ricin phenotypes for Retro-2 and candidate genes. (a) Reproducibility of genes’ knockdown effect on ricin resistance in the presence of Retro-2 for the CRISPRi single-gene screen. The value is calculated by comparing the enrichment of guides targeting each gene between the Retro-2 treated and ricin plus Retro-2 treated conditions. (b) Modifiers of Retro-2 activity. The x-axis indicates the change in growth phenotypes of gene knockdowns in the presence of Retro-2, calculated by comparing enrichment of guides between untreated and Retro-2 treated conditions. A positive value indicates that the gene knockdown is less toxic in the presence of Retro-2, and a negative value indicates that the gene knockdown is more toxic in the presence of Retro-2. The y-axis indicates the change in protective activity of a gene knockdown in the presence of Retro-2, normalized for the change in gene toxicity, calculated by comparing enrichments of guides between ricin and ricin plus Retro-2 treated conditions, normalizing for the guide enrichment between untreated and Retro-2 treated conditions. A positive value indicates that a knockdown is more protective against ricin in the presence of Retro-2, while a negative value indicates that a knockdown is less protective against ricin in the presence of Retro-2. (c) Summary of single-gene CRISPRi results. The y-axis indicates the ricin phenotype of genes in the presence of 10 µM Retro-2, calculated by comparing the enrichment of guides targeting each gene between the Retro-2 and Retro-2 plus 2.5 ng/µL ricin conditions. A positive value indicates the gene knockdown is protective against ricin and a negative value indicates the gene knockdown is sensitizing against ricin. The x-axis is similarly the change in ricin phenotype in the presence/absence of 10 µM Retro-2, calculated by comparing the ricin treated conditions to the ricin plus Retro-2 treated conditions. A positive value indicates that the gene knockdown is more protective or less sensitizing to ricin in the presence of Retro-2 while a negative value indicates the gene knockdown is less protective or more sensitizing in the presence of Retro-2. Top 100 genes are determined by the absolute value of the x-axis. ASNA1 is highlighted in red. (d,e) Reproducibility of genetic profiles. Genetic profiles for genes can be determined using single replicates of the paired-gene CRISPRi screen. The genetic profile measured by (d) replicate 1 or (e) replicate 2 can then be compared to the profile of Retro-2 from the single-gene CRISPRi screen. (f) Reproducibility of double phenotypes measured in the paired-CRISPRi screen. Positive scores indicate that the pair of knockdowns is protective against ricin; negative scores indicates sensitization. (g) Summary of paired-gene screen. The y-axis shows all 105 genes and their phenotypic profiles in the pairwise CRISPR experiment as well as the phenotypic profile of Retro-2 in the single-gene experiment. The x-axis shows all 101 genes tested in both experiments. Each entry is the normalized ricin phenotype of the double gene knockdown (or the single gene knockdown in the Retro-2 treated sample). The normalized ricin phenotype was calculated as the enrichment for all 9 pairs of sgRNAs targeting both genes (3×3 per gene pair) comparing untreated and ricin treated samples. The median ricin phenotype for each row was then subtracted from each entry. Order of rows indicates the rank of correlation of each gene to Retro-2. Order of columns is the ranked order of scores for the top correlate, WRB.
Figure S5.1: (Continued)
Figure S5.2: ASNA1 knockout and Retro-2/DHQZ36.1 treatment destabilize the TA reporter. (a) Left: Whole-cell lysates were prepared from wildtype and ASNA1KO HEK293T cells and subjected to SDS-PAGE and visualized by immunoblotting (IB). SQSTM1 was used as the loading control. ASNA1KO clone #2 (marked in red) was carried forward for the rest of the experiments. Right: Sequencing for ASNA1KO clone #2 at the ASNA1 locus. The line represents the sgRNA used to make the deletion. (b) Top: Chemical structures for Retro-2, DHQZ36.1 and DHQZ5. Bottom: Wildtype and ASNA1KO HEK293T cell lines expressing the GFP-2A-RFP-SEC61B_TMD reporter were pre-treated with the indicated compound for 1 hour prior to induction with dox for approximately 18 hours and FACS analysis. Cells were treated with 10 µM Retro-2, 3 µM DHQZ36.1 or 10 µM DHQZ5. Shown are bar graphs of reporter RFP to GFP ratios as relative means ± standard deviation (3 experiments) to mock-treated wildtype cells. C) Wildtype HEK293T cells expressing the GFP-2A-RFP-SEC61B_TMD reporter were pre-treated with Retro-2 (black circles) or DHQZ36.1 (red squares) for 1 hour prior to induction with dox for approximately 18 hours and FACS analysis. Shown are the dose-response curves for the reporter RFP to GFP ratios as relative means (3 experiments) to the mock-treated cells. The dose response was modeled using the four parameter logistic regression to determine the half maximal effective concentration (EC50 ± standard error). Error bars for the means represent the standard error calculated from the four parameter logistic regression.
Figure S5.3: Effect of ASNA1 knockdown or Retro-2 and DHQZ36.1 in HeLa cells. (a) Dose curves for the protective effect of Retro-2 against ricin cytotoxicity in HeLa cells expressing either shRNAs targeting ASNA1 or scrambled negative controls. Cells are pretreated with the indicated drug concentration, then treated with ricin for 24 hours. Ricin was washed out and cells were imaged for 72 hours on an Incucyte. Confluency of cells was calculated using phase images and is presented as 100 minus the percent confluency. (b) Dose curves for DHQZ36.1 relative to Retro-2. Cells were treated and imaged as above. (c) Additional confocal images of HeLa cells expressing either ASNA1-targeting or scrambled control (shCtrl) shRNAs, treated with DMSO, 10 µM Retro-2 or 3 µM DHQZ36.1, which were used for quantitative analysis in Figure 5.3b. Cells were fixed and imaged as in Figure 5.3a. Scale bar represents 25 µm and applies to all images.
Figure S5.4: Replication and validation of CRISPR-X screen. (a) Allelic fraction of ASNA1 mutations post-selection for second replicate. The frequency of mutations across the gene-body of ASNA1 in the second replicate of the selected population. Top mutation in both replicates is C460T, which results in an alanine to valine coding mutation (A149V). Allelic fraction was calculated by determining the per-base variant frequency i.e. the number of reads which contain a mutated base at a given position vs the number of reads which contain the wildtype base at that position. (b) A149V substitution was installed using homology directed repair (HDR) in a Cas9-expressing K562 line. As negative controls, the synonymous, PAM-breaking mutant C461T (A149A) was also installed, along with a no template, a safe-targeting guide, mock, and parent control line. HDR and knockout frequencies were then monitored via Sanger sequencing of pooled cells and quantified using ICE. Alleles were considered wildtype* if they did not contain the intended mutation and had either no change or a frame-preserving insertion/deletion. (c) ASNA1 A149V is resistant to DHQZ36.1-induced toxicity. Indicated pooled cell populations were treated with 20 µM DHQZ36.1 for four days and live cells were counted using forward/side scatter. Error bars indicate standard error of the mean from three technical replicates. (d) Indicated cell populations were treated with ricin toxin in the presence of 10 µM Retro-2 or DMSO. Viability of treated cells was measured by cytometry on the basis of forward/side-scatter. Shown are bar graphs of the mean with standard error from three technical replicates. Also shown are data for five control lines treated in the same way.
Figure S5.4: (Continued)
Figure S5.5: In vitro analysis of DHQZ36.1’s effect on ER targeting. (a) Crude RRL lysate was size-separated on a 5-25% sucrose gradient. The fractions were subjected to SDS-PAGE and visualized by immunoblotting for BAG6, ASNA1, SGTA, and GET4. (b) In vitro translation of SEC61B in the presence of $^{35}$S-methionine and 15 µM DHQZ36.1 in crude rabbit reticulate lysate (RRL). Completed lysate reactions were subjected to size fractionation by centrifugation in a 5-25% sucrose gradient and their individual fractions chemically crosslinked with 0.250 mM bismaleimidohexane (BMH). Crosslinked samples were resolved by SDS-PAGE and visualized by autoradiography. Adducts to SEC61B are denoted with ×. (c) $^{35}$S-methionine-labeled SEC61B, which has C-terminal V5 epitope, was translated in crude RRL lysate in the presence of 15 µM of the indicated compounds. Translation reactions were subjected to chemical crosslinking (XL) with 0.250 mM BMH. Non-crosslinked samples were directly analyzed by SDS-PAGE. Crosslinked adducts to the TA protein substrate or ASNA1 were analyzed after denaturing immunoprecipitation (IP) with α-V5 or α-ASNA1 antibodies, respectively. For crosslinked products to GET4, samples were subjected to a non-denaturing IP with α-BAG6 antibody, which maintains the BAG6/GET4 interaction. Samples were visualized by autoradiography. Adducts to SEC61B are denoted with ×. (d) Representative data for SGTA~TA protein complex formation. In vitro translation of model TA substrate STX5TMD in the presence of $^{35}$S-methionine in PURE system supplemented with recombinant SGTA (final concentration of ~14 µM). Completed reactions were subjected to size fractionation by centrifugation in a 5-25% sucrose gradient. Fractions were analyzed by SDS-PAGE and visualized by Coomassie blue staining (top) and autoradiography (bottom). Fractions 3, 4, and 5 were pooled together and used as source of SGTA~STX5TMD in Figure 5.5E. * is a component of the PURE system. (e) The complex of $^{35}$S-methionine labeled SEC61B and SGTA (SGTA~SEC61B) was generated using the PURE system. The transfer reactions were assembled with recombinant TRC pathway components in the presence of 15 µM of the indicated compounds and subjected to chemical crosslinking (XL) with 0.250 mM BMH. Samples were directly analyzed by SDS-PAGE, and autoradiography to visualize TA protein crosslinks (top), or Coomassie blue stain to detect the input proteins (bottom). Adducts to SEC61B are denoted with ×.
Figure S5.5: (Continued)