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1 **A common mechanism of Sec61 translocon inhibition by small molecules**

2

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20

21 **Abstract**

22 The Sec61 complex forms a protein-conducting channel in the endoplasmic reticulum (ER)
23 membrane that is required for secretion of soluble proteins and production of many membrane
24 proteins. Several natural and synthetic small molecules specifically inhibit the Sec61 channel,
25 generating cellular effects that are potentially useful for therapeutic purposes, but their inhibitory
26 mechanisms remain unclear. Here we present near-atomic-resolution structures of the human
27 Sec61 channel inhibited by a comprehensive panel of structurally distinct small molecules—
28 cotransin, decatransin, apratoxin F, ipomoeassin F, mycolactone, cyclotriazadisulfonamide
29 (CADA) and eeyarestatin I (ESI). Remarkably, all inhibitors bind to a common lipid-exposed
30 pocket formed by the partially open lateral gate and plug domain of the channel. Mutations
31 conferring resistance to the inhibitors are clustered at this binding pocket. The structures
32 indicate that Sec61 inhibitors stabilize the plug domain of Sec61 in a closed state, thereby
33 preventing the protein-translocation pore from opening. Our study reveals molecular interactions
34 between Sec61 and its inhibitors in atomic detail and offers the structural framework for further
35 pharmacological studies and drug design.

36

37 Introduction

38 The universally conserved heterotrimeric Sec61 complex (SecY in prokaryotes) plays essential
39 roles in biosynthesis of more than one third of proteins in all species (for review, see ref. ¹⁻⁴). In
40 eukaryotes, secretory proteins are first translocated into the ER by the Sec61 complex before
41 reaching the cell surface by vesicular trafficking. The Sec61 complex also mediates membrane
42 integration of many proteins, including most cell surface receptors and cell adhesion molecules.
43 The Sec61/SecY channel has an hourglass-like structure with a pore constriction (termed the
44 pore ring) halfway across the membrane, which is gated by a movement of a plug-like ER-
45 luminal (or extracellular in SecY) domain of the channel⁵. In addition, the channel has a seam
46 (lateral gate) in the wall that can open laterally in the plane of the membrane to release
47 transmembrane segments (TMs) of membrane protein clients into the lipid phase. Concerted
48 opening of the luminal and lateral gates is also required for initial insertion of the client protein's
49 hydrophobic signal sequence or anchor into the channel (Fig. 1a).

50 The Sec61/SecY channel translocates polypeptides either co-translationally by docking a
51 translating ribosome or post-translationally by engaging a fully synthesized polypeptide client. In
52 eukaryotes, the post-translational mode is enabled by association of the channel with two
53 additional membrane proteins Sec63 and Sec62 (ref. ⁶⁻⁸). X-ray crystallography and cryo-
54 electron microscopy (cryo-EM) have visualized structures of the Sec61/SecY channel in
55 different functional states and revealed how it is gated and engages with client proteins^{5,9-18}. The
56 current model posits that association of a ribosome or Sec63 slightly perturbs ("primes") or
57 partially opens the lateral gate^{11,15,16} (Fig. 1a). Insertion of the client polypeptide needs further
58 widening of the lateral opening and a displacement of the plug away from the pore, which occur
59 in a cooperative manner. In cotranslational translocation, these conformational changes are
60 presumed to be induced by an interaction between the channel and the signal
61 sequence/anchor^{11,13}, whereas in post-translational translocation, they seem to be mediated by
62 Sec62¹⁷.

63 Several natural and synthetic small molecules bind to Sec61 and inhibit protein translocation
64 (for review, see ref. ¹⁹⁻²²). These inhibitors have been investigated as potential anticancer,
65 antiviral, and/or immunosuppressive agents²³⁻²⁷. Inhibition of Sec61 leads to downregulation of
66 disease-related and clinically-relevant proteins, such as cytokines, cell surface receptors, and
67 viral membrane proteins. Indeed, one such Sec61 inhibitor is currently being tested in a phase-I
68 clinical trial for treatment of solid tumor malignancies²⁸. A founding class of Sec61 inhibitors is a
69 group of fungal-derived cyclic heptadepsipeptides named cotransins²⁹⁻³¹. Other naturally
70 occurring inhibitors discovered to date are decatransin, mycolactone, apratoxins, coibamide A,
71 and ipomoeassin F, which are produced by certain fungal, bacterial, and plant species³²⁻³⁸. In
72 addition, two synthetic compounds CADA and ESI have also been shown to inhibit the Sec61
73 channel^{39,40}. These inhibitors are structurally unrelated to each other, but several of them have
74 been suggested to bind to an overlapping site in the Sec61 channel based on their abilities to
75 compete for Sec61 binding. Remarkably, cotransin and CADA inhibit Sec61 in a client-specific
76 manner^{29,30,41}, whereas other inhibitors act more broadly independent of clients. Biochemical
77 data suggest that cotransin likely interacts with the lateral gate and/or the plug of Sec61 (ref. ⁴²).
78 However, key information regarding the actions of these inhibitors remains unavailable,
79 including molecular details about Sec61-inhibitor interactions, which specific steps along the

80 translocation process are inhibited, and what underlies client-specific versus broad-spectrum
81 inhibition. This has limited our capability to design or discover additional therapeutically
82 promising small-molecule agents that target Sec61.

83

84 **Results and Discussion**

85 **Experimental design and cryo-EM analysis of inhibitor-bound Sec61**

86 To understand the mechanism of Sec61 inhibition, we sought to determine high-resolution
87 structures of inhibitor-bound Sec61 using cryo-EM. To date, all mammalian Sec61 structures
88 have been obtained from ribosome-bound cotranslational complexes^{11,12,43}. However, due to
89 flexibility of Sec61 with respect to the ribosome, this approach limits the resolution of Sec61 to
90 only ~5 Å, a resolution that is impractical to model protein side chains and small ligands¹¹. This
91 problem also exists in a recent cryo-EM structure of a mycolactone-treated Sec61-ribosome
92 complex⁴⁴. By contrast, we previously attained 3.1–3.7-Å resolution structures of the Sec61
93 channel from fungal post-translational translocation complexes^{15,17} (termed the Sec complex),
94 which contained Sec62, Sec63 and fungal-specific nonessential Sec71 and Sec72 in addition to
95 the three (α , β , and γ) subunits of Sec61. Thus, we reasoned that use of the Sec complex would
96 be an effective approach to study Sec61 inhibitors.

97 To enable high-resolution cryo-EM analysis of inhibitor-bound human Sec61, we designed a
98 chimeric Sec complex, whose transmembrane and cytosolic domains are derived from the
99 human and yeast proteins, respectively (Fig. 1b). Our initial efforts employing the entirely yeast
100 or human Sec complex were unsuccessful. The yeast Sec complex incubated with cotransin
101 failed to show any cotransin-like feature in the cryo-EM map (Supplementary Fig. S1 a and b).
102 This could be due to a lower binding affinity of cotransin towards yeast Sec61 compared to
103 mammalian Sec61³², the presence of detergent in the sample, or both. While we could see a
104 putative cotransin density in a cryo-EM structure of the human Sec complex lacking Sec62, the
105 resolution could not be improved beyond ~5 Å, probably due to high flexibility of the cytosolic
106 domain of human Sec63 (Supplementary Fig. S1 c–f). On the other hand, the human-yeast
107 chimeric Sec complex reconstituted into a peptidisc⁴⁵ yielded structures at overall 2.5 to 2.9-Å-
108 resolution with most side-chain densities well defined (Fig 1c, and Supplementary Figs. S2–S4
109 and Table S1). In the absence of inhibitors, particle images could be sorted into two three-
110 dimensional (3-D) classes with minor differences (Supplementary Fig. S2 b–g). In both classes,
111 the Sec61 channel adopts a similar conformation, including a partially open lateral gate and a
112 closed plug, as expected for a complex lacking Sec62 (ref. 17). However, the two classes
113 showed slightly different arrangements of Sec61 with respect to Sec63-Sec71-Sec72 due to a
114 loose contact between the engineered L6/7 loop of Sec61 α and the FN3 domain of yeast Sec63
115 in Class 2 (Supplementary Fig. S2 g and h).

116 For inhibitor-bound structures, we used five naturally occurring inhibitors, cotransin, decatransin,
117 apratoxin F, ipomoeassin F, and mycolactone; and two designed synthetic compounds CADA
118 and ESI. Focused refinement masking out the cytosolic domains of Sec63-Sec71-Sec72 further
119 improved the map of the Sec61 complex (at overall resolution of 2.6 to 3.2 Å) showing clear,
120 well-defined density features for the added inhibitor (Fig. 1 d–k, and Supplementary Figs. S3

121 and S4). Local resolution around the inhibitor-binding region was on par with or better than the
122 overall resolution owing to relatively uniform resolution distributions (Supplementary Fig. S3f).
123 Reliable atomic models of inhibitor molecules could be built into the densities of inhibitors based
124 on their two-dimensional (2-D) chemical structures (Fig. 1 d–k). However, we note that positions
125 and orientations of certain atoms and bonds may deviate from their true structures as our
126 structures do not resolve individual atoms.

127

128 Inhibitor-binding site

129 Despite their diverse chemical structures, all analyzed inhibitors are found to bind essentially to
130 the same site in the Sec61 channel (Figs. 1 and 2, and Supplementary Fig. S5). The pocket is
131 formed at the partially open lateral gate, approximately halfway across the membrane. The
132 inhibitors commonly interact with lateral gate helices TMs 2b, 3, and 7 of the Sec61 α subunit.
133 However, it should be noted that the actual structure of the pocket substantially varies
134 depending on the bound inhibitor because the lateral gate adopts different degrees of opening
135 (Fig. 2, and Supplementary Fig. S5). The width of the lateral gate opening is widest in the
136 cotransin-bound structure and narrowest in the ipomoeassin F-bound structure. During protein
137 translocation, the lateral gate of the Sec61/SecY channel dynamically adopts closed or variable
138 open states by a relative motion between the N- and C- terminal halves of the α subunit^{5,9-18}.
139 Our structures show that inhibitors bind to the lateral gate in one of these partially open states to
140 form a tight fit with the pocket. Compared to natural inhibitors, the interfaces of CADA and ESI
141 to Sec61 seem less extensive, possibly explaining for lower (micromolar-range) affinities of
142 these synthetic inhibitors (Supplementary Fig. S5).

143 In addition to the lateral gate, the plug and pore ring critically participate in binding of all
144 inhibitors. The partially open lateral gate of inhibited Sec61 is reminiscent of conformations
145 observed with substrate-engaged Sec61. In fact, the inhibitor binding site largely coincides with
146 where a signal sequence docks upon the insertion of a substrate protein into the channel^{13,14,46}.
147 However, one crucial difference exists between polypeptide substrates and inhibitors: unlike the
148 signal sequence, all inhibitors also form a direct contact with the plug in a closed position
149 through hydrophobic moieties (Figs. 1 and 3). Many inhibitors even further intercalate into the
150 dilated, crescent-shaped pore ring and interact with pore-ring residues (Ile81, Val85, Ile179,
151 Ile183, Ile292, and/or Ile449). In the cases of mycolactone and ESI, their extended chain
152 penetrates deeply into the channel interior and occupies a substantial space of the channel's
153 cytosolic funnel (Fig. 3 and Supplementary Figs. S5 and S6). These parts of mycolactone and
154 ESI are known to be critical for their inhibitory activity^{23,40}.

155

156 Structures of Sec61 inhibitors and interactions with Sec61

157 Except for cotransin and apratoxin, the structures of which were determined in organic solvents
158 by NMR spectroscopy or X-ray crystallography⁴⁷⁻⁴⁹, 3D structures of most Sec61 inhibitors were
159 unknown. Our cryo-EM structures now reveal their 3D structures in association with the Sec61
160 channel. Notably, conformations of cotransin and apratoxin F in our cryo-EM structures are

161 highly similar to those structures determined in organic solvent⁴⁷⁻⁴⁹. This might be because the
162 inhibitor-binding site in Sec61 forms a markedly hydrophobic environment. Particularly, the
163 pocket is open towards the lipid phase (Figs. 1 and 2), and thus, all inhibitors are expected to
164 interact with hydrocarbon tails of membrane lipids. The lipid-exposed parts of inhibitors are
165 predominantly hydrophobic (Fig. 3). Similarly, the parts of inhibitors that face the Sec61 channel
166 are mostly hydrophobic as they form contacts with hydrophobic side chains from the lateral
167 gate, plug, and pore ring of Sec61 α .

168 While van der Waals interactions between apolar groups of inhibitors and Sec61 seem to be
169 dominant contributors to inhibitor binding, our cryo-EM structures also show a recurring pattern
170 of polar interactions between Sec61 and inhibitors. In the closed channel, the lateral gate
171 contains a conserved polar cluster halfway across the membrane, formed mainly by the side
172 chain amide groups of Gln127 (Q127) in TM3 and Asn300 (N300) in TM7. Mutations in this
173 polar cluster has been shown to affect the energetics of channel gating⁵⁰. In the inhibitor-bound
174 structures, Q127 and N300 are separated by lateral gate opening, but instead they do form
175 polar interactions with certain oxygen and nitrogen atoms in the backbones of the inhibitors.
176 Given that these prong-like polar interactions are present in a predominantly hydrophobic milieu,
177 it is likely that they substantially strengthen inhibitor binding at the pocket (see below).

178

179 **Mutations in Sec61 conferring resistance to inhibitors**

180 Several point mutations in Sec61 α have been found to confer resistance to Sec61
181 inhibitors^{32,33,36-38,42,44}. These mutations are mostly located in the plug and the lateral gate. Given
182 the direct interactions between inhibitors and these parts, disruption of the inhibitor binding
183 surface could be a mechanism for these mutations. However, it has also been suggested that
184 mutations may work indirectly through altering the conformation of the channel⁴⁴. Indeed,
185 extensive biochemical studies of the Sec61/SecY complexes have well established that
186 mutations in the lateral gate, plug, and pore ring often change the gating behavior of the
187 channel⁵⁰⁻⁵². The best-known examples are *prl* mutations that give rise to relaxed client
188 selectivity through increased propensity of channel opening. Thus, this phenotypic complexity
189 has obscured how Sec61 mutations confer resistance to inhibitors. Moreover, positions of the
190 identified mutations were often redundant and sparse, limiting detailed investigation of their
191 mechanisms.

192 To biochemically probe inhibitor-binding sites in the Sec61 complex, we conducted a
193 comprehensive mutational analysis fully blinded from our cryo-EM study. We focused on two
194 inhibitors cotransin and ipomoeassin F, which were readily available to us. In addition to anti-
195 proliferation activities on mammalian cancer cell lines, these compounds also cause growth
196 retardation of yeast cells in a Sec61 α (Sec61p)-specific manner³². Therefore, we tested 84 point
197 mutations on 34 amino acid positions in yeast Sec61 α for their half-maximal growth inhibitory
198 concentration (IC₅₀) (Supplementary Table S2). Positions were mainly chosen from the cytosolic
199 funnel and lateral gate as they were likely candidates to bind inhibitors (each site was typically
200 mutated to either Asp or Trp). This led us to identify 19 and 14 new resistance-conferring
201 positions for cotransin and ipomoeassin F, respectively.

202 We then mapped the mutation positions onto the cryo-EM structures. The results clearly show
203 that most resistance mutations are clustered around bound cotransin or ipomoeassin F ([Figure](#)
204 [4 a and b](#)), suggesting that their primary mechanism is through directly impairing the inhibitor-
205 binding surface. However, some mutations (e.g., mutations equivalent to R66I/G and E78K in
206 human Sec61 α) are located at distal sites in the plug, and they may act through a
207 conformational change in the plug domain. The plug makes a substantial contact with all tested
208 inhibitors and is one of the most mobile parts of Sec61. Thus, altered dynamics of the plug may
209 explain for weakened inhibitor binding.

210 Our mutational analysis also confirms importance of polar interactions at the binding site. We
211 found that an N302L mutation in yeast Sec61 α (equivalent to N300L in human Sec61 α) confers
212 strong resistance to cotransin, decatransin, and ipomoeassin F ([Fig. 4 c–e](#)). A Q129L mutant
213 (equivalent to Q127L in human Sec61 α) showed strong to intermediate resistance to
214 decatransin and ipomoeassin F while only little effects on cotransin. This difference may be
215 explained in part by the structural observation that the side-chain amide of N300 more directly
216 faces toward these inhibitors than that of Q127 ([Supplementary Fig. S6](#)).

217

218 **Mechanism of Sec61 inhibition and discussion**

219 Our study reveals how Sec61 inhibitors interact with the channel and block the protein
220 translocation. Remarkably, all seven tested inhibitors were found to bind to the same site in the
221 channel formed by a partially open lateral gate and the fully closed plug domain, suggesting that
222 this mode of interaction provides possibly the most effective mechanism for small molecules to
223 inhibit the Sec61 channel. Among all known major Sec61 inhibitors to date, coibamide A is the
224 only compound that was not included in the present study. However, given the previous
225 observations that it competes with apratoxin A and mycolactone for Sec61 binding and that its
226 resistant mutation could be found also in the plug³⁸, coibamide A is likely to bind to the same or
227 an overlapping site. We also note that the mycolactone model proposed in the previous
228 medium-resolution cryo-EM study⁴⁴ differs in both position and conformation from those we
229 found in our study. Our data suggest that the density feature previously assigned as
230 mycolactone is unlikely to be mycolactone. During the preparation of this manuscript, a medium-
231 resolution cryo-EM structure of the mammalian Sec61 channel in association with a ribosome
232 and a cotransin derivative has been reported⁵³. While the overall structure of the channel and
233 the location of the binding pocket seem consistent with ours, we note that the orientation of the
234 inhibitor model is substantially different from that of cotransin from our study. This discrepancy
235 is more likely due to a limited map resolution of the ribosome-Sec61 structure, although we
236 cannot rule out a possibility that it may originate from minor structural differences between the
237 two compounds.

238 Despite their distinct chemical structures, some common features among the inhibitors could be
239 derived from our results. First, the inhibitors have two major clusters of hydrophobic moieties,
240 one arranged to interact with the plug and the lateral gate, and the other with membrane lipids.
241 The Sec61-facing sides are characterized by strong surface complementarity for the binding
242 pocket, while the lipophilicity of the other parts would also contribute to efficient binding as the

243 pocket exists within the plane of the membrane. Second, all inhibitors form polar interactions
244 between their backbone and the side chains of the lateral gate (Asn300, Gln127, and/or Thr86
245 of Sec61 α). We found that this is crucial for Sec61 binding affinity. These polar groups of
246 inhibitors would also provide some water solubility of the compounds. Third, certain inhibitors,
247 such as mycolactone and ESI, further penetrate the cytosolic funnel of Sec61 forming additional
248 polar and hydrophobic interactions therein. These interactions likely contribute to more stable
249 binding and broad-spectrum inhibition.

250 Our data indicate that all known Sec61 inhibitors block the protein translocation process
251 commonly by locking both lateral and luminal gates of Sec61 into translocation-incompetent
252 conformations (Fig. 4f). Although the lateral gate stays partially open, it does not provide a
253 sufficient space for a signal sequence/anchor to pass. Importantly, the luminal gate, i.e., the
254 plug, remains fully closed such that the client polypeptide cannot insert into the pore. Overall, all
255 three key gating elements—the lateral gate, plug, and pore ring—are cemented together by the
256 inhibitor at their interface, thereby prohibiting their concerted opening required for the client
257 protein insertion.

258 Although further investigations would be necessary, our comparative analysis also hints at why
259 certain inhibitors exhibit client-dependent translocation inhibition. Cotransin and CADA have
260 been shown to be less effective in blocking translocation of client proteins containing a stronger
261 targeting signal, such as a signal sequence with higher hydrophobicity or a TM signal anchor⁵⁴⁻
262 ⁵⁸. Our structures show that in the cotransin-bound structure, the lateral gate adopts a relatively
263 more open conformation on the cytosolic side (Fig. 2). This may allow certain hydrophobic
264 interactions between the lateral gate and the incoming signal sequence/anchor (Fig. 4g). A
265 stronger interaction exerted by a stronger targeting signal probably tends to further pry open the
266 lateral gate, promoting the inhibitor to be released. Although the lateral gate of the CADA-bound
267 structure is not as wide as that of cotransin-bound Sec61, its relatively low binding affinity (~0.2
268 μ M) might facilitate certain hydrophobic signals to overcome inhibition. On the other hand, those
269 inhibitors that deeply insert into the pore and cytosolic funnel of the channel may tend to exert
270 broad-spectrum inhibition by additionally impeding client insertion into the pore. Mycolactone
271 and ESI fall into such a category.

272 It remains unclear whether binding of an inhibitor requires prior opening of the Sec61 channel.
273 In our chimeric complex, the lateral gate is partially opened by Sec63. In co-translational
274 translocation, it has been generally thought that the ribosome docking alone does not open the
275 lateral gate to a considerable extent¹¹, which seems necessary for inhibitor binding. However, a
276 transient breathing motion of the channel might allow inhibitors to bind. Single-molecule
277 fluorescence studies of the bacterial SecY channel have indicated that the lateral gate
278 spontaneously fluctuates between closed and open states without any binding partner^{59,60}.
279 Thus, it is possible that inhibitor binding may not require priming or partial opening of the
280 channel induced allosterically by the ribosome or Sec63.

281 Lastly, the rich structural and mechanistic knowledge we provide here can facilitate structure-
282 guided design of Sec61 inhibitors. The Sec61 channel has been considered as a promising
283 target for therapeutic intervention due to its essential role in production of many cytokines,
284 surface receptors, and cell adhesion molecules that are clinically relevant. Nevertheless,

285 currently available Sec61 inhibitors would need further structural optimizations to improve their
286 effectiveness and pharmacological properties while reducing undesired cytotoxicity. Our new
287 approach enabling high-resolution structural analysis of human Sec61 and bound ligands would
288 accelerate efforts to understand the mechanisms of new Sec61 inhibitors and optimize
289 previously identified molecules.

290

291

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302

303 **Author contributions**

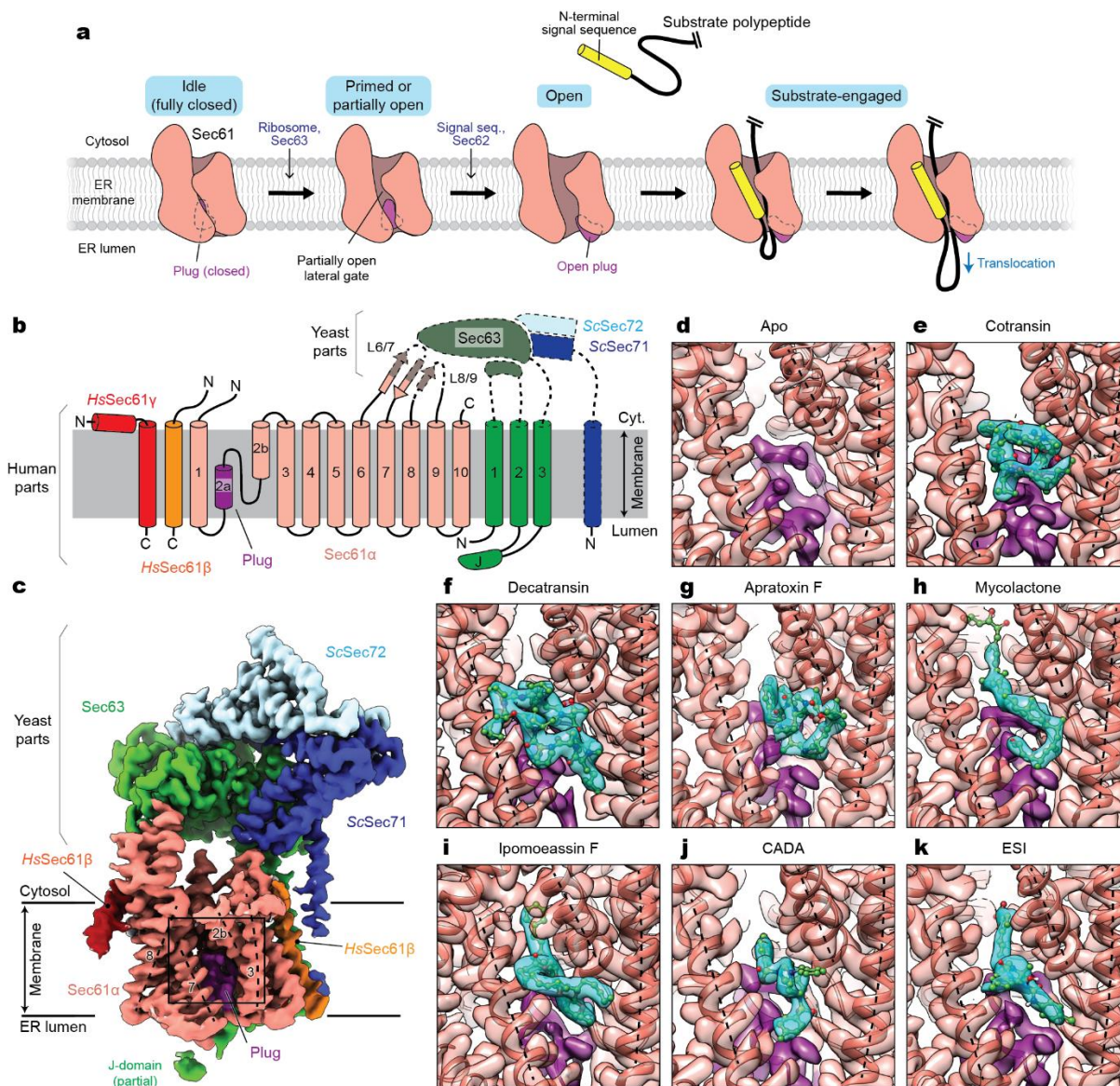
304 E.P. conceived the project and supervised the cryo-EM study. L.W. and S.I. cloned the chimeric
305 Sec construct and prepared protein samples. S.I., L.W., and E.P. collected and analyzed cryo-
306 EM data and built atomic models. R.S. helped purification of the human Sec complex and
307 cloning of the chimeric Sec complex. T.J., M.S., and D.H. performed the yeast mutational study.
308 D.H. provided cotransin and decatransin. C.F. and L.X. provided apratoxin F. W.S. provided
309 ipomoeassin F. N.B. provided mycolactone. All authored contributed to interpret results. E.P.
310 wrote the manuscript with input from all authors.

311

312 **Competing interests**

313 The remaining authors declare no competing interests.

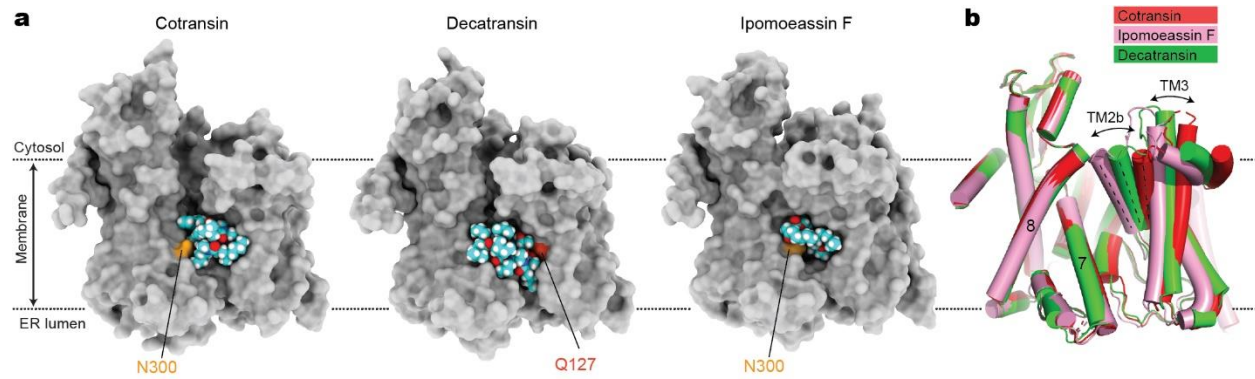
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315

316 **Figure 1. Cryo-EM structures of the human Sec61 complex inhibited by various small-molecule**
 317 **inhibitors.** **a**, Architecture of the Sec61 channel and overall model for gating and substrate engagement.
 318 **b**, Design of a human-yeast chimeric Sec complex. Parts derived from human and yeast proteins are
 319 outlined with solid and dashed lines, respectively. Note that except for the cytosolic L6/7 and L8/9 loops,
 320 Sec61 α is from the human sequence (SEC61A1). *Hs*, *Homo sapiens*; *Sc*, *Saccharomyces cerevisiae*; J,
 321 J-domain. **c**, 2.7-Å-resolution cryo-EM map of the chimeric Sec complex in an apo state (Class 1,
 322 unsharpened map). The lateral gate helices are indicated by dashed lines and TM numbers. The region
 323 outlined by a rectangle indicates the inhibitor-binding site (also see **d–k**). **d–k**, Views into the inhibitor-
 324 binding site of Sec61 α of apo and inhibitor-bound structures. Cryo-EM maps (semi-transparent surface)
 325 and atomic models were overlaid. Inhibitor and plug densities are shown in cyan and purple, respectively.
 326 Dashed lines indicate lateral gate helices TMs 2b, 3, and 7 as in **c**.

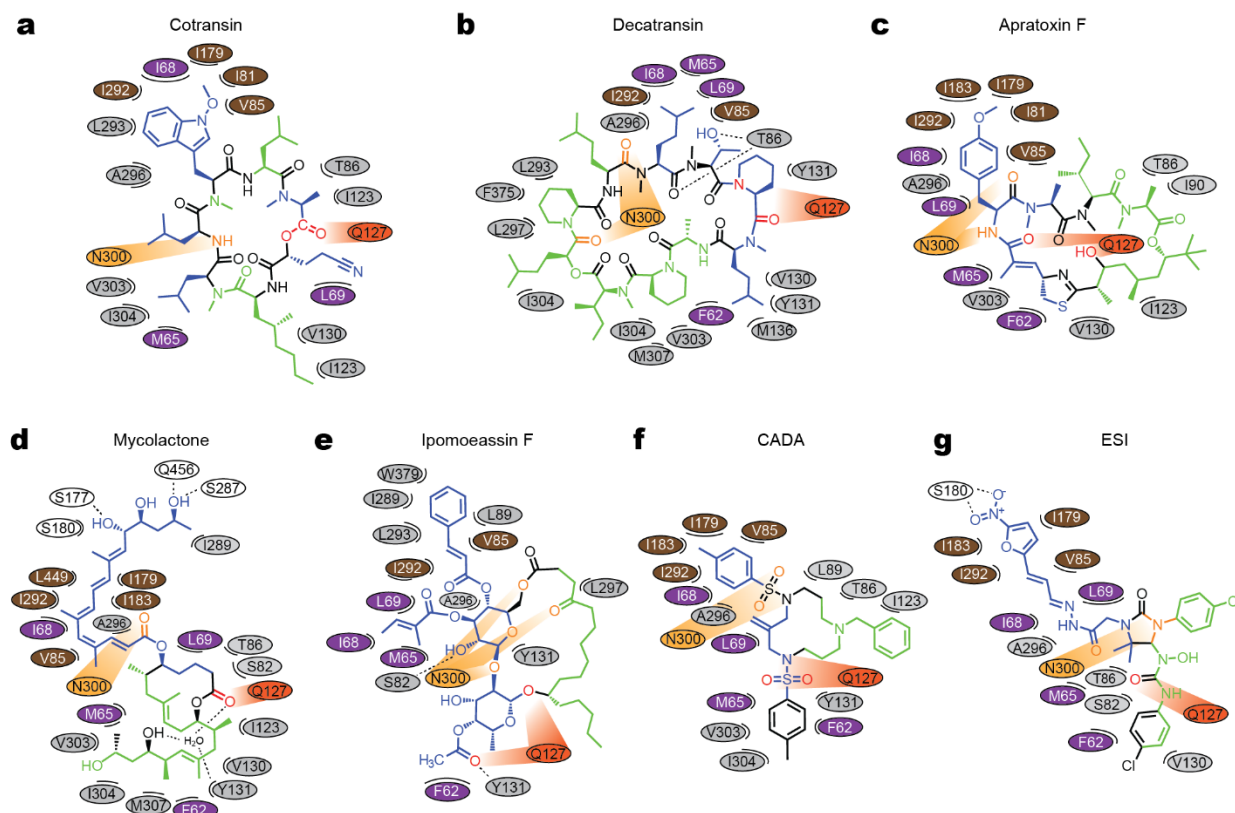
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328

329 **Figure 2. Structural plasticity of the inhibitor-binding pocket.** a, The inhibitor-binding pocket of Sec61
330 and bound inhibitors are shown in surface (protein) and sphere (inhibitors) representations. Conserved
331 polar amino acids N300 and Q127 at the inhibitor binding site (also see Fig. 3) are indicated in light and
332 dark orange, respectively. Note that part (cinnamate moiety) of ipomoeassin is deeply buried inside the
333 channel and invisible in this representation. b, Superposition of the Sec61 structures shown in a. Note
334 differences in the lateral gate opening due to the varying position of the N-terminal half of Sec61 α ,
335 particularly TMs 2b and 3. For other inhibitors, see [Supplementary Fig. S5](#).

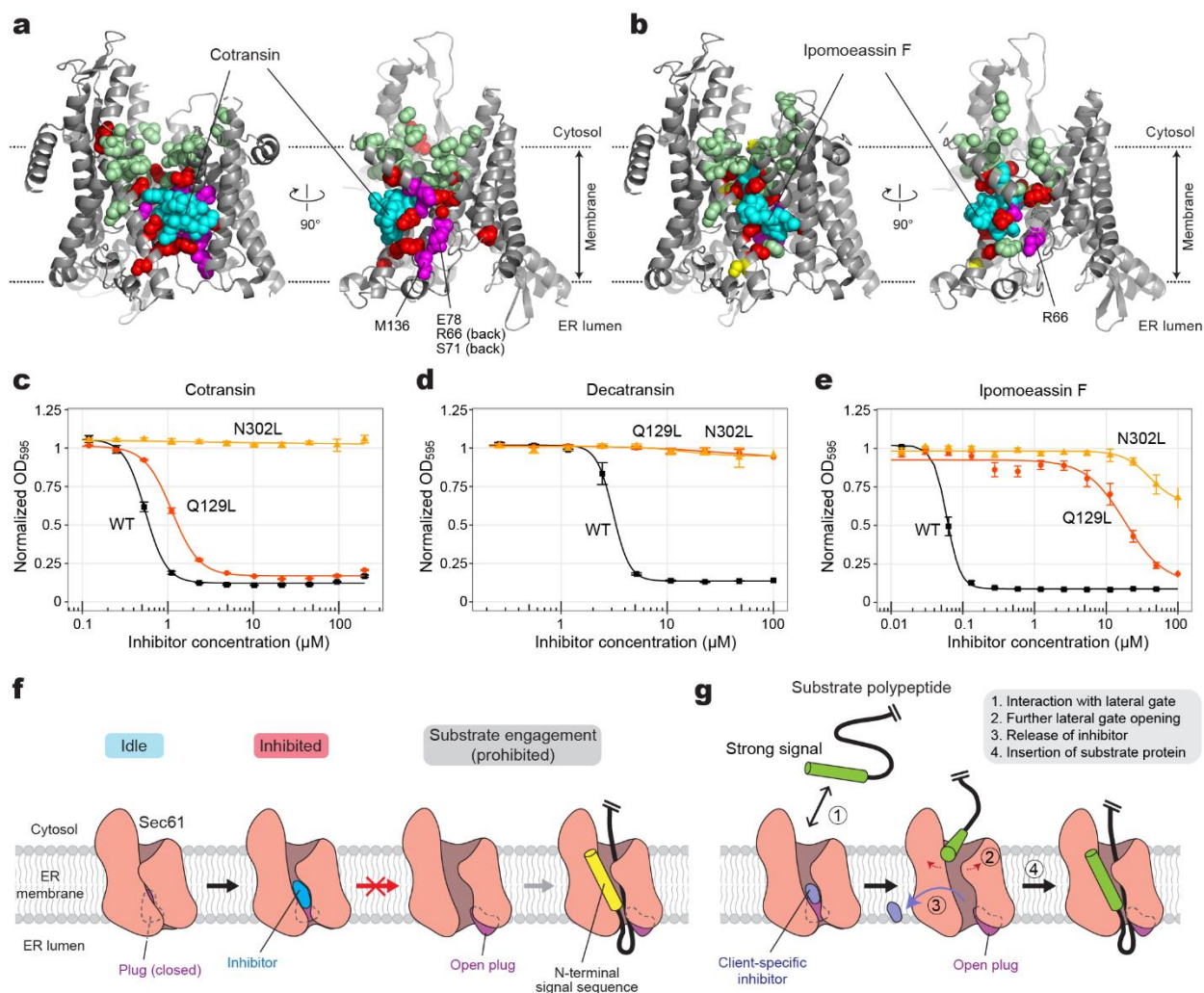
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337

338 **Figure 3. Maps for interactions between Sec61 and inhibitors.** Chemical structures of inhibitors and
 339 amino acids (ovals) in the immediate vicinity are drawn in a two-dimensional representation. For actual
 340 3D structures, see [Supplementary Fig. S6](#). Different colors were used for ovals to indicate regions in
 341 Sec61a: purple–plug, brown–pore ring, gray–lateral gate, light and dark oranges–polar cluster
 342 Q127/N300, and white–others. In chemical diagrams of the inhibitors, main lipid-exposed parts are in
 343 green whereas channel-facing parts are in blue. Dashed lines indicate putative hydrogen bonds. Note that
 344 in the mycolactone-bound structure, a water molecule coordinated by Sec61 and mycolactone was
 345 observed in the pocket.

346



347

348 **Figure 4. Inhibitor-resistant mutations and a model for Sec61 inhibition.** **a**, Positions of mutations
 349 tested with yeast Sec61 were mapped onto the cotransin-bound structure (also see [Supplementary Table](#)
 350 [S2](#)). Left, front view; right, cutaway side view. Cotransin (cyan) and amino acid side chains are shown as
 351 spheres. Red and pale green spheres indicate positions in which mutation to Asp or Trp develops high
 352 and no cotransin resistance, respectively. Magenta, positions of other resistant mutations previously
 353 reported^{32,42}. **b**, as in **a**, but with ipomoeassin-F-resistant mutations. Yellow spheres additionally show
 354 positions that give rise to moderate ipomoeassin F resistance. **c–e**, Effects of Sec61 lateral gate polar
 355 amino acid mutations on sensitivity to cotransin, decatransin, and ipomoeassin F. Dose-response curves
 356 were generated based on the yeast growth assay (residue numbers are according to yeast Sec61). We
 357 note that the assay could not be performed for other inhibitors due to their poor response in yeast. **f**,
 358 General model for the mechanism of Sec61 inhibitors. Inhibitors bind to Sec61 in a partially open
 359 conformation and precludes the plug from opening. This prevents substrate polypeptide insertion. **g**, A
 360 proposed model for client-specific inhibition. Certain client-specific inhibitors may allow an interaction
 361 between strong signals (e.g., TM signal anchors) and the channel such that the signal sequence/anchor
 362 is wedged into the partially open lateral gate. This would further open the lateral gate to cause release of
 363 the inhibitor. Inhibitors forming less interactions with the pore and plug, rendering the lateral gate into a
 364 more open conformation, and/or displaying a weaker overall affinity are likely to be overcome by this way.

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366 **Legends for Supplementary Figures**

367 **Supplementary Figure S1. Cryo-EM analysis of the yeast and human Sec complexes.**

368 **a**, A schematic of the single-particle cryo-EM analysis of the yeast Sec (ScSec) complex
369 incubated with cotransin. Note that the particles were sorted into two 3D classes, with and
370 without Sec62, due to partial occupancy of Sec62. **b**, 3D reconstructions of the ScSec complex
371 with and without ScSec62 (shown in yellow). No cotransin-like density was observed in either
372 class. For this experiment, we used a pore ring mutant (PM; M90L/T185I/M294I/M450L) that
373 stabilize the plug towards a closed conformation. **c**, Purification of the human Sec (*HsSec*)
374 complex. Shown is a Superose 6 size-exclusion chromatography elution profile with fractions
375 analyzed on a Coomassie-stained SDS gel. Note that under the used purification condition,
376 *HsSec62* does not co-purify at a stoichiometric ratio or stably comigrate with the Sec61–Sec63
377 complex. The fractions indicated by gray shade were used for cryo-EM. MW standards: Tg,
378 thyroglobulin; F, ferritin; Ald, aldolase. **d**, A schematic of the single-particle analysis of *HsSec*
379 complex incubated with cotransin. Due to a poor refinement result from nonuniform refinement
380 in cryoSPARC, the final reconstruction was obtained by the ab-initio refinement function of
381 cryoSPARC (see **f**). **e**, Representative 2D classes of the *HsSec* complex. Diffuse cytosolic
382 features of Sec63 (green arrowheads) suggest its flexibility or disorderedness. **f**, The 3D
383 reconstruction of the *HsSec* complex. A putative cotransin feature (cyan) is visible at the lateral
384 gate.

385 **Supplementary Figure S2. Cryo-EM analysis of the chimeric Sec complex in an apo form.**

386 **a**, Purification of the chimeric Sec complex reconstituted in a peptidisc. Left, Superose 6 elution
387 profile; right, Coomassie-stained SDS gel of the peak fraction. The fraction marked by gray
388 shade was used for cryo-EM. Asterisks, putative species of glycosylated ScSec71. **b**, A
389 schematic of the cryo-EM analysis of the chimeric Sec complex in an apo state. **c** and **d**,
390 Distributions of particle view orientations in the final reconstructions of Classes 1 (**c**) and 2 (**d**). **e**
391 and **f**, Fourier shell correlation (FSC) curves and local resolution maps of the final
392 reconstructions. **g**, Superimposition of the Class 1 and 2 atomic models (based on the cytosolic
393 domains) shows a slight difference in relative positions between Sec63–Sec71–Sec72 and the
394 Sec61 complex. **h**, Side views showing the contact between the engineered cytosolic loops of
395 Sec61 α and the FN3 domain of ScSec63. Note that in Apo Class 2, the contact is more poorly
396 packed than Class 1.

397 **Supplementary Figure S3. Cryo-EM analysis of the chimeric Sec complex in an inhibitor**
398 **(apratoxin F)-bound form.**

399 **a**, Images of a representative micrograph and particles of the apratoxin F-bound chimeric Sec
400 complex. Scale bar, 10 nm. **b**, A schematics of the cryo-EM analysis of the apratoxin F-bound
401 chimeric Sec complex. **c**, Representative 2D classes of the apratoxin F-bound Sec complex. **d**,
402 Distribution of particle view orientations in the final reconstruction. **e**, The FSC curve and local
403 resolution map of the final reconstruction (full Sec complex map). **f**, As in **e**, but for the map from
404 focused (local) refinement. **g**, Segmented density maps of the apratoxin F-bound Sec61 α
405 subunit. **h**, Segmented density features of bound natural inhibitors.

406 **Supplementary Figure S4. FSC curve and local resolution maps of inhibitor-bound Sec**
407 **complexes.**

408 As in [Supplementary Figure S3 e and f](#), but for all other inhibitor-bound structures.

409 **Supplementary Figure S5. Variation in the extent of lateral gate opening in inhibitor-**
410 **bound structures.**

411 As in [Fig. 2 a and b](#), but showing other inhibitor-bound structures. In all panels showing a lateral
412 gate comparison, cylindrical representations in red and pink are the cotransin- and ipomoeassin
413 F- bound structures, respectively, whereas the representation in green is the structure with the
414 indicated inhibitor.

415 **Supplementary Figure S6. 3D maps for interactions between Sec61 and inhibitors.**

416 Shown are stereo-views into the inhibitor-binding site. Inhibitors and adjacent protein side
417 chains are shown in a stick representation together with C α traces for TM2b, TM3, TM7, and the
418 plug. The views are roughly similar between the different structures but adjusted for each
419 structure for more clear representations. The following colors are used to differentiate parts:
420 brown, pore ring residues; magenta, plug; lighter orange; N300, darker orange, Q127. All
421 inhibitors are shown in cyan with certain atom-dependent coloring (nitrogen-blue, oxygen-red,
422 sulfur-yellow, and chlorine-green).

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435 **Materials and Methods**

436 **Sec61 Inhibitors**

437 Isolation of cotransin (previously referred to as “Compound 2”) and decatransin from fugal
438 species have been described previously³². For apratoxin F, ipomoeassin F, and mycolactone,
439 we used synthetic versions. Synthesis of apratoxin F (ref. ^{61,62}), ipomoeassin F (ref. ^{63,64}),
440 mycolactone (ref. ⁶⁵) has been as described previously. We note that apratoxin F and its more
441 commonly studied analog apratoxin A have only a minor structural difference and both are
442 known to exhibit comparable IC₅₀ values on mammalian cancer cell lines⁶². We also note that
443 the used synthetic mycolactone is a 4:1 mixture of two epimers at C12 in favor of the natural
444 configuration. CADA and ESI were purchased from Calbiochem. Inhibitors were dissolved in
445 dimethyl sulfoxide (DMSO) at 10 mM (for decatransin, ipomoeassin F, and mycolactone), 20
446 mM (for cotransin, apratoxin F, and CADA), or 50 mM (for ESI) before use.

447 **Plasmid constructs for cryo-EM studies**

448 The plasmids and yeast strain to express the *S. cerevisiae* Sec complex have been described
449 previously^{15,17}. To express the human Sec complex in *Spodoptera frugiperda* (Sf9) cells, we
450 modified a Bac-to-Bac baculovirus expression vector (Invitrogen) adapting the multigene-
451 expression approach from MoClo Yeast ToolKit (YTK)⁶⁶ as follows. First, we generated part
452 plasmids for a baculovirus polyhedrin (PH) promoter and a SV40 polyA signal, and an acceptor
453 plasmid (pBTK1) consisting of the backbone of pFastBac-1 (including a Tn7L element, an
454 ampicillin resistance gene, a pUC *E. coli* origin of replication, a Tn7R element and a gentamycin
455 resistance gene) and a *BsmBI*–superfolder GFP (sfGFP)–*BsmBI* acceptor cassette from
456 pYTK096 (ref. ⁶⁶). Gene fragments encoding human Sec subunits were chemically synthesized
457 and individually cloned into the entry plasmid pYTK001 as coding sequence (CDS) parts. Amino
458 acids sequences of human (denoted by “Hs”) Sec61, Sec62, and Sec63 subunits are from the
459 following entries in UniProt: P61619 (S61A1_HUMAN) for *HsSec61α*, P60468
460 (SC61B_HUMAN) for *HsSec61β*, P60059 (SC61G_HUMAN) for *HsSec61γ*, Q99442
461 (SEC62_HUMAN) for *HsSec62*, and Q9UGP8 (SEC63_HUMAN) for *HsSec63*. For the
462 pYTK001-*HsSec63* plasmid, a DNA segment encoding a human rhinovirus (HRV) 3C-cleavable
463 linker (amino acid sequence: GAGSNSLEVLFGQPTAAAA; italic, HRV 3C cleavage site) and an
464 enhanced green fluorescence protein (eGFP) were inserted immediately before the stop codon
465 of *HsSec63*. To generate single Sec gene expression cassettes, each Sec subunit CDS was
466 assembled with connectors (from pYTK003–007 and pYTK067–072), the PH promoter, and the
467 SV40 terminator into pYTK095 using *BsaI* Golden Gate cloning. Then, all Sec subunit
468 expression cassettes were assembled into pBTK1 using *BsmBI* Golden Gate cloning. In this
469 multigene plasmid, the expression cassettes were arranged in the following order: PH-
470 *HsSec61α*-SV40 | PH-*HsSec61γ*-SV40 | PH-*HsSec61β*-SV40 | PH-*HsSec63*-3C-eGFP-SV40 |
471 PH-*HsSec62*-SV40.

472 The plasmid expressing the human-yeast chimeric Sec complex were made similarly to the
473 human Sec complex plasmid with modifications of pYTK95 *HsSec61α* and *HsSec63* expression
474 constructs as follows. To modify *HsSec61α*, two substitution mutations were introduced in cytosolic
475 loops of *HsSec61α* using PCR to replace (1) amino acid residues 263–278
476 (VDLPIKSARYRGQYNT) with the corresponding yeast sequence (residues 265–280;
477 YELPIRSTKVRGQIGI) and (2) amino acid residues 394–411 (LKEQQMVMRGRHRETSMVH)
478 with amino acids 395–412 of *ScSec61* (FKDQGMVINGKRETSIYR;

479 “Sc” denotes *Saccharomyces cerevisiae*). The substitutions in *HsSec63* were introduced using
480 Gibson assembly by first substituting amino acid residues 30–96 (ATY...VKK) with amino acids
481 29–93 of *ScSec63* (MTL...RRN), followed by substitution of residues 215 to the C-terminus
482 (SIR...stop) with the corresponding sequence from *ScSec63* (residues 246–stop; TQS...stop).
483 Fragments of *ScSec63* were amplified from genomic DNA of yeast strain BY4741. In the
484 multigene pBTK1 construct of the chimeric Sec complex, *HsSec62* cassette was omitted, and
485 instead, the cassettes for *ScSec71* (PH-*ScSec71*-SV40) and *ScSec72* (PH-*ScSec72*-SV40)
486 were added. The CDS fragments of *ScSec71* and *ScSec72* were amplified by PCR from
487 genomic DNA of yeast strain BY4741 and cloned into pYTK001. Like other single subunit
488 expression plasmids, *ScSec71* and *ScSec72* CDSs were assembled into pYTK095 together
489 with the PH promoter and the SV40 polyA signal before use for the *BsmBI* assembly.

490 Protein Expression

491 Baculovirus bacmids encoding the human or chimeric Sec complex were generated by
492 transforming the respective pBTK1 plasmid into the DH10Bac *E. coli* cells (Invitrogen) according
493 to the manufacturer’s instructions. Bacmids were isolated using a DNA midiprep kit (Epoch Life
494 Science). 40 mL of a Sf9 suspension culture were prepared in ESF921 medium (Expression
495 Systems) to a density of ~1.5 M/mL. 40 µg bacmid DNA were mixed with 80 µg PEI Max
496 transfection reagent (PolySciences) in 4 mL Dulbecco’s phosphate-buffered saline (DPBS).
497 After incubating at 22°C for 20–30 minutes, the DNA:PEI mixture was added to the culture.
498 Supernatant containing P1 virus was harvested ~4 days post transfection and stored at 4°C
499 after supplementing 5% FBS (Gibco). Expression of the Sec complex was carried out by adding
500 0.5 mL P1 virus to 0.7 L of Sf9 cells at density of ~1.5 M/ml that were prepared in a 2-L baffled
501 flask. Cells were harvested by centrifugation typically two to three days post-infection upon
502 verifying uniform expression of green fluorescence under microscope. Cell pellets were frozen
503 in liquid nitrogen and stored at –80°C until use.

504 Protein Purification

505 The yeast Sec complex was purified from yeast strain ySI8 (ref. ¹⁷). This strain expresses a
506 “pore mutant (PM)” version of *ScSec61*, the pore ring residues of which were mutated to amino
507 acids corresponding to *HsSec61α* (M90L/T185I/M294I/M450L). The yeast Sec complex was
508 purified as described previously^{15,17}. After Superose 6 (GE Life Sciences) size-exclusion
509 chromatography, the purified protein was concentrated to ~4 mg/mL in 20 mM Tris pH 7.5, 100
510 mM NaCl, 1mM EDTA, 2 mM DTT, and 0.02% glycol-diosgenin (GDN; Anatrace) and mixed
511 with 100 µM cotransin for 0.5–1 h before preparing cryo-EM grids.

512 To purify the human Sec complexes, Sf9 cell pellets were first resuspended in lysis buffer
513 containing 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 2 mM dithiothreitol (DTT), 1 mM
514 ethylenediaminetetraacetic acid (ETDA) supplemented with protease inhibitors (5 µg/ml
515 aprotinin, 5 µg/ml leupeptin, 1 µg/ml pepstatin A, and 1.2 mM PMSF). All subsequent steps
516 were carried out in ice or at 4°C. The cells were broken with a glass Dounce homogenizer using
517 ~100 strokes. After removing large debris by brief centrifugation (4,000 g, 10 min), membranes
518 were pelleted by ultracentrifugation for 1.5 h (125,000 g, Beckman Type 45 Ti). The membrane
519 pellet was resuspended in ~10 pellet volumes of lysis buffer supplemented with 5 µM cotransin.
520 Membranes were solubilized by an addition of 1% lauryl maltose neopentyl glycol (LMNG;
521 Anatrace) and 0.2% cholesteryl hemisuccinate (CHS; Anatrace) for 2 h. Then, the lysate was
522 clarified by ultracentrifugation at 125,000 g for 1 h. The clarified lysate was then supplemented

523 with 2 μg *Serratia marcescens* nuclease and incubated with home-made anti-GFP nanobody
524 Sepharose beads for 1.5 h. Beads were washed with wash buffer containing 25 mM Tris-HCl pH
525 7.5, 100 mM NaCl, 2 mM DTT, 1 mM EDTA, 0.02% GDN, and 5 μM cotransin (hereafter, 5 μM
526 cotransin was included in all buffers). The complex was eluted by incubating beads with the
527 HRV 3C protease overnight. The eluate was collected and concentrated to ~ 10 mg/ml by
528 Amicon Ultra (cutoff 100 kDa). The sample was then injected to a Superose 6 increase column
529 (GE Life Sciences) equilibrated with the wash buffer. Peak fractions were pooled and
530 concentrated to ~ 6 mg/mg, before preparing cryo-EM grids.

531 The chimeric Sec complex was purified similarly using the method to purify the human Sec
532 complex but with minor modifications. First, the Sec complex was purified without
533 supplementing Sec61 inhibitors during purification (inhibitors were added to the purified Sec
534 complex before cryo-EM grid preparation). Second, to solubilize membranes, 1% n-dodecyl- β -
535 D-maltopyranoside (DDM; Anatrace) and 0.2% CHS was used instead of LMNG/CHS. For
536 column wash, the buffer contained 0.02% DDM and 0.004% CHS instead of GDN. Third, the
537 Sec complex was reconstituted into a peptidisc⁴⁵ as follows. After concentrating the eluate from
538 GFP-nanobody beads to ~ 10 mg/ml, the Sec complex was mixed with the peptidisc protein
539 (Peptidisc Lab) at a weight ratio of 1.5:1 (peptidisc to Sec). After incubating for 1 h, the mixture
540 was injected into a Superose 6 Increase column equilibrated with 25 mM Tris-HCl pH 7.5, 100
541 mM NaCl, 2 mM DTT and 1 mM EDTA. Peak fractions were pooled and concentrated to ~ 10
542 mg/ml (~ 52 μM), and one of the Sec61 inhibitors was added for ~ 1 h before preparing cryo-EM
543 grids. The inhibitor concentrations used were: 100 μM for cotransin, 100 μM for decatransin,
544 100 μM for apratoxin F, 100 μM for ipomoeassin F, 100 μM for mycolactone, 200 μM for CADA,
545 and 500 μM for eeyarestatin I.

546 **Cryo-EM data acquisition**

547 Immediately prior to preparing cryo-EM grids, 3 μM Fos-Choline-8 (Anatrace) was added to the
548 protein sample. The sample was then applied to a gold Quantifoil R 1.2/1.3 holey carbon grid
549 (Quantifoil) that was glow discharged for 35 sec using PELCO easiGlow glow discharge
550 cleaner. The grid was blotted for 3–4 sec using Whatman No. 1 filter paper and plunge frozen
551 using Vitrobot Mark IV (FEI) set at 4°C and 100% humidity.

552 The yeast Sec complex dataset (1,578 movies) was acquired on FEI Talos Arctica electron
553 microscope operated at an acceleration voltage of 200 kV, with Gatan K2 Summit direct
554 electron detector. A magnification of 36,000x under super resolution mode (with the physical
555 pixel size of 1.14 Å) was used with a nominal defocus range that was set between -0.8 to -2.2
556 μm . Each micrograph was composed of 42 frames with total exposure of 50 e^-/pixel .

557 The human Sec complex dataset (3,499 movies) was collected on FEI Titan Krios G2
558 microscope operating at an acceleration voltage of 300 kV and equipped with a Gatan Quantum
559 Image Filter (slit width of 20 eV) and a Gatan K3 direct electron detector. A magnification of
560 64,000x under the super-resolution mode (with physical pixel size of 0.91 Å) was used at a
561 defocus range that was set between -0.8 and -2.0 . Each micrograph was composed of 42
562 frames with total exposure of 50 e^-/pixel . Exposures were performed with beam shifts onto 9
563 holes (3 by 3) per stage movement.

564 All chimeric Sec complex datasets were acquired on an FEI Titan Krios G3i electron microscope
565 operating at an acceleration of 300 eV, with a Gatan K3 Summit direct electron detector and a
566 Gatan Quantum Image Filter (with 20 eV slit width). A magnification of 81,000x under the super-

567 resolution mode (with physical pixel size of 1.05 Å) was used at a defocus range that was set
568 between -0.8 and -2.0. Each micrograph was composed of 50 frames with a total exposure of
569 50 e⁻/pixel. Exposures were performed with beam shifts onto 9 holes (3 by 3) per stage
570 movement (often acquiring movies for two non-overlapping areas per hole). All datasets were
571 acquired using SerialEM software⁶⁷.

572 **Cryo-EM image analysis**

573 Preprocessing of the movies and particle image extraction were done using Warp⁶⁸. Motion
574 correction and CTF estimation were performed on images divided to 7 × 5 tiles, and particles
575 (256 x 256 pixels) were picked by the BoxNet algorithm in Warp. All subsequent image
576 processing procedures were performed using cryoSPARC v3.3 (ref. ⁶⁹).

577 Cotransin treated pore mutant ScSec complex: A data processing flowchart diagram is shown in
578 [Supplementary Fig. S1a](#). A dataset of 528,128 auto-picked particles was classified into fifty 2-D
579 class averages. Using visual inspection of the output, classes that represented empty micelles
580 or poor-quality classes were removed and particles grouped into well resolved classes
581 corresponding to a single copy of the full Sec complex were selected (385,686 particles). Three
582 ab-initio 3-D maps were then generated in cryoSPARC using the selected particles, followed by
583 heterogeneous refinement. One 3-D class with 274,913 particles refined to a density map
584 exhibiting defined Sec complex features. Non-uniform refinement of the particles in this class
585 yielded a consensus map with 3.9-Å overall resolution. The particles were further separated into
586 two 3-D classes using a heterogeneous refinement with inputs of the consensus map and the
587 consensus map with manually erased Sec62. After a subsequent round of non-uniform
588 refinement 174,058 particles yielded a map of ScScSec complex with Sec62 at 4.0-Å overall
589 resolution, and 100,855 particles yielded a map of ScSec complex without Sec62 at 4.2-Å
590 overall resolution.

591 Cotransin-bound wildtype HsSec complex: A data processing flowchart diagram is shown in
592 [Supplementary Fig. S1d](#). A dataset of 601,465 auto-picked particles was classified into seventy
593 2-D class averages. Selected classes yielded 330,005 particles that were then reconstructed
594 into three 3-D classes using ab-initio reconstruction followed by heterogeneous refinement. One
595 major class, with 202,946 particles, was selected for further refinement. Non-uniform refinement
596 of this class resulted in a reconstruction only at 7.4-Å resolution due to poor image alignment.
597 Thus, for the final map, we used the ab-initio reconstruction method (without splitting the particle
598 sets for half maps) with the maximal refinement resolution manually set to 5.0-Å
599 ([Supplementary Fig. S1f](#)).

600 Apo chimeric Sec complex: A data processing flowchart diagram is shown in [Supplementary](#)
601 [Fig. S2b](#). Using 2-D classifications starting with 616,121 auto-picked particles, we selected
602 363,027 particles for 3-D reconstruction. Following an ab-initio refinement step generating four
603 initial maps and a heterogeneous refinement step we identified two major 3-D classes with
604 distinguishable full Sec complex features. Each of these classes were refined using non-uniform
605 refinement, local CTF refinement, and another round of non-uniform refinement, yielding full
606 maps of the chimeric Sec complex at overall resolutions of 2.7 and 2.8 Å from 188,637 particles
607 (Class 1) and 147,081 particles (Class 2), respectively. The Sec61 channel was further refined
608 by masking out the cytosolic domains of the complex and performing local refinement, yielding
609 overall channel resolutions of 3.0 Å (Class 1) and 3.4 Å (Class 2).

610 Apratoxin F-bound chimeric Sec complex: A data processing flowchart diagram is shown in
611 [Supplementary Fig. S3b](#). Using 2-D classifications starting with 910,463 auto-picked particles,
612 we selected 534,411 particles for 3-D reconstruction. Following an ab-initio refinement step
613 generating four initial maps and a heterogeneous refinement step we identified two structurally
614 indistinguishable major 3-D classes with defined full Sec complex features. The particles from
615 the two classes were combined and refined using non-uniform refinement, local CTF
616 refinement, and another round of non-uniform refinement, yielding a full map of the apratoxin F
617 bound chimeric Sec complex at an overall resolution of 2.5 Å from 497,555 particles. The Sec61
618 channel was further refined by masking out the cytosolic domains of the complex and
619 performing local refinement, producing an overall channel resolution of 2.6 Å.

620 All other inhibitor-bound datasets were processed using a workflow described for Apratoxin F-
621 bound structure with minor variations in the numbers of classes in 2-D and 3-D classification
622 procedures. For details, see [Supplementary Figs. S7 and S8](#). Statistics for final refined maps
623 are shown in [Supplementary Fig. S4 and Supplementary Table S1](#).

624 **Model building and refinement**

625 Atomic model building and refinement were performed using Coot⁷⁰ and Phenix⁷¹. An initial
626 model was built by docking an ScSec complex model (PDB ID 7KAH; ref. ¹⁷) into the cryo-EM
627 map of the cotransin-bound complex using UCSF chimera⁷² and rebuilding the polypeptide
628 chains. For building and refining of Sec61 and inhibitor models, we used maps from focused
629 (local) refinements as they typically showed better protein side-chain and inhibitor features than
630 full maps. The initial model was further improved by using our highest-resolution map, which
631 was obtained from the apratoxin F-bound complex. This model was then used to build atomic
632 models for apo and other inhibitor-bound complexes by docking the model to the map using
633 UCSF chimera and locally adjusting it into the map in Coot. The restraint models of inhibitors
634 were generated from SMILES strings of inhibitors using the Grade web server
635 (<http://grade.globalphasing.org>) or the eLBOW tool of Phenix. The atomic models of inhibitors
636 were then fitted into the cryo-EM map in Coot. We note that stereochemistry of decatransin has
637 not been experimentally determined. We assumed that all amino acid residues of decatransin
638 are in an L or S configuration based on an observation that no epimerase was found in the
639 biosynthetic gene cluster of decatransin. The configuration of C α of the homoleucine-derived 2-
640 hydroxy carboxylic acid remains ambiguous³², but we also assumed that it is in an S
641 configuration. The resulting model could be fitted well into the cryo-EM map. The atomic models
642 were refined with Phenix real-space refinement using maps that were sharpened with B-factors
643 estimated based on the Guinier plots and low-pass-filtered at their overall resolution. The
644 refinement resolution was also limited to the overall resolution of the maps in Phenix. Structural
645 validation was performed using MolProbity⁷³. UCSF Chimera, ChimeraX (ref. ⁷⁴), and PyMOL
646 (Schrödinger) were used to prepare figures in the paper.

647 **Mutagenesis of yeast Sec61 α and IC₅₀ measurements**

648 Except for the experiment shown in Fig. 4c, cotransin IC₅₀ measurements were based on the
649 yeast strain RSY1293 (*mat α* , *ura3-1*, *leu2-3,-112*, *his3-11,-15*, *trp1-1*, *ade2-1*, *can1-*
650 *100*, *sec61::HIS3*, [pDQ1]) (ref. ⁷⁵). In strain RSY1293URA, pDQ1, i.e., YCplac111 (*LEU2 CEN*)
651 containing the gene of an N-terminally His-tagged, otherwise wild-type Sec61 α (Sec61p) with its
652 own promoter, was exchanged for YCplac33 (*URA3 CEN*) containing the same insert. Mutations
653 in *sec61* were introduced in pDQ1 by PCR and transformed into RSY1293URA, followed by

654 elimination of the *URA3* plasmid containing wild-type using 5-fluoro-orotic acid. Finally, the
655 presence of the mutation was confirmed by sequencing.

656 For experiments shown in Fig. 4 c–e and ipomoeassin IC₅₀ measurements, we used the yeast
657 strain BY4743Δ8a (*mat a*, *ura3Δ0*, *leu2Δ0*, *his3Δ1*, *lys2Δ0*, *snq2::KanMX4*; *pdr3::KanMX4*;
658 *pdr5::KanMX4*; *pdr1::NAT1*; *yap1::NAT1*; *pdr2Δ*; *yrm1Δ*; *yor1Δ*) lacking eight genes involved in
659 drug resistance (efflux pumps *SNQ2*, *PDR5*, and *YOR1*, and transcription factors *PDR1*, *PDR2*,
660 *PDR3*, *YAP1*, and *YRM1*)⁷⁶. This strain showed higher sensitivity to ipomoeassin F compared to
661 RSY1293. YCplac33 containing (untagged) *SEC61* with 200 bp of its own upstream and 205 bp
662 of its own downstream sequence was transformed into BY4743Δ8a. Genomic *SEC61* together
663 with 194 bp 5'- and 204 bp 3'-noncoding sequence was replaced with a hygromycin resistance
664 cassette using pAG32 (*HphMX4*) (ref. ⁷⁷) resulting in the strain BY4743Δ9aURA
665 (*sec61::HphMX4* [pSEC61-YCplac33]). Finally, pDQ1 containing the mutated *sec61* versions
666 were transformed and the wild-type *SEC61* URA3 plasmid counterselected. Plasmid exchange
667 was validated by PCR. The IC₅₀ measurements for cotransin in the BY4743Δ9aURA
668 background paralleled those in the RSY1293.

669 IC₅₀ measurements were performed as described previously³² by testing log-phase cultures in
670 96-well microtiter plates in YPD medium with serial dilutions of the compound. The assay
671 volume was 120 μl/well, start OD₆₀₀ was 0.05, DMSO was normalized to 2%. Curves were
672 calculated by taking the 19 h OD₆₀₀ measurements and applying a log regression curve fit in
673 TIBCO Spotfire v3.2.1.

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