

1 **A baton-relay and proofreading mechanism for selective ER retrieval signal**
2 **capture by the KDEL receptor**

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15

16 **ABSTRACT**

17 The KDEL-retrieval pathway captures escaped ER proteins with a KDEL or variant
18 C-terminal signal at acidic pH in the Golgi and releases them at neutral pH in the ER.
19 To address the mechanism of signal binding and the molecular basis for differences
20 in signal affinity, we determined the HDEL and RDEL bound structures of the KDEL-
21 receptor. Affinity differences are explained by interactions between the variable -4
22 position of the signal and W120, whereas initial capture of retrieval signals by their
23 carboxyl-terminus is mediated by a baton-relay mechanism involving a series of
24 conserved arginine residues in the receptor. This explains how the signal is first
25 captured and then pulled into the binding cavity. During capture, retrieval signals
26 undergo a selective proofreading step involving two gatekeeper residues D50 and
27 E117 in the receptor. These mechanisms operate upstream of the pH-dependent
28 closure of the receptor and explain the selectivity of the KDEL-retrieval pathway.

29
30 **INTRODUCTION**

31 Stable maintenance of the luminal composition of the endoplasmic reticulum (ER) is
32 crucial for the function of the secretory pathway (Ellgaard and Helenius, 2003).
33 Because of the continuous flow of material from the ER to the Golgi, the essential
34 chaperones and redox enzymes needed for protein folding in the ER lumen undergo
35 dynamic retrieval from the Golgi apparatus (Gomez-Navarro and Miller, 2016).
36 Conversely, secretory proteins destined for secretion and integral membrane
37 proteins intended for other cellular compartments are not retained. This separation of
38 secreted and retained cargo proteins involves signal-mediated sorting, whereby
39 folded proteins destined for exit from the ER have active transport or exit signals,
40 and proteins to be retained in the ER have signals for retrieval (Barlowe, 2003;
41 Gomez-Navarro and Miller, 2016). For membrane proteins, cytoplasmic signals can

42 directly engage with the selective vesicle coat complexes required for transport
43 between the ER and Golgi. For luminal proteins, this information has to be relayed
44 by a transmembrane receptor that serves as an intermediary to the cytoplasmic coat
45 protein complexes (Dancourt and Barlowe, 2010). In the archetypal KDEL-retrieval
46 system, a 7-transmembrane receptor captures escaped ER luminal proteins carrying
47 a C-terminal KDEL or variant tetrapeptide sequence in the acidic pH of the Golgi
48 (Munro and Pelham, 1987; Semenza et al., 1990). Signal binding to a luminal cavity
49 in the receptor triggers a conformational change in its cytoplasmic face that exposes
50 a lysine motif recognised by the COP I coat complex (Bräuer et al., 2019). Release
51 of the signal in the neutral pH environment of the ER results in a reversal of this
52 conformational change, burying the lysine motif, and exposing a patch of aspartate
53 and glutamate residues presumed to form a COPII-binding ER exit signal for the
54 receptor (Bräuer et al., 2019; Newstead and Barr, 2020). Hence, the KDEL receptor
55 cycles between the ER and Golgi capturing escaped ER proteins in a dynamic
56 retrieval process (Dean and Pelham, 1990; Lewis and Pelham, 1992; Townsley et
57 al., 1993; Zagouras and Rose, 1989). The rapid recycling of the receptor means it
58 does not need to be stoichiometric with the ER concentration of retained proteins,
59 only present at levels sufficient to capture escaped proteins that reach the Golgi
60 (Newstead and Barr, 2020). However, ER resident proteins differ widely in
61 abundance, yet, remarkably, this does not pose a problem for efficient retention of
62 low abundance proteins. One possible explanation for this is the presence of HDEL
63 and RDEL variants of the canonical KDEL signal with different binding affinities
64 (Scheel and Pelham, 1998; Wilson et al., 1993). However, despite extensive
65 mutation and structural analysis the molecular basis and functional significance of
66 these affinity differences remains unclear (Bräuer et al., 2019; Townsley et al.,

67 1993). Complicating this picture, in some organisms including the yeasts
68 *Kluyveromyces lactis* and *Schizosaccharomyces pombe*, DDEL and ADEL variants
69 are used as ER retrieval signals (Pidoux and Armstrong, 1992; Semenza and
70 Pelham, 1992). Comparative analysis of the budding yeast *Saccharomyces*
71 *cerevisiae* HDEL- and *K. lactis* DDEL-receptors implicated a luminal region including
72 a key variant residue, D50 in the human receptor, in selectivity for DDEL (Lewis et
73 al., 1990; Semenza et al., 1990; Semenza and Pelham, 1992). Mutation of D50 to
74 cysteine in the human receptor resulted in reduced binding affinity for KDEL, RDEL
75 and HDEL (Scheel and Pelham, 1998). However, recent structure determination of
76 the chicken receptor with a bound TAEKDEL peptide indicates this residue sits on
77 the luminal surface of the receptor and does not make contact with any portion of the
78 signal (Bräuer et al., 2019). Thus, although it is clear that the specificity of ER
79 retrieval is encoded by the KDEL receptor, the molecular basis for the recognition of
80 different signal variants remains unclear.

81 Our previous work has shown the KDEL receptor has a transporter-like
82 architecture and undergoes pH-dependent closure around cognate retrieval signals
83 (Bräuer et al., 2019; Newstead and Barr, 2020). However, the molecular basis for
84 affinity differences for retrieval signal variants and any functional significance these
85 differences may create, was not explained by that work or other previous studies.
86 Furthermore, how signals are initially captured and selected from other sequences
87 remains enigmatic. To answer these related questions, we solved structures of the
88 human KDEL receptor in complex with both HDEL and RDEL retrieval signals, and
89 performed a combination of computational and cell biological analysis. Based on this
90 data, we can break down the retrieval signal recognition process into a series of
91 steps for signal proofreading and initial capture of the free carboxyl terminus,

92 followed by full engagement with the binding cavity and finally pH-dependent closure
93 and activation of the receptor.

94

95 **RESULTS**

96 **ER retrieval signals in mammalian cells**

97 To understand how the KDEL-receptor differentiates between cargo proteins, we first
98 sought to define the major signal variants used in mammalian cells. For this purpose,
99 we exploited luminal ER proteome datasets to investigate the relative abundance of
100 retrieval signal variants (Itzhak et al., 2017; Itzhak et al., 2016). This confirmed that
101 KDEL, HDEL and RDEL are the major variants in mammals, and the frequency of
102 ER resident proteins with these variants of the retrieval signal at the -4 position is
103 approximately equal (Figure 1a). However, this does not reflect the abundance of the
104 proteins carrying the signal. Strikingly, the total concentration of KDEL bearing
105 proteins is over five-fold higher than either HDEL or RDEL (Figure 1b). This largely
106 reflects a small number of highly abundant ER-resident chaperones, BIP, PDI and
107 calreticulin (Figure 1 – supplement 1a and b). Each of these proteins is present in
108 the 5-10 μM range, far more abundant than the dominant KDEL receptor 2
109 (KDEL2) species which is estimated to be 0.2-0.3 μM (Figure 1 – supplement 1c).
110 In total, the concentration of retrieval signals thus exceeds that of the receptor by at
111 least two orders of magnitude. In good agreement with previously reported studies
112 on the mammalian KDEL receptor (Scheel and Pelham, 1998; Wilson et al., 1993),
113 we found that HDEL has the highest affinity for the receptor K_D 0.24 μM , followed by
114 KDEL K_D 1.94 μM and RDEL K_D 2.71 μM (Figure 1c). Previous work has suggested
115 DDEL binds to semi-purified human KDEL receptors in membrane fractions and can
116 function as a retrieval signal when the receptor is overexpressed at high level in

117 COS7 cells (Lewis and Pelham, 1992; Wilson et al., 1993). However, we find that
118 DDEL binds with 60-fold lower affinity than HDEL (K_D 14.9 μ M) (Figure 1c), in
119 agreement with other data for purified KDEL receptors (Scheel and Pelham, 1998).
120 Thus, the receptor binds to the HDEL sequence with one order of magnitude greater
121 affinity than the canonical KDEL ligand present on the most abundant ER resident
122 proteins. Despite this difference in affinities, mScarlet fusions with KDEL, RDEL or
123 HDEL signals all triggered similar changes to the steady-state distribution of the
124 KDEL receptor in cells, driving almost complete retrieval from the Golgi to the ER
125 (Figure 1d and 1e). By contrast, expression of ADEL or DDEL had little effect on the
126 Golgi-ER distribution of the receptor (Figure 1d and 1e). In line with these effects on
127 the receptor, the mScarlet-KDEL, RDEL and HDEL ligands were retrieved to the ER,
128 whereas ADEL and DDEL showed predominantly Golgi and punctate localisation
129 consistent with secretion (Figure 1d). These latter observations explain why there
130 are no verified examples of endogenous proteins using ADEL and DDEL retrieval
131 signals in mammalian cells.

132 Given its higher affinity, why then is HDEL not the dominant ER retrieval
133 signal, especially for crucial ER proteins such as BIP, PDI and calreticulin? We
134 tested the idea that due to its higher binding affinity, increasing the concentration of
135 HDEL bearing proteins would effectively compete for KDEL receptors in the Golgi,
136 and prevent efficient ER retrieval of KDEL and RDEL containing proteins. To do this
137 we used our series of variant xDEL signals, where x at the -4 position is either K, R,
138 H, A or D. When expressed in cells, KDEL, RDEL and HDEL are retained in the cell,
139 whereas ADEL and DDEL are mostly secreted (Figure 1 – supplement 1d and e).
140 With the exception of HDEL this is broadly in line with their respective binding
141 affinities. Despite binding to the receptor with a higher affinity (Figure 1c), HDEL was

142 less efficiently retained than either KDEL or RDEL (Figure 1 – supplement 1d and e).
143 We then examined the effect of these ligands on the major ER proteins BIP and PDI
144 as well as the less abundant chaperones ERP72 and ERP44 ((Figure 1 –
145 supplement 1a). As predicted, ADEL and DDEL had little effect on ER retention,
146 while HDEL caused secretion of all four proteins (Figure 1 – supplement 1f).

147 These results indicate that the retrieval system is selective yet not optimised
148 for binding affinity, and instead has evolved to ensure optimal retrieval of a broad
149 cohort of proteins of widely differing abundance. In human cells, ADEL and DDEL do
150 not bind to the receptor with high affinity and do not function as retrieval signals,
151 suggesting specific recognition of the -4 position is a key determinant for binding.
152 Previously, it has been suggested that complementary charges at receptor position
153 50 and the -4 position of the signal explain this specificity (Lewis and Pelham, 1992;
154 Semenza and Pelham, 1992). However, this mechanism does not obviously explain
155 how ADEL, with no charged residue at the -4 position, functions as a signal in some
156 organisms. How signal selectivity is achieved is therefore a crucial question we need
157 to answer.

158

159 **HDEL and RDEL signals bind similarly to the canonical KDEL variant**

160 To understand the molecular basis for the affinity differences between retrieval signal
161 variants, we determined structures for the chicken KDELR2 bound to HDEL and
162 RDEL signals. These structures with TAEHDEL and TAERDEL peptides have
163 resolutions of 2.24 and 2.31 Å, respectively (Figure 2a-2c and Table S1). In both
164 instances the overall structure of the receptor is similar to our previous complex with
165 the TAEKDEL peptide (Figure 2d), with a root mean square deviation (R.M.S.D.) of
166 0.223 and 0.153 Å over 200 C_α atoms for the HDEL and RDEL structures,

167 respectively. Both HDEL and RDEL peptides are bound in a vertical orientation with
168 respect to the membrane, with the side chains clearly resolved in the electron
169 density map (Figure 2 – supplement 1a and b). Both the HDEL and RDEL peptides
170 interact with the receptor through the same salt bridge interactions seen for the
171 KDEL peptide (Figure 2b-2d). Superimposing the three peptides reveals little
172 movement of the peptide at the -1 and -2 positions when bound to the receptor
173 (Figure 2e). For RDEL, we observe slight movement of the backbone C_α atom of the
174 peptide to accommodate the larger arginine side chain, resulting in a minor
175 repositioning of the glutamate at the -3 position in the receptor. Nonetheless, the
176 position of the positive charge at the -4 position on all three peptides is identical
177 relative to E117 and W120 within the receptor, supporting the view that a salt bridge
178 is formed with E117 on TM5. D50 previously proposed to be important for
179 recognition of the -4 position is at >5 Å distance, outside the region depicted in the
180 figures, indicating it is unlikely to form a salt bridge and directly contribute to binding
181 of the retrieval signal. Some studies have suggested the core tetrapeptide retrieval
182 motif should be extended to include the -5 and -6 positions (Alanen et al., 2011).
183 However, these positions are not conserved in retained ER luminal proteins (Figure
184 1a). In our structures, the glutamate at the -5 position sits close to S54, but would not
185 obviously increase the binding affinity, whereas no contacts are made to the -6
186 position. In all cases, the -1 position leucine residue and free carboxy terminus form
187 interactions to R47 and Y48 on TM2, as well as R159 and Y162 on TM6. The
188 glutamate at position -2 forms a further salt bridge interaction to R5 on TM1 and a
189 hydrogen bond to W166 on TM6, whereas the aspartate at -3 forms a salt bridge
190 with R169, also on TM6. For the histidine side chain at the -4 position of HDEL, the
191 imidazole group is predicted to form a π - π stacking interaction with W120 (Figure

192 [2b](#)). In comparison, the RDEL arginine side chain sits in the same position as the
193 amine group of the KDEL sequence and could thus interact with W120 via a cation- π
194 interaction and E117 via a classical salt bridge interaction ([Figure 2c](#)). We therefore
195 conclude that both E117 and W120 play a role in retrieval signal binding, and the
196 only major difference between the HDEL, RDEL and KDEL signals is the precise
197 nature of the interaction with W120 indicating that this may be a critical residue to
198 explain the affinity of different signals.

199

200 **Probing the importance of E117 and W120 for signal binding**

201 To directly test the requirement for E117 and W120 in signal recognition, ligand
202 binding assays using recombinant chicken wild type, E117 or W120 mutant KDELR2
203 were performed (Bräuer et al., 2019). All proteins had similar thermal stability
204 indicating they were correctly folded. For the wild type receptor at pH 5.4, K_D for
205 KDEL and HDEL signals were $1.9 \pm 0.46 \mu\text{M}$ and $0.26 \pm 0.04 \mu\text{M}$, respectively
206 ([Figure 3a and 3b](#)). Conservative substitution of E117 with aspartate resulted in a
207 slight reduction in binding for both KDEL and HDEL, K_D of $2.1 \pm 0.33 \mu\text{M}$ and $0.52 \pm$
208 $0.02 \mu\text{M}$, respectively ([Figure 3a and 3b](#)). Substitution of E117 with alanine had a
209 greater effect on KDEL binding, $K_D \sim 9.3 \pm 1.0 \mu\text{M}$, compared to HDEL, $K_D 0.52 \pm$
210 $0.02 \mu\text{M}$ ([Figure 3a and 3b](#)). This suggested that any salt bridge to E117 plays a
211 greater role for KDEL than HDEL signals.

212 We next examined the contribution of W120 to signal recognition. Tryptophan
213 side chains have long been recognized as important contributors in protein ligand
214 interactions, as they are capable of interacting with ligands via both aromatic and
215 charged forces (Dougherty, 1996; Liao et al., 2013; Okada et al., 2001). Our
216 structures show that the -4 position histidine, arginine or lysine side chain of the

217 human retrieval signal variants can in principle interact favourably with W120 via
218 cation- π interactions. We also reasoned that given the additional π - π stacking
219 observed with the imidazole group in the crystal structure, this interaction might
220 explain the increased affinity observed for the HDEL signal variant. Mutation of
221 W120 to alanine resulted in loss of binding to the KDEL peptide and it was not
222 possible to calculate a K_D (Figure 3a). For the HDEL peptide, binding was reduced to
223 20% confirming that W120 plays an important role in mediating receptor-peptide
224 interactions (Figure 3b). Consistent with the hypothesis that the -4 histidine of HDEL
225 undergoes π - π stacking interactions with W120, conserved substitution to
226 phenylalanine supported 50% HDEL binding with K_D $5.5 \pm 0.57 \mu\text{M}$, whereas no
227 interaction was observed with the KDEL peptide (Figure 3a and 3b). Thus, W120
228 plays a crucial role in binding of both KDEL and HDEL signals and may explain the
229 higher affinity of the receptor for HDEL. By contrast, E117 is less important and it is
230 unclear why it is a conserved feature of the binding site.

231 To analyse whether the properties measured using purified components *in*
232 *vitro* reflect the behaviour of the KDEL receptor and retrieval system *in vivo*, we
233 analysed the ability of these same variants in the human KDEL receptor to
234 differentiate between human retrieval signal sequences in a cellular ER retrieval
235 assay. All the receptor mutants tested reached the Golgi apparatus supporting the
236 view they are able to fold and exit the ER (Figure 3c, -Ligand, and Figure 3 –
237 supplement 1a). The WT receptor showed robust retrieval to the ER in response to
238 model cargo proteins bearing KDEL, RDEL or HDEL retrieval sequences (Figure 3c
239 and Figure 3 – supplement 1a-d). Receptors with conservative (E117Q and E117D)
240 or non-conservative (E117A) substitutions at E117 were efficiently retrieved to the
241 ER with KDEL, RDEL or HDEL signal variants (Figure 3c and Figure 3 – supplement

242 [1b-d](#)). By contrast, receptors with mutations at W120A and W120F did not respond
243 to KDEL and RDEL signals and showed greatly reduced response to HDEL ([Figure](#)
244 [3c and Figure 3 – supplement 1b-d](#)). The residual response to HDEL was abrogated
245 in a double E117A/W120A mutant receptor ([Figure 3c and Figure 3 – supplement](#)
246 [1b-d](#)). This *in vivo* behaviour is in good agreement with the changes to affinity
247 measured using *in vitro* binding assays ([Figure 3a and 3b](#)), and supports the view
248 that W120 is of greater importance for ligand binding and ER retrieval.

249 To provide further support for this conclusion, we investigated the free energy
250 of interaction between the histidine side chain of the retrieval signal and W120 of the
251 receptor. Protonation of the HDEL histidine is a crucial consideration since retrieval
252 signal binding to the receptor occurs at acidic pH in the Golgi. We therefore asked if
253 the protonation state of the histidine is important for binding affinity. Molecular
254 mechanics-based alchemical transformation was used to compute the free energy
255 difference of changing the lysine in KDEL to different protonation states of the
256 histidine in HDEL. The binding free energy of HDEL is -1.8 ± 1.4 kcal.mol⁻¹ stronger
257 than the KDEL signal ([Table S2](#)), which is in good agreement with the expected -1.3
258 kcal/mol free energy difference derived from measured K_D values for KDEL and
259 HDEL. The preference for HDEL of -1.9 ± 0.2 kcal.mol⁻¹ is mainly attributed to the
260 protonated histidine which makes favourable cation- π interactions with W120 ([Table](#)
261 [S2, HIP](#)). In agreement with the experimental data ([Figure 3b and 3c](#)), the W120F
262 mutation, which is anticipated to preserve the cation- π interactions, reduced but did
263 not abolish the preference for HDEL to -0.7 ± 1.6 kcal.mol⁻¹, notwithstanding the
264 large error on this calculation. Furthermore, the W120A mutation which eliminates
265 the cation- π interactions, greatly reduced the preference for HDEL to -0.3 ± 0.9
266 kcal.mol⁻¹.

267 To quantify the strength of the π - π and cation- π interactions between W120
268 variants and the histidine, we decomposed the interactions using symmetry-adapted
269 perturbation theory from quantum mechanics. Although both W120 and W120F form
270 π - π and cation- π interactions with protonated histidine, W120F exhibits ~ 1.5 kcal/mol
271 weaker π - π interactions and ~ 0.5 kcal/mol weaker cation- π interactions with the
272 histidine (Figure 3d and Table S3). The consequence of these changes is that for
273 W120F higher root mean squared fluctuations are seen (Figure 3e), indicative of less
274 rigid binding. These fluctuations are further increased for W120A (Figure 3e),
275 consistent with its greater effect on signal binding. These results support the
276 hypothesis that the π - π interactions between the protonated histidine sidechain and
277 W120 explain the higher affinity observed for HDEL signals. Further support for this
278 interpretation comes from *in vitro* analysis of the pH-dependence of HDEL binding.
279 At pH6.4 HDEL shows $\sim 60\%$ maximal binding to the receptor (Figure 3f), compared
280 to $<20\%$ seen at the same pH for KDEL (Bräuer et al., 2019). The level of HDEL
281 binding seen at pH 7 would saturate the KDEL receptor in the ER if the most
282 abundant luminal proteins such as BIP carried this signal variant. Our observation
283 that W120 is also necessary for recognition of KDEL indicates that cation- π
284 interactions to W120, rather than a salt bridge to E117, is the crucial determinant for
285 recognition of the -4 position.

286

287 **E117 plays a role in KDEL receptor selectivity**

288 This mode of signal binding involving W120 is different than previously proposed,
289 where charge complementarity between D50 in the receptor and the -4 position of
290 the signal was thought to be a key determinant of specificity in ER retrieval (Lewis
291 and Pelham, 1992; Scheel and Pelham, 1998; Semenza and Pelham, 1992).

292 However, as our crystal structures show, D50 is outside the immediate binding
293 region for all retrieval signal variants and therefore unlikely to directly contribute to
294 binding. Thus, the precise roles of D50 and E117 remain enigmatic. In this regard
295 the behaviour of ADEL signals is noteworthy due to the simple methyl side chain.
296 Comparison of different retrieval signals shows that ADEL does not activate the wild
297 type human KDEL receptor ([Figure 1d and 1e](#)). The simplest explanation for this
298 finding is that the -4 position is crucial for high affinity binding of retrieval signals to
299 the human receptor. However, this view is unlikely to be correct. First, the KDEL,
300 RDEL and HDEL bound receptor structures do not support the view that recognition
301 of the -4 position requires D50, and instead provide an alternative possibility where
302 E117 fulfils this role. However, our biochemical and functional data show that E117
303 does not contribute greatly to signal binding affinity or retrieval in cells ([Figure 3a-](#)
304 [3c](#)). Therefore, rather than selecting for the sequence, E117 may be more important
305 to select against unwanted signal variants. To test this idea, we examined the
306 response of E117A mutant receptors to variant ADEL and DDEL signals.
307 Remarkably, the E117A mutant receptor relocated to the ER in response to both
308 KDEL and ADEL, but not DDEL signals ([Figure 4a and 4b](#)). In *S. pombe* and *K.*
309 *lactis*, organisms where ADEL and DDEL are used for ER retrieval, the E117
310 position is either an asparagine or a glutamine residue, and we therefore tested
311 E117N and E117Q mutants next. Similar to the results with E117A, E117Q and
312 E117N receptors move to the ER in response to KDEL or ADEL signals, yet
313 interestingly still failed to respond to DDEL ([Figure 4a and 4b](#)). Ligand expression
314 was in a similar range in all instances ([Figure 4 – supplement 1](#)), and in the absence
315 of ligand all three mutant receptors localised to the Golgi with a low ER background
316 indicating normal folding and ER exit ([Figure 4a](#)).

317 Thus, E117 is important for determining which signals are rejected by the wild
318 type human receptor based on the -4 position of the signal, but does not appear to
319 play a major role in binding affinity. ADEL must bind to the E117A mutant receptors
320 via the “DEL” tri-carboxylate portion of the retrieval signal, suggesting this region
321 may be the major contributor to binding affinity for all signal variants. For HDEL, the
322 protonated histidine side chain makes additional π - π interactions with W120 to bind
323 with higher affinity. Importantly, the lack of response to DDEL shows that signal
324 selection and recognition must involve additional features in the *S. pombe* and *K.*
325 *lactis* receptor, and we investigated this question further.

326

327 **A charge screening mechanism for signal differentiation by the KDEL receptor**

328 To identify additional features that might play a role in signal selection, we performed
329 a comparison of the receptors and most abundant cognate ligands of the HSPA5/BIP
330 family of ER resident proteins in different species. Although most regions of the
331 receptor are highly conserved, as noted previously (Semenza and Pelham, 1992),
332 sequence alignment reveals two regions where there is covariation that may be
333 related to the cognate tetrapeptide retrieval signal (Figure 5a). In receptors
334 recognising ADEL and DDEL, D50 is changed for asparagine, E117 for glutamine or
335 asparagine, and position 54 is a positively charged arginine or lysine rather than a
336 polar side chain (Figure 5a). To understand the consequences of these changes we
337 examined their positions relative to the bound TAEHDEL signal (Figure 5b). This
338 reveals that E117 and S54 sit close to the -4 histidine and -5 glutamate, respectively
339 and D50 is over 5 Å away from any residue in the signal in the final bound state
340 (Figure 5b). Analysis of the charge distribution across the surface of the receptor
341 shows a negatively charged feature above the positively charged binding cavity

342 occupied by the DEL portion of the signal, with the -4 residue sited at the boundary
343 to these two regions (Figure 5c). Strikingly, progressive introduction of changes in
344 the human receptor to mimic the *K. lactis* receptor, D50N S/N54K E117Q erodes the
345 negatively charged luminal feature (Figure 5d).

346 One simple explanation for this feature is that it extends the binding site to
347 impart specificity for the region upstream of the core KDEL signal. However, analysis
348 of different classes of ER luminal proteins from yeast and animal cells does not
349 provide strong support for this possibility. The upstream sequences of many
350 abundant ER proteins including human and yeast HSPA5/BIP homologues are acidic
351 in nature, and not basic (Figure 5a and Figure 5 – supplement 1a), making any
352 interaction unfavourable. For the human signal, the -4 position is crucial and
353 mutation to A or D abolishes ER retrieval of the receptor (Figure S5b and Figure 5 –
354 supplement 1d). Conversely, *S. pombe* and *K. lactis* BIP ADEL and DDEL signals
355 become functional with the human receptor if the -4 position is changed to lysine
356 confirming this is the critical residue, independent of upstream sequences (Figure 5
357 – supplement 1c and d). In *K. lactis* BIP the -5 position is a bulky aromatic residue
358 rather than a charged residue. Previous work has suggested that the budding yeast
359 FEHDEL signal with a bulky aromatic residue at the -6 position does not function in
360 mammalian cells (Wilson et al., 1993), however consistent with our other data we
361 find this HDEL variant is also functional (Figure 5 – supplement 1c). Extending this
362 analysis to human FKBP family proteins with even more diverse upstream
363 sequences reveals no obvious pattern of conservation other than the canonical C-
364 terminal HDEL or HEEL retrieval signal (Figure 5 – supplement 1e).

365 To directly test the role of the charged luminal surface in signal selection, we
366 made a series of mutants introducing the changes seen in *K. lactis* and *S. pombe*

367 into the human receptor and tested these against KDEL, ADEL and DDEL signals. A
368 single D50N mutation abolished the response to all signal variants and the receptor
369 remained in the Golgi ([Figure 6a and 6b](#)). Thus, like E117, D50 is not the sole
370 determinant of signal selectivity. Similarly, N54K reduced the response to KDEL but
371 did not result in ADEL or DDEL recognition ([Figure 6a and 6b](#)). A D50N N54K double
372 mutant showed a loss of specificity and gave an intermediate response to KDEL,
373 ADEL and DDEL signals, showing that it is possible to uncouple binding from
374 selectivity at the -4 position. We then combined D50N or N54K with E117Q
375 mutations. These double mutant receptors showed switched specificity towards
376 ADEL and DDEL with only a residual response to KDEL ([Figure 6a and 6b](#)).
377 Combination of D50N N54K and E117Q improved the response to ADEL and DDEL
378 and further reduced that towards KDEL ([Figure 6a and 6b](#)). Comparable results were
379 obtained with a *S. pombe* like D50N N54R E117N triple mutant receptor ([Figure 6 –](#)
380 [supplement 1a and b](#)). Both these altered specificity receptors responded to the
381 cognate ADEL or DDEL variant of BIP for that organism, a response that was
382 abolished solely by mutation of the -4 position of the signal ([Figure 6 – supplement](#)
383 [1a and b](#)).

384 These results indicate that the -4 position of the signal is read out during initial
385 signal binding and is important for exclusion of unwanted signals, but is less
386 important for binding affinity. We therefore tested whether the mode of ADEL and
387 DDEL binding to the switched specificity receptors still involves W120. A D50N N54K
388 E117Q W120A mutant receptor does not relocate from the Golgi to the ER with
389 KDEL and ADEL signals and shows only a small response to the DDEL signal
390 ([Figure 6a and 6b](#)). Together, these findings suggest a common mode of binding for
391 all retrieval signal variants through residues conserved in all species. Specificity for

392 the -4 position is largely achieved through a proofreading mechanism involving two
393 gatekeeper residues, D50 and E117, as the signal enters the ligand binding cavity.
394 An E117A substitution partially uncouples this mechanism and allows ADEL binding,
395 whereas both D50 and E117 residues have to be changed to allow DDEL binding.
396 Bringing together all our observations to this point, we conclude that the luminal
397 surface of the receptor plays a crucial role in signal selectivity prior to adoption of the
398 final activated state, perhaps by determining the rate of signal association from
399 solution.

400

401 **Initial retrieval signal capture by the free carboxyl terminus**

402 To explore the initial interaction of retrieval signals with the KDEL receptor we
403 simulated an all-atom model of the KDEL signal with a free C-terminal carboxylate
404 engaging with the receptor ([Supplemental movie 1](#)). This simulation shows that the
405 signal initially encounters the receptor through a salt bridge interaction from its C-
406 terminal carboxyl group with R169 on TM6 of the receptor ([Figure 7a, i.](#)). The C-
407 terminal carboxyl group then moves to engage R5 ([Figure 7a, ii.](#)), shortly followed by
408 interaction of the -2 glutamate with R169 ([Figure 7a, iii.](#)). Finally, the C-terminus
409 engages with R47 on TM4 enabling the -3 aspartate to interact with R169 ([Figure 7a,](#)
410 [iv.](#)). Thus, the carboxy-terminus of the retrieval signal sequentially engages R169,
411 R5 and finally R47 ([Figure 7c](#)). Movement of the -4 position lysine towards E117 is
412 concomitant with the final engagement of the carboxyl-terminus of the signal by R47,
413 whereas D50 does not come in close proximity to the KDEL signal and there is only
414 a transient interaction of S54 with the -5 position ([Figure 7d](#)).

415 We therefore propose a carboxyl-handover model for signal capture mediated
416 by the ladder of arginine residues in the binding pocket ([Figure 7b](#)). As the carboxyl-

417 terminus progresses further into the receptor binding site, the carboxylate groups at
418 positions -2 and -3 engage their respective positions in the D- and E-sites
419 respectively. Only the final stage of the binding, where the receptor closes around
420 the signal locking it in place is pH dependent, all other stages are predicted to be
421 freely and rapidly reversible. Because many proteins have a free C-terminal
422 carboxylate, this highlights the importance of an initial proofreading stage where non-
423 cognate signals are rejected, as we have already argued, due to their net charge.

424 To test these ideas, we investigated the importance of the retrieval signal C-
425 terminus and R169 in the receptor using *in vitro* binding assays and functional
426 experiments in cells. First, we synthesised C-terminally amidated HDEL and KDEL
427 peptides and assayed their ability to bind to wild-type receptors ([Figure 7e](#)). Blocking
428 the C-terminal carboxylate in this way completely abolished binding to KDEL and
429 reduced the affinity for the HDEL peptide by two orders of magnitude from 19 ± 1.3
430 μM to 1.7 ± 0.1 mM. For HDEL, this residual affinity suggests the peptide still enters
431 and exits the binding pocket, but fails to trigger the final pH dependent capture. Next,
432 we performed binding assays with R169 variant receptors. Comparable results to the
433 C-amidated peptide binding assays were obtained with R169A, which showed no
434 binding to KDEL and greatly reduced binding to HDEL ligands ([Figure 7f](#)).
435 Conservative substitution to R169K greatly reduced binding of both HDEL and KDEL
436 in line with predictions ([Figure 7f](#)). Finally, we tested the R169 variants in ER
437 retrieval assays. R169A mutant receptors showed no response to KDEL and only
438 ~10% response to HDEL signals ([Figure 7g](#), [Figure 7 – supplement 1a and b](#)). By
439 contrast, the conservative substitution R169K showed an attenuated response to
440 both signals, in agreement with the simulation and reduced binding affinity ([Figure](#)
441 [7g](#), [Figure 7 – supplement 1a and b](#)). We therefore conclude that the interaction of

442 receptor R169 with the C-terminal carboxylate of the retrieval signal plays an
443 important role in initial signal capture.

444
445 **DISCUSSION**

446 **A baton-relay mechanism for initial signal capture by the KDEL receptor**

447 Canonical ER retrieval signals can be broken down into two components: the -4
448 position, which enables the receptor to distinguish between different populations of
449 ER proteins, and a tri-carboxylate moiety formed by the -3 aspartate, -2 glutamate
450 and -1 C-terminal carboxylate. We propose a baton-relay handover mechanism for
451 capture of this signal by the KDEL receptor wherein a ladder of three arginine
452 residues in the receptor pairs with the three-carboxyl groups of the signal ([Figure 8](#)).
453 During cargo capture, the receptor engages the retrieval signal in a stepwise
454 process, with the C-terminal carboxyl group of the cargo protein moving between
455 these three interaction sites. At neutral pH, C-terminal sequences will rapidly sample
456 the binding site, a process that we imagine will occur in both the ER and Golgi
457 apparatus. Only at acidic pH are cargo proteins captured once the C-terminus of the
458 signal engages with R47 in the receptor, which then undergoes a conformational
459 change to close around the signal, locking it in place, as we have explained
460 previously (Bräuer et al., 2019). This mechanism explains why the retrieval signal
461 must be located at the C-terminus of the cargo protein, and the defined requirement
462 for either glutamate or aspartate residues at the -2 and -3 positions due to their
463 carboxyl group containing side chains. Variation at the -4 position would not directly
464 alter this initial capture mechanism, possibly explaining why it is the key determinant
465 for signal selectivity.

466 The structures we have obtained for the KDEL receptor with bound HDEL,
467 RDEL or KDEL signals reveal that the side chains at the -4 position form a salt

468 bridge interaction with E117 but, crucially, not D50 as previously proposed.
469 Unexpectedly, the salt bridge interaction between E117 and the -4 position of the
470 retrieval signal makes only a limited contribution to binding affinity and does not
471 explain the higher affinity for HDEL. Our other data show that the higher affinity for
472 HDEL is due to the stronger π - π interaction between the histidine of the -4 position of
473 the retrieval signal and W120 in the receptor. However, because E117A mutant
474 receptors have expanded specificity and can recognise ADEL, we conclude that the
475 side chain at the -4 position is unlikely to play a major role in binding affinity for
476 signals other than HDEL. For these reasons, we refer to the -4 position as the
477 passkey residue, important for the signal selection only. By determining net charge
478 on the signal it may thus play a greater role in initial binding kinetics, rather than
479 affinity per se.

480 Previous work has suggested that the -5 and -6 positions of the retrieval
481 signal also play a key role in signal binding (Alanen et al., 2011), and that the
482 individual human KDEL receptors have unique specificity (Raykhel et al., 2007).
483 However, these properties are not completely consistent with the structures, pattern
484 of sequence conservation, or wider analysis presented here. It is noteworthy that
485 both those studies used a bimolecular fluorescence complementation approach
486 where the signal and receptor are dimerised by a split YFP molecule that will likely
487 contribute to the observed signal binding affinity. This will interfere with the initial
488 proofreading mechanism described here, making comparison with our data difficult.
489 Since the three human KDEL receptors share identical ligand binding residues and
490 only differ in conservative substitutions at position 54, we believe they will have the
491 same or closely-related ligand binding properties. Based on the structures it seems
492 reasonable that the -5 position may contribute to signal proofreading in some cases.

493 However, as we show, a wide variety of signals that lack any obvious conserved
494 features upstream of the canonical tetrapeptide function efficiently to trigger ER
495 retrieval of the receptor ([Figure 1a and S5b-S5e](#)), suggesting the -5 position
496 modulates but does not play an essential role in signal recognition. Taken together,
497 these data support a model for retrieval sequence recognition that explains both the
498 importance of the free C-terminal carboxyl group and how changes at the -4 position
499 can modulate binding to the receptor.

500

501 **The important role of luminal pH in regulating HDEL-mediated ER retrieval.**

502 One important outcome from our work is the idea that KDEL receptors are not
503 optimised for an individual signal and must retain the ability to differentiate variant
504 high and low affinity ER retrieval signals. We propose that cells exploit these
505 properties to maximise the retrieval efficiency of a broad range of ER resident
506 proteins with widely different abundance, over 2 or 3 orders of magnitude. This idea
507 explains an explanation for the functional significance of the affinity differences of
508 retrieval signal variants in mammalian cells. The most abundant proteins use the
509 KDEL retrieval signal, whereas lower abundance proteins tend to carry the HDEL
510 signal. By artificially increasing the concentration of HDEL proteins we can show that
511 this effectively poisons the ER retrieval system, leading to the secretion of normally
512 retained ER chaperones. This behaviour is reminiscent of other cellular regulatory
513 systems, where substrate or signal binding properties are optimised for rate and
514 turnover, rather than for the highest affinity which can reduce throughput of the
515 pathway. Indeed, in some cases electrostatic properties are exploited to create
516 rapid-binding high-affinity inhibitors that outcompete substrates (Cundell et al., 2016;
517 Schreiber and Fersht, 1996). This may explain why histidine has been selected for

518 the highest affinity variant of the signal to counteract this effect. For the HDEL variant
519 of the retrieval signal, protonation of both the receptor histidine 12 and signal peptide
520 histidine -4 favour binding to the receptor in the Golgi. However, deprotonation of
521 both the retrieval signal and receptor at pH 7.0 enable rapid release in the ER, and
522 hence receptor recycling to the Golgi. Thus, HDEL binds more tightly than KDEL in
523 the Golgi, but still releases rapidly in the ER. A signal with the same affinity as HDEL
524 that was not proton dependent would strongly inhibit retrieval even at low
525 concentration due to slow release at neutral pH. An alternative mechanism to
526 capture low abundance ER proteins would have been to increase the cellular
527 concentration of the KDEL receptor from the observed low levels. That would require
528 receptors to be nearly stoichiometric with cargo, a problematic proposition
529 considering the millimolar concentration of ER chaperones. These potential traps are
530 avoided by the combination of pH-regulation of both the receptor and the high affinity
531 HDEL signal. Thus, the versatile binding site architecture of a single KDEL receptor
532 enables differentiation of both high and low affinity signals, thereby enabling efficient
533 ER retrieval of both low and high abundance proteins in eukaryotic cells.

534

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541 PhD student (203741/Z/16/A)

542

543 The authors declare no competing financial interests.

544

545 **AUTHOR CONTRIBUTIONS**

546 FAB, SN, PB, AG and JLP designed the experiments. PB, AG, TS, JLP, FAB and SN

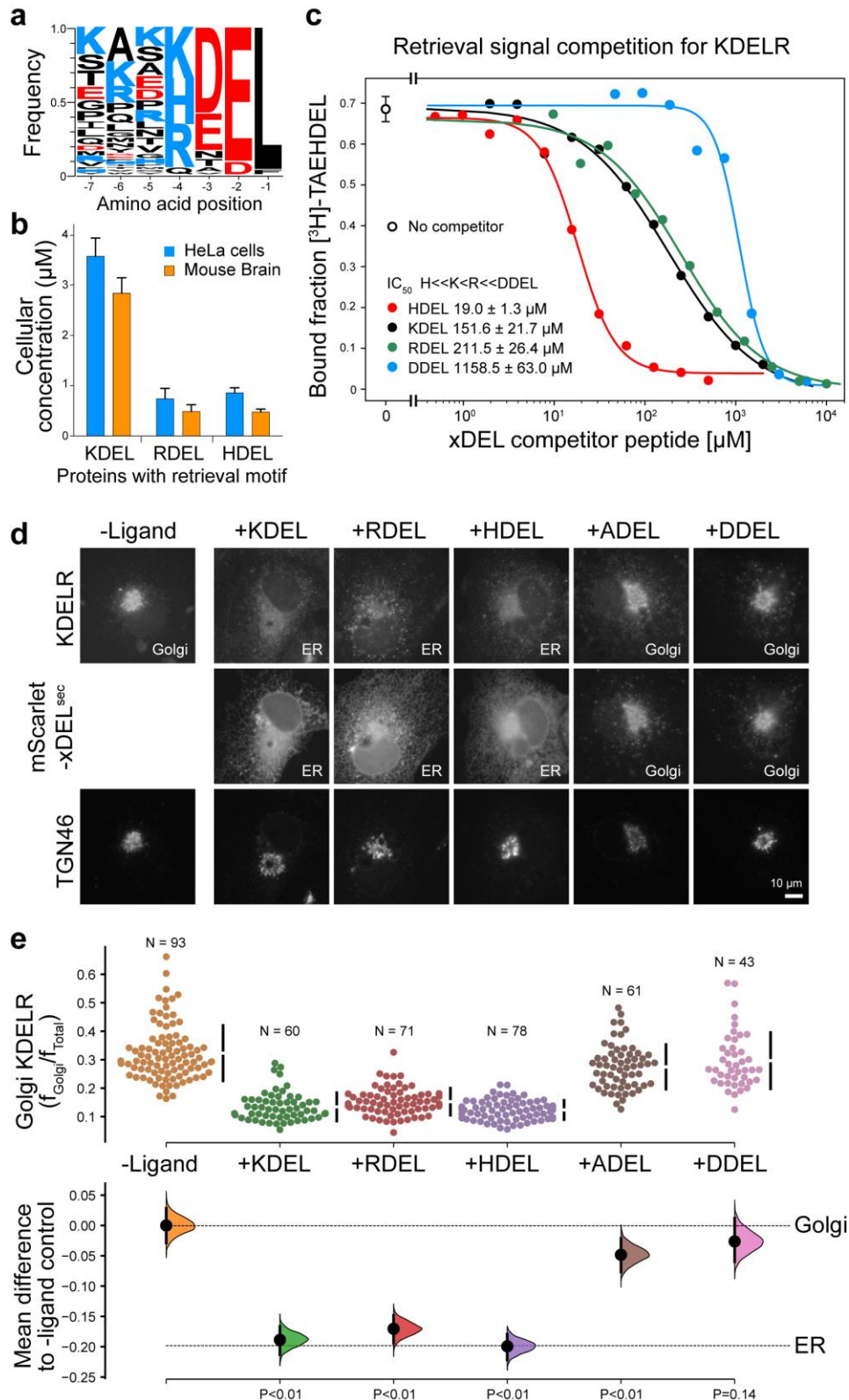
547 carried out experiments and interpreted the data. ZW and PCB designed and

548 performed the computational analysis. FAB and SN wrote the paper with input from

549 all authors.

550

551 **MAIN FIGURES**



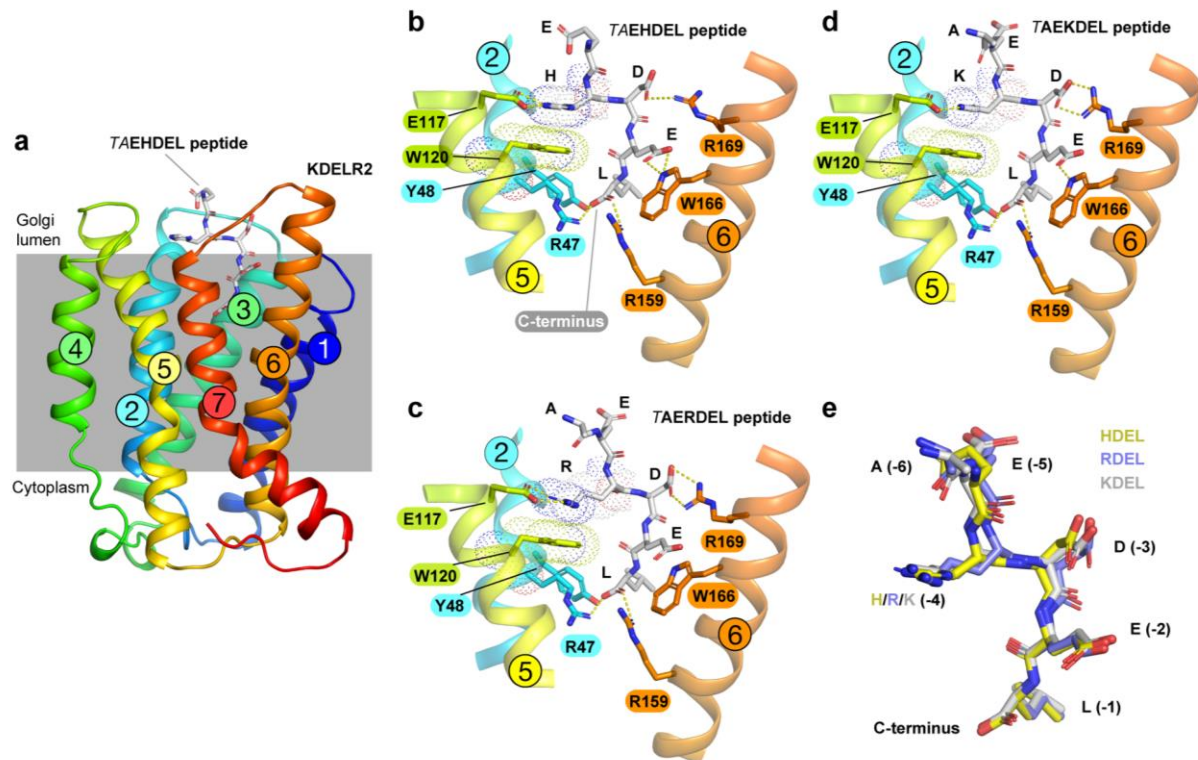
552

553 **Figure 1. ER retrieval signal abundance and affinity are not correlated. a.**

554 Sequence logos for ER resident proteins with C-terminal KDEL retrieval signals and

555 variants thereof calculated using frequency or protein abundance (Itzhak et al., 2017;

566 Itzhak et al., 2016). **b.** Combined cellular concentrations of ER resident proteins with
567 canonical KDEL, RDEL and HDEL retrieval sequences in HeLa cells and mouse
568 brain. **c.** Competition binding assays for [³H]-TAEHDEL and unlabelled TAEKDEL,
569 TAERDEL and TAEHDEL to the KDEL receptor showing IC₅₀ values for the
560 competing peptides. These were used to calculate apparent K_D using the Cheng-
561 Prusoff equation (Cheng and Prusoff, 1973). **d.** Endogenous KDEL receptor
562 redistribution was measured in the absence (-ligand) or presence of K/R/H/A/DDEL
563 (mScarlet-xDEL^{sec}). TGN46 was used as a Golgi marker. Scale bar is 10µm. **e.** The
564 mean difference for K/R/H/A/DDEL comparisons against the shared no ligand control
565 are shown as Cummings estimation plots. The raw data for the fraction of KDEL
566 receptor fluorescence in the Golgi is plotted on the upper axes with sample sizes and
567 p values.
568



569

570 **Figure 2. Structures of the KDEL receptor bound to HDEL and RDEL retrieval**

571 **signals. a.** Crystal structure of chicken KDELR2 viewed from the side with the

572 transmembrane helices numbered and coloured from N-terminus (blue) to C-

573 terminus (red). The predicted membrane-embedded region of the receptor is

574 indicated by a grey shaded box, with labels at the luminal and cytoplasmic faces.

575 The TAEHDEL peptide is shown in stick format, coloured grey. **b.** Close up views of

576 bound TAEHDEL (this study), **c.** TAERDEL (this study) and **d.** TAEKDEL

577 (PDB:6I6H) peptides bound to the receptor are shown with contributing side chains

578 labelled. Hydrogen bonds are indicated as dashed lines. The molecular orbitals of

579 W120 and the -4 histidine on the peptide are shown as a dotted surface. **e.**

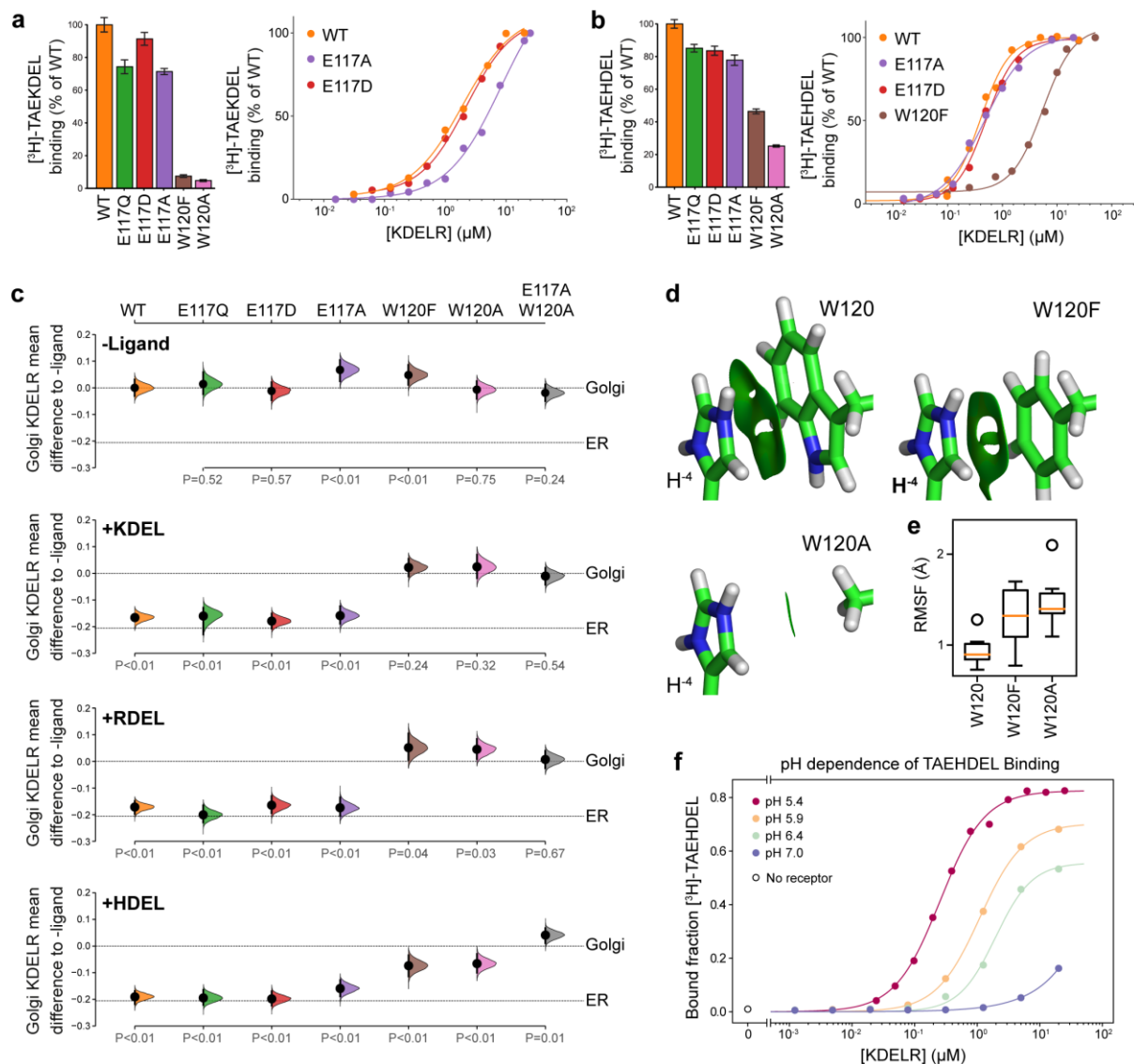
580 Superposition of the HDEL, RDEL and KDEL peptides reveals near identical binding

581 position within the receptor. Retrieval signal side chains are numbered counting

582 down from the C-terminus.

583

584



585

586 **Figure 3. Roles of KDEL receptor E117 and W120 in retrieval signal binding**

587 **and function in cells. a.** Normalised binding of [³H]-TAEKDEL and **b.** [³H]-

588 TAEHDEL signals to purified WT and the indicated E117 and W120 mutant variants

589 of chicken KDELRL2. Bar graphs show mean binding ± SEM (n=3). Line graphs show

590 titration binding assays. **c.** The fraction of WT, E117 and W120 mutant KDEL

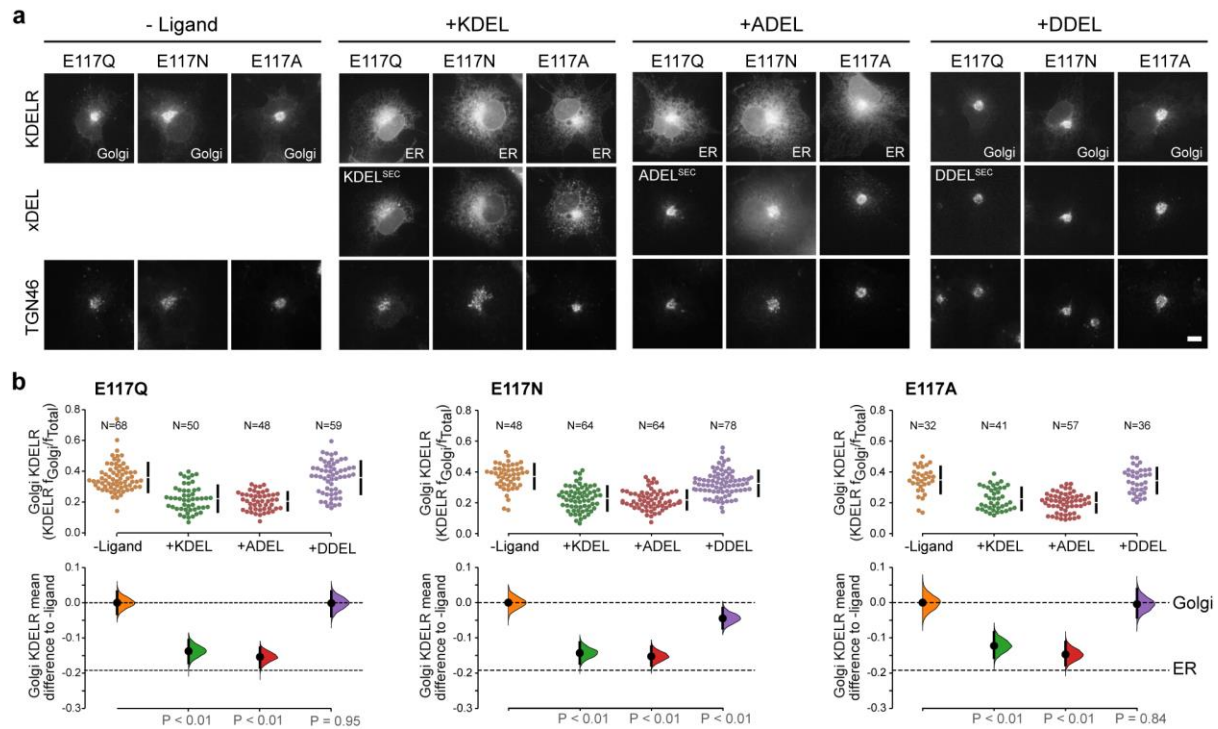
591 receptor localised to the Golgi was measured before (no ligand) and after challenge

592 with different retrieval signals (K/R/HDEL) as indicated. Effect sizes are shown as

593 the mean difference for K/R/HDEL comparisons against the shared -ligand control

594 with sample sizes and p-values. **d.** The π - π interactions between W120 and the
595 histidine were visualised using reduced density gradient analysis. The wild-type
596 W120 exhibit stronger π - π interactions compared with W120F, while W120A shows
597 no π - π interactions. **e.** When W120 is changed to phenylalanine, the protonated
598 histidine has a higher root mean squared fluctuation (RMSF) in the binding pocket,
599 which is further increased for the W120A substitution. **f.** Binding of [³H]-TAEHDEL to
600 the KDEL receptor was measured at pH5.4-7.0 and is plotted as a function of
601 receptor concentration.

602



603

604 **Figure 4. KDEL receptor E117 mutants show reduced selectivity for retrieval**

605 **signals. a.** E117Q, E117N or E117A mutant KDEL receptors were tested for

606 K/A/DDEL-induced redistribution from Golgi to ER. KDEL receptor distribution was

607 followed in the absence (-ligand) or presence of K/A/DDEL^{SEC}. TGN46 was used as a

608 Golgi marker. Scale bar is 10 μ m. **b.** The fraction of E117Q, E117N or E117A mutant

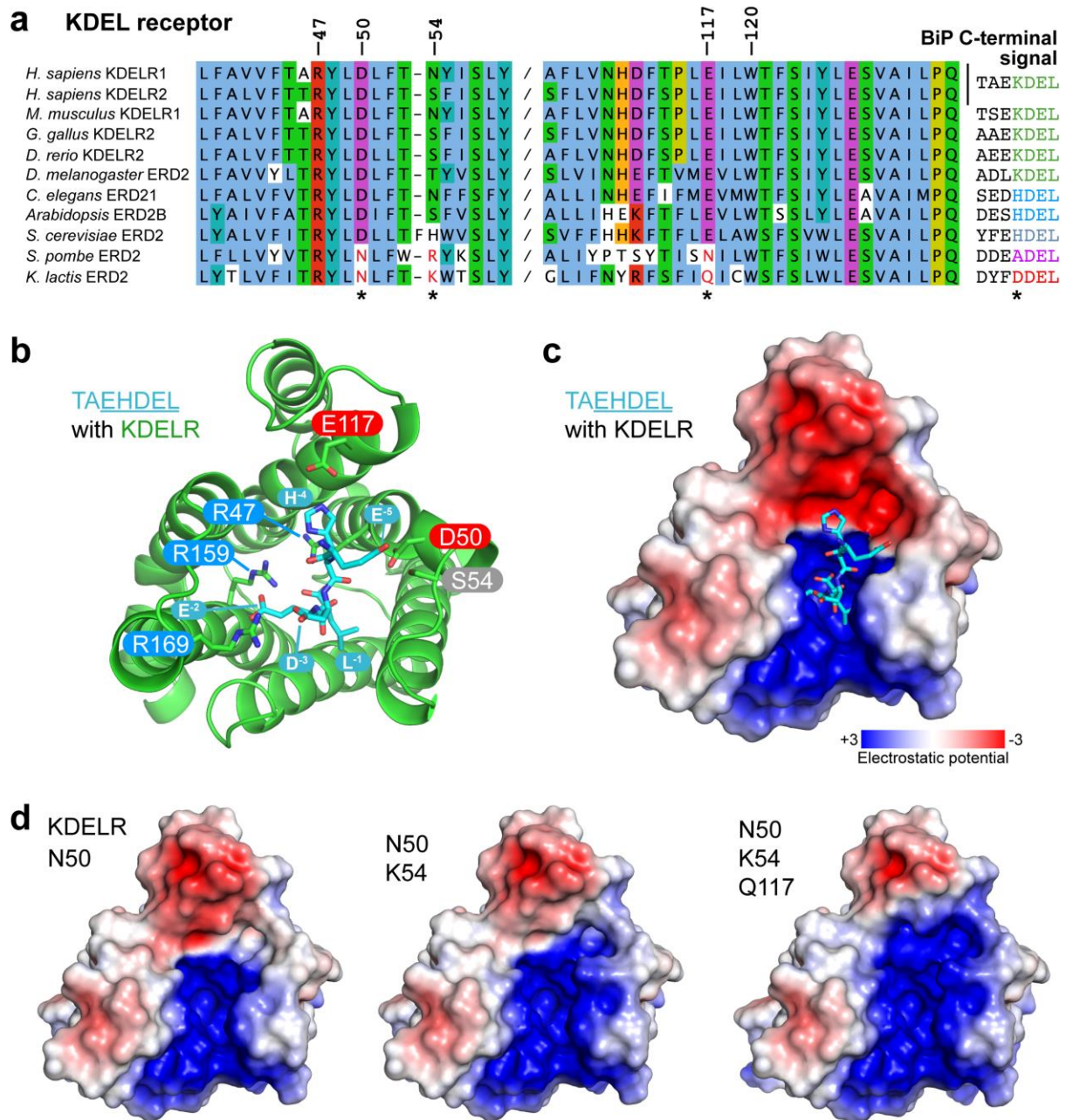
609 KDEL receptor localised to the Golgi was measured before (no ligand) and after

610 challenge with different retrieval signals (K/A/DDEL). Effect sizes are shown as the

611 mean difference for K/A/DDEL comparisons against the shared -ligand control with

612 sample sizes and p values.

613



614

615

Figure 5. Charge distribution across the luminal opening of the KDEL receptor

616

binding pocket. a. KDEL receptor sequence alignment showing two regions centred

617

around amino acid D50 and W120 of the human proteins. Cognate retrieval signal

618

variants are shown to the right of the alignment. **b.** The structure of the KDEL

619

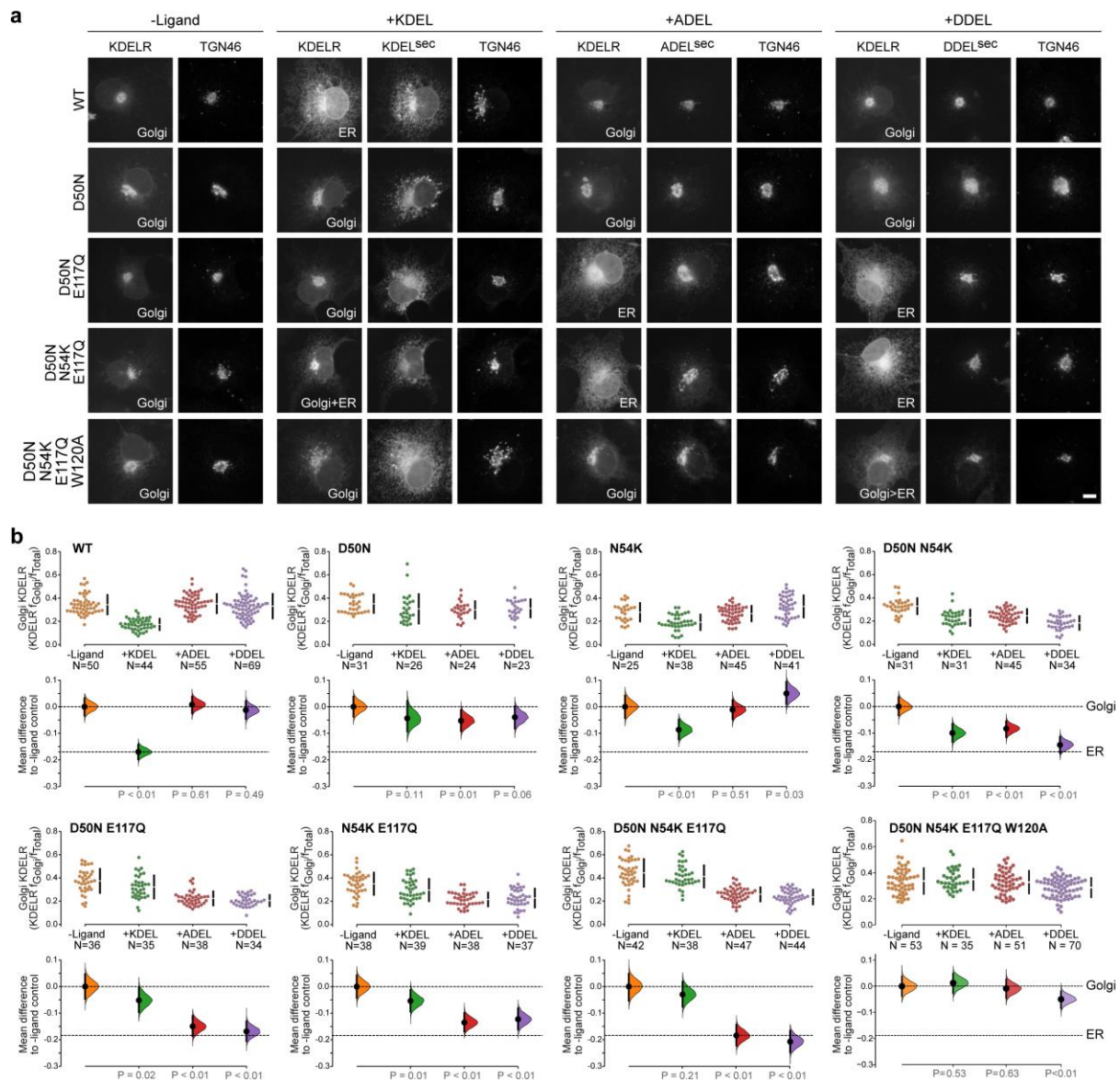
receptor with bound TAEHDEL highlighting key residues involved in ligand binding

620

and variant residues D50, N54 and E117. **c.** The charged surface for the WT KDEL

621

receptor and **d.** N50, N50/K54 and N50/K54/Q117 mutants is shown.



622

623 **Figure 6. Re-engineering the selectivity of the human KDEL receptor for ADEL**

624 **and DDEL signals. a.** WT and a series of “*K. lactis*”-like mutant KDEL receptors

625 were tested for K/A/DDEL-induced redistribution from Golgi to ER. KDEL receptor

626 distribution was followed in the absence (-ligand) or presence of K/A/DDEL^{sec}.

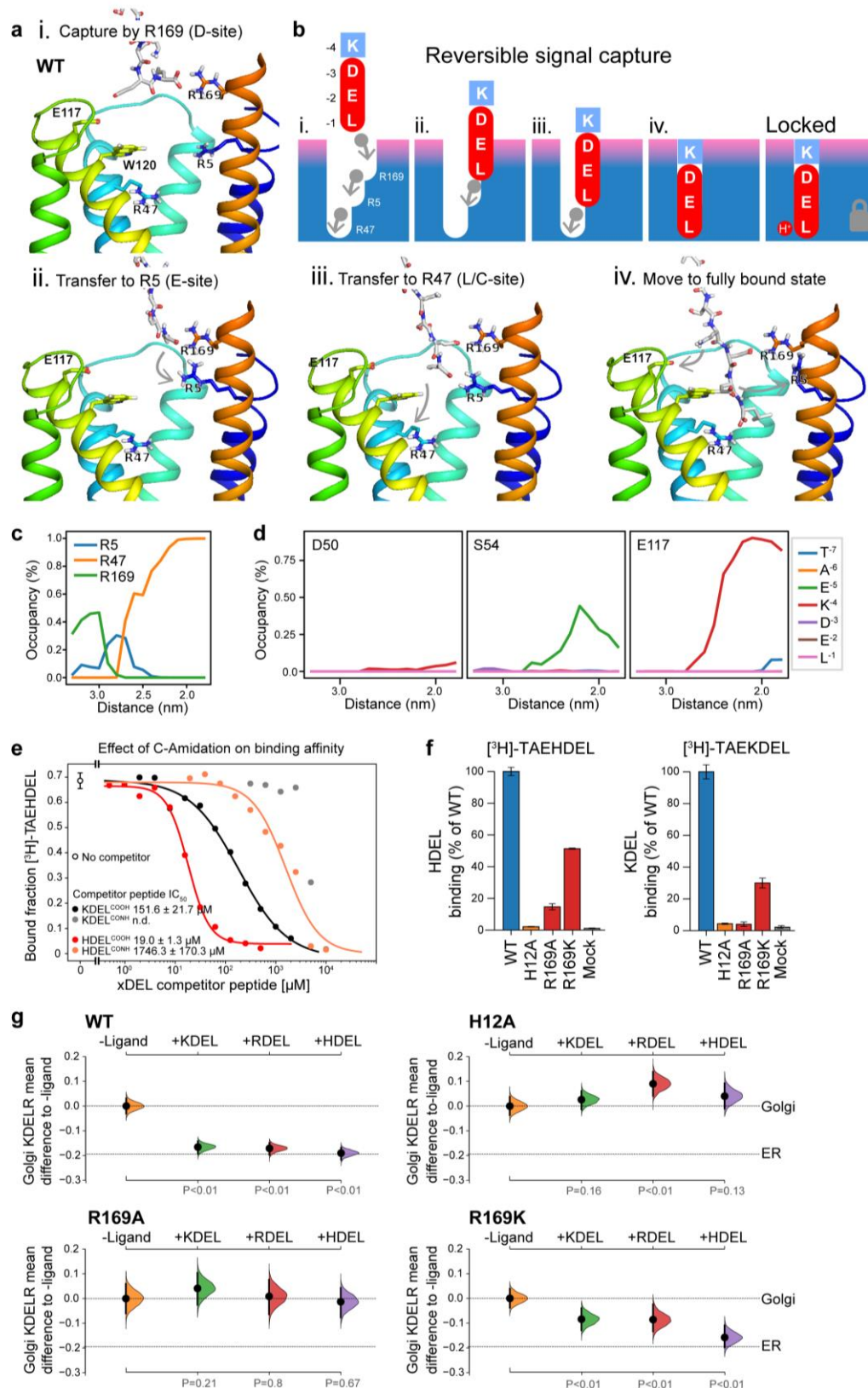
627 TGN46 was used as a Golgi marker. Scale bar is 10 μ m. **b.** The fraction of WT and

628 mutant KDEL receptor localised to the Golgi was measured before (no ligand) after

629 challenge with different retrieval signals (K/A/DDEL). Effect sizes are shown as the

630 mean difference for K/A/DDEL comparisons against the shared -ligand control with

631 sample sizes and p values.



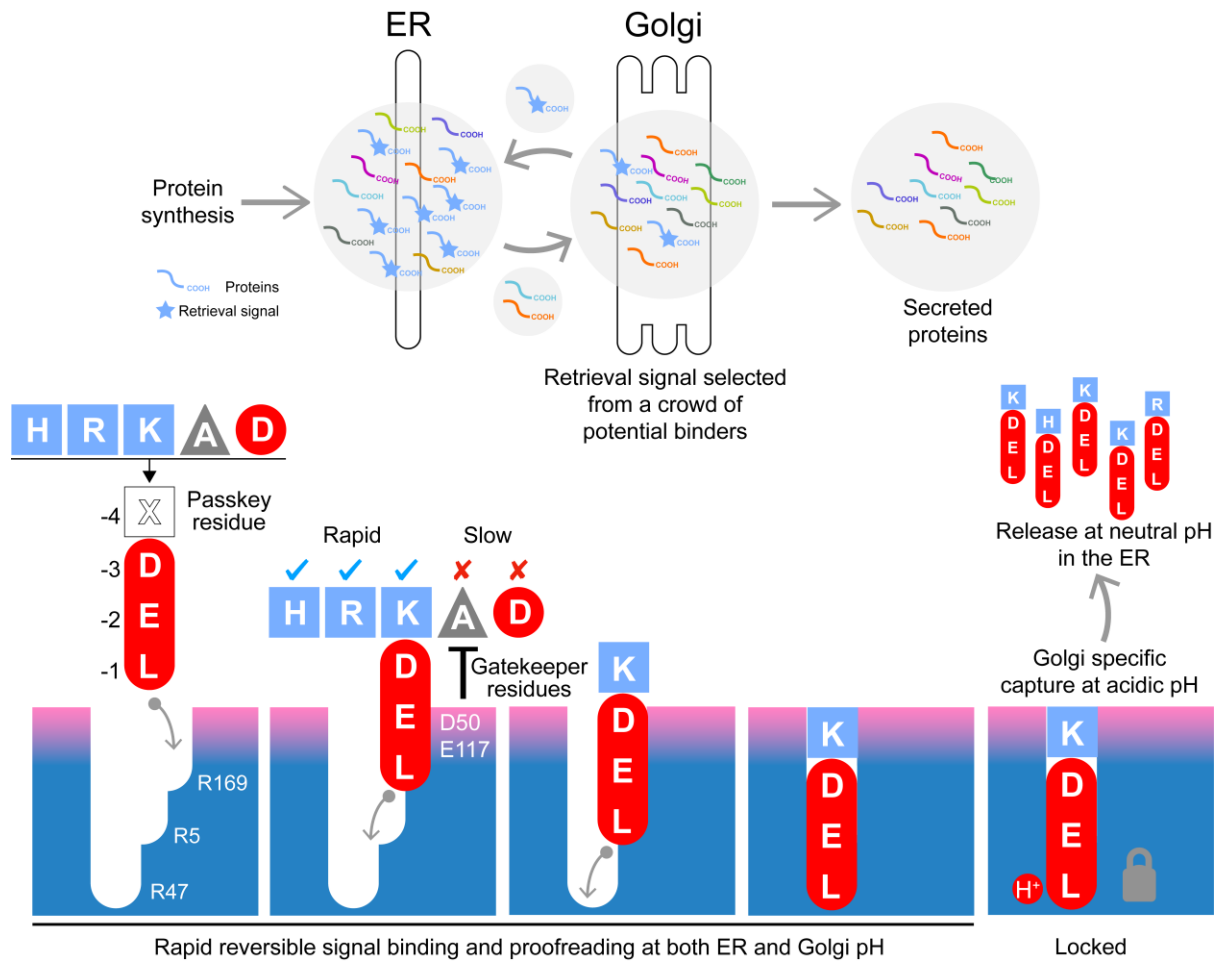
632

633 **Figure 7. A baton-relay mechanism for initial retrieval signal capture by the**

634 **KDEL receptor. a.** Images depicting the key stages (i.-iv.) of KDEL binding to the

635 wild-type (WT) human receptor simulated using molecular dynamics. Initial
636 engagement of the C-terminus to R169 (i) is followed by transfer to R5 (ii), shortly
637 followed by interaction of E -2 with R169 (iii). Finally, R47 engages the C-terminus
638 allowing D -3 to interact with R169 (iv). **b.** A cartoon model depicting the key stages
639 of retrieval signal binding and final pH-dependent locked state. **c.** Occupancy of the
640 hydrogen bonds between the C-terminus of the KDEL retrieval signal and R5, R47
641 and R169 is plotted as a function of signal position within the binding pocket. **d.** The
642 occupancy of potential hydrogen bonds between the different positions of the KDEL
643 retrieval signal and D50, S54 and E117 is plotted as a function of signal position
644 within the binding pocket. **e.** Competition binding assays for [³H]-TAEHDEL and
645 unlabelled TAEKDEL and TAEHDEL with a free (COOH) or amidated (CONH) C-
646 terminus to chicken KDEL R2 showing IC₅₀ values for the competing peptides. **f.**
647 Normalised binding of [³H]-TAEHDEL and [³H]-TAEKDEL signals to the purified WT
648 H12A, R169A or R169K mutant chicken KDEL R2. A mock binding control with no
649 receptor indicates the background signal. **g.** Distribution of WT, H12A, R169A and
650 R169K KDEL receptors was measured in the absence (-ligand) or presence of
651 K/R/HDEL^{sec}. The mean differences for K/R/HDEL comparisons against the shared
652 no ligand control are shown with sample sizes and p values.

653



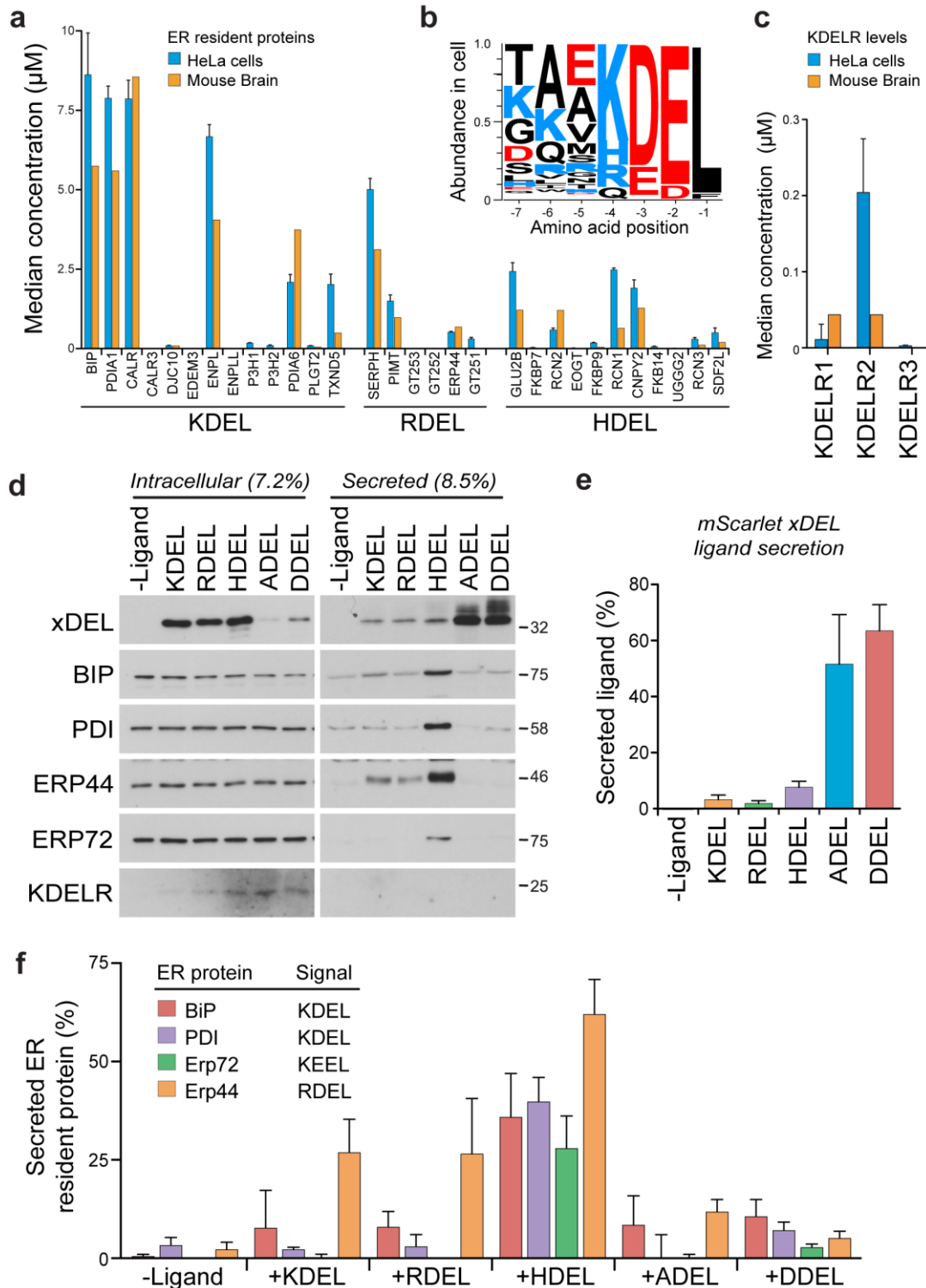
654

Rapid reversible signal binding and proofreading at both ER and Golgi pH

Locked

655 **Figure 8. A combined proofreading and baton-relay handover model for initial**
 656 **signal capture by the KDEL receptor.** Newly synthesized secretory and ER luminal
 657 proteins are translocated into the ER and on to the Golgi. Those proteins with C-
 658 terminal retrieval signals are captured by the KDELR receptor and returned to the
 659 ER. Other proteins with different C-terminal sequences move on to be secreted. The
 660 retrieval signal can be broken down into two sections: the variable -4 passkey
 661 position and the -1 to -3 positions with free carboxyl-terminus. Signals are initially
 662 captured through their free carboxyl-terminus by the receptor R169. This is then
 663 handed over to R5 and finally R47 in a baton-relay mechanism. Sequences are
 664 proofread for the residue at the -4 position by gatekeeper residues D50 and E117.
 665 Unwanted signal variants are rejected. Only signals that completely enter the binding
 666 pocket and engage R47 can undergo pH dependent capture and return to the ER.

667 **FIGURE SUPPLEMENTS**



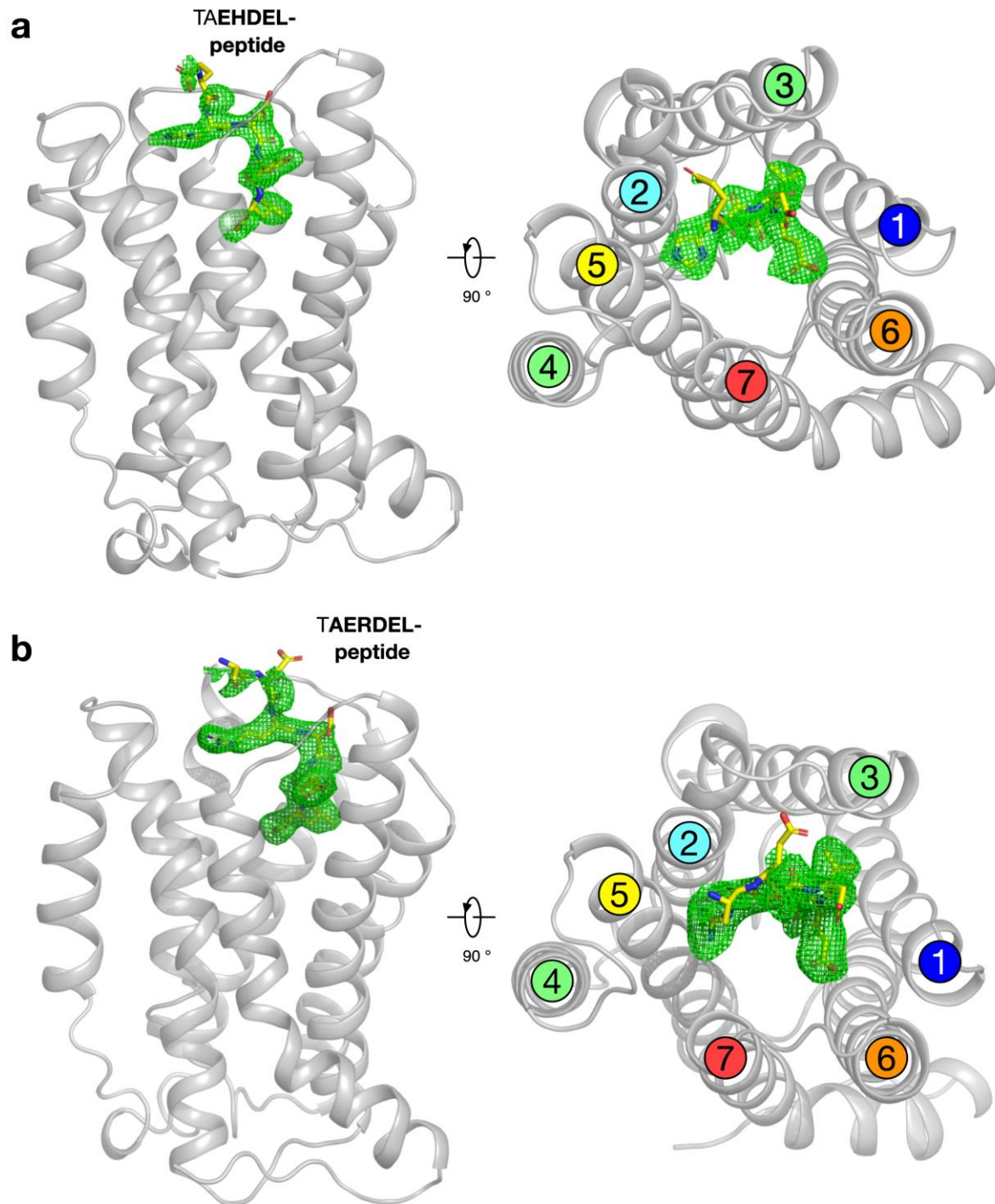
668

669 **Figure 1 – supplement 1. Abundance of ER resident proteins and chaperones**

670 **in human cells and mouse brain. a.** The mean concentration of ER resident

671 **chaperones with the indicated ER retrieval sequence variant is plotted in the bar**

672 graph (Itzhak et al., 2017; Itzhak et al., 2016). **b.** Combined cellular concentrations of
673 ER resident proteins with canonical KDEL, RDEL and HDEL retrieval sequences in
674 HeLa cells and mouse brain. **c.** The mean concentration of KDELR1, KDELR2 and
675 KDELR3 in HeLa cells and mouse brain is plotted in the bar graph (Itzhak et al.,
676 2017; Itzhak et al., 2016). **d.** Cells and media collected from cultures expressing the
677 xDEL variants (mScarlet-xDEL^{sec}) indicated in the figure were Western blotted for
678 resident ER chaperones and KDELR. **e.** A bar graph of xDEL secretion showing
679 mean \pm SEM (n=3). **f.** Endogenous ER chaperone secretion was measured by
680 western blotting after challenge with different retrieval signals, and plotted as a bar
681 graph showing mean \pm SEM (n=3).



682

683

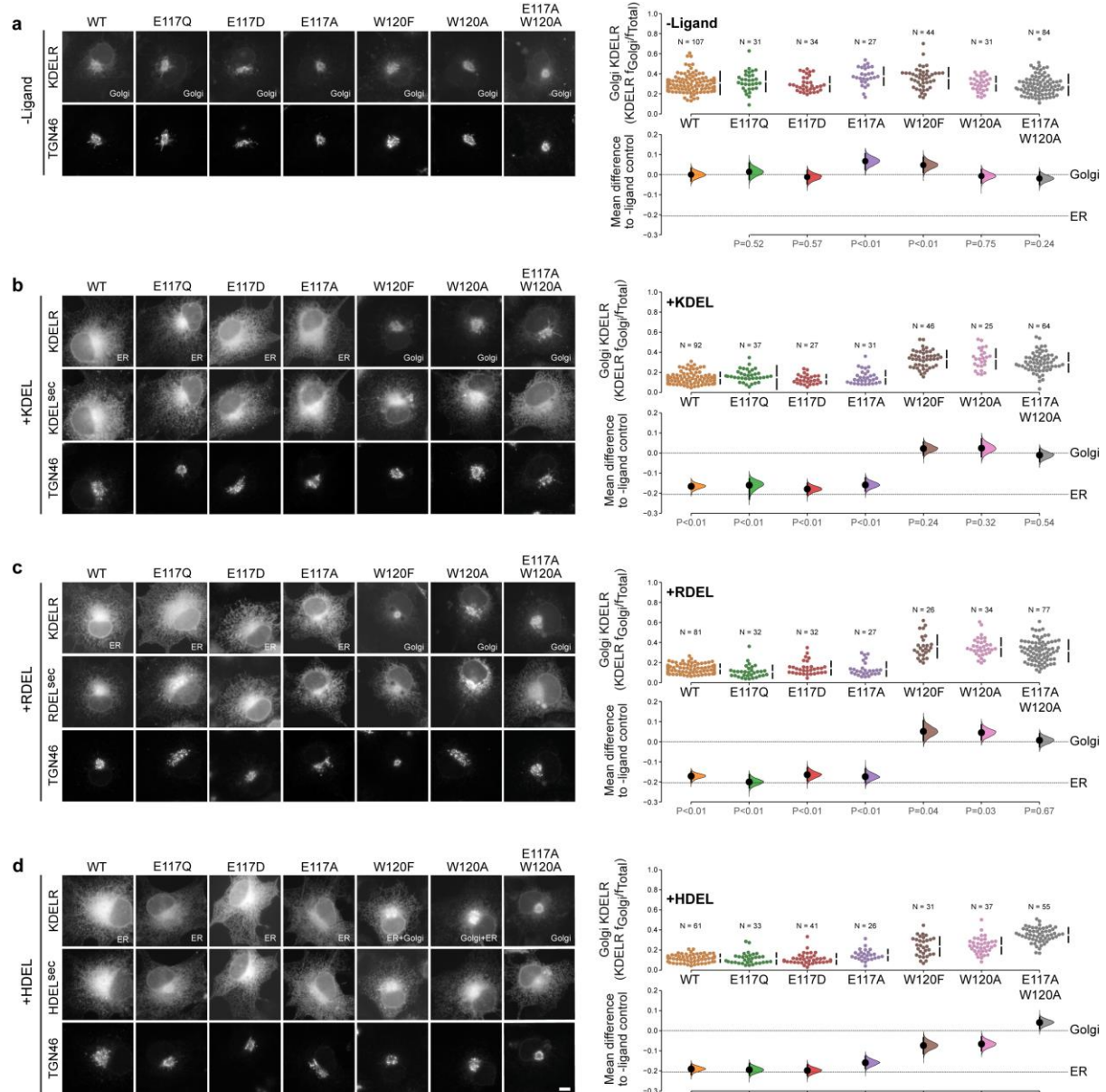
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687

Figure 2 – supplement 1. Polder difference density electron density maps for HDEL and RDEL peptides. a. The structure of the KDELR bound to the TAEHDEL peptide is shown as in Figure 2a. The *mFo-DFc* difference electron density used for model building is displayed (green mesh), contoured at 3σ . **b.** Equivalent maps calculated for the RDEL peptide.

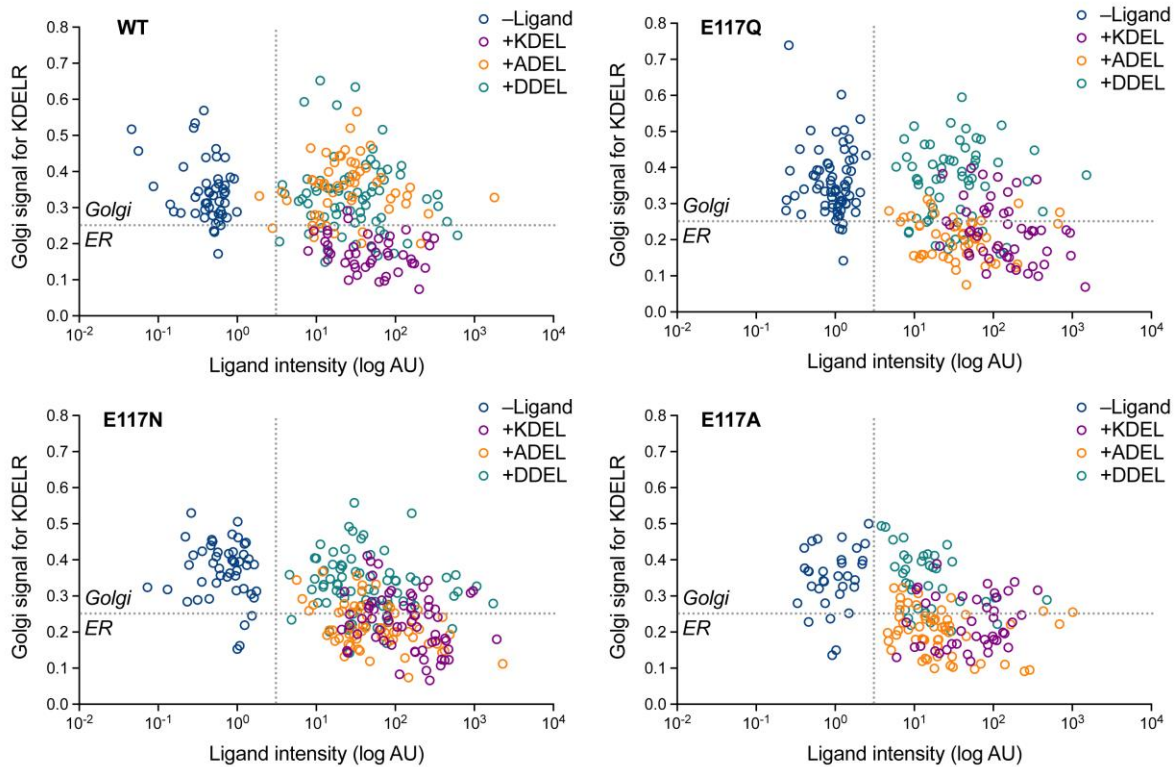


688

689 **Figure 3 – supplement 1. Effect of KDEL receptor E117 and W120 mutants on**
 690 **retrieval signal function in cells. a.** Distribution of WT, E117 and W120 mutant
 691 KDEL receptors was measured in the absence (-ligand) or presence of **b. KDEL, c.**
 692 RDEL or **d. HDEL** retrieval signals (K/R/HDEL^{sec}). TGN46 was used as a Golgi
 693 marker. Scale bar is 10µm. The fraction of WT, E117 and W120 mutant KDEL
 694 receptor localised to the Golgi was measured before (no ligand) and after challenge
 695 with different retrieval signals (K/R/HDEL) as indicated. Golgi signal for KDEL
 696 receptor and ligand intensity are shown on the scatter plots with sample sizes. Effect

697 sizes are shown as the mean difference for K/R/HDEL comparisons against the
698 shared -ligand control with sample sizes and p-values. The Cumming estimation
699 plots for this data are used in main Figure 3c.

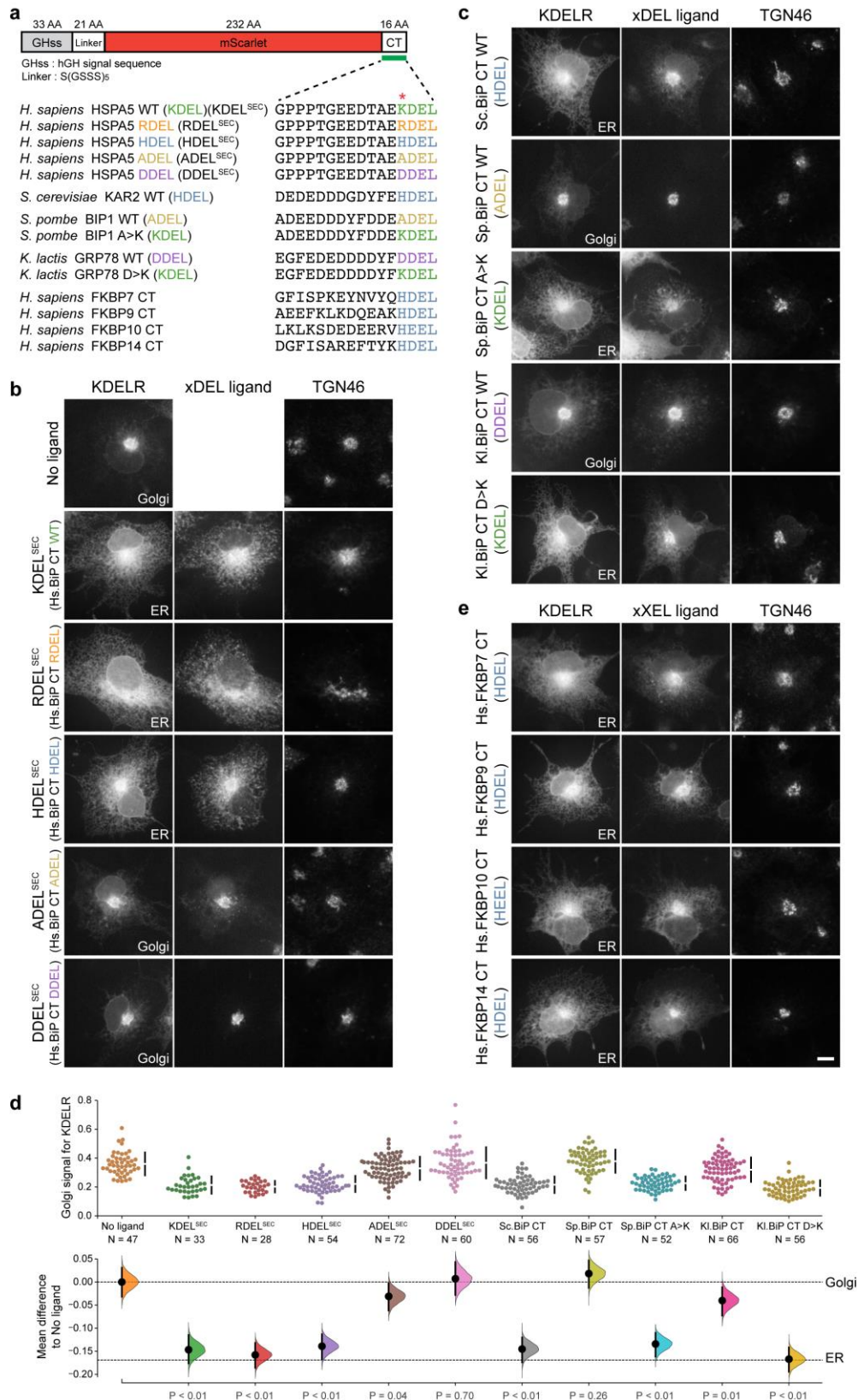
700



701

702 **Figure 4 – supplement 1. Effect of ligand levels on the response of KDEL**

703 **receptor E117 mutants to KDEL, ADEL and DDEL signals.** Distribution of WT,
704 E117A, E117Q and E117N mutant KDEL receptors was measured in the absence (-
705 ligand) or presence of KDEL, ADEL and DDEL retrieval signals. The fraction of WT,
706 E117 mutant KDEL receptor localised to the Golgi was measured before (no ligand)
707 and after challenge with different retrieval signals. Ligand intensity was also
708 measured and plotted against the Golgi fraction of KDEL receptor.



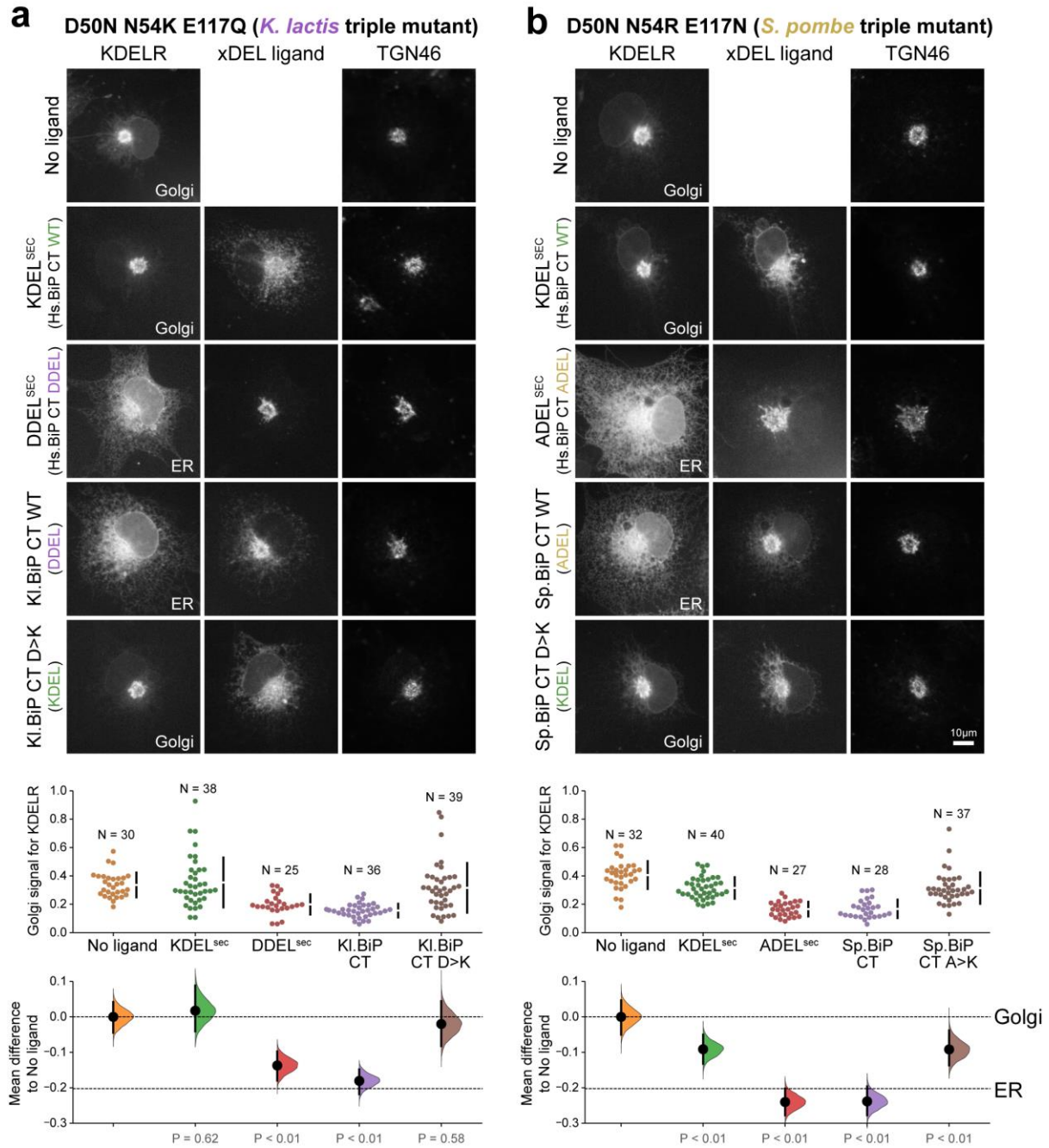
709

710 **Figure 5 – supplement 1. Comparison of human and yeast ER retrieval signals.**

711 **a.** Schematic of the ER retrieval construct showing the human growth hormone

712 signal sequence (hGHss), linker, mScarlet fluorescent protein, and 16 amino acid

713 extension carrying a C-terminal (CT) retrieval signal from known human and yeast
714 ER proteins. A sequence alignment shows the conservation of the retrieval signal. **b.**
715 WT KDEL receptor distribution was followed in the absence (-ligand) or presence of
716 human BIP derived signals (K/R/H/A/DDEL^{sec}). **c.** WT KDEL receptor distribution
717 was followed in the absence (-ligand) or presence of yeast BIP derived signals
718 K/R/HA/DDEL^{sec}. **d.** The fraction of KDEL receptor localised to the Golgi was
719 measured before (no ligand) and after challenge with the different retrieval signals
720 tested in b. and c. Effect sizes are shown as the mean difference for retrieval signal
721 comparisons against the shared -ligand control with sample sizes and p-values. **e.**
722 WT KDEL receptor distribution was followed in the absence (-ligand) or presence of
723 ER retrieval signals from human FKBP family proteins. In all image panels, TGN46
724 was used as a Golgi marker and the scale bar is 10µm.



725

726 **Figure 6 – supplement 1. Retrieval specificity of “*K. lactis*” and “*S. pombe*”**

727 **triple mutant KDEL receptors. a.** Triple mutant “*K. lactis*” and **b.** “*S. pombe*”-like

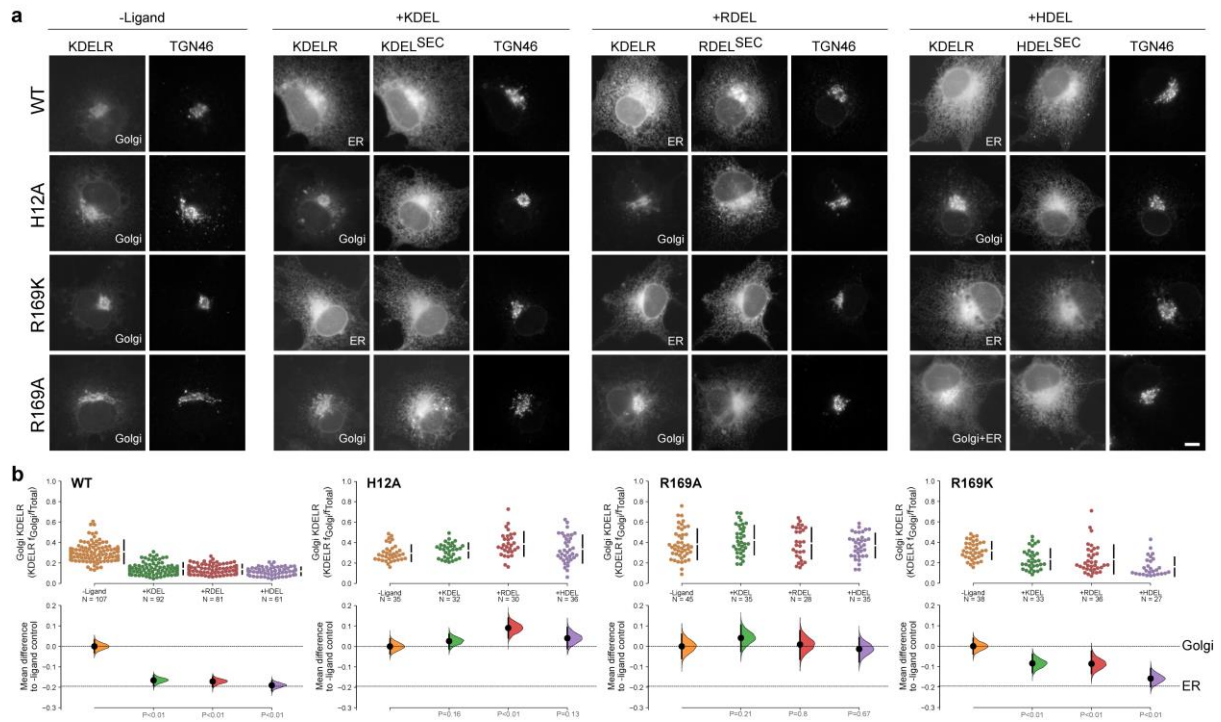
728 KDEL receptors were tested for K/A/DDEL-induced redistribution from Golgi to ER.

729 KDEL receptor distribution was followed in the absence (-ligand) or presence of the

730 indicated ligands. TGN46 was used as a Golgi marker. Scale bar is 10µm. The

731 fraction of WT and mutant KDEL receptor localised to the Golgi was measured

732 before (no ligand) after challenge with different retrieval signals.



733

734 **Figure 7 – supplement 1. R169 plays a crucial role in signal recognition. a.**

735 Distribution of WT, H12A, R169A and R169K KDEL receptors was measured in the

736 absence (-ligand) or presence of K/R/HDEL (mScarlet-xDEL^{SEC}). TGN46 was used

737 as a Golgi marker. Scale bar is 10µm. **b.** The raw data for the fraction of KDEL

738 receptor fluorescence in the Golgi is plotted on the upper axes with sample sizes,

739 with effect sizes shown in the lower graphs.

740

741 **MATERIALS & METHODS**

742 **RESOURCES TABLE**

REAGENTS	SOURCE	IDENTIFIER
Antibodies		
TGN46 sheep polyclonal antibody	Bio-rad (AbD Serotec)	AHP500G
GRP78 BiP rabbit polyclonal antibody	Abcam	ab21685
PDI rabbit polyclonal antibody	ProteinTech	#11245-1
ERp72 rabbit monoclonal antibody	Cell Signalling Technology	#5033S
ERp44 rabbit monoclonal antibody	Cell Signalling Technology	# 3798S
KDEL receptor mouse monoclonal antibody	Enzo Life Sciences	ADI-VAA-PT048
Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Invitrogen	A-21202
Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	Invitrogen	A-21448
Peroxidase-AffiniPure Donkey Anti-Rabbit IgG (H+L)	Jackson ImmunoResearch	711-035-152-JIR
Peroxidase-AffiniPure Donkey Anti-Mouse IgG (H+L)	Jackson ImmunoResearch	711-035-152-JIR
Peroxidase-AffiniPure Donkey Anti-Sheep IgG (H+L)	Jackson ImmunoResearch	713-035-147-JIR
Bacterial and Yeast Strains		
XL1-Blue Competent Cells	Agilent Technologies	200249
<i>Saccharomyces cerevisiae</i> Bj5460	ATCC	208285
Chemicals and Peptides		
Dulbecco's modified Eagle's medium	ThermoFisher Scientific	31966-047
Fetal Bovine Serum	Sigma	F9665
TrypLE Express Enzyme	ThermoFisher Scientific	12605036
Opti-MEM	ThermoFisher Scientific	11058021
Mirus TransIT-LT1	Mirus Bio LLC	MIR 2306
GE Healthcare Amersham ECL	GE Healthcare	RPN2106
Sodium phosphate monobasic (NaH ₂ PO ₄)	Sigma	S8282
Sodium phosphate dibasic (Na ₂ HPO ₄)	Sigma	71640
Sodium periodate (NaIO ₄)	Sigma	311448
16% Formaldehyde	Thermo	28908
Saponin	Sigma	S7900
L-Lysine monohydrochloride	Sigma	62929
Mowiol 4-88	Millipore	475904
Trichloroacetic acid	Sigma	T6399
DDM	Glycon	D97002-C
CHS	Sigma	C6512
Monoolein	Sigma	M7765
HisPur™	Thermo Scientific	25214
HisTrap HP	Cytiva	17-5248-01
Superdex 200 Increase 10/300 GL	Cytiva	28-9909-44
Ultra-15 Centrifugal Filter Unit, 50,000 NMWC	Amicon	UFC905024
Yeast Drop Out media -Ura	Formedium	DCS0169
Yeast Drop Out media -Leu	Merck	Y1376-20G
Ultima Gold Scintillation Fluid	Perkin Elmer	6013326

3H-TAEHDEL	Cambridge Research Biochemicals	185 MBq 106 Ci/mmol
3H-TAEKDEL	Cambridge Research Biochemicals	185 MBq 128 Ci/mmol
TAEHDEL	Cambridge peptides	custom synthesis
TAEKDEL	Cambridge peptides	custom synthesis
TAERDEL	Cambridge peptides	custom synthesis
TAEDDEL	Cambridge peptides	custom synthesis
TAEKDELCOOH	Cambridge peptides	custom synthesis
TAEKDELCONH	Cambridge peptides	custom synthesis
TAEHDELCOOH	Cambridge peptides	custom synthesis
TAEHDELCONH	Cambridge peptides	custom synthesis
Structures		
HDEL structure	This paper	6Y7V
RDEL structure	This paper	6ZXR
Mammalian Cell Lines		
COS-7	ATCC	CRL-1651
HeLa S3	ATCC	CCL-2.2
Plasmids		
pcDNA3.1 hGHss-mScarlet- <i>H. sapiens</i> BiP ₆₃₉₋₆₅₄ (KDEL ^{SEC})	Bräuer et al. 2019	pFB9692
pcDNA3.1 hGHss-mScarlet- <i>H. sapiens</i> BiP ₆₃₉₋₆₅₄ K651R (RDEL ^{SEC})	This paper	pFB9712
pcDNA3.1 hGHss-mScarlet- <i>H. sapiens</i> BiP ₆₃₉₋₆₅₄ K651H (HDEL ^{SEC})	This paper	pFB9713
pcDNA3.1 hGHss-mScarlet- <i>H. sapiens</i> BiP ₆₃₉₋₆₅₄ K651A (ADEL ^{SEC})	This paper	pFB9714
pcDNA3.1 hGHss-mScarlet- <i>H. sapiens</i> BiP ₆₃₉₋₆₅₄ K651D (DDEL ^{SEC})	This paper	pFB9715
pcDNA3.1 hGHss-mScarlet- <i>S. cerevisiae</i> BiP ₆₆₇₋₆₈₂	This paper	pFB9716
pcDNA3.1 hGHss-mScarlet- <i>S. pombe</i> BiP ₆₄₈₋₆₆₃	This paper	pFB9717
pcDNA3.1 hGHss-mScarlet- <i>S. pombe</i> BiP ₆₄₈₋₆₆₃ A660K	This paper	pFB9718
pcDNA3.1 hGHss-mScarlet- <i>K. lactis</i> BiP ₆₆₄₋₆₇₉	This paper	pFB9719
pcDNA3.1 hGHss-mScarlet- <i>K. lactis</i> BiP ₆₆₄₋₆₇₉ D676K	This paper	pFB9720
pcDNA3.1 hGHss-mScarlet- <i>H. sapiens</i> FKBP7 ₂₀₇₋₂₂₂	This paper	pFB9721
pcDNA3.1 hGHss-mScarlet- <i>H. sapiens</i> FKBP9 ₅₅₅₋₅₇₀	This paper	pFB9722
pcDNA3.1 hGHss-mScarlet- <i>H. sapiens</i> FKBP10 ₅₆₇₋₅₈₂	This paper	pFB9723
pcDNA3.1 hGHss-mScarlet- <i>H. sapiens</i> FKBP14 ₁₉₆₋₂₁₁	This paper	pFB9724
pEF5/FRT human KDELR1-GFP	Bräuer et al. 2019	pFB9693
pEF5/FRT human KDELR1 H12A-GFP	Bräuer et al. 2019	pFB9694
pEF5/FRT human KDELR1 D50N-GFP	This paper	pFB9695
pEF5/FRT human KDELR1 N54K-GFP	This paper	pFB9696
pEF5/FRT human KDELR1 E117Q-GFP	This paper	pFB9697
pEF5/FRT human KDELR1 E117D-GFP	This paper	pFB9698
pEF5/FRT human KDELR1 E117A-GFP	This paper	pFB9699
pEF5/FRT human KDELR1 E117N-GFP	This paper	pFB9700
pEF5/FRT human KDELR1 W120F-GFP	This paper	pFB9701
pEF5/FRT human KDELR1 W120A-GFP	This paper	pFB9702
pEF5/FRT human KDELR1 R169K-GFP	This paper	pFB9703
pEF5/FRT human KDELR1 R169A-GFP	This paper	pFB9704

pEF5/FRT human KDELR1 E117A/W120A-GFP	This paper	pFB9705
pEF5/FRT human KDELR1 D50N/N54K-GFP	This paper	pFB9706
pEF5/FRT human KDELR1 D50N/E117Q-GFP	This paper	pFB9707
pEF5/FRT human KDELR1 N54K/E117Q-GFP	This paper	pFB9708
pEF5/FRT human KDELR1 D50N/N54K/E117Q-GFP	This paper	pFB9709
pEF5/FRT human KDELR1 D50N/N54R/E117N-GFP	This paper	pFB9710
pEF5/FRT human KDELR1 D50N/N54K/E117Q/W120A-GFP	This paper	pFB9711
pDDGFP-Leu2d-GgKDELR2	Addgene	123618
pDDGFP-Leu2d-GgKDELR2_H12A	This paper	KDELR2_H12A
pDDGFP-Leu2d-GgKDELR2_E117A	This paper	KDELR2_E117A
pDDGFP-Leu2d-GgKDELR2_E117D	This paper	KDELR2_E117D
pDDGFP-Leu2d-GgKDELR2_E117Q	This paper	KDELR2_E117Q
pDDGFP-Leu2d-GgKDELR2_E127A	This paper	KDELR2_E127A
pDDGFP-Leu2d-GgKDELR2_E127Q	This paper	KDELR2_E127Q
pDDGFP-Leu2d-GgKDELR2_W120A	This paper	KDELR2_W120A
pDDGFP-Leu2d-GgKDELR2_W120F	This paper	KDELR2_W120F
pDDGFP-Leu2d-GgKDELR2_R169A	This paper	KDELR2_R169A
pDDGFP-Leu2d-GgKDELR2_R169K	This paper	KDELR2_R169K
Computer Software		
Metamorph 7.5	Molecular Dynamics Inc	www.moleculardevices.com
Fiji 2.0.0-rc-49/1.52i	NIH Image	http://fiji.sc/
Prism 7	GraphPad Software	www.graphpad.com
Adobe Illustrator CS3 13.0.2	Adobe Systems Inc	www.adobe.com
Adobe Photoshop CS3 10.0.1	Adobe Systems Inc	www.adobe.com
Phenix (Afonine et al., 2018)	Phenix	https://www.phenix-online.org
COOT (Emsley and Cowtan, 2004)		https://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot
PyMOL	Schrodinger	https://pymol.org/2
Buster	Global Phasing	https://www.globalphasing.com
Other		
TEV Protease	Merck	T4455-10KU
Tunair Flasks	Sigma	Z710822-4EA

743

744 **Mammalian cell lines**

745 HeLa cells were cultured at 37°C and 5% CO₂ in DMEM containing 10% [vol/vol]

746 foetal bovine serum (Invitrogen). For passaging, cells were washed in PBS, and then

747 removed from the dish by incubation with TripLE Express (Thermo Fisher Scientific).

748

749 **ER retrieval and secretion assays**

750 *Homo sapiens* KDELR1 (Uniprot: P24390) was cloned into the pEF5/FRT low level
751 mammalian expression vector with a C-terminal 20 amino acid linker made up of 5
752 copies of Gly-Ser-Ser-Ser followed by GFP to create KDELR-GFP. Specific point
753 mutations in the ligand binding site, described in the figures, were introduced using
754 the Quickchange protocol (Stratagene). To create the mScarlet-KDEL^{sec} ligand
755 construct, mScarlet with the N-terminal hGH signal peptide and the 16 C-terminal
756 residues of human BiP at its C-terminus, containing the KDEL signal, was cloned
757 into the pcDNA3.1 vector. This was modified using site-directed mutagenesis or
758 annealed oligo ligation to create C-terminal retrieval signal variants from known
759 human and yeast ER proteins. COS-7 cells were grown on 10 mm diameter 0.16-
760 0.19 mm thick glass coverslips in DMEM containing 10% [vol/vol] bovine calf serum
761 at 37°C and 5% CO₂. Cells were plated at 50,000 cells per well of a 6-well plate,
762 each well containing 2 coverslips. For ER retrieval assays, the cells were transfected
763 after 24 h with 0.25 µg KDELR-GFP and 0.5 µg mScarlet-ligand (+ xDEL ligand) or
764 0.25 µg KDELR-GFP and 0.5 µg pcDNA3.1 (- ligand) diluted in 100µl Optimem and
765 3 µl Mirus LT1 (Mirus Bio LLC). After a further 18 h, cells were washed twice with 2
766 mL of PBS, then fixed for 2 h in 2 mL 2% wt/vol) formaldehyde in 87.5 mM lysine,
767 87.5 mM sodium phosphate pH 7.4, and 10 mM sodium periodate. Subsequently,
768 coverslips were washed three times in 2 mL permeabilization solution 100 mM
769 sodium phosphate pH 7.4, then permeabilised in 1 mg mL⁻¹ BSA, 0.12 mg mL⁻¹
770 saponin, and 100 mM sodium phosphate pH 7.4 for 30 min. Primary and secondary
771 antibody staining was performed for 60 min in permeabilization solution at 22°C.
772 Commercially available antibodies were used to detect the Golgi protein TGN46
773 (sheep; AbD Serotec). Coverslips were mounted on glass slides in Mowiol 4-88 and

774 imaged with a 60×/1.35 NA oil immersion objective on an Olympus BX61 upright
775 microscope (with filtersets for DAPI, GFP/Alexa-488, -555, -568, and -647 (Chroma
776 Technology Corp.), a 2048x2048 pixel CMOS camera (PrimΣ; Photometrics), and
777 MetaMorph 7.5 imaging software (Molecular Dynamics Inc.). Illumination was
778 provided by a wLS LED illumination unit (QImaging). Image stacks of 3-5 planes with
779 0.3 μm spacing through the ER and Golgi were taken. The image stacks were then
780 maximum intensity projected and the selected channels merged to create 24-bit
781 RGB TIFF files in MetaMorph. To produce the figures, images in 24-bit RGB format
782 were cropped in Photoshop to show individual cells and then placed into Illustrator
783 (Adobe Systems Inc.). To determine ER retrieval efficiency, the Golgi signal
784 (integrated pixel intensity) for the KDEL receptor was measured (Schindelin et al.,
785 2012) in the region defined by the Golgi marker antibody in the presence (+) and
786 absence (-) of ligand. Golgi signal was normalised to the ER signal, to account for
787 different expression levels.

788 For ER secretion assays, upon transfection HeLa S3 cells were allowed to
789 express the proteins for 24 h. The media were TCA precipitated and both cell and
790 media were resuspended and boiled in SDS-PAGE sample buffer. All samples were
791 analysed by Western blotting for xDEL ligand, resident ER chaperones BIP (rabbit
792 #ab21685, Abcam), PDI (rabbit #11245-1, ProteinTech), ERP72 (rabbit #5033S, Cell
793 Signalling Technology), ERP44 (rabbit #3798S, Cell Signalling Technology) and the
794 KDEL receptor (mouse ADI-VAA-PT048, Enzo Life Sciences).

795

796 **Statistical analysis of ER retrieval and secretion**

797 To estimate the effect sizes and significance of receptor mutations for ligand-
798 mediated ER retrieval, data was analysed in R using the open-source package

799 dabestr (Ho et al., 2019; Team, 2017; Wickham, 2010). Data are presented as
800 Cumming estimation plots, where the raw data is plotted on the upper axes and
801 mean differences are plotted as bootstrap sampling distributions on the lower axes
802 for 5000 bootstrap samples. Each mean difference is depicted as a dot. Each 95%
803 confidence interval is indicated by the ends of the vertical error bars; the confidence
804 interval is bias-corrected and accelerated. The p values reported are the likelihood of
805 observing the effect size, if the null hypothesis of zero difference is true. For each
806 permutation p value, 5000 reshuffles of the control and test labels were performed.

807

808 **KDEL receptor crystallisation and structure determination**

809 Gg KDELR2 was expressed and purified as described previously (Bräuer et al.,
810 2019), concentrated to 14.5 mg mL⁻¹ and incubated with 6.4 mM TAEHDEL peptide
811 on ice for one hour prior to crystallisation. Crystals were set up at 20 °C as above
812 using precipitant 30% (v/v) PEG 600, 100 mM MES pH 6.0, 100 mM Sodium Nitrate.
813 Phases were determined via molecular replacement using Phaser and employing
814 PDB:6I6H as the search model with the TAEKDEL peptide removed from the search
815 model. The TAEHDEL peptide was built into difference density using Coot (Emsley
816 et al., 2010), followed by refinement in BUSTER (Blanc et al., 2004).

817

818 **Retrieval signal binding assays**

819 Binding assays were performed in 20 mM MES pH 5.4, 40 mM Sodium Chloride,
820 0.01% DDM 0.0005% CHS unless stated otherwise. 5 µL of ³H-TAEK/HDEL
821 (Cambridge peptides, UK) at 20 nM was incubated with 5 µL of Gg KDELR or
822 variants thereof at the desired concentration at 20 °C for 10 min. The reaction was
823 then filtered through a 0.22 µm mixed cellulose ester filters (Millipore, USA) using a
824 vacuum manifold. Filters were then washed with 2 x 500 µL buffer. The amount of

825 peptide remaining bound was measured using scintillation counting in Ultima Gold
826 (Perkin Elmer). Experiments were performed a minimum of three times to generate
827 an overall mean and standard deviation. Data was normalised to the maximal
828 binding at pH 5.4 and fit with a four-parameter logistic non-linear regression model.

829

830 **Relative binding free energy calculations**

831 To compute the free energy of the deprotonation of the histidine or lysine and the
832 mutation of lysine to histidine, molecular mechanics based alchemical transformation
833 was performed. The free energy difference was taken as the difference in the free
834 energy of the transformation between the protein-peptide complex and the peptide in
835 solution. The KDEL receptor in the protein-peptide complex was taken from the
836 crystal structure (KDEL: 6I6B (Bräuer et al., 2019); HDEL: 6Y7V). The C-terminus of
837 the receptor was modelled to full length using Modeller 9.21 (Webb and Sali, 2016);
838 100 models were created and the one with the best DOPE score was selected (Shen
839 and Sali, 2006). The protein was then embedded into a lipid membrane containing
840 186 DMPC lipids using the procedure described by us previously (Wu et al., 2019).
841 The system of peptide in solution was constructed by taking the coordinates of the
842 peptide from the crystal structure and placing in a box, where the box edge was at
843 least 2 nm from the peptide. Both systems were solvated and neutralised to final salt
844 concentration of 150 mM NaCl. For the deprotonation calculations, the change in
845 charge in the system was counteracted by simultaneously charging a sodium ion in
846 the corner of the box (ie at the start of the process the charge was zero and by the
847 end it was +1). To minimise the interactions between the histidine (or lysine) and
848 this alchemical sodium ion, the histidine/lysine residues were restrained to the centre
849 of the box via their C α atom using a harmonic restraint of 1000 kJ/mol/nm and the

850 alchemical sodium ion was either restrained to the edge of the box for the peptide in
851 solution or restrained to the z-axis in the case of the peptide-protein complex.

852 The Amber ff14SB force field (Maier et al., 2015) was used to describe the
853 protein and alchemical transformation was done with pmx (Gapsys et al., 2015).
854 Lipids were described by LIPID17, which was ported from amber to Gromacs by us
855 (Wu and Biggin (2020). GMX_lipid17.ff: Gromacs Port of the amber LIPID17 force
856 field. Zenodo. <http://doi.org/10.5281/zenodo.3610470>). The simulations were run
857 with GROMACS 2018 (Abraham et al., 2015). The simulation input parameters were
858 set according to recommendations suggested by pmx. Since the equilibrium method
859 was used, the sc-alpha and sc-sigma parameters were set to 0.5 and 0.3
860 respectively. For the lysine to histidine transformation, a total of 21 lambda windows
861 with 0.05 equal spacing were used to transform the charge and the vdw parameters
862 at the same time. A soft-core potential was used for the coulombic interactions to
863 avoid singularity effects. For the deprotonation calculations, 11 equally spaced
864 windows were used to change the partial charge and an addition window was used
865 to complete the transformation. After energy minimisation, each window was run for
866 200 ps in the NVT ensemble and 1 ns in an NPT ensemble with position restraints of
867 1000 kJ/mol to reach a final temperature of 310 K and 1 bar. 30 ns production runs
868 with replicate exchanges at intervals of 1 ps were then performed. Data were
869 analysed using the Multistate Bennett Acceptance Ratio with alchemical analysis
870 with the first 5 ns discarded (Klimovich et al., 2015). For each transformation, three
871 replicates were performed and the result was given as the mean and standard
872 deviation. For the LYS/HIP transformation, since both HDEL-bound and KDEL-
873 bound structure were available, six simulations (three starting from KDEL-bound
874 structure and three from HDEL-bound structure) were used to produce the results.

875 To compute the free energy difference of KDEL to HDEL transformation, the
 876 total free energy difference of alchemically changing KDEL to HDEL is computed as

$$\Delta G_{KDEL \rightarrow HDEL} = \Delta G_{LYS \rightarrow LYS/N} + \Delta G_{LYS \rightarrow HIP} - \Delta G_{HIP \rightarrow HIP/D/E}$$

877 Where $\Delta G_{HIP \rightarrow LYS}$ is the free energy difference of converting KDEL to HDEL when
 878 both lysine and histidine are in the protonated form. $\Delta G_{HIP \rightarrow HIP/D/E}$ and $\Delta G_{LYS \rightarrow LYS/N}$ is
 879 the free energy of converting protonated histidine or lysine from the protonated to an
 880 ensemble of protonated and deprotonated forms (for example we might calculate the
 881 energy to go from 100% protonated to an ensemble of 40% protonated and 60%
 882 deprotonated):

$$\Delta G_{LYS \rightarrow LYS/N} = w_{LYS}0 + w_{LYN}(\Delta G_{LYS \rightarrow LYN} - \Delta G_{LYS_{offset}}) - T\Delta S$$

$$\Delta G_{HIP \rightarrow HIP/D/E}$$

$$= w_{HIP}0 + w_{HID}(\Delta G_{HIP \rightarrow HID} - \Delta G_{HIP_{offset}}) + w_{HIE}(\Delta G_{HIP \rightarrow HIE} - \Delta G_{HIP_{offset}}) - T\Delta S$$

883 The $\Delta G_{HIP_{offset}}$ and $\Delta G_{LYS_{offset}}$ are terms to calibrate the computational protonation
 884 free energy to the experimental microscopic pka (histidine: 6.0; lysine: 8.95) and
 885 were defined as:

$$\Delta G_{offset} = 2.303RT \times (7.0 - pka)$$

886 w is the Boltzmann weight of each protonation state and is computed as:

$$w = \frac{e^{\frac{-\Delta G}{RT}}}{\sum e^{\frac{-\Delta G}{RT}}}$$

887 and ΔS is the configurational entropy and is defined as:

$$\Delta S = -R \sum w \ln w$$

888

889 **Quantum mechanical calculations for the effect of HDEL protonation**

890 To explore the interactions between the signal and receptor, the histidine of the
891 HDEL signal and tyrosine (W120) of the receptor were taken from the crystal
892 structure and capped at both ends (with acetyl and amide groups the N and C-
893 termini respectively). The hydrogens were added to the complex and the three
894 different protonation states of the histidine were constructed with Maestro 2019.2.
895 The capped three amino acid complex were geometry minimised with non-hydrogen
896 atoms constrained at the RI-B3LYP-D3(BJ)/def2-TZVP theory level with geometry
897 counterpoise (Grimme et al., 2010; Grimme et al., 2011; Kruse and Grimme, 2012;
898 Weigend, 2006; Weigend and Ahlrichs, 2005) using ORCA 4.2.0 (Neese, 2012). The
899 interactions between the three different protonation states of the histidine and W120
900 were computed at the SAPT2+/jun-cc-pVDZ (Parker et al., 2014) theory level from
901 the geometry optimised structure using psi4 1.3.2 (Parrish et al., 2017).

902

903 **Simulation of signal engagement with the binding site**

904 To obtain a converged view of how the KDEL peptide enters the KDEL receptor,
905 umbrella sampling was used to enhance the sampling of the behaviour of the C-
906 terminus in the binding pocket. The initial frames were generated by pulling the N-
907 terminus of the KDEL peptide out of the binding pocket using a moving restraint
908 (Gromacs 2019.4/plumed 2.6.0) (consortium, 2019). The collective variable (CV) was
909 defined as the distance between the N-terminus of the KDEL peptide (N atom) and
910 the centre of the binding pocket, which was defined as the centre of the C α atoms of
911 residue 9, 44, 64, 124 and 162. Pulling was performed using a CV=1.8 nm to 3.3 nm
912 with a restraint strength of 1000 kcal/mol/nm for 100 ns. To prevent the complete
913 dissociation of the peptide from the receptor, a one-side distance restraint was

914 applied on the distance between the C-terminus of the peptide (atom C) and the
915 binding pocket at 1.7 nm with a strength of 1000 kJ/mol/nm. Sixteen windows were
916 set up where the CV was varied from 1.8 nm to 3.3 nm with a step of 0.1 nm and
917 were run for 500 ns. The results were analysed with MDAnalysis 1.0
918 (https://conference.scipy.org/proceedings/scipy2016/oliver_beckstein.html).

919

920 **Quantification and statistical analysis**

921 Details of the number of experimental repeats, numbers of cells analysed and the
922 relevant statistics are detailed in the figure legends and specific method details.

923

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