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Publication Date

2019

DOI

10.7554/elife.46595

Peer reviewed

1 **Ceapins block the unfolded protein response sensor ATF6 α by inducing a neomorphic**
2 **inter-organelle tether**

3

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22 **Abstract**

23 The unfolded protein response (UPR) detects and restores deficits in the endoplasmic
24 reticulum (ER) protein folding capacity. Ceapins specifically inhibit the UPR sensor ATF6 α , an
25 ER-tethered transcription factor, by retaining it at the ER through an unknown mechanism. Our
26 genome-wide CRISPR interference (CRISPRi) screen reveals that Ceapins function is
27 completely dependent on the ABCD3 peroxisomal transporter. Proteomics studies establish that
28 ABCD3 physically associates with ER-resident ATF6 α in cells and *in vitro* in a Ceapin-
29 dependent manner. Ceapins induce the neomorphic association of ER and peroxisomes by
30 directly tethering the cytosolic domain of ATF6 α to ABCD3's transmembrane regions without
31 inhibiting or depending on ABCD3 transporter activity. Thus, our studies reveal that Ceapins
32 function by chemical-induced misdirection which explains their remarkable specificity and opens
33 up new mechanistic routes for drug development and synthetic biology.

34

35 **Introduction**

36

37 The endoplasmic reticulum (ER) is the site of folding and assembly of secreted and
38 transmembrane proteins. When ER homeostasis is disturbed, misfolded proteins accumulate and
39 activate the unfolded protein response (UPR) (Walter and Ron, 2011). One of the ER-resident
40 UPR sensors, ATF6 α , is an ER-tethered transcription factor that is cytoprotective and necessary
41 for cell survival when cells experience ER stress (Wu et al., 2007; Yamamoto et al., 2007).
42 Under stress conditions, ATF6 α traffics to the Golgi apparatus, where it undergoes
43 intramembrane proteolysis, releasing a bZIP transcription factor domain that moves to the
44 nucleus and activates transcription (Haze et al., 1999; Yoshida et al., 1998). The events leading to
45 ATF6 α activation and trafficking remain poorly understood, but require the Golgi-resident
46 proteases S1P and S2P and general components involved in COPII trafficking (Nadanaka et al.,
47 2004; Okada et al., 2003; Schindler and Schekman, 2009; Ye et al., 2000) that are not specific to
48 ATF6 α .

49

50 Using a cell-based high-throughput screen, we recently identified a series of selective
51 small-molecule inhibitors of ATF6 α signaling, termed Ceapins (from the Irish verb “ceap”
52 meaning “to trap”) (Gallagher et al., 2016). Ceapins act on the most upstream step of ATF6 α
53 activation by retaining ATF6 α at the ER and excluding it from ER exit sites during ER stress.
54 When this trafficking requirement is removed by collapsing the Golgi apparatus into the ER,
55 making ATF6 α accessible to S1P and S2P, ATF6 α is still cleavable by the proteases in the
56 presence of Ceapin. Upon Ceapin treatment, ATF6 α rapidly and reversibly forms foci without
57 requiring new protein synthesis (Gallagher et al., 2016; Gallagher & Walter, 2016). The

58 molecular target(s) of Ceapins, let alone how Ceapins specifically inhibit ATF6 α , especially in
59 light of the fact that activation depends on components that are shared by other cellular process,
60 have remained an enigma.

61

62 To identify the molecular target of Ceapin, we carried out an unbiased genome-wide
63 screen and proteomic analysis. Our approaches converged on a single target, the peroxisomal
64 transporter ABCD3. ATF6 α and ABCD3 normally do not interact and, indeed, localize to
65 different parts of the cell. Ceapins induce these novel physical associations between ATF6 α and
66 ABCD3 in cells and *in vitro*. Our results indicate that Ceapins achieve their remarkable
67 specificity through an unprecedented mechanism of small molecule induced inter-organelle
68 tethering.

69

70 **Results**

71

72 **ABCD3 KD desensitizes cells to Ceapin-A7**

73

74 To decipher the molecular mechanism of Ceapins, we carried out a genome-wide
75 CRISPR interference (CRISPRi) screen to identify genes whose knockdown (KD) resulted in
76 reduced or enhanced sensitivity to the drug. To this end, we screened a genome-wide sgRNA
77 library (Horlbeck et al., 2016) in K562 cells that stably expressed dCas9-KRAB and an mCherry
78 transcriptional reporter dependent on ATF6 α activation (Figure 1A). Treatment with tunicamycin
79 (Tm), which blocks N-linked glycosylation, activates ATF6 α signaling leading to a two-fold
80 reporter induction that was completely dependent on ATF6 α (Figure 1A). As a positive control,
81 knocking down *MBTPS2*, one of the Golgi proteases that processes ATF6 α , also inhibited

82 induction of the reporter (Figure 1- figure supplement 1A), whereas knocking down *HSPA5*,
83 encoding the major Hsp70-type ER chaperone BiP (Binding Protein), induced ER stress and the
84 reporter constitutively (Figure 1 - figure supplement 1B).

85

86 To carry out our genome-wide screen, we transduced the K562 ATF6 α reporter cell line
87 and selected for sgRNA expressing cells. We then induced ER stress with Tm in the presence or
88 absence of Ceapin-A7, a potent member of the Ceapin family, and sorted cells by FACS
89 (fluorescence-activated cell sorting). We isolated populations with decreased or increased
90 ATF6 α signaling (bottom 30% and top 30% of the reporter signal distributions, respectively) and
91 used next-generation sequencing to quantify frequencies of cells expressing each sgRNA in both
92 pools to evaluate how expression of each individual sgRNA affects activation of the ATF6 α
93 reporter (Adamson et al., 2016; Sidrauski et al., 2015) (Figure 1B).

94

95 As expected, KD of *ATF6 α* or *MBTPS2* (encoding S2P) inhibited reporter induction
96 (Figure 1C). Knocking down abundant ER quality control components such as *HSPA5*, induced
97 ER stress and turned on the reporter independently of Ceapin treatment (labeled in *red* in Figure
98 1C, Figure 1 - figure supplement 1C-D). Ceapin independent genes localized to the diagonal
99 because their knockdown changed the expression of the reporter to the same degree in both
100 treatments (labeled in red in Figure 1 - figure supplement 1C). Of particular interest were genes
101 whose KD specifically made cells insensitive to Ceapin treatment allowing activation of the
102 reporter by Tm in the presence of Ceapin (labeled in black in Figure 1 - figure supplement 1C).
103 Two genes, *ABCD3* and *PEX19*, robustly retested among the more than twenty hits from the
104 genetic screen we individually knocked down and tested in the ERSE reporter cell line.

105 ABCD3, which encodes a peroxisomal ABC transporter involved in long-chain fatty acid
106 import into peroxisomes, desensitized cells to Ceapin treatment (Figure 1C, Figure 1 - figure
107 supplement 1C-D). Additionally, *PEX19*, which is necessary for chaperoning and targeting
108 ABCD3 to the peroxisome, also desensitized cells to Ceapin treatment (Figure 1C, Figure 1 -
109 figure supplement. 1C-D). We knocked down these candidates individually and performed
110 ERSE-mCherry dose response assays using Tm. Retesting of these candidates revealed that
111 *ABCD3* and *PEX19* KD cells remained completely insensitive to Ceapin-A7 at saturating
112 concentrations (Figure 1D, Figure 2 - figure supplement 3A). To determine if ATF6 α trafficking,
113 processing, or activation is altered in *ABCD3* KD cells, we then measured ATF6 α nuclear
114 translocation (Figure 1 - figure supplement 2) and the downstream ATF6 α -N activation of the
115 reporter and endogenous ATF6 α target genes HSPA5 and HSP90B1 (Figure 1E-G). In the
116 absence of ER stress, *ABCD3* or *PEX19* KD cells also do not cause constitutive nuclear
117 translocation nor activate ATF6 α (Figure 1E-G, Figure 1 - figure supplement 2, Figure 2 - figure
118 supplement 3B). Furthermore, in the presence of ER stress, *ABCD3* or *PEX19* KD alone did not
119 impede ATF6 α nuclear translocation nor activation (Figure 1E-G, Figure 2 - figure supplement
120 3B). These results indicate that neither ABCD3 nor PEX19 have direct roles in ATF6 α signaling,
121 posing the question of how Ceapins functionally connect proteins that reside in separate
122 organelles.

123

124 **ABCD3 is required for Ceapin-induced ATF6 α foci**

125

126 Ceapin treatment induces rapid and reversible formation of ATF6 α foci that are retained
127 in the ER (Figure 2A) (Gallagher et al., 2016; Gallagher & Walter, 2016). We next tested if

128 ABCD3 was directly involved in the formation of these foci and would colocalize with ATF6 α .
129 Indeed, in Ceapin-treated cells, ATF6 α colocalized with ABCD3 as visualized by
130 immunofluorescence (Figure 2A-B). This result was surprising because newly synthesized
131 ABCD3 is inserted directly into the peroxisomal membrane using PEX19 as import receptor
132 (Imanaka et al., 1996; Biermanns and Gärtner, 2001; Kashiwayama et al., 2007; Kashiwayama et
133 al., 2005; Sacksteder et al., 2000). ABCD3 is not co-translationally translocated into the ER,
134 indicating there is not a pool of ABCD3 in the ER (Figure 2 - figure supplement 1) (Jan et al.,
135 2014); indeed, it is commonly used as a reliable marker for peroxisomes (Uhlén et al., 2015).
136 Since both ABCD3 and PEX19 scored as hits in our screen, it seemed plausible that Ceapin
137 induces ATF6 α colocalization with peroxisomal ABCD3. We next tested whether ATF6 α also
138 colocalized with other peroxisomal markers, peroxisomal membrane protein PEX14 and
139 peroxisomal matrix protein Thiolase (a marker for mature import competent peroxisomes). In the
140 absence of Ceapin, ATF6 α and PEX14 or Thiolase did not colocalize (Figure 2A, C, Figure 2 –
141 figure supplement 2). By contrast, in the presence of Ceapin, we observed ATF6 α and PEX14
142 and Thiolase colocalization (Figure 2A, C Figure 2 – figure supplement 2). Furthermore, in
143 ABCD3 KD cells treated with Ceapin, ATF6 α no longer formed foci or colocalized with
144 peroxisomes (Figure 2A, C). This result was consistent in PEX19 KD cells, where peroxisome
145 biogenesis is affected and ABCD3 is no longer chaperoned and targeted to the peroxisome
146 (Kashiwayama et al., 2007; Kashiwayama et al., 2005; Sacksteder et al., 2000), ATF6 α no longer
147 formed foci in the presence of Ceapin (Figure 2 - figure supplement 3C). Thus, peroxisomes
148 interact with Ceapin-induced ATF6 α foci in an ABCD3-dependent fashion to sequester ATF6 α
149 at the ER.
150

151 After prolonged ER stress, ATF6 α attenuates and forms foci that are reminiscent of
152 Ceapin induced foci (Gallagher & Walter, 2016). We next asked whether Ceapin was acting on
153 the normal mechanism of ATF6 α attenuation by testing ABCD3 colocalization with stress
154 attenuated ATF6 α foci. To induce stress attenuated ATF6 α foci, we treated U2OS cells
155 expressing GFP-ATF6 α with ER stress, Tm or Tg (thapsigargin, which inhibits the ER calcium
156 pump) for 2 and 4 hours. In positive control cells treated with Ceapin, ATF6 α colocalized with
157 ABCD3 and PEX14. In stress induced cells, attenuated ATF6 α foci did not colocalize with
158 ABCD3 or PEX14 by immunofluorescence (Figure 2D-E). Thus, Ceapin does not act on the
159 ATF6 α pathway by stabilizing the attenuated ATF6 α state. The stress attenuated foci and Ceapin
160 induced foci are distinct.

161

162 **Ceapin treatment does not inhibit ABCD3 activity**

163

164 Since Ceapin treatment inhibits ATF6 α , we next tested whether Ceapin treatment also
165 inhibits ABCD3. ABCD3 knockout mice and hepatocytes display defects in bile acid
166 biosynthesis (Ferdinandusse et al., 2015). To test if Ceapin treatment affects ABCD3 activity, we
167 measured bile acid levels in a liver cancer cell line (HepG2) after Ceapin treatment and *ABCD3*
168 KD. As expected, in ABCD3 KD cells, bile acid levels were decreased (Figure 3). In control
169 cells treated at the EC₅₀ and ten-times the EC₅₀ of Ceapin, bile acid levels were similar to cells
170 treated with vehicle only (Figure 3). Thus, Ceapin does not inhibit ABCD3 activity in cells.

171

172 **Ceapin-induced ATF6 α -ABCD3 interaction does not require known ER-peroxisome** 173 **tethers**

174

175 The tight association between the ER and peroxisome is mediated by ER-peroxisome
176 tethers, VAPA and VAPB on the ER and ACBD4 and ACBD5 on the peroxisomes (Costello et
177 al., 2017; Costello et al., 2017; Hua et al., 2017). While the ER components are redundant,
178 ACBD5 KD or overexpression alone decreases or increase ER-peroxisome contacts, respectively
179 (Costello et al., 2017; Hua et al., 2017). To determine whether proximity between the ER and
180 peroxisomes induced by these tethers is required for Ceapin-induced foci formation, we
181 knocked-down these known ER-peroxisome tethers. In tether KD cells treated with Ceapin,
182 ATF6 α foci still formed and ATF6 α colocalized with ABCD3 (Figure 4A-B). Additionally,
183 tether KD cells were not resistant to Ceapin treatment (Figure 4C), consistent with the results
184 from our screen in which these components also did not score as hits.

185

186 **Ceapin-induced interactions do not require ER localized ATF6 α nor ABCD3 transporter**
187 **activity**

188

189 We next tested if ER membrane association of ATF6 α is required for Ceapin induced
190 foci. To this end, we knocked down endogenous ATF6 α and FACS sorted for a narrow, low
191 level of GFP expression for truncated variants of ATF6 α containing its cytosolic regions without
192 the transmembrane and ER-luminal domains (Figure 5A). We found that GFP-ATF6 α (2-302),
193 which was retained in the cytosol with a nuclear exit signal and was no longer associated with
194 the ER, colocalized with ABCD3 and formed foci (Figure 5A-B). Further truncations showed
195 that only the first 89 amino acids of ATF6 α were both necessary and sufficient for Ceapin-

196 dependent foci formation and colocalization with ABCD3 and peroxisomes (Figure 5A-B, Figure
197 5 - figure supplement 1).

198

199 Since ABCD3 is a transporter, we then tested if ABCD3 catalytic activity was required
200 for Ceapin action. Similarly to our ATF6 α truncations, we also knocked down endogenous
201 ABCD3 and FACS sorted for low level GFP expression of constructs with mutations of ABCD3
202 residues that mediate ATP binding (G478R) and hydrolysis (S572I) or a deletion of the entire
203 catalytic domain (Roerig et al., 2001). There is one reported patient with a C terminal truncation
204 of ABCD3 in which a reduced number of import competent peroxisomes are present
205 (Ferdinandusse et al., 2015). Similarly, GFP-ABCD3 Δ NBD cells, with a deletion of the entire
206 catalytic domain, have reduced, enlarged peroxisomes (Figure 5C, Figure 5 - figure supplement
207 2). We also confirmed correct localization of the GFP-ABCD3 constructs to the peroxisome
208 (Figure 5 - figure supplement 2). As a positive control, ABCD3 KD cells complemented with the
209 full length ABCD3 construct were able to colocalize with and form ATF6 α foci when treated
210 with Ceapin (Figure 5C-D). In our catalytic activity mutants, we found that ABCD3 ATP
211 binding or hydrolysis was not required for Ceapin-induced foci formation (Figure 5C-D).

212 Although there are fewer larger peroxisomes in GFP-ABCD3 Δ NBD cells, peroxisomal ABCD3
213 still induced foci formation and colocalized with ATF6 α in the presence of Ceapin (Figure 5C-
214 D). These results indicate that Ceapin-induced interactions do not require ER localized ATF6 α
215 nor ABCD3 transporter activity.

216

217 **Ceapin drives ATF6 α -ABCD3 interaction in cells and *in vitro*.**

218

219 To identify components physically associating with ATF6 α in the presence of Ceapin, we
220 carried out native immunoprecipitation – mass spectrometric (IP-MS) analyses. We treated
221 3xFLAG-ATF6 α HEK293 cells with Ceapin-A7 or an inactive analog, Ceapin-A5, in the
222 presence of stress (Tg) and found that ABCD3 co-purified as the top hit with epitope-tagged
223 ATF6 α selectively in the presence of active Ceapin-A7 but not inactive Ceapin-A5 (Figure 6A-
224 B). The native reciprocal affinity purification with full-length GFP-ABCD3 cells confirmed
225 these results (Figure 6C). Furthermore, GFP-ABCD3 Δ NBD, lacking the entire nucleotide
226 binding domain, also physically associated with ATF6 α in the presence of Ceapin (Figure 6C).

227

228 We then tested if the minimal cytosolic domain of ATF6 α , GFP-ATF6 α (2-90), physically
229 associated with peroxisomal ABCD3. We immunoprecipitated GFP-ATF6 α (2-90) from
230 detergent solubilized lysates and specifically enriched ABCD3 in the presence of active Ceapin-
231 A7 but not inactive Ceapin-A5 (Figure 6D). Thus, consistent with the above experiments where
232 organelle tethering was not required, these results confirm that no other ER proteins are required
233 for Ceapin-A7 induced ATF6 α and ABCD3 physical association.

234

235 Finally, we tested whether purified ATF6 α and ABCD3 were sufficient for Ceapin-
236 induced tethering. In a binding assay with purified ATF6 α (2-90) and ABCD3, our vehicle
237 (DMSO) and inactive Ceapin-A5 controls did not induce ATF6 α (2-90) and ABCD3 binding
238 (Figure 6E). In the presence of Ceapin-A7, however, the cytosolic domain of ATF6 α (2-90) and
239 ABCD3 associated in solution (Figure 6E). Thus, Ceapin is directly responsible for tethering
240 ABCD3 to ATF6 α .

241

242 **Discussion**

243

244 Ceapins, named for their ability to trap ATF6 α in the ER, act with exquisite selectivity;
245 they do not affect signaling of ATF6 α 's close homolog ATF6 β or SREBP (sterol response
246 element binding protein) (Gallagher et al., 2016), which depend on broadly used vesicular
247 trafficking ER-Golgi pathways and are activated by the same Golgi-resident proteases
248 (Nadanaka et al., 2004; Okada et al., 2003; Schindler and Schekman, 2009; Ye et al., 2000).
249 Here we discovered the basis of this specificity. Ceapins induce neomorphic inter-organelle
250 junctions, forcing interactions between the cytosolic domain of ER-tethered ATF6 α and the
251 peroxisomal transmembrane protein ABCD3 to sequester ATF6 α from its normal trafficking
252 route (Figure 7), and do so without interfering with or depending on ABCD3's normal function.
253 Since ABCD3 protein expression is ten-fold higher than ATF6 (Hein et al., 2015), it is likely
254 ABCD3 is not saturated. Ceapin induced interaction of ABCD3 with the most N-terminal region
255 of ATF6 α also clarifies how ATF6 α foci are excluded from COPII trafficking, while the
256 transmembrane region of ATF6 α remains accessible to protease cleavage. Mechanistically,
257 Ceapins could act as molecular staples that physically bridge the respective proteins or bind to
258 one or the other inducing allosteric changes that promote their association; but in either case,
259 Ceapin is responsible for tethering ABCD3 to ATF6 α .

260

261 Remarkably, in the absence of Ceapins, ATF6 α and ABCD3 localize to different parts of
262 the cell and are not known to interact physically or functionally. Indeed, an 89-amino acid
263 fragment of ATF6 α fused to GFP is sufficient to recruit GFP to peroxisomes, ruling out the need
264 for endogenous inter-organellar tethers. This Ceapin-induced tethering enables an "anchor away"

265 strategy but one that uses an abundant, ubiquitously expressed endogenous acceptor protein.
266 There has been increasing interest in small molecules that induce novel protein-protein
267 interactions with therapeutic potential (De Waal et al., 2016; Han et al., 2017; Krönke et al.,
268 2014; Lu et al., 2014; Petzold et al., 2016; Uehara et al., 2017). Ceapins provide a novel example
269 of such molecules and increase the repertoire to include the induction of inter-organellar
270 connections, opening new mechanistic routes for drug development and synthetic biology by
271 broadly enabling control of protein function through chemical-induced misdirection.

272

273 Understanding the mechanism of action of a chemical modulator of cellular stress and
274 establishing that it is acting directly and specifically is critical for exploiting the utility of any
275 stress modulators either as research or potential therapeutic agents. Our identification of the
276 mechanism by which Ceapins achieve their remarkable specificity forms a foundation to explore
277 the utility of ATF6 α inhibition in the treatment of cancers, such as squamous carcinomas, in
278 which ATF6 α signaling protects dormant tumor cells from classical chemotherapies (Schewe and
279 Aguirre-Ghiso, 2008).

280

281 **Acknowledgment**

282 We thank Marco Jost for critical reading of the manuscript; Jeff Kelly and Luke Wiseman for
283 advice on target identification and mass spectrometry; Mable Lam, James Nuñez, and Elif
284 Karagöz for advice on protein purification; Nico Stuurman and Vladislav Belyy for advice on
285 fluorescence microscopy; John Christianson for advice on solubilization of intact membrane
286 protein complexes; and members of the Walter and Weissman labs for helpful discussions. This
287 research was supported by Collaborative Innovation Awards from the Howard Hughes Medical
288 Institute (HHMI) and by NIH/NIGMS New Innovator Award DP2 OD021007 (MK). Research
289 of MG and RMS is supported by NIH GM111126 to RMS. PW and JSW are Investigators of the
290 HHMI.

291 **Competing Financial Interest**

292 The authors declare no competing financial interests.

293

294

295 **Figures**

296

297 **Figure 1. ABCD3 KD desensitizes cells to Ceapin-A7.**

298 (A) Schematic of the ER stress element (ERSE) reporter cassette. K562 ERSE reporter cells
299 were transduced with the indicated sgRNAs and treated with vehicle (DMSO) or tunicamycin
300 (Tm) (6 $\mu\text{g/ml}$) for 16 h. (B) Schematic of the CRISPRi screen to identify the target of Ceapin.
301 K562 cells expressing the ERSE reporter were transduced with the sgRNA library. The
302 population was then divided into two subpopulations, which were treated with Tm or Tm plus
303 Ceapin-A7 at EC_{90} (3 μM) for 16 h. Cells in the top and bottom thirds of mCherry fluorescence
304 of each subpopulation (Tm-treatment and Tm + Ceapin-treatment) were collected by FACS and
305 processed to measure the frequencies of sgRNAs contained within each. (C) Volcano plot of
306 gene-reporter phenotypes and p values from CRISPRi screen. Negative control sgRNA targeted
307 genes (*grey*), Ceapin-independent genes (*red*), genes with growth phenotypes (*blue*), and Ceapin
308 hits (*black*) are indicated. ✦ denotes chromatin architecture and remodeling related genes that
309 impact reporter transcription. The reporter phenotypes and p values for genes in CRISPRi screen
310 are listed in Figure 1- source data 1. (D) K562 ERSE reporter cells with individual ABCD3
311 sgRNAs or control sgRNA (NegCtrl) were treated with Tm and increasing concentrations of
312 Ceapin-A7 for 16 h. Reporter fluorescence was measured by flow cytometry and median values
313 were plotted (N = 3, \pm SD). (E) K562 ERSE reporter *ABCD3* and NegCtrl KD cells were treated
314 with DMSO or Tm and reporter activation was measured as in (D). (F and G) qPCR analysis of
315 ATF6 α target genes *HSPA5* and *HSP90B1*, respectively. HepG2 CRISPRi NegCtrl and ABCD3
316 KD cell lines were treated with DMSO, thapsigargin (Tg) (100 nM), and Tg with Ceapin (6
317 μM). Tg blocks the ER calcium pump and induces ER stress. Data plotted are mRNA levels for

318 *HSPA5* and *HSP90B1* normalized to GAPDH and then compared to unstressed NegCtrl cells \pm
319 standard deviation of duplicate technical replicates of two biological replicates.

320

321 **Figure 1 – source data 1. Reporter phenotypes and p values for genes in CRISPRi screen.**

322

323 **Figure 1 - figure supplement 1. Genome-scale CRISPRi screen to identify molecular target**
324 **of Ceapin**

325 (A and B) K562 ERSE reporter cells were transduced with the indicated sgRNAs and treated

326 with vehicle (DMSO) or tunicamycin (Tm) (6 μ g/ml) for 16 h. (C) Reporter phenotypes from

327 CRISPRi screens treated with ER stress in the absence (x-axis) and presence (y-axis) of

328 Ceapin. Ceapin-independent genes (labeled in red) are genes whose knockdown changed the

329 expression of the reporter to the same degree in both treatments and localized to the diagonal.

330 Genes with growth phenotypes of at least -0.19 in previous growth screens (12) are labeled in

331 blue. ✦ denotes chromatin architecture and remodeling related genes that impact reporter

332 transcription. Negative control genes are labeled in grey. (D) Volcano plot of gene-reporter

333 phenotypes and p values from CRISPRi screen described in (Figure 1C) and shown on y-axis of

334 (A) with additional genes labeled. The reporter phenotypes and p values for genes in CRISPRi

335 screen are listed in Figure 1- source data 1.

336

337 **Figure 1 - figure supplement 2. ABCD3 KD does not affect ATF6 α nuclear translocation.**

338 Quantification of nuclear translocation of ATF6 α . Endogenous ABCD3 was knocked-down in

339 3xFLAG-ATF6 α HEK293 CRISPRi cells and full length GFP-ABCD3 construct was added back

340 by FACS sorting for narrow, low GFP levels. Data plotted is the ratio of ATF6 α signal intensity

341 of nucleus to ER per cell, from one of three independent experiments and with at least twenty
342 cells per condition. Statistical analysis used unpaired two-tailed t-tests, **** indicates $p <$
343 0.0001.

344

345 **Figure 2. ABCD3 is required for Ceapin-induced ATF6 α foci.**

346 (A) HEK293 CRISPRi cells stably expressing doxycycline inducible 3xFLAG-ATF6 α with
347 ABCD3 or NegCtrl KD were treated either with DMSO or Ceapin (6 μ M) for 30 min prior to
348 fixation, staining with anti-ABCD3 and/or anti-PEX14, and confocal fluorescent imaging. Scale
349 bar, 10 μ m. Images are representative of two independent experiments, in which we imaged at
350 least 20 positions per well for each experiment. (B and C) Plotted is the mean and standard
351 deviation of the mean per cell correlation of 3xFLAG-ATF6 α and ABCD3 or PEX14 from (A)
352 with at least 30 cells imaged per condition. All cells imaged in ABCD3 KD (96% KD), including
353 wildtype cells, were used in quantification. Statistical analysis used unpaired two-tailed t-tests,
354 **** indicates $p < 0.0001$. (D) U2-OS cells stably expressing GFP-ATF6 α were treated either
355 with vehicle (DMSO), Tg (100 nM), Tm (2 μ g/ml), or Ceapin (6 μ M) for 2 h or 4 h (shown)
356 prior to fixation, co-staining with anti-ABCD3 and anti-PEX14, and fluorescent imaging. Stress
357 attenuated GFP-ATF6 α foci are indicated by arrowheads. Scale bar, 10 μ m. (E) Quantification of
358 correlation of GFP-ATF6 α and ABCD3 within PEX14 sites.

359

360 **Figure 2 - figure supplement 1. ABCD3 is not co-translationally translocated into the ER.**

361 Data from Jan et al. 2014 is plotted. Gene enrichments obtained with the general BirA-Sec61 β
362 ER marker in HEK293 cells and SS annotations predicted by SignalP. ABCD4 was previously

363 annotated to be peroxisomal, but recently shown to be ER localized. PEX16 has been previously
364 shown to be co-translationally translocated. PXP2 is a peroxisomal membrane protein.

365

366 **Figure 2 - figure supplement 2. Ceapin-induced ATF6 α foci colocalize with peroxisomal**
367 **matrix protein Thiolase.**

368 (A) 3xFLAG-ATF6 α HEK293 CRISPRi cells with NegCtrl KD were treated and fixed as in
369 Figure 2A and stained for Thiolase. Scale bar, 10 μ m. (B) Quantification of the correlation of
370 ATF6 α and Thiolase from (A) and plotted as in Figure 2B.

371

372 **Figure 2 - figure supplement 3. PEX19 KD desensitizes cells to Ceapin and is required for**
373 **Ceapin-induced ATF6 α foci**

374 (A) K562 ERSE reporter cells with NegCtrl or PEX19 sgRNA KD were treated with ER stressor
375 (6 μ g/ml Tm) and increasing concentrations of Ceapin-A7 for 16 h. Reporter fluorescence was
376 measured by flow cytometry and median values were plotted (N = 3, \pm SD). (B) K562 ERSE
377 reporter *PEX19* and NegCtrl KD cells were treated without (DMSO) or with ER stressor (Tm)
378 and reporter activation was measured as in (A). (C) 3xFLAG-ATF6 α HEK293 CRISPRi cells
379 with *PEX19* sgRNA or NegCtrl KD were treated, fixed, and stained as in Figure 2A. Scale bar,
380 10 μ m.

381

382 **Figure 3. Ceapin treatment does not inhibit ABCD3 activity**

383 Bile acid levels were measured in HepG2 CRISPRi cells with NegCtrl or ABCD3 KD treated
384 with vehicle (DMSO), EC₅₀ of Ceapin (600nM), and ten times the EC₅₀ of Ceapin-A7 (6 μ M).

385

386 **Figure 4. Ceapin-induced ATF6 α -ABCD3 interaction does not require known ER-**
387 **peroxisome tethers**

388 (A) ER tether components, VAPA and VAPB, and peroxisome tether components, ACBD4 and
389 ACBD5, were individually knocked-down in 3xFLAG-ATF6 α HEK293 CRISPRi cell line,
390 treated, fixed, and stained as in Figure 2A prior to fluorescence imaging. Scale bar, 10 μ m. (B)
391 Quantification of the correlation of ATF6 α and ABCD3 from (A) and plotted as in Figure 2B.
392 (C) K562 ERSE reporter cells with NegCtrl or indicated knockdowns were treated with Tm and
393 increasing concentrations of Ceapin-A7 for 16 h. Reporter fluorescence was measured by flow
394 cytometry and median values were plotted (N = 3, \pm SD).

395

396 **Figure 5. Ceapin-induced interactions do not require ER localized ATF6 α nor ABCD3**
397 **transporter activity**

398 (A) Diagram of GFP-ATF6 α constructs tested. A nuclear exit signal (NES) was added to ATF6 α
399 truncated constructs to retain ATF6 α in the cytosol. Endogenous ATF6 α was knocked-down in
400 3xFLAG-ATF6 α HEK293 CRISPRi cells grown without doxycycline, so that only GFP-ATF6 α
401 constructs were expressed. GFP-ATF6 α -truncated cell lines were treated with DMSO or Ceapin-
402 A7, fixed and stained for ABCD3. Scale bar, 10 μ m. (B) Quantification of the correlation of
403 GFP-ATF6 α within ABCD3 sites. (C) Diagram of the GFP-ABCD3 mutants and truncations
404 tested. Endogenous ABCD3 was knocked-down in 3xFLAG-ATF6 α HEK293 CRISPRi cells so
405 only GFP-ABCD3 constructs were expressed. GFP-ABCD3 cell lines were treated with DMSO
406 or Ceapin-A7, fixed and stained for FLAG(ATF6 α) (shown) and PEX14. Scale bar, 10 μ m. (D)
407 Quantification of the correlation of GFP-ABCD3 and 3xFLAG-ATF6 α within PEX14 sites.

408

409 **Figure 5 - figure supplement 1. ATF6 α (2-90) colocalizes with peroxisomal matrix protein**
410 **Thiolase**

411 (A) Endogenous ATF6 was knocked-down in U2OS Flp-InTM CRISPRi cells and FACS sorted
412 for narrow, low GFP levels so only GFP-ATF6 α (2-90) construct was expressed. Cells were
413 treated and fixed as in Figure 2A and stained for Thiolase. Scale bar, 10 μ m. (B) Quantification
414 of the correlation of ATF6 α and Thiolase from (A) and plotted as in Figure 2B.

415

416 **Figure 5 - figure supplement 2. ABCD3 constructs localization to peroxisome.**

417 Endogenous ABCD3 was knocked-down in 3xFLAG-ATF6 α HEK293 CRISPRi cells and FACS
418 sorted for narrow, low GFP levels so only GFP-ABCD3 constructs were expressed. GFP-
419 ABCD3 cell lines were treated with DMSO or Ceapin-A7, fixed and stained for PEX14 (shown)
420 and FLAG(ATF6 α). Scale bar, 10 μ m.

421

422 **Figure 6. Ceapin drives ATF6 α -ABCD3 interaction in cells and *in vitro*.**

423 (A and B) Proteomic analysis and immunoblot (IB) of anti-FLAG affinity purification from
424 3xFLAG-ATF6 α HEK293 cells treated with stress (100nM Tg) and inactive Ceapin-A5 analog
425 (6 μ M) or active Ceapin-A7 (6 μ M) with two replicates for each treatment condition. The
426 proteins identified with affinity-purified FLAG-ATF6 treated with ER stress and Ceapin-A5 or
427 Ceapin-A7 are listed in Figure 6 – source data 1. SQSTM1 KD (✦) was the top second hit in
428 proteomics, however, SQSTM1 KD in the K562 ATF6 reporter cell line did not render cells
429 resistant to Ceapin treatment and retained a similar response to negative control cells. I, input;
430 FT, flow-through; E, elution. (C) Immunoprecipitation of full-length GFP-ABCD3 and GFP-
431 ABCD3 Δ NBD from cells treated with DMSO or Ceapin-A7. (D) Detergent solubilized GFP-

432 ATF6 α (2-90) or GFP-only cell lysates were incubated with Ceapin-A7 or inactive analog
433 Ceapin-A5 and affinity purified with anti-GFP. (*) Indicates a degradation product. (E) Purified
434 ATF6 α -MBP and ABCD3-GFP were incubated with inactive Ceapin-A5 or active Ceapin-A7
435 and affinity purified with anti-MBP antibody.

436

437 **Figure 6 – source data 1. Excel spreadsheet showing all the proteins identified with affinity-**
438 **purified FLAG-ATF6 treated with ER stress and Ceapin-A5 or Ceapin-A7.**

439

440 **Figure 7. Model for Ceapin induced ATF6 α inhibition.**

441 Ceapins sequester ATF6 α into a transport-incompetent pool during ER stress by tethering
442 ATF6 α to peroxisomal ABCD3. ATF6 α is occluded from COPII trafficking, while its
443 transmembrane domain remains accessibly to protease cleavage.

444 **Material and Methods**

445

446 *Cell culture and experimental reagents*

447 U2OS, 293TREx, and HepG2 cells were cultured in DMEM. K562 cells were cultured in RPMI.
448 Culture media was supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1%
449 penicillin/streptomycin (ThermoFisher). U2-OS cells stably expressing GFP-HsATF6 α were
450 purchased from Thermo Scientific (084_01) and - figure supplemented with 500 μ g/ml G418 to
451 maintain GFP-HsATF6 α expression. HeLa CRISPRi cells expressing SFFV-dCas9-BFP-KRAB
452 were previously described (Jost et al., 2017). Tunicamycin and thapsigargin were purchased
453 from Sigma. Antibodies used were rabbit anti-GFP (ThermoFisher A11122), mouse anti-FLAG
454 M2 (Sigma F1804), rat anti-GRP94 9G10 (abcam ab2791), rabbit anti-pmp70 (ab109448) for

455 PFA fixation and (PA1-650) for methanol fixation, mouse anti-pmp70 (ab211533) for PFA
456 fixation and (SAB4200181) for methanol fixation.

457

458 *Generation of constructs and cell lines*

459 To generate CRISPRi knockdown cell lines, SFFV-dCas9-BFP-KRAB (Addgene 46911) or
460 UCOE-EF1 α -dCas9-BFP-KRAB (Jost et al., 2017) were stably transduced and FACS-sorted for
461 BFP positive cells. 293 TReX cells expressing doxycycline-inducible 6xHis-3xFLAG-HsATF6 α
462 (Gallagher et al., 2016) were infected with SFFV-dCas9-BFP-KRAB and sorted twice for BFP
463 expressing cells. HepG2 cells from ATTC (CRL-10741) were infected with UCOE-EF1 α -dCas9-
464 BFP-KRAB and FACS sorted for BFP expression. The ERSE reporter construct was generated
465 by subcloning mCherry into the ERSE.Fluc.pcDNA3.1 (Mortenson et al. 2017) using the
466 polymerase incomplete primer extension method to replace the FLuc gene. This construct was
467 then subcloned into ClaI and EcoRI digested pLenti6.V5.GFP. K562 cells stably expressing
468 dCas9-KRAB (Gilbert et al., 2014) were stably transduced with the ERSE reporter construct and
469 a monoclonal line was selected and expanded to generate K562 ERSE reporter cell line.

470

471 Individual gene knockdowns were carried out by selecting sgRNA protospacers from compact
472 hCRISPRi-v2 library and cloning into lentiviral plasmid pU6-sgRNA EF1 α -puro-t2a-
473 BFP (Addgene 60955) as previously described (Horlbeck et al., 2016). Protospacer sequences
474 used for individual knockdowns are listed in Table 1. The resulting sgRNA expression vectors
475 were packaged into lentivirus by transfecting HEK293T with standard packaging vectors using
476 TransIT®-LTI Transfection Reagent (Mirus, MIR 2306). The viral supernatant was harvested 2-

477 3 days after transfection and frozen prior to transduction into CRISPRi knockdown cell lines
478 described above.

479

480 3xFLAG-ATF6 α HEK293 CRISPRi described above was stably transduced with sgRNA
481 knockdown of endogenous ATF6 α or ABCD3 and grown without doxycycline to ascertain that
482 only truncation constructs would be expressed. ATF6 α truncation constructs were generated by
483 Gibson assembly of IDT gblock of sfGFP and ATF6 α PCR amplified from pGFP-HsATF6 α
484 (Addgene #32955) into inserted into BamHI/NotI digested pHR-SFFV-Tet3G (Gilbert et al.,
485 2014). ATF6 α truncations were PCR amplified with reverse primers containing nuclear exit
486 signal (NES) (NES, CTGCCCCCCTGGAGCGCCTGACCCTG; NES_REV,
487 CCCCTGCAGCTGCCCCCCTGGAGCGGCTGACCCTG) to retain ATF6 α in the cytosol.

488 Full length GFP-ABCD3 and GFP-ABCD3 Δ NBD (2-416) were cloned by Gibson assembly of
489 ABCD3 PCR amplified from cDNA and IDT gblock of sfGFP into BamHI/NotI digested pHR-
490 SFFV-Tet3G (Gilbert et al., 2014). ABCD3 G478R and S572I mutations were generated by site
491 directed mutagenesis (QuikChange Lightning Agilent) of full-length GFP-ABCD3 construct.

492 ATF6 α and ABCD3 truncation vectors were packaged into lentivirus as described above, stably
493 transduced, and FACS sorted for a narrow and low level of GFP expression.

494

495 U2OS Flp-In™ cells were infected with UCOE-EF1 α -dCas9-BFP-KRAB and FACS sorted for
496 BFP expression. They were then stably transduced with sgRNA knockdown of endogenous
497 ATF6 α and GFP-ATF6 α (2-90) construct, and FACS sorted for a narrow level of GFP
498 expression. Parental cell lines and commercially available cell lines were authenticated by STR
499 analysis and tested negative for mycoplasma contamination.

500

501 ***Genome-scale CRISPRi screen***

502 Reporter screens were carried out using protocols similar to those previously described
503 (Adamson et al., 2016; Gilbert et al., 2014; Sidrauski et al., 2015). The compact (5 sgRNA/gene)
504 hCRISPRi-v2 (12) sgRNA libraries were transduced into ERSE reporter cells at a MOI <1 (55%
505 BFP+ cells). Cells were grown in spinner flasks for 2 days without selection, selected with 2
506 $\mu\text{g/ml}$ puromycin for 2 days, and allowed to recover for 3 days. Cells were then split into two
507 populations, which were treated for 16 h with 6 $\mu\text{g/ml}$ tunicamycin alone or 6 $\mu\text{g/ml}$ tunicamycin
508 and 3 μM Ceapin (EC90). Cells were then sorted based on reporter fluorescence using BD FACS
509 Aria2. Cells with the highest (~30%) and lowest (~30%) mCherry expression were collected and
510 frozen after collection. Approximately 20 million cells were collected per bin. Genomic DNA
511 was isolated from frozen cells, and the sgRNA-encoded regions were enriched, amplified, and
512 prepared for sequencing.

513

514 Sequenced protospacer sequences were aligned and data were processed as described (Gilbert et
515 al., 2014; Horlbeck et al., 2016) with custom Python scripts (available at
516 <https://github.com/mhorlbeck/ScreenProcessing>). Reporter phenotypes for library sgRNAs were
517 calculated as the \log_2 enrichment of sgRNA sequences identified within the high-expressing
518 mCherry over the low-expressing mCherry cells. Phenotypes for each transcription start site
519 were then calculated as the average reporter phenotype of all 5 sgRNAs. Mann-Whitney test p-
520 values were calculated by comparing all sgRNAs targeting a given TSS to the full set of negative
521 control sgRNAs. For data presented in Figure 1B, screen hits are defined as those genes where
522 the absolute value of a calculated reporter phenotype over the standard deviation of all evaluated

523 phenotypes multiplied by the \log_{10} of the Mann-Whitney p-value for given candidate is greater
524 than 7. Growth screen data (Horlbeck et al., 2016) was used to label genes with growth
525 phenotype of at least -0.19. Ceapin independent genes are defined as genes that were hits in
526 tunicamycin alone and tunicamycin with Ceapin treatment since their phenotype was
527 independent of Ceapin treatment. Genes involved in chromatin remodeling and architecture have
528 been previously described in UPR screens to act downstream and directly affect expression of
529 the reporter (Jonikas et al., 2012). Chromatin related genes that impact reporter expression are
530 labeled with (✱) in Figure 1 - figure supplement 1C-D.

531

532 ***Bile Acid Assay***

533 HepG2 CRISPRi ABCD3 KD and NegCtrl cells were treated with DMSO or Ceapin at 600 nM
534 or 6 μ M for 24 h. Cells were harvested in scrapping buffer (cold PBS with 10 μ M MG132 and
535 1X protease inhibitor), spun down, resuspended in lysis buffer (50 mM Tris pH 7.4, 150 mM
536 NaCl, 5 mM EDTA, 1X protease inhibitor, and 1% LMNG), and spun down at 10,000 x g for 10
537 min. The supernatant was used for bile acid assay (Cell Biolabs STA-631) as described by the
538 manufacturer.

539

540 ***Quantitative RT-PCR***

541 Cells were harvested and total RNA was isolated using the NucleoSpin RNA II (Macherey-
542 Nagel) according to manufacturer's instructions. RNA was converted to cDNA using AMV
543 reverse transcriptase under standard conditions with oligo dT and RNasin (Promega, Life
544 Technologies). Quantitative PCR reactions were prepared with a 2x master mix according to the
545 manufacturer's instructions (KAPA SYBR FAST qPCR Kit). Reactions were performed on a

546 LightCycler thermal cycler (Roche). Primers used were against HSPA5 (forward,
547 TGTGCAGCAGGACATCAAGT: reverse, AGTTCCAGCGTCTTTGGTTG) and HSP90B1
548 (forward, GGCCAGTTTGGTGTTCGGTTT ; reverse, CGTTCCCCGTCCTAGAGTGTT).

549

550 *Immunofluorescence*

551 Fluorescence confocal imaging was carried out as described in (10,11). 293 TReX, U2OS,
552 HepG2, and HeLa cells were plated in 8-well ibiTreat μ Slide (ibidi 80826) at 20-25,000
553 cells/well. In 3xFLAG-ATF6 α imaging experiments (Figure 2A-C, Figure 2 – figure supplement
554 2-3, Figure 4A-C, Figure 5A-D, Figure 5 – figure supplement 2), 3xFLAG-ATF6 α HEK293
555 CRISPRi cells were plated and induced with 50nM doxycycline on the same day. On the
556 following day, cells were treated with DMSO or 6 μ M Ceapin for 30 min and then fixed with
557 cold methanol or 4% PFA. For cold methanol fixation, media was removed, cold ethanol was
558 added for 3 min at -20 $^{\circ}$ C, washed, and permeabilized with PHEM (60 mM PIPES, 25 mM
559 HEPES, 10 mM EGTA, 2 mM MgCl₂, pH 6.9) with 0.1% Triton X-100, and washed twice with
560 PHEM. For PFA fixation, media was removed from slides, 4% PFA (EMS) was added for 10
561 min at room temperature, washed, permeabilized as above, and washed with PHEM. Slides were
562 then treated with blocking buffer (5% goat serum (Jackson ImmunoResearch) in PHEM) for 1 h
563 at room temperature. Antibodies were diluted in blocking buffer and incubated with cells at 4 $^{\circ}$ C
564 overnight. After three washes with PHEM, cells were incubated with secondary antibodies
565 conjugated to Alexa 488, Alexa 568, and/or Alexa 633 (Invitrogen) for 1 h at room temperature.
566 Slides were imaged on a spinning disk confocal with Yokogawa CSUX A1 scan head, Andor
567 iXon EMCCD camera and 100x ApoTIRF objective NA 1.49 (Nikon). Linear adjustments were
568 made using ImageJ. Quantification of correlation between ATF6 α with ABCD3 and/or PEX14

569 was calculated using CellProfiler 2.1.1. ABCD3 or PEX14 images were used to identify objects,
570 a background threshold for ATF6 α images was set to 1.2, and clumped objects were separated
571 based on intensity. The resulting ABCD3 or PEX14 outlines were used as masks to count the
572 ATF6 α intensity within ABCD3 or PEX14. Data from CellProfiler was imported into GraphPad
573 Prism version 6.0 for statistical analysis and plotting.

574

575 *Nuclear Translocation Assay*

576 3xFLAG-ATF6 α HEK293 CRISPRi cells with ABCD3 KD and ABCD3 KD complemented
577 with full length GFP-ABCD3 construct were plated in ibidi 96-well ibiTreat μ -plate (ibidi
578 89626) and induced with 50nM doxycycline on the same day. On the following day, cells were
579 treated with DMSO or 100 nM Tg for 2 hours and then fixed with 4% PFA as described above.
580 The plates were then treated with blocking buffer (5% goat serum (Jackson ImmunoResearch) in
581 PHEM) for 1 h at room temperature. Primary antibodies, mouse anti-FLAG M2 (Sigma F1804)
582 and rat anti-GRP94 9G10 (abcam ab2791), were diluted in blocking buffer and incubated with
583 cells at 4 $^{\circ}$ C overnight. After three washes with PHEM, cells were incubated with secondary
584 antibodies conjugated to Alexa 568 and Alexa 633 (Invitrogen) and nuclear stain (DAPI,
585 Molecular Probes D-1306, 5 μ g/mL) for 1 h at room temperature. Quantification ATF6 α signal
586 in ER and nucleus was calculated using CellProfiler 2.1.1 as described in (Gallagher et al. 2016).
587 DAPI images were used to identify primary objects and clumped objects were distinguished
588 based on fluorescence intensity. The GRP94 images were then used to generate secondary
589 objects from primary objects using global Otsu two-class thresholding with weighted variance.
590 The final ER mask was generated by subtracting the nuclear area from the ER area. Lastly, the
591 FLAG-ATF6 α images were used to calculate FLAG-ATF6 α intensity in the nucleus and ER and

592 determine the nucleus to ER ratio of each cell. Data from CellProfiler was exported as a
593 MATLAB file for analysis and plotted on GraphPad Prism version 6.0.

594

595 *Immunoprecipitation and Immunoblot Analysis*

596 Cells were grown in 100 mm plates with two replicates for each treatment condition, treated with
597 50 nM doxycycline the following day, treated with 100nM Tg and 6uM Ceapin-A5 or 6uM
598 Ceapin-A7 for 30 min on the day of harvest, and harvested in scrapping buffer (cold PBS with 10
599 μ M MG132 and 1X protease inhibitor). Ceapin A-7, inactive analog Ceapin A-5, or DMSO
600 were kept in scrapping and lysis buffers throughout IP. Cells were lysed for 1 h at 4° C in lysis
601 buffer (50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1X protease inhibitor, and 1% LMNG
602 (Anatrace NG322)). The lysates were cleared by centrifugation at 17,000 x g for 30 min.

603 Dynabeads Protein-G (ThermoFisher) were bound with Sigma FLAG M2 antibody for 1 h at 4°
604 C and crosslinked with 100 μ M BS3 crosslinker for 30 min. 293 TREx 3XFLAG cell lysates
605 were then incubated with these FLAG beads for 2 h at 4° C. IP beads were washed with wash
606 buffer (lysis buffer without LMNG) and boiled and eluted in buffer containing 50 mM Tris pH
607 6.8, 300 mM NaCl, 2% SDS, and 10 mM EDTA. Protein samples were then precipitated, trypsin
608 digested, labeled with tandem mass tags (TMT), and analyzed by liquid chromatography-mass
609 spectrometry using Multidimensional Protein Identification Technology (MuDPIT) , as described
610 previously (Mortenson et al. 2017; Plate et al. 2018). TMT intensities for proteins detected in
611 each channel were normalized to the respective TMT intensity of ATF6 α . TMT ratios for
612 individual proteins were then calculated between Tg+Ceapin-A7/DMSO treatment or
613 Tg+Ceapin-A5/DMSO treatment.

614

615 The reciprocal affinity purification with full-length GFP-ABCD3 or GFP-ABCD3 Δ NBD cells
616 was carried out by culturing, treating, and lysing cells as described above. 293 TReX 3XFLAG
617 GFP-ABCD3 clarified cell lysate was then incubated with GFP-Trap_MA ChromoTek beads for
618 2 h at 4° C. IP beads were washed with wash buffer (lysis buffer without LMNG) and boiled in
619 SDS sample buffer for 10 min.

620

621 Cells for *in vitro* incubation were lysed with lysis buffer containing LMNG (described above)
622 and cleared by centrifugation at 17,000 x g for 30 min in the absence of any drug. Cleared
623 supernatant was then incubated with Ceapin A-7 or inactive analog Ceapin A-5 for 30 min at
624 room temperature, bound to GFP-Trap_MA ChromoTek beads for 1 h at 4° C, washed with wash
625 buffer containing Ceapin A-7 or Ceapin A-5, and eluted by boiling in SDS sample buffer.

626

627 For *in vitro* binding studies with purified components, 6.25 nM 3XFLAG-ATF6 α (2-90)-TEV-
628 MBP- HIS_{6X} and 100 nM ABCD3-eGFP- HIS_{8X} were incubated in lysis buffer (50 mM Tris pH
629 7.4, 150 mM NaCl, 5 mM EDTA, 1X protease inhibitor, 0.001% LMNG) with 15-90 μ M
630 Ceapin-A7 or inactive Ceapin-A5 for 30 min at room temperature. Samples were then incubated
631 with MBP-Trap_A ChromoTek beads for 1 h at 4° C, washed with same buffer containing
632 Ceapin A-7 or A-5 and 300 mM NaCl, and eluted by boiling in SDS sample buffer.

633

634 Samples were run on a precast 4%–12% Bis-Tris polyacrylamide gel (Life Technologies) under
635 denaturing conditions and transferred to nitrocellulose membrane. Antibodies described above
636 for FLAG, GFP, and Pmp70 (SAB4200181) were used to detect proteins and blots were imaged

637 for chemiluminescence detection using a ChemiDoc™ XRS+ Imaging System (Bio-Rad)
638 (Figure 6B, 6D-E) or LICOR system (Figure 6C).

639

640 ***Generation of recombinant proteins***

641 Human ATF6 α (2-90) with an N-terminal 3XFLAG was cloned into pET16b-TEV-MBP-HIS_{6X}
642 (Novagen) using Gibson assembly. The construct was expressed in BL21-Gold(DE3) E. coli
643 cells, grown to 0.6-0.8 OD₆₀₀, and induced overnight at 16° C with 0.25 mM IPTG (Gold
644 Biotechnology). The cells were harvested and resuspended in buffer containing 50 mM HEPES
645 pH 7, 150 mM NaCl, 10% glycerol, 2 mM TCEP, and complete EDTA-free protease inhibitor
646 cocktail (Roche). After lysis by sonication, the lysate was clarified at 30,000 x g for 30 min at 4°
647 C. The clarified lysate was loaded onto a HisTrap HP 5 ml column, washed in binding buffer (50
648 mM HEPES, pH 7, 300 mM NaCl, 1 mM TCEP, 10% glycerol, and 25 mM imidazole), and
649 eluted with a linear gradient of 25 mM to 1M imidazole in the same buffer. The ATF6 α fractions
650 eluted at 240 mM imidazole were collected and concentrated with an Amicon Ultra-15
651 concentrator (EMD Millipore) with a 30,000-dalton molecular weight cutoff. The ATF6 α
652 concentrated fraction was loaded onto a Mono Q HR16/10 column (GE Healthcare), washed in
653 Buffer A (50 mM HEPES, pH 7.5, 100 mM NaCl, 10% glycerol, and 1 mM DTT) and eluted
654 with a linear gradient of 100 mM to 1M NaCl in the same buffer. Fractions were collected,
655 concentrated as above, and loaded onto a Superdex 200 10/300 GL column (GE Healthcare)
656 equilibrated with buffer containing 30 mM HEPES, pH 7.5, 300 mM NaCl, 5% glycerol, and 1
657 mM DTT.

658

659 Expression and purification of human ABCD3: Full-length human ABCD3 isoform I was
660 synthesized and cloned into modified pFastBac1 plasmid with a C-terminal -eGFP -8XHis-tag
661 for baculoviral expression in *Spodoptera frugiperda* SF9 cells. Bacmid DNA was produced by
662 transforming the recombinant pFastBac1 plasmid into *E. coli* DH10Bac strain. To express the
663 protein, SF9 cells were infected with the bacmid made from recombinant pFastBac1 plasmid at
664 multiplicity of infection (MOI) = 2 for 48 h at 27° C. The cells were harvested and resuspended
665 in lysis buffer (50 mM Tris Cl, pH 7.5, 100 mM NaCl, 100 mM MgCl₂, 10% glycerol)
666 containing complete EDTA-free protease inhibitor cocktail (Roche), and lysed by sonication.
667 The lysate was centrifuged at 186,010 x g for 2 h to extract the membrane fraction. 3 g of the
668 membrane was solubilized in 30 ml of lysis buffer containing 1% w/v lauryl maltose neopentyl
669 glycol (LMNG) (Anatrace): 0.1% w/v cholesteryl hemisuccinate (CHS) (Anatrace) overnight.
670 Solubilized membrane was clarified by centrifugation at 104,630 x g for 30 min with 5 mM
671 imidazole added. A HiTrap™ TALON® crude 1 ml column (GE Healthcare) was equilibrated
672 with the lysis buffer containing 5 mM imidazole and solubilized membrane loaded onto the
673 column. After binding the column was washed with 15 ml of 10 mM imidazole, 0.02% glyco-
674 diosgenin (GDN) (Anatrace) in lysis buffer. The protein was eluted from the column with 10 ml
675 of 150 mM imidazole, 0.02% GDN containing lysis buffer. The protein obtained was
676 concentrated using Amicon Ultra-15 centrifugal filter units (MilliporeSigma) and size exclusion
677 chromatography was done to further purify the protein in SEC buffer (20 mM HEPES, pH 7.5,
678 100 mM NaCl, 2 mM MgCl₂, 2% glycerol and 0.02% GDN).

679

680 Table 1. Protospacer sequence of sgRNAs

Gene	Protospacer
NegCtrl	GCGCCAAACGTGCCCTGACGG
ATF6	GTGGGATCTGAGAATGTACCA
ABCD3-1	GGTACCAGCGAGCCGGCGAG
ABCD3-2	GACTGCCGGTACCAGCGAGC
PEX19-1	GGCCGAAGCGGACAGGGAAT
PEX19-2	GGAGGAAGGCTGTAGTGTCG
ACBD4	GCCGGCCCTGCTGGACCCCG
ACBD5	GGGAGCCGCTCTCCCACCCT
VAPA	GCACCGAACCGGTGACACAG
VAPB	GCGGGGGTCCTCTACCGGGT

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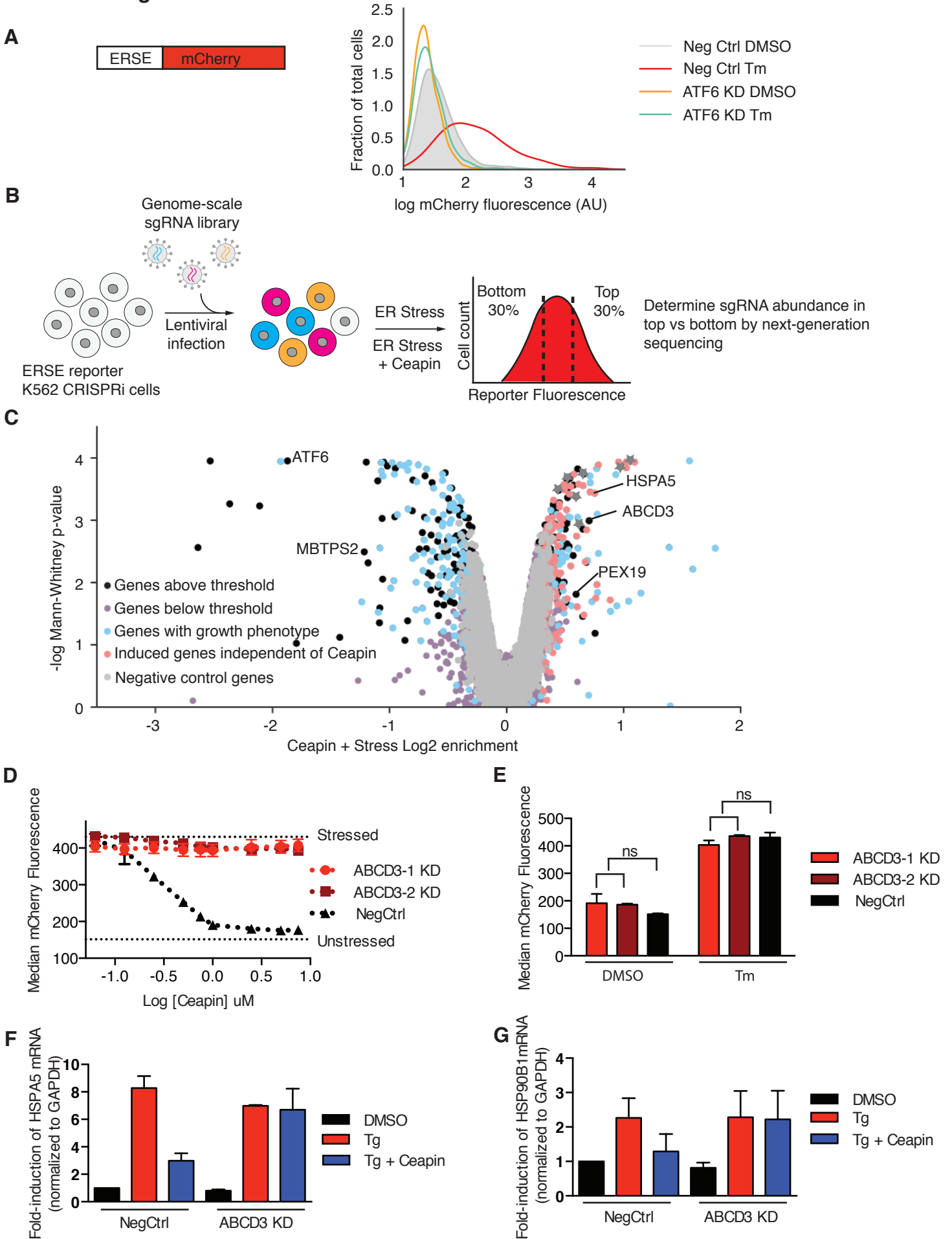
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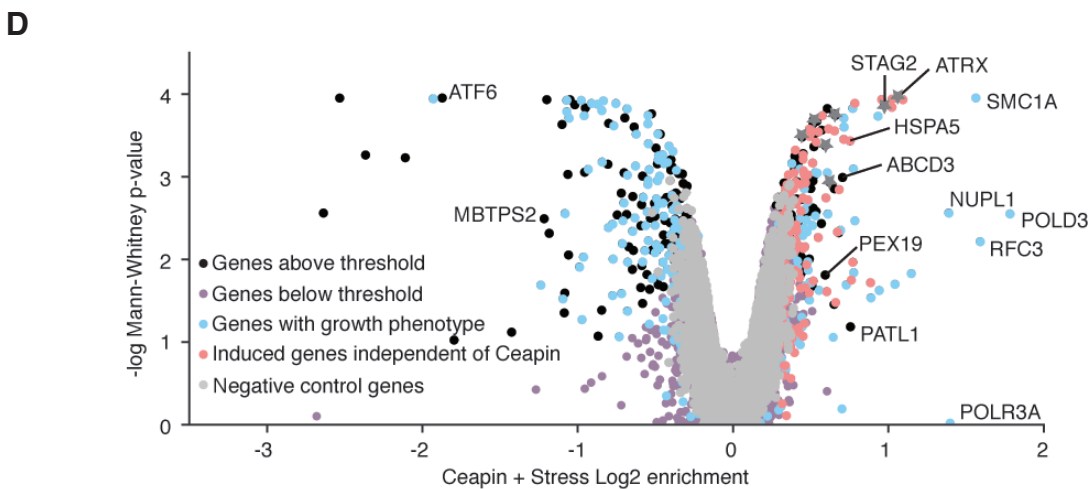
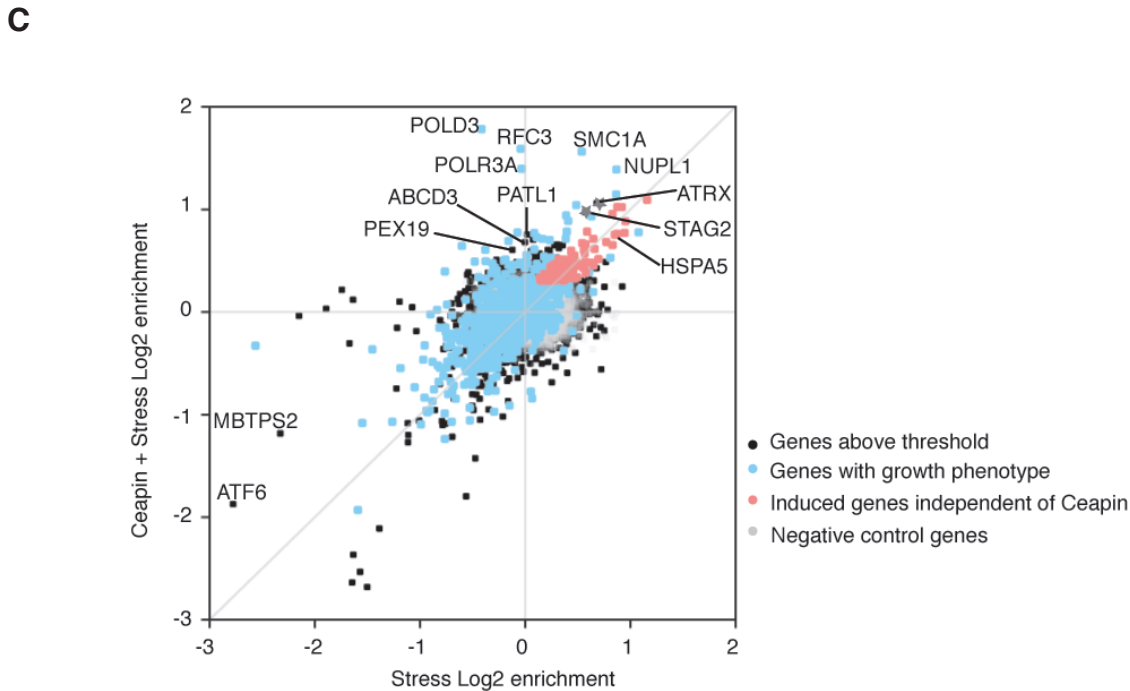
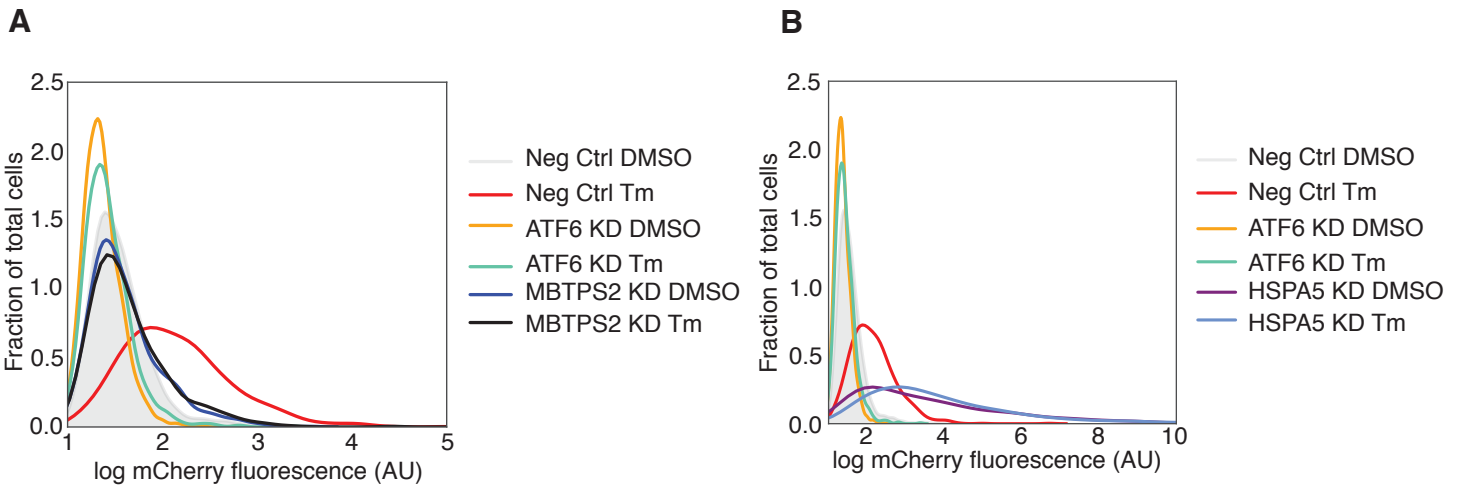
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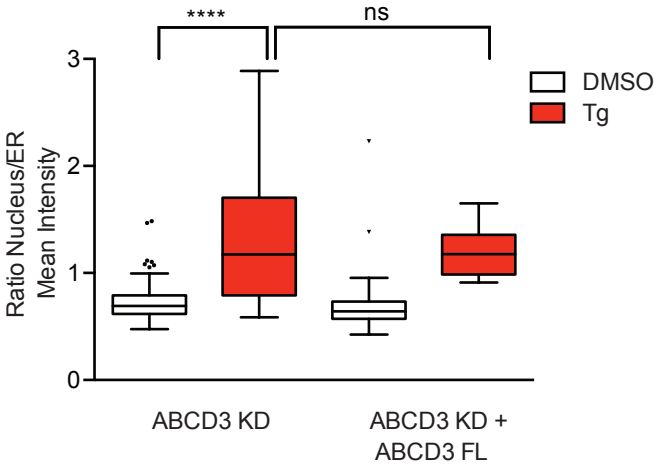
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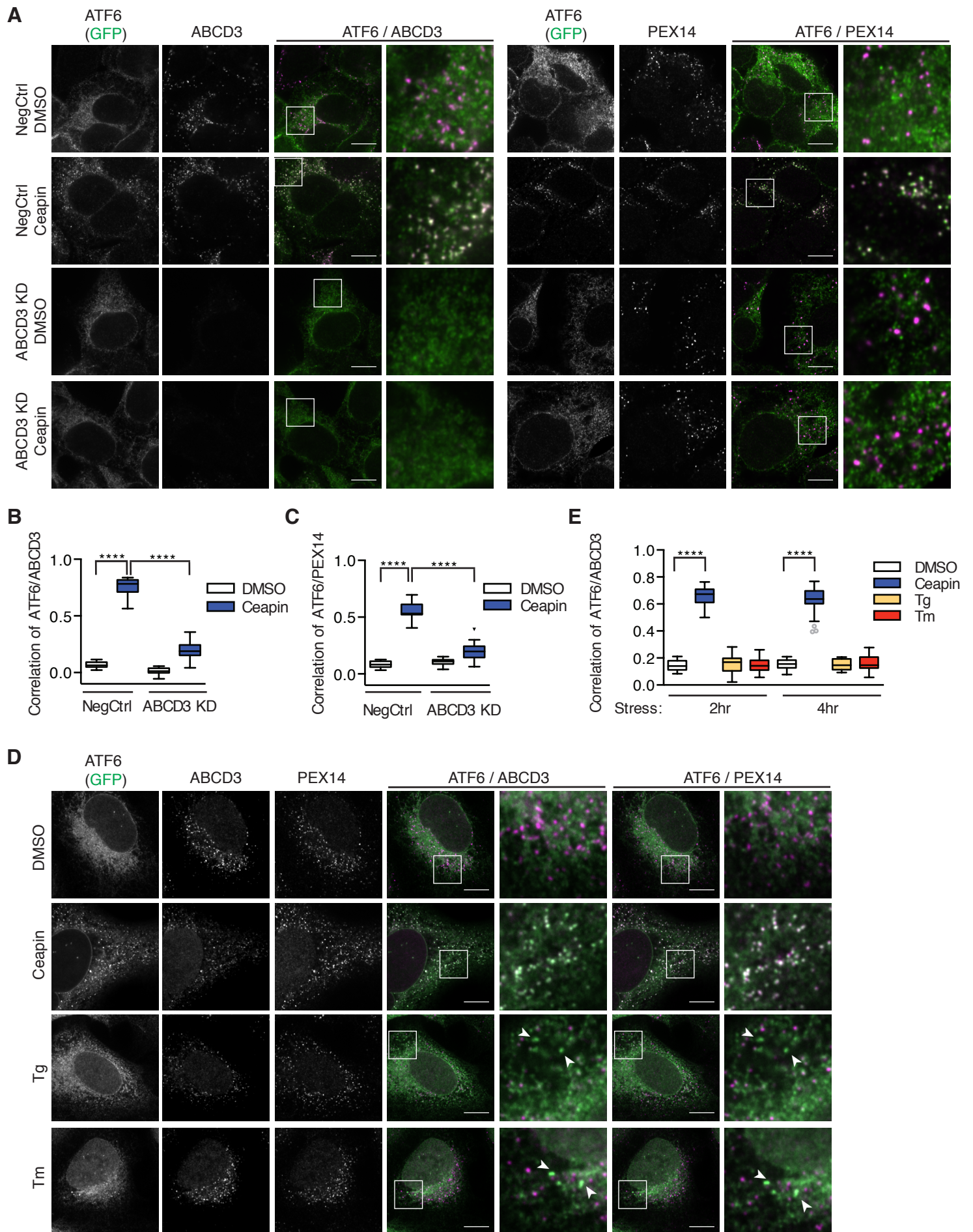
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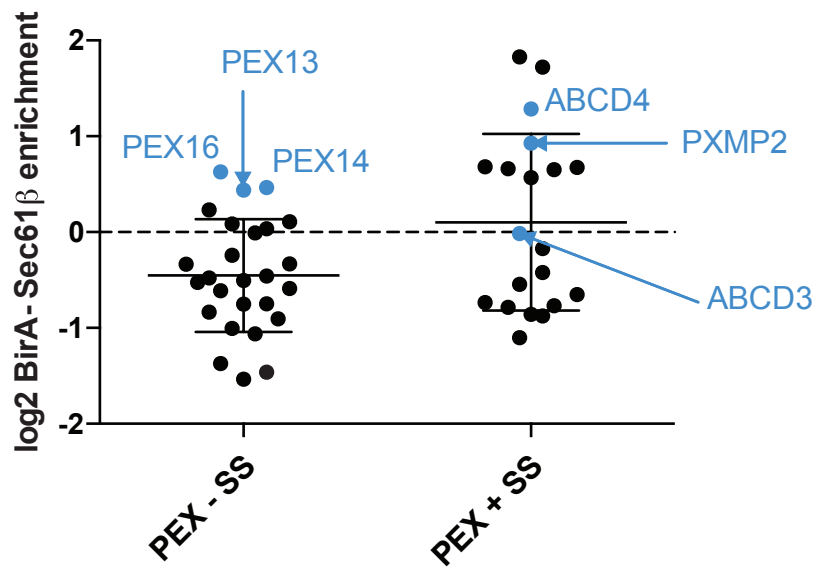
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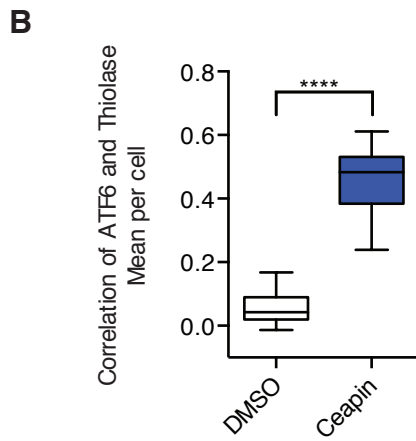
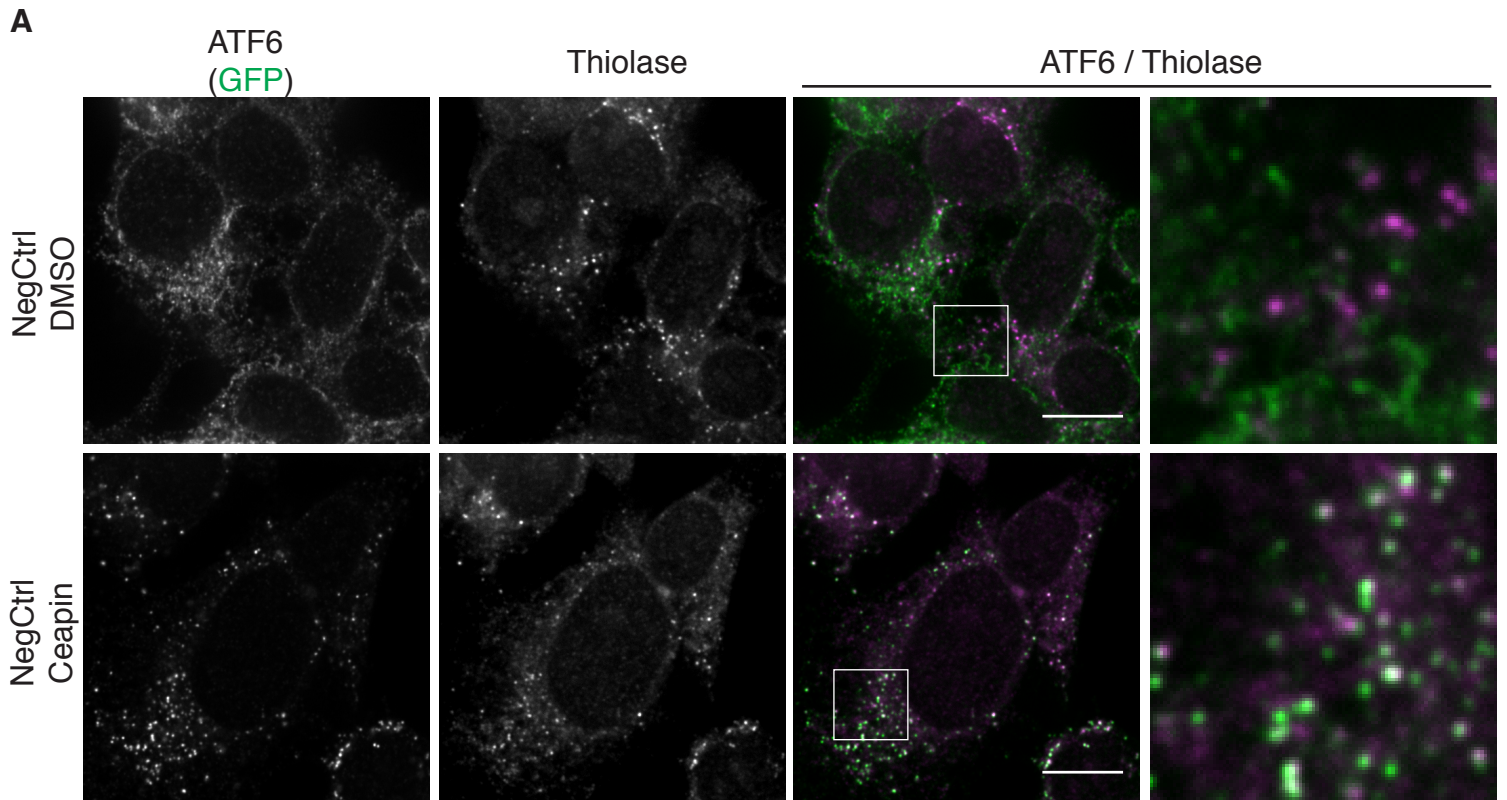
Torres et al. Figure 2



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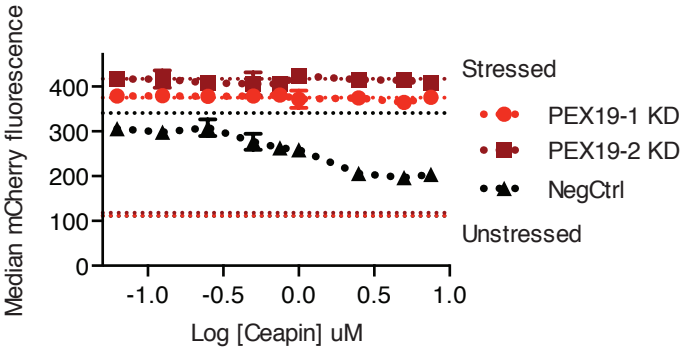


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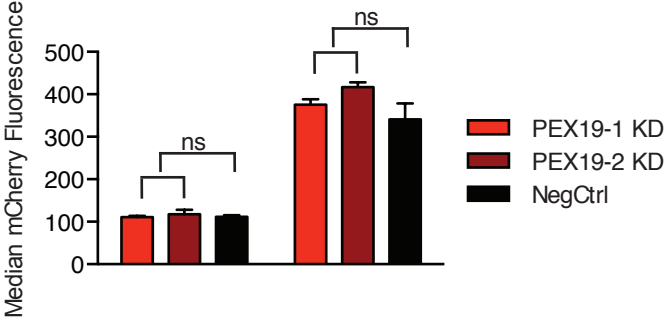


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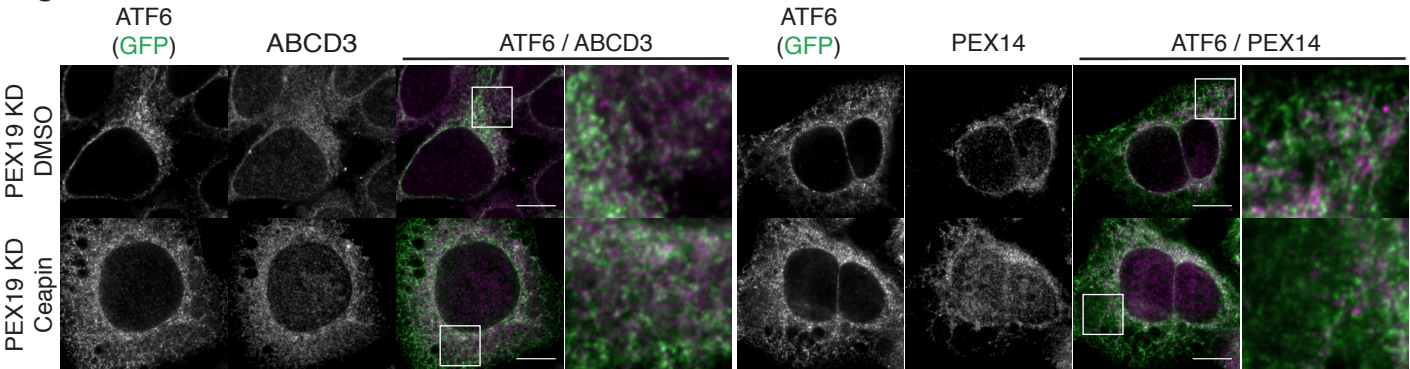
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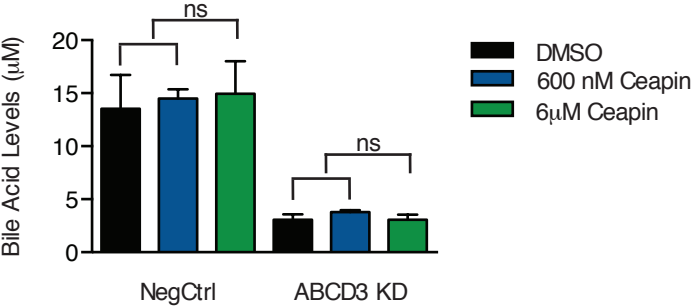
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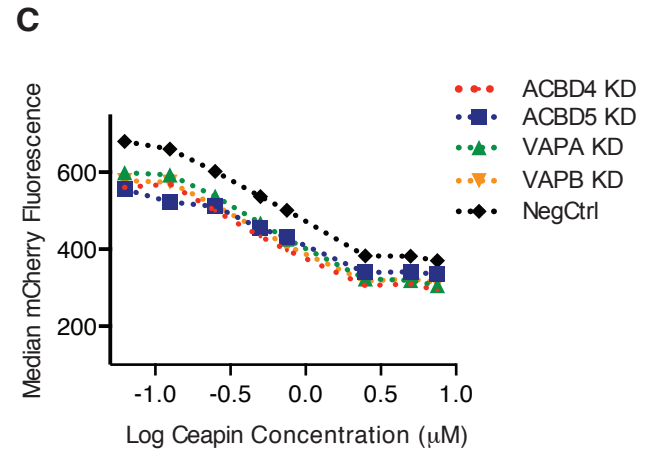
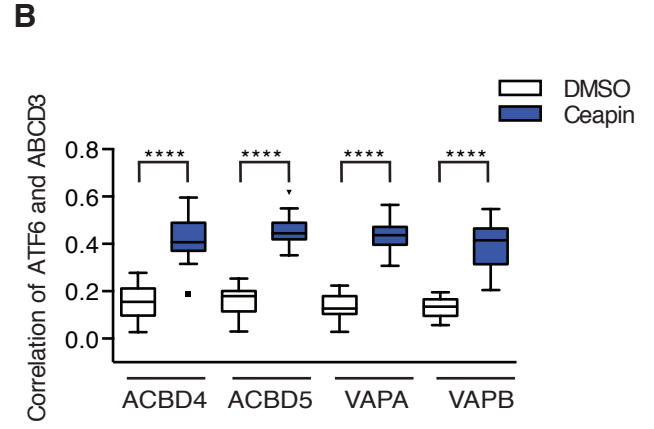
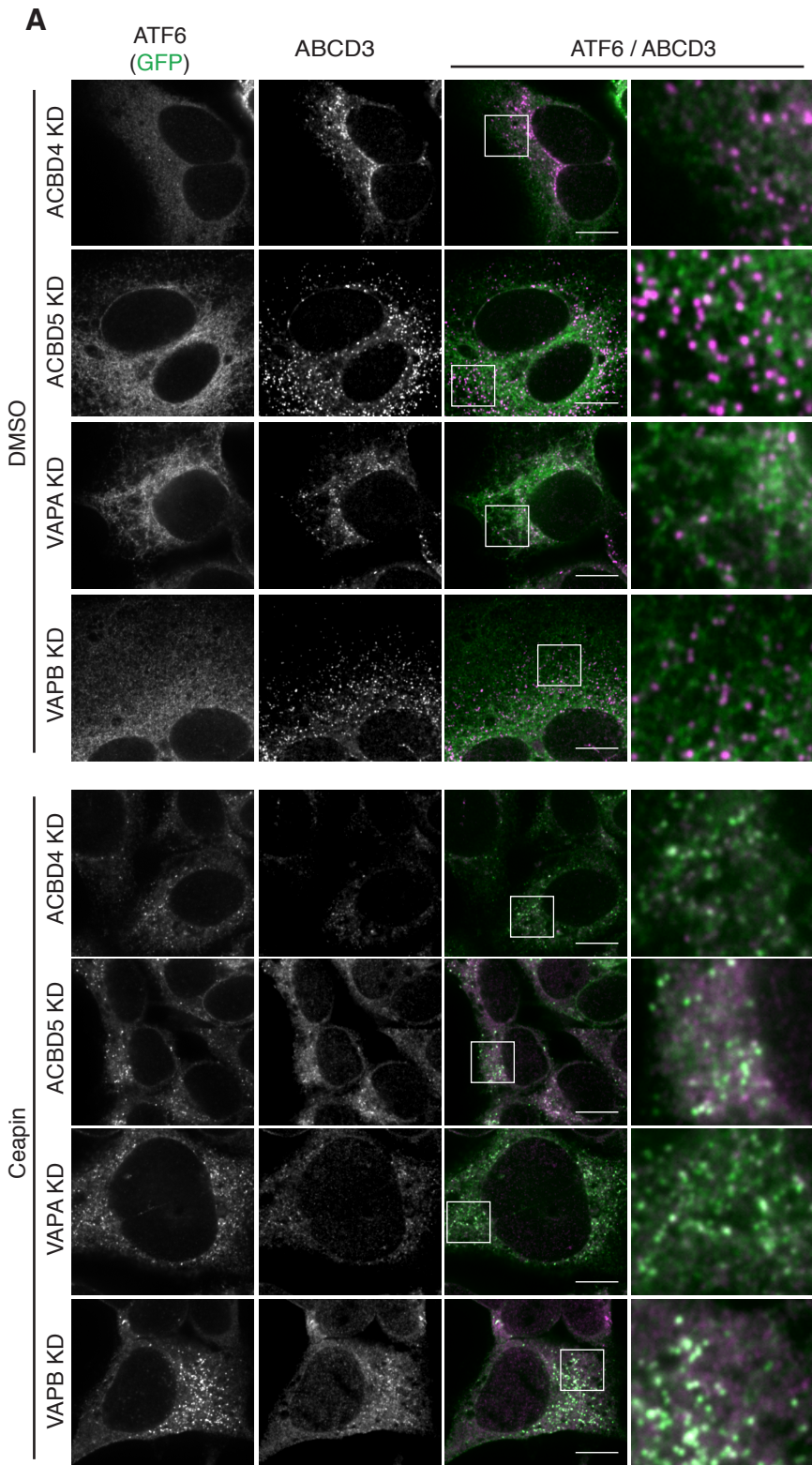
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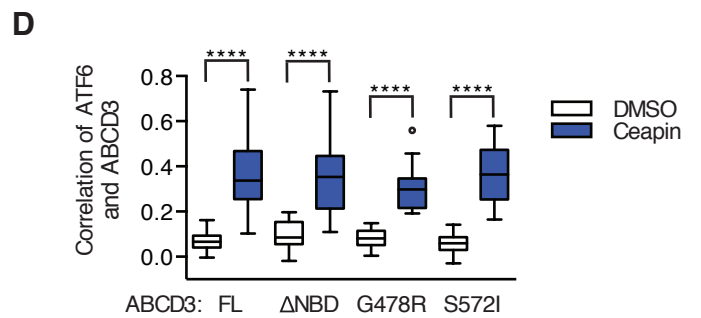
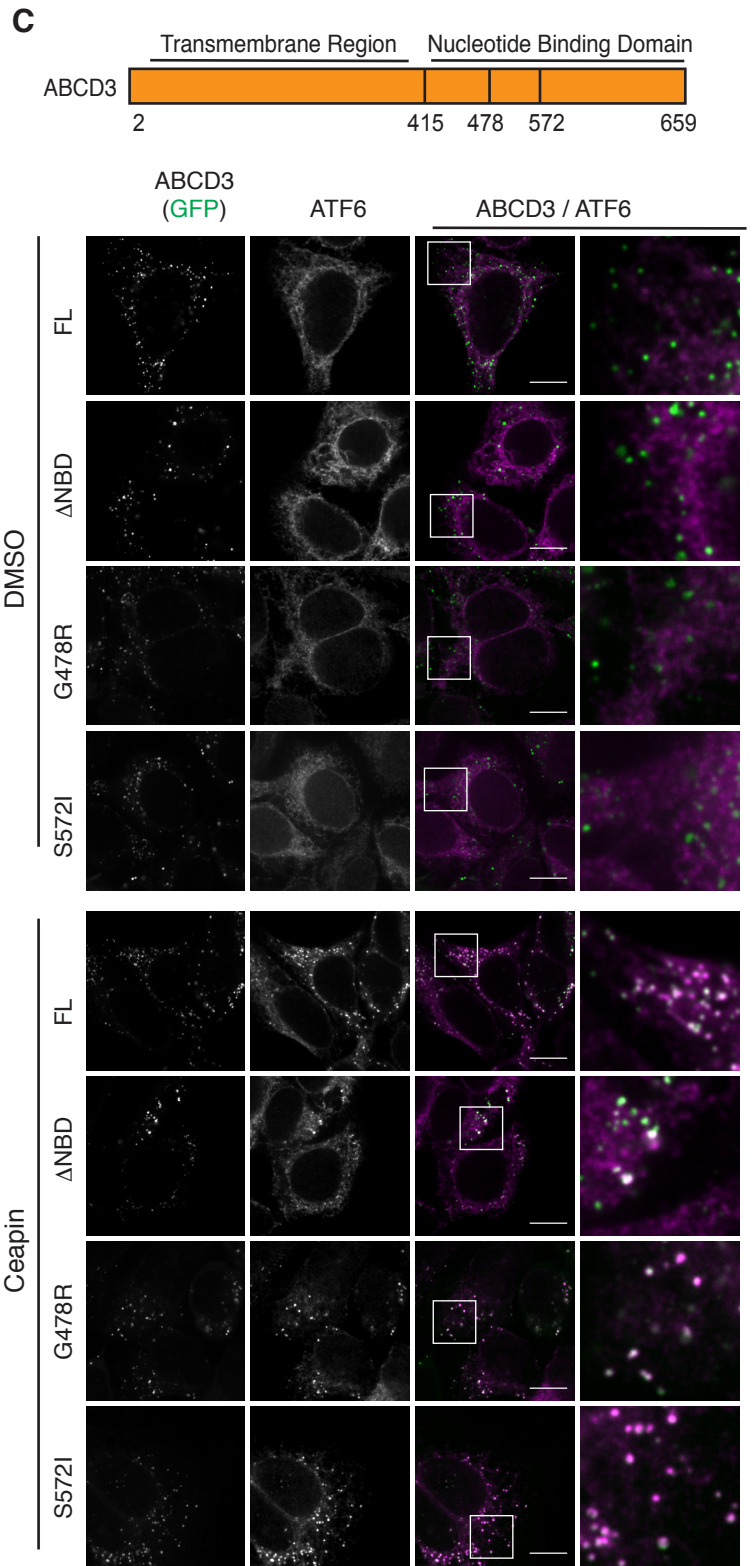
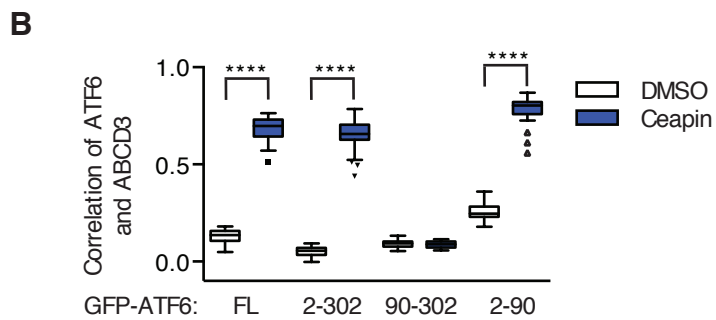
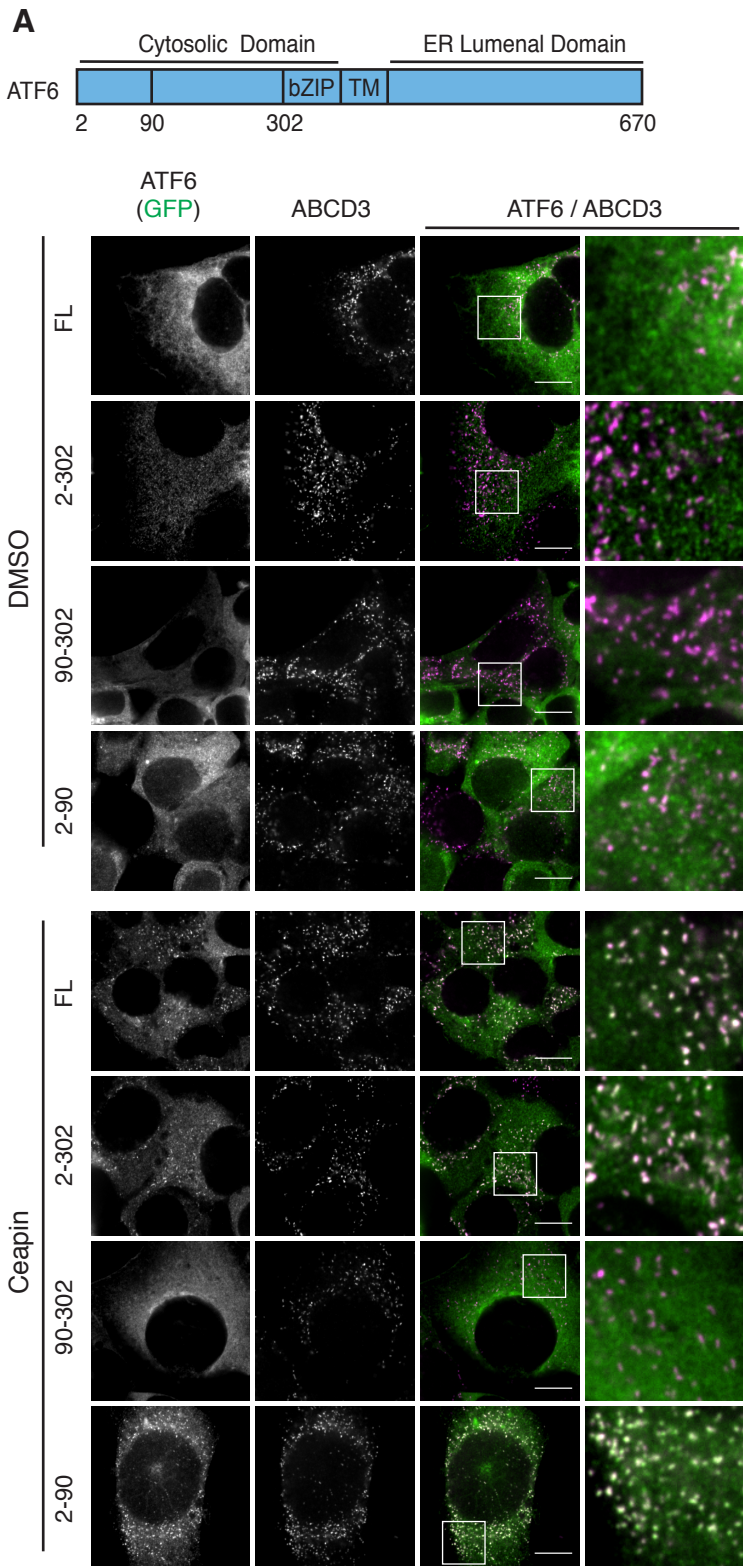
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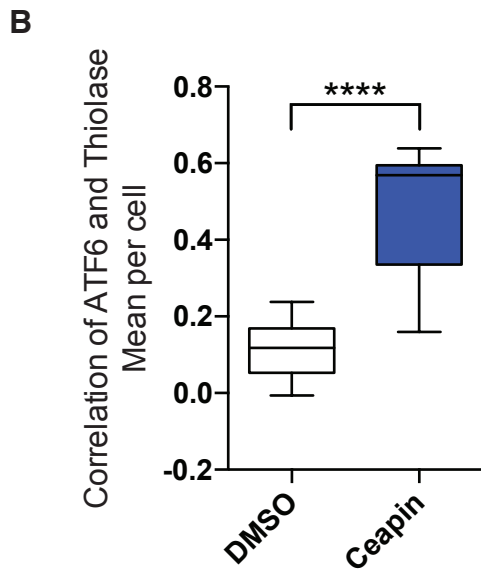
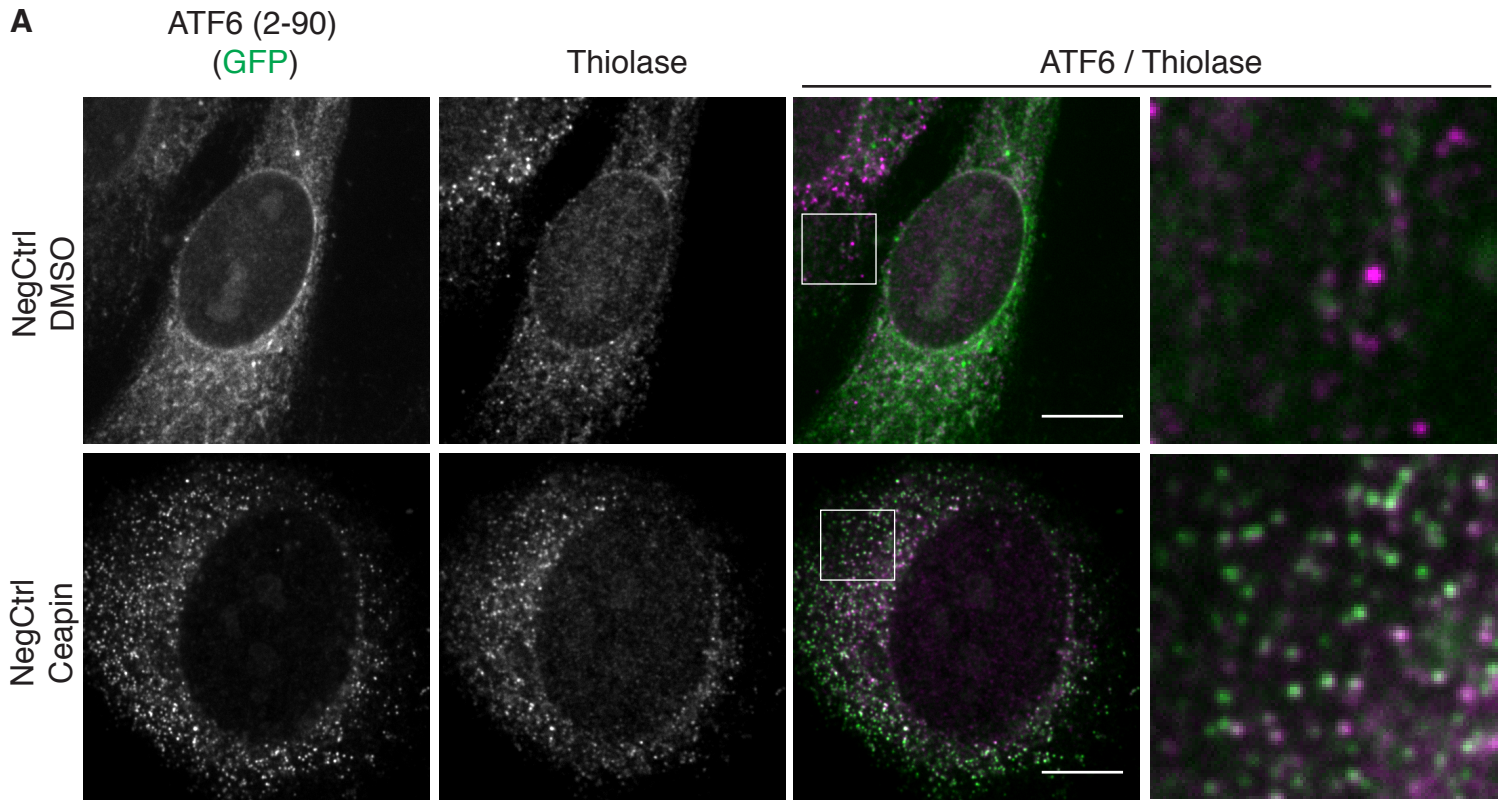
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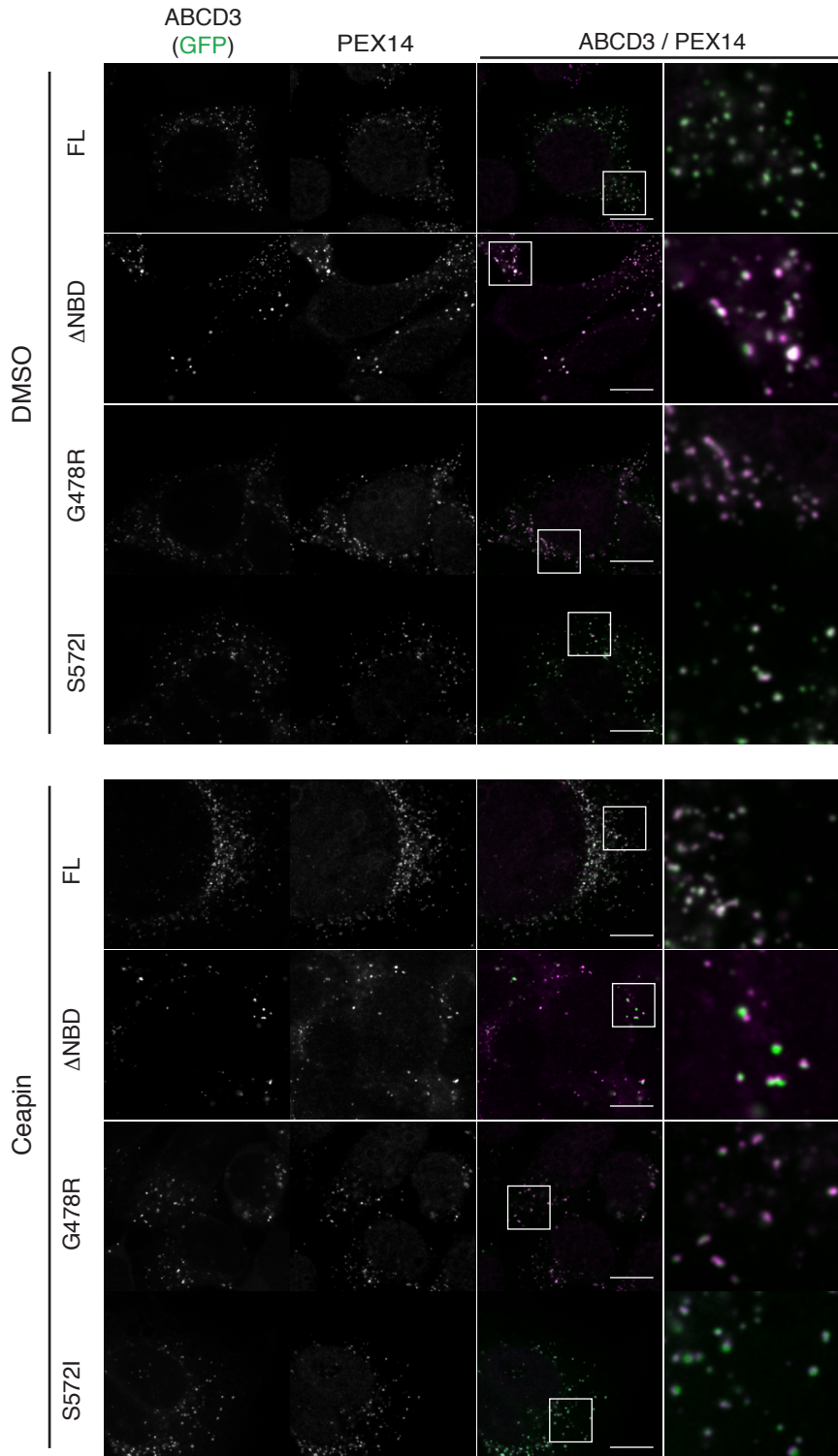
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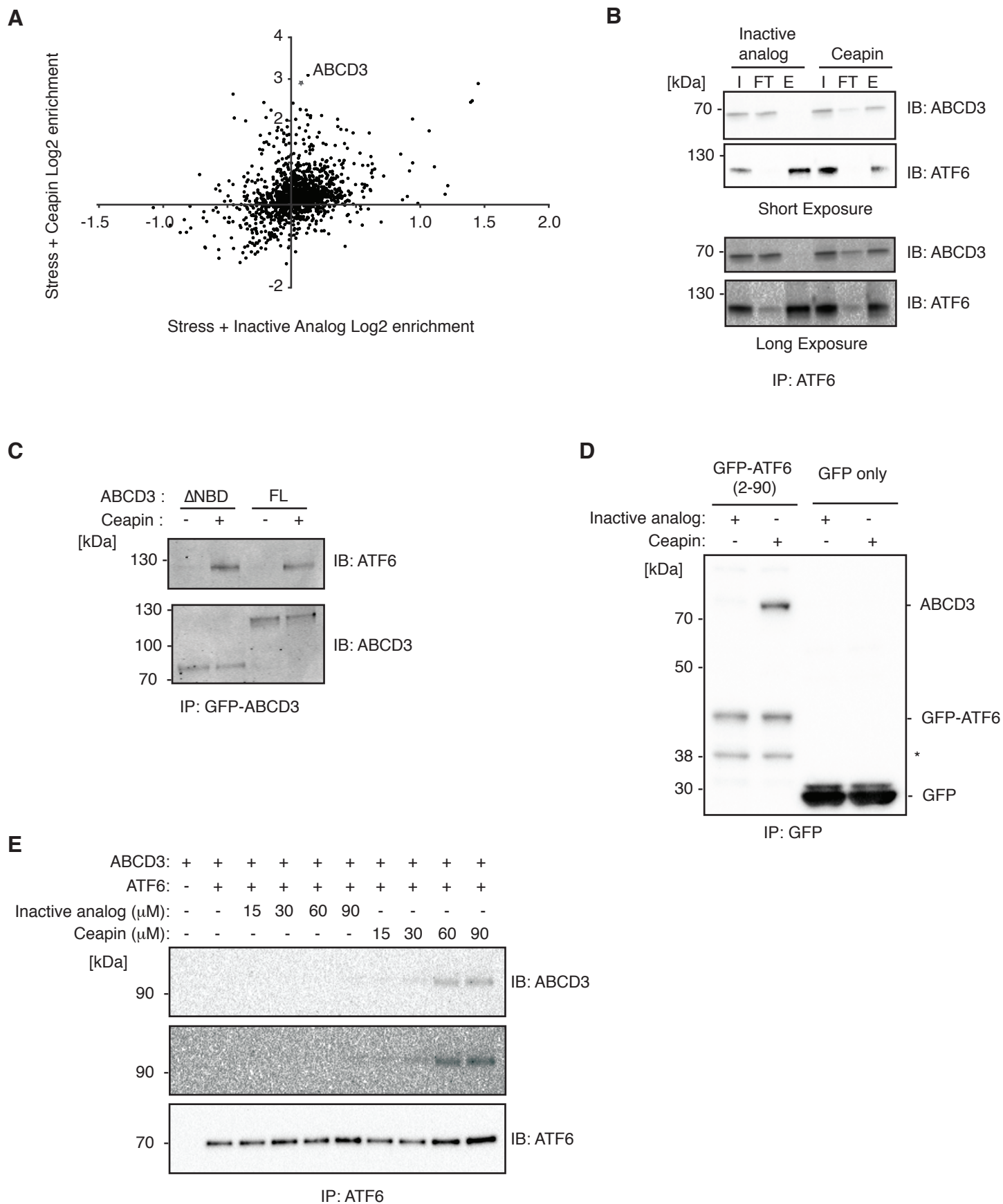
Torres et al. Figure 5 - figure supplement 1



Torres et al. Figure 5 - figure supplement 2



Torres et al. Figure 6



Torres et al. Figure 7

