Compromised ESCRT signalling is sufficient for resistance to the Target of Rapamycin Complex inhibitor Torin1 in fission yeast

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Abstract

Background

Fission yeast cells defective in Golgi-endosomal sorting display high resistance to Torin1, a pan-Target of Rapamycin (TOR) inhibitor. TOR complexes regulate the ESCRT system to integrate nutrient availability with cell division. TOR activity is frequently deregulated in cancer, making it an attractive therapeutic target. Deregulated ESCRT activity has also been associated with cancer, but its role in mediating drug resistance is not fully understood. Herein, we have investigated the role of the ESCRT system in regulating sensitivity to Torin1.

Methods

Growth assays were used to monitor the growth of yeast cells. The effect of Torin1 on protein expression was monitored by immunoblotting. Fluorescence microscopy was used to investigate the action of Torin1 on protein localization.

Results

The ESCRT system mediates Torin1-induced degradation of amino acid and glucose transporters. The expression of these transporters at the plasma membrane is not abolished in ESCRT mutants. Mutants unable to effectively ubiquitylate these transporters are also resistant to Torin1. Impaired ESCRT-mediated protein degradation is associated with strong resistance to Torin1.

Conclusions

Mutations in genes encoding ESCRT components have been reported in cancer. We present evidence that compromised ESCRT signalling is sufficient for resistance to Torin1. Cells defective in ESCRT signalling or ubiquitin homeostasis are highly resistant to Torin1. Our studies demonstrate that defective ESCRT-mediated proteolysis can suppress sensitivity to Torin1.

Keywords: TORC1**,** TORC2, ESCRT, Gsk3, fission yeast, nutrient transport, cell division, cancer, caffeine, rapamycin, Torin1, drug resistance

Background

The Target of Rapamycin Complexes, TORC1 and TORC2, integrate nutrient availability and environmental stress conditions with cell metabolism, growth and division. They, thus, play important roles in cancer development and drug resistance, and dual complex inhibitors are in clinical development. Crosstalk between the two complexes following ligand-receptor activation complicates the effective use of TORC inhibitors as anti-cancer agents. As with all

49 anti-cancer therapeutics, *de novo* or acquired resistance limits the efficacy of pan-TORC
50 inhibitors (1) (2). We previously reported that *Schizosaccharomyces pombe* (*S. pombe*) fission
51 yeast mutants defective in Endosomal Sorting Complex Required for Transport (ESCRT)
52 signalling display strong resistance to the pan-TORC inhibitor Torin1 (3). Herein, we have
53 investigated the links between deregulated ESCRT signalling and Torin1 resistance in *S.*
54 *pombe*.
55
56 The ESCRT pathway is an ancient, evolutionarily conserved pathway consisting of
57 multicomponent complexes that execute various cellular functions, such as membrane
58 modification, endosomal sorting, cytokinesis, membrane repair, nuclear pore sealing and viral
59 budding in all organisms (4,5). Deregulated ESCRT activity has also been linked to genomic
60 instability and resistance to Epidermal Growth Factor Receptor (EGFR) inhibitors and
61 Oxaliplatin in cancer cells (6–8). In fission yeast, the TORC1 and TORC2 complexes regulate
62 nutrient transporter localization and stability (9–11). Additionally, these complexes also
63 regulate amino acid transporter expression by Isp7 a member of the 2-oxoglutarate-Fe(II)-
64 dependent oxygenase gene family (12). Ubiquitylation of the Aat1 transporter by the HECT-
65 type Pub1 E3 ligase is required for its localization to the Golgi-endosome. In the absence of
66 Pub1, Aat1 is localized to the plasma membrane (PM). Arrestin-related molecular adaptors
67 such as Any1 (Arn1) and Aly3 are required for Pub1-mediated ubiquitylation of nutrient
68 transporters. When TORC1 is inhibited, or *pub1* is deleted, Aat1 localizes to the PM and is
69 then degraded via the ESCRT pathway (9,10). Aly3 similarly regulates ubiquitylation of the
70 Ght5 high-affinity hexose transporter. The inhibition of TORC2 abolishes Gad8-dependent
71 phosphorylation of Aly3. This permits ubiquitylation of Ght5 by Pub1 and results in the
72 transport of Ght5 to the endosomes for degradation in lysosomes (10,11). Inhibition of TORC2
73 also results in the activation of the downstream Gsk3 kinase, which is itself implicated in
74 cancer development and progression. Gsk3 mutants are also highly resistant to Torin1.
75 Phosphorylation of Pub1 by Gsk3 has been reported to regulate its activity (3,13). Interestingly,
76 Pub1 also regulates Cdc25 stability, targeting it for ubiquitin-dependent proteasomal

77 degradation. Cdc25 is a phosphatase that regulates cell cycle progression by counteracting
78 the inhibitory phosphorylation of Cdc2 by Wee1(14). Cdc25 degradation is required for
79 cytokinesis and proper cell division following mitosis (15). Wee1 has been reported to delay
80 cell cycle progression in G2 following glucose withdrawal, and *wee1* mutants lose viability
81 under these conditions (16). Inhibition of Cdc2 activity leads to arrest in the G1, S or G2 phases
82 of the cell cycle in a context-dependent manner (14).
83 The expression of PM nutrient transporters is regulated by TORC1 and TORC2 according to
84 nutrient availability. In the absence of nitrogen, cells rapidly divide and exit the cell cycle or
85 undergo mating. In contrast, glucose limitation induces a transient cell cycle arrest in G2 as
86 Ght5 expression is upregulated (11,16,17). Exposure of fission yeast cells to caffeine or
87 rapamycin inhibits TORC1 but not TORC2, driving entry into mitosis without subsequent cell
88 cycle arrest. In contrast, exposure to Torin1 inhibits TORC1 and TORC2, which drives cells
89 into mitosis and cell cycle arrest in G2 (17,18). TORC1 and TORC2, together with Gsk3 and
90 Pub1, thus regulate Cdc25 activity, integrating nutrient availability with cell cycle regulation.
91 Furthermore, Torin1 induces Cdc25 degradation and cell cycle arrest due to the inhibition of
92 Cdc2 activity by Wee1 (17,19). We hypothesized that Torin1 induces the global degradation
93 of amino acid and glucose transporters, leading to cell cycle arrest due to Cdc2 inhibition.
94 Mutations that confer resistance to Torin1 would need to rewire cell signalling pathways to
95 reactivate or prevent the inactivation of Cdc2.
96 We report that Torin1 treatment results in the degradation of the Aat1 and Cat1 amino acid
97 transporters as well as the Ght5 glucose transporter in an ESCRT-dependent manner.
98 Furthermore, Pub1 is required for the degradation of these transporters following exposure to
99 Torin1. In *pub1*Δ and ESCRT mutants, Aat1, Cat1 and Ght5 expression is not abolished in
100 response to Torin1. Interestingly, suppression of global protein ubiquitylation also confers
101 resistance to Torin1. We propose that fission yeast strains defective in ESCRT-mediated
102 protein degradation maintain the expression of nutrient transporters at the PM following
103 exposure to Torin1. Deregulated ESCRT activity can thus contribute to Torin1 resistance by
104 preventing cell cycle exit.

105 ## Methods

106 ### Strains, media, and reagents

107 Strains are listed in Additional file 1. Cells were grown in yeast extract plus supplements
108 medium (YES). For experiments in the absence of nitrogen, we used EMM2-N medium
109 (Formedium, Hunstanton, United Kingdom). Stock solutions of caffeine (Sigma Aldrich,
110 Gillingham, United Kingdom) (100 mM) were prepared in water stored at -20°C. Rapamycin
111 (Tocris, Abingdon, United Kingdom) was dissolved in DMSO (500µg/mL) and stored at -
112 20°C. Torin1 (Tocris) was dissolved in DMSO (3.3mM) and stored at -20°C.

113

114 ### Microscopy

115 Fluorescent microscopy of GFP-expressing strains was performed using an EVOS M5000
116 and Leica DMRA2 epi-fluorescent microscope attached to a monochrome Orca-ER camera
117 from Hamamatsu Photonics.

118

119 ### Immunoblotting

120 Antibodies directed against GFP (11814460001 Roche) were purchased from Sigma-Aldrich
121 (Gillingham, United Kingdom). Secondary antibodies directed against mouse (ab205719)
122 IgG were purchased from Abcam (Abcam, Cambridge, United Kingdom). Cell pellets were
123 prepared for SDS-PAGE and treated as previously reported (Alao et al., 2025). Where
124 deemed appropriate, images were quantified using Image J software and Student's t-test for
125 statistical analyses.

126

127 ## Results

128 ### Torin1 and nitrogen withdrawal induce Ght5 degradation

129 We previously identified genes encoding ESCRT pathway components in screens for Torin1
130 resistance (3,21). As part of this study, we also identified *hse1* (ESCRT 0) and *sst6* (*vps23*)

131 (ESCRT I) deletion mutants as being resistant to the drug. We also identified *atg6* and *vps38*
132 (required for the activation of ESCRT signalling) but not *atg36* (regulates the activation of
133 autophagy) mutants in our screens, indicating that the ESCRT pathway confers sensitivity to
134 Torin1 (Fig. 1A (Light green and dark green ovals, respectively) and Fig. 1B). *S. pombe* cells
135 cannot efficiently degrade GFP, which accumulates as Ght5 is degraded (22,23). We thus
136 investigated the effect of Torin1 on transmembrane nutrient transporters using strains
137 expressing Ght5-GFP (11). Exposure to 5 μM Torin1 but not 10 mM caffeine for 3h induced
138 Ght5-GFP degradation independently of Tor1. We noted, however, that Ght5-GFP degradation
139 was impaired in the *tor1*Δ mutant compared to *wt* cells (See discussion) (Fig. 1C and 1D).
140 Exposure to 100 ng/ mL rapamycin slightly induced Ght5-GFP degradation, as indicated by
141 the accumulation of GFP. Exposure to caffeine and rapamycin induced Ght5-GFP degradation
142 to an extent like that of Torin1. It is unclear if this effect is due to the induction of autophagy
143 rather than Tor1 inhibition and degradation *via* ESCRT signalling (Fig. 1E). While we identified
144 *atg6* as the only autophagy-regulating gene in our screen for Torin1 resistance, exposure to
145 the drug also induces autophagy as detected by GFP-Atg8 cleavage (Additional file 1A). In
146 contrast to glucose deprivation, exposure to Torin1 thus mimics the effects of nitrogen
147 withdrawal on Ght5-GFP (Fig. 1F). Microscopic analyses demonstrated that exposure to 5 μM
148 Torin1 induced Ght5-GFP vacuolar accumulation after 1h, followed by degradation (1h vs 3h)
149 (Fig. 1C and 1G). Similar results were observed only when caffeine was combined with
150 rapamycin. Caffeine and rapamycin also exerted differential effects on Ght5 localization, which
151 was more diffuse than the latter. This is consistent with their mechanisms of TORC1 inhibition.
152 We also noted that in contrast to cells exposed to Torin1, cells exposed to caffeine with or
153 without rapamycin were still undergoing division after 3h (Fig. 1G). Since TORC2 prevents
154 Ght5-GFP ubiquitylation, we conclude that caffeine combined with rapamycin induces its
155 degradation independently of the ESCRT system. In contrast, Torin1 induces the degradation
156 of Ght5-GFP via the ESCRT pathways by inhibiting TORC2. Pub1 and Pub3 have been
157 identified as Ght5 E3 ligases (10). The deletion of *pub1* clearly suppressed the degradation of
158 Ght5-GFP, confirming our previous findings. Ght5-GFP degradation was not abolished in this

159 mutant, likely due to residual Pub3 activity (Additional file 1C). Torin1 thus arrests cell division
160 in G2, in part by preventing glucose import via the Ght5 transporter at the PM (10,17).

161 **Defective ESCRT signaling attenuates Torin1-mediated Ght5 degradation**

162 Next, we investigated the effect of Torin1 on Ght5-GFP expression in ESCRT mutants. In
163 contrast to wt cells, Torin1 failed to induce Ght5-GFP degradation in *hse1*Δ mutants. We noted
164 that this mutation did not abolish Ght5-GFP degradation as GFP still accumulated. Deletion of
165 *sst4* did not prevent Ght5-GFP degradation, although basal levels were higher, and we did not
166 observe the accumulation of GFP in this mutant (Fig. 2A to 2C). Exposure to Torin1 did not
167 induce Ght5-GFP degradation in *vps25*Δ mutants. GFP still accumulated in these mutants,
168 suggesting the degradation of Ght5-GFP is not completely abolished (Fig. 2D and 2E).
169 Inhibition of Tor1 or Gad8 activates Gsk3, which regulates Pub1 and Pub3 activity (ref) (Fig.
170 2F). We deleted *gsk3* in the Ght5-GFP mutant to investigate its role in regulating Ght5 stability.
171 However, deletion of *gsk3* did not affect Ght5-GFP expression or following exposure to Torin1
172 (Fig. 2G to 2I, Additional file 1B). The resistance of *gsk3* mutants to Torin1, thus, appears to
173 be unrelated to its regulation of Pub1/ Pub3 expression and Ght5-GFP ubiquitylation/
174 degradation (ref, ref). Our results identify the ESCRT system as being required for Torin1-
175 induced Ght5 degradation.

176 **ESCRT mutants maintain Ght5 expression at the PM following exposure to Torin1**

177 We next compared the expression of Ght5-GFP in wt and ESCRT pathway mutants following
178 exposure to 5 μM Torin1. In untreated cells, Ght5-GFP was expressed at the PM in bright
179 patches with little expression in the cytoplasm. Exposure to Torin1 induced the rapid import
180 of Ght5-GFP in a speckled pattern and subsequent degradation within the vacuole after 3h
181 (Fig. 1 and 3A). ESCRT mutants also expressed Ght5 at the PM, but the expression pattern
182 differed in different mutants. Ght5-GFP was visible in a granular cytoplasmic pattern even in
183 untreated *hse1*Δ, *vps25*Δ mutants and strongly at the cell membrane in *sst4*Δ, and *sst6*Δ cells
184 (Fig. 3B to 3E). Defective TORC2 and ESCRT-mediated protein degradation thus result in the

185 accumulation of Ght5 (ref). We noted by immunoblotting that basal Ght5-GFP levels were not
186 identical in these mutants (not shown). Following exposure to Torin1, Ght5-GFP was still
187 expressed at the PM in *hse1*Δ, *vps25*Δ, *sst4*Δ, and *sst6*Δ mutants. Torin1 exposure did not
188 affect the expression of Ght5 in *sst4*Δ mutants (Fig. 3D). Exposure to Torin1 appeared to
189 increase the expression of Ght5 at the PM in *sst6*Δ mutants (Fig. 3E). We concluded that,
190 unlike wt cells, *hse1*Δ, *vps25*Δ, *sst4*Δ, and *sst6*Δ mutants continue to express Ght5-GFP at
191 the PM following exposure to Torin1 (Fig. 3A to 3E). Glucose is required for cell division, and
192 low concentrations strongly induce the expression of Ght5 at the PM (Fig. 1F) (11). We
193 investigated whether growth on 0.03 % glucose induces resistance to Torin1. The Torin1
194 sensitivity of the *wt*, *tor1*Δ and ESCRT mutants rather increased under these conditions
195 (Additional file 1B). Thus, overexpression of Ght5 alone is insufficient to suppress Torin1
196 sensitivity. Torin1 induces Ght5 degradation, and its expression is maintained at the cell
197 membrane of ESCRT mutants. These findings partly explain the different effects of caffeine,
198 rapamycin and Torin1 on cell cycle progression in fission yeast (17,24).

199

200 **Deletion of *pub1* abolishes Torin1- induced Aat1 degradation**

201 TORC1-mediated ubiquitylation of the amino acid transporter Aat1 by Pub1 is required for its
202 retention at the Golgi/ endosomes (ref). We investigated the effect of Torin1 on Aat1 in wt and
203 *pub1*Δ mutants. As observed with Ght5-GFP, exposure to 5 μM Torin1 and nitrogen withdrawal
204 induced the degradation of Aat1-GFP. The effect was not observed following exposure to
205 caffeine and rapamycin, which induced Aat1-GFP degradation (Fig. 4A). This effect of Torin1
206 on Aat1 was abolished when *pub1* was deleted (Fig. 4B and 4D). Unlike Ght5-GFP, exposure
207 to caffeine and rapamycin did not induce Aat1-GFP degradation (Fig. 1C and 4A). Interestingly,
208 exposure to rapamycin induced the accumulation of Aat1-GFP in *pub1*Δ mutants. TORC1
209 negatively regulates Aat1 via Isp7 at the transcriptional level (12). Inhibition of TORC1 with
210 rapamycin may induce *aat1* expression and accumulation of Aat1 in *pub1* mutants, as it cannot
211 be degraded. (Fig. 4B). These findings also highlight significant differences between caffeine

212 and rapamycin in terms of TORC1 inhibition (19,24). The deletion of *gsk3* did not abolish the
213 Torin1-induced degradation of Aat1-GFP. Our observations suggest, however, that it does
214 affect the dynamics of Aat1-GFP degradation following exposure to Torin1 (Fig. 4E and 4F).

215 We next examined the effect of Torin1 on Aat1 expression by fluorescence microscopy. In *wt*
216 cells, exposure to Torin1 induced the transport of Aat1-GFP at the Golgi/ endosomes and
217 subsequent degradation (Fig. 4G). Deletion of *pub1* resulted in the constitutive expression of
218 Aat1-GFP at the PM as previously reported. Aat1-GFP also accumulated at the septa of
219 dividing cells following exposure to Torin1. Pub1 has been reported to be partly localized to
220 the septum in *S. pombe* (25). These findings demonstrate that Pub1 is required for the Torin1-
221 induced degradation of Aat1-GFP (Fig. 4G). It was also observed that *pub1* mutants continued
222 cell division, albeit asynchronously, following exposure to Torin1 (Fig. 4G). The larger cell size
223 of *pub1*Δ mutants has been previously reported (25,26). Studies using Aat1-GFP *gsk3*Δ
224 mutants produced variable results. In our experiments, the localization of Aat1-GFP in *gsk3*Δ
225 mutants mimicked that observed in *pub1*Δ mutants (Fig. 4H). We noted, however, that Aat1-
226 GFP exhibits a differential localization pattern in *gsk3*Δ mutants (Fig. 4G, 4H and I). These
227 findings are consistent with previous reports linking TORC2 and Gsk3 to Pub1 stability and
228 regulation of Aat1 expression (13). The inhibition of TORC2 induces Aat1 degradation.
229 Furthermore, Pub1 but not Gsk3 is required for the Torin1-induced degradation of Aat1 in
230 fission yeast. Both Pub1 and Gsk3 regulate Aat1 expression.

231

232 **Deletion of *pub1* abolishes Torin1- induced Cat1 degradation**

233 As the PM amino acid transporter Cat1 is regulated like Aat1, we next investigated the effect
234 of Torin1 on its expression. Exposure to 5 μM Torin1 induced Cat1-GFP degradation as
235 observed in Aat1-GFP and Ght5-GFP expressing strains (Fig. 1, 2 and 5A). Cat1-GFP
236 appeared to be more sensitive to exposure to 10 mM caffeine alone or with 100 ng/ mL
237 rapamycin than Aat1-GFP (Fig. 5B). Deletion of *pub1*Δ abolished the effect of Torin1 on Cat1-

238 GFP expression. We noted, however, that GFP still accumulated, indicating Pub1-independent
239 alternate Cat1-GFP degradation mechanisms. Pub3 may thus substitute for Pub1 in mediating
240 Cat1 but not Aat1 degradation (Fig. 5C). As observed with Aat1-GFP, deletion of *gsk3*Δ
241 delayed but did not abolish Torin1-induced Cat1-GFP degradation in this strain (Fig. 5C and
242 5D). This observation resembles that observed with the *aat1-GFP* strain (Fig. 4E and 4F).
243 Fluorescence microscopy revealed that exposure to Torin1 induced the relocation of Cat1-
244 GFP from the Golgi/ endosomes to vacuoles. In untreated wt cells, Cat1 was localized to the
245 Golgi/ endosomes. Exposure to Torin1 resulted in a diffuse appearance in the cytoplasm with
246 bright speckles (Fig. 5Ea and 5Eb). Deletion of *pub1* resulted in the dispersion of Cat1-GFP
247 from the Golgi/ endosomes to vacuoles to the cytoplasm, PM and septum of dividing cells (Fig.
248 5Ec and Ed). Deletion of *pub1*Δ prevented the degradation of Cat1-GFP following exposure
249 to Torin1. While exposure to Torin1 in wt cells resulted in a speckled appearance, this was not
250 observed in *pub1*Δ mutants, which fail to degrade Cat1 (Fig. 5B). Furthermore, *pub1* mutants
251 continued to express Cat1-GFP at the PM following exposure to Torin1 (Fig. 5Ec and 5Ed).
252 We next examined the effect of deleting *gsk3* in the *aat1-GFP, cat1-GFP, and Ght5-GFP*
253 strains. The deletion of *gsk3* did not affect the localization of *cat1* -GFP as observed in pub1Δ
254 mutants (Figure 5E and 5F). The deletion of *gsk3*, similarly, did not affect the expression of
255 Ght5-GFP in untreated cells. Together, our findings suggest that while Pub1 regulates the PM
256 expression of both Aat1 and Cat1, Gsk3 regulates only the localization of the former (Fig. 5F).
257 This observation is consistent with previous reports linking Gsk3 to Pub1 activity and Aat1
258 expression. Pub1 is, however, required for the degradation of Aat1, Cat1 and Ght5 following
259 exposure to Torin1. In this model, the deletion of *gsk3* would limit the availability of Pub1 and
260 suppress sensitivity to Torin1 by maintaining nutrient transporter expression (13). Fission yeast
261 cells can thus selectively fine-tune the expression of nutrient transporters under various
262 conditions in a TORC1 and TORC2-dependent manner.

263

264 **The ubiquitin system is required for Torin1 sensitivity and links TORC1 and TORC2**
265 **signalling with cell division**

266 Torin1 induces the ubiquitin-dependent degradation of nutrient transporters and Cdc25 by
267 Pub1 (Fig. 1, 2, 3, 4 and 5) (19). E3 ligases such as Pub1 regulate multiple targets and
268 pathways. Loss of Pub1 expression would prevent the degradation of Cdc25 and numerous
269 nutrient transporters (9,9,10,19). The deletion of *pub1* did not confer resistance to caffeine
270 combined with rapamycin or Torin1 but rather increased sensitivity to these drugs (Fig. 6A).
271 Mutants lacking Pub1 are sensitive to various stresses and exhibit cell cycle defects, which
272 may override its effect on Torin1 sensitivity (27). Gsk3 activity is elevated in *gad8*Δ mutants.
273 These cells displayed increased sensitivity to caffeine combined with rapamycin or Torin1,
274 consistent with TORC2 regulating sensitivity to these agents. As previously reported, *gsk3*Δ
275 mutants are highly resistant to Torin1 (3). The deletion of *pub2* did not affect sensitivity to either
276 caffeine plus rapamycin or Torin1. When both *pub2* and *pub3* were deleted, the cells exhibited
277 resistance to Torin1 but not caffeine combined with rapamycin. A WD-repeat protein Lub1, the
278 loss of which was found to show resistance to Torin1, is required for adequate ubiquitin
279 expression in fission yeast, and *lub1*Δ mutants exhibit low global ubiquitylated protein levels
280 (28). These mutants are highly resistant to Torin1, presumably due to their inability to
281 sufficiently ubiquitylate target proteins (Fig. 6A). These findings identify a novel ubiquitin-
282 dependent, ESCRT-regulated degradation system required for sensitivity to Torin1. We
283 deleted *gsk3* in a strain expressing a Cdc25-GFP variant that lacks negative phosphorylation
284 sites (29). In the Cdc25(12A)-GFP *gsk3*Δ mutant, expression of the phosphatase was
285 extremely low and cleaved GFP was not detectable. Furthermore, exposure to Torin induced
286 Cdc25(12A) degradation in wt but not *gsk3*Δ cells. Surprisingly, Torin1 induced Cdc25
287 accumulation in the absence of Gsk3 (Fig. 6B). This ESCRT-dependent system thus
288 integrates nutrient transport with cell division through dual regulation of PM nutrient transporter
289 and Cdc25 stability dependent on Pub1 (Fig. 6C).

290

291 **Discussion**

292 We have identified an ESCRT-dependent protein degradation system that selectively
293 regulates sensitivity to Torin1 in fission yeast. E3 ligases target nutrient transporters for
294 degradation in lysosomes via the ESCRT pathway, which differs mechanistically from
295 autophagy (4,30). TOR inhibitors have drawn considerable attention as anticancer agents, due
296 to their central role in cellular metabolism, autophagy and cell division (1,2). Their use as
297 anticancer agents has been limited by modest anti-tumor activity and various drug resistance
298 mechanisms. Current studies investigate the impact of cell metabolism on the inhibitory
299 activities of these drugs and the mechanisms that underlie resistance to them (2). We present
300 evidence that suppression of Torin1-induced degradation of nutrient transporters is sufficient
301 for resistance to this drug. To our knowledge, such a resistance mechanism has not been
302 previously reported for TOR inhibitors. Clinically, deregulated ESCRT signalling has been
303 proposed as a potential mechanism of drug resistance. Compromised EGFR sorting to
304 lysosomes via the ESCRT pathway could potentially result in resistance to EGF receptor
305 signalling. Thus, drug resistance need not be necessarily linked to mutations altering the drug
306 affinity of a target protein or the activity of downstream effectors. Our findings in an
307 evolutionarily conserved model system confirm the need for further investigations into
308 deregulated ESCRT signalling as a mechanism of TORC inhibitor drug resistance (6,31).
309 Crucially, this system does not mediate Torin1 resistance due to a single deregulated protein.
310 Rather, compromised individual proteins at any stage of the ESCRT-mediated degradation
311 pathway, including low ubiquitin levels, confer resistance to Torin1. We identified only *atg6* and
312 *vps38* mutants as exhibiting Torin1 resistance in the study by Rodríguez-López *et al*., (27).
313 Atg6 and Vps38 direct protein degradation via the ESCRT pathway, while Atg38 regulates
314 autophagy. We also noted that the majority of *atg* gene deletions strongly enhanced sensitivity
315 to Torin1 (21). Thus, defective ESCRT signalling but not autophagy induces resistance to
316 Torin1 (Fig. 1A).

317 TORC1 and 2 regulate the expression of nutrient transporters through the ESCRT system,
318 which is dependent on the E3 ligases Pub1 and Pub3 (10,11,13,32). Cells ensure the
319 adequate import of nutrients by selectively adjusting the expression levels of nutrient
320 transporters, including Aat1, Cat1 and Ght5. On nitrogen withdrawal, membrane transporters
321 relocate to the PM and/ or are directly degraded via the ESCRT system. Glucose deprivation
322 induces G2 arrest, and both conditions lead to cell cycle exit and induction of autophagy
323 (11,32–34). TORC2 positively regulates protein expression levels by preventing their
324 degradation by the ESCRT system. Exposure to Torin1 but not caffeine or rapamycin induces
325 cell cycle arrest. Thus, Torin1 inhibits fission yeast proliferation by inhibiting TORC2. We have
326 demonstrated that exposure to Torin1 mimics nitrogen withdrawal by inducing the degradation
327 of amino acid and glucose transporters. This degradation requires the prior arrestin-related
328 adaptor-dependent ubiquitylation of substrate proteins by Pub1 and Pub3 (10,11,25). We have
329 shown that mutants defective in ESCRT-mediated degradation display strong resistance to
330 and fail to degrade Ght5 in response to Torin1. Hence, Torin1 sensitivity correlates strongly
331 with uncompromised ESCRT signalling. ESCRT mutants such as *hse1*Δ and *vps25*Δ fail to
332 degrade Ght5 following exposure to Torin1 and are highly resistant to the drug. Aat1, Cat1 and
333 Ght5 mutants lacking *pub1* fail to degrade these transporters following Torin1 exposure.
334 Indeed, the degradation of Aat1 does not occur in response to nitrogen withdrawal in *pub1*Δ
335 mutants. In contrast to *wt* cells, therefore, ESCRT and *pub1*Δ mutants continue to express
336 nutrient transporters at the PM. This may permit the sufficient importation of nutrients to
337 prevent cell cycle exit. The deletion of *pub*1 did not confer resistance to Torin1. This may reflect
338 its dual role in negatively regulating Cdc25. We noted that Torin1 failed to arrest but induced
339 asynchronous cell division in these mutants. Interestingly, low endogenous ubiquitin levels are
340 also associated with Torin1 resistance. In these mutants, insufficient ubiquitin availability
341 prevents the degradation of target proteins. Mutants that fail to de-ubiquitylate target proteins
342 are also resistant to this agent, likely due to ubiquitin sequestration by target proteins, which
343 accumulate within the cell or on the PM (3,7). Our study identifies an ESCRT system that
344 includes proteins maintaining ubiquitin homeostasis, arrestin-related adaptors, Pub1 and

345 Pub3, and vacuolar biosynthesis whose expression is required for Torin1 sensitivity. TORC1
346 and 2 integrate the availability of nutrients with cell division through this system by co-
347 regulation of nutrient transporters and Cdc25 (ref, ref) (Fig. 6C)

348 While caffeine in combination with rapamycin does slow cell division, our studies suggest that
349 the underlying mechanism differs substantially from that of Torin1. Torin1 directs Ght5 for
350 degradation through the ESCRT system. In contrast, caffeine plus rapamycin induces the
351 degradation of Cat1 and Ght5 but not Aat1. Significantly, rapamycin induced Aat1
352 accumulation in *pub1*Δ mutants. We noted that cleavage of Aat1-, Cat1- and Ght5-GFP still
353 occurs in *pub1* mutants. Furthermore, ESCRT mutants activate autophagy in the presence of
354 Torin1. Caffeine combined with rapamycin may target Cat1 and Ght5 for degradation via
355 autophagy. The co-deletion of *pub2* and *pub3* conferred resistance to Torin1 but not caffeine
356 and rapamycin. Low ubiquitin expression in *lub1* mutants presumably induces Torin1
357 resistance by suppressing Pub1 and Pub3 activity (28). These mutants exhibited resistance
358 to caffeine plus rapamycin and Torin1, but there is no correlation in drug resistance between
359 them. The ESCRT system thus mediates sensitivity to Torin1 but not caffeine when combined
360 with rapamycin. Caffeine has been shown to indirectly inhibit TORC1 by inducing the
361 activation of AMPK. These findings suggest that caffeine, a well-characterised drug, can
362 suppress resistance to TOR inhibitors. Caffeine in combination with rapamycin may trigger
363 nutrient transporter degradation independently of ESCRT signalling. We previously reported
364 that mutants unable to transfer substrate proteins from the Golgi/ endosomes to lysosomes
365 are also resistant to Torin1. Future studies will investigate the role of the ESCRT system in
366 mediating sensitivity to Rapalink-1 (3,35).

367 **Conclusions**

368 We have identified an ESCRT-dependent pathway required for sensitivity to Torin1. A role for
369 this pathway in mediating resistance to TOR inhibitors has not been previously reported.
370 Mutants unable to degrade PM nutrient transporters continue to undergo cell division in the
371 presence of Torin1. Our findings partly explain why Torin1, but not caffeine, combined with

372 Rapamycin induces cell cycle arrest. Crucially, ubiquitin homeostasis is similarly required for
373 sensitivity to Torin1.

374

375 **List of abbreviations**

376 EGFR- Epidermal Growth Factor Receptor

377 ESCRT- Endosomal Sorting Complex Required for Transport

378 GFP- Green Fluorescent Protein

379 PM- Plasma Membrane

380 TORC1- Target of Rapamycin Complex 1

381 TORC2- Target of Rapamycin Complex 2

382 **Declarations**

383 **Consent for publication**

384 All authors consented to the publication of the manuscript**.**

385 **Availability of data and materials**

386 Data and yeast strains are available on request, subject to permission of the creators.

387 **Competing interests**

388 The authors declare no competing interests.

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394

395 **Authors' contributions**

396 J.P.A. and C.R. designed the study. J.P.A. and R.A.I. carried out experiments. Y.T. prepared
397 the yeast mutants. J.P.A., Y.T. and S.S. and C.R. analyzed the data. J.P.A. wrote the
398 manuscript. J.P.A., R.A.I., C.R., Y.T. and S.S. revised and edited the manuscript

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402

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509 fission yeast. Commun Biol. 2025 Sept 29;8(1):1364.
510
511
512 **Figure legends**
513 **Fig. 1** Torin1 induces Ght5 degradation. **A** Mutations in ESCRT complex protein subunits
514 induce resistance to Torin1. Subunits in dark green were identified independently of our drug
515 screening. The resistance of *atg6* and *vps38*, but not *atg38* mutants links ESCRT signalling to
516 Torin1 sensitivity. **B** Wt, *hse1*Δ, *vps25*Δ and *vps28*Δ mutants were grown to stationary phase
517 and then cultured in fresh YES media for at least 2h. Cells were adjusted to an $O.D._{600}$ of 0.3
518 to 0.5 and plated on YES agar with or without 5μM Torin1 and incubated at 32°C for 2-3 days.
519 **C** Cells expressing Ght5-GFP were grown to log phase and exposed to 5μM Torin1 for 3h or
520 as indicated. Cell pellets were stored at -80° C and analysed by SDS-PAGE. GFP expression
521 was monitored by immunoblot analysis using monoclonal antibodies directed against GFP.
522 Full-length Ght5-GFP and cleaved GFP is indicated. Ponceau S was used to monitor gel
523 loading. **D** Quantification of Torin1-induced Ght5-GFP degradation. Experiments were carried
524 out as in **C** (*n*= 3). **E** Cells were grown to log phase and incubated with 10mM caffeine,
525 100ng/mL Rapamycin alone or in combination or 5μM Torin1 for 3h. **F** Cells were grown to log
526 phase and transferred into fresh media with 3% glucose, 0.03% glucose or EMM2-N for 3h.

527 Samples were analysed as in D. **G** Cells expressing Ght5-GFP were grown to log phase,
528 exposed to 5μM Torin1 for 1 and 3h and examined by fluorescent microscopy. White
529 arrowheads indicate septa. Scale bars indicate 10μm.
530
531 **Fig. 2** Torin1 induces ESCRT-dependent Ght5 degradation. **A** *Wt* cells expressing Ght5-GFP
532 were exposed to 5μM Torin1 for 3h and samples analysed by immunoblot analysis. Numbers
533 indicate relative expression levels. **B** *hse1*Δ and *sst4*Δ mutants were exposed to 5μM Torin1
534 for 3h and analysed as in A. **C** Quantification of Torin1-induced Ght5 degradation in wt and
535 *hse1*Δ cells (*n*=3). **D** Wt and *vps25*Δ mutants were treated as in A. **E** Quantification of Torin1-
536 induced Ght5 degradation in wt and *vps*Δ cells (*n*=3). **F** Regulation of nutrient transporter
537 stability by TORC2. Torin1 inhibits TORC2, blocks Gad8 activity and induces Gsk3 activation.
538 Gsk3 stabilizes Pub1, which targets Cdc25 for ubiquitin-dependent proteasomal degradation.
539 Inhibition of Gad8 facilitates arrestin-related adaptor-mediated ubiquitylation of Aat1, Cat and
540 Ght5 by Pub1 and Pub3. TORC2 also regulates Aat1 and Cat1 expression by regulating the
541 activity of Isp7. **G** *Wt* and *gsk3*Δ mutants were exposed to 5μM Torin1 as indicated. Samples
542 were processed as in A. **H** Quantification of Torin1-induced Ght5 degradation in *wt* and *gsk3*Δ
543 mutants (*n*=1). **I** *Wt* and *gsk3*Δ mutants expressing Ght5-GFP were exposed to 5μM Torin1 for
544 3h and examined by fluorescent microscopy. Scale bars indicate 10μm.
545
546 **Fig 3.** ESCRT mutants maintain Ght5 expression at the PM following exposure to Torin1. **A**
547 *Wt* **B** *hse1*Δ, **C** *vps25*Δ, **D** *sst4*Δ and **E** *sst6*Δ strains were examined by fluorescent microscopy.
548 Cells were exposed to 5μM Torin1 for 3h. White arrowheads indicate Ght5 expression at the
549 PM. Scale bars indicate 10μm.
550
551 **Fig 4.** Torin1 induces Aat1 degradation in an ESCRT-dependent manner. **A** Cells expressing
552 Aat1-GFP were exposed to 10mM caffeine, 100ng/mL Rapamycin alone or in combination,
553 5μM Torin1 or EMM2-N for 3h. Samples were analysed by immunoblotting. Full-length Aat1-
554 GFP and cleaved GFP are indicated. **B** *pub1*Δ mutants expressing Aat1-GFP were treated as

555 in A. **C** Wt cells expressing Aat1-GFP were exposed to 5μM Torin1 for the indicated timepoints
556 and treated as in A. **D** *Wt* and *pub1*Δ mutants expressing Aat1-GFP were treated as in C. **E**
557 *Wt* and *gsk3*Δ mutants expressing Aat1-GFP were treated as in C. **F** Quantification of Aat1-
558 GFP expression in *wt* and *gsk3*Δ mutants exposed to 5μM Torin1 for 3h (n=3). **G** *Wt* and *pub1*Δ
559 mutants expressing Aat1-GFP were exposed to 5μM Torin1 for 1 to 3h and examined by
560 fluorescent microscopy. Scale bar indicates 10μm. **H** *Wt* and *gsk3*Δ mutants expressing Aat1-
561 GFP were examined by fluorescent microscopy. Scale bar indicates 10μm. **I** *Wt, gsk3*Δ and
562 *pub1*Δ mutants expressing Aat1-GFP were examined by fluorescent microscopy. White
563 arrowheads indicate septa. Scale bar indicates 10μm.
564
565 **Fig 5.** Torin1 induces Cat1 degradation in a Pub1-dependent manner. **A** Cells expressing
566 Cat1-GFP were exposed to 5μM Torin1 for the indicated time points. Alternatively, the cells
567 were exposed to 10mM caffeine, 100ng/mL Rapamycin alone or in combination, 5μM Torin1
568 or EMM2-N for 3h. Samples were analysed by immunoblotting. Full-length Cat1-GFP and
569 cleaved GFP are indicated. **B** *Wt* and *pub1*Δ mutants expressing Cat1-GFP were exposed to
570 5μM Torin1 for the indicated timepoints. Samples were treated as in A. **C** *Wt* and *gsk3*Δ
571 mutants expressing Cat1-GFP were exposed to 5μM Torin1 for the indicated timepoints.
572 Samples were treated as in A. **D** Quantification of Aat1-GFP expression in *wt* and *gsk3*Δ
573 mutants exposed to 5μM Torin1 for the indicated timepoints (n=3). **E** *Wt* and *pub1*Δ mutants
574 expressing Cat1-GFP were exposed to 5μM Torin1 for 3h and examined by fluorescent
575 microscopy. White arrowheads indicate expression of Cat1 at the PM. Scale bar indicates
576 10μm.
577
578 **Fig 6.** TORC2 and Pub1 integrate nutrient availability with cell division. **A** *Wt*, *gad8*Δ, *gsk3*Δ,
579 *pub1*Δ, *pub2*Δ, *pub2*Δ *pub3*Δ strains were grown to stationary phase, diluted into fresh media
580 at an $O.D._{600}$ of 0.3 and incubated for 2h. Cells were serially diluted and plated on YES media
581 with 10mM caffeine and 100ng/mL rapamycin or 5μM Torin1 and incubated for 3-5 days. **B**
582 *Wt* and *gsk3*Δ *Cdc25(12A)-GFP* strains were incubated with 5μM Torin1 for the indicated time

583 points. Cell lysates were analyzed by immunoblotting. Full-length Cdc25(12A)-GFP and
584 cleaved GFP are indicated. **C** TORC1 and TORC2 adjust nutrient transporter expression to
585 cell cycle regulation. Environmental and nutrient stress inhibit TORC1-mediated Gad8
586 inhibition, resulting in Gsk3 activation. Gsk3 stabilises Pub1 which is required for the
587 ubiquitylation and degradation of PM nutrient transporters. Gad8 inhibits the arrestin-related-
588 mediated ubiquitylation of Ght5 by Pub1. Low endogenous ubiquitin levels prevent optimal
589 target protein degradation. TORC2 also positively regulates Cdc25 stability and is required for
590 cell cycle re-entry following activation of DNA damage checkpoints. TORC1 and TORC2 also
591 interact with Ssp1 and Ssp2 which also regulate cell cycle kinetics.
592
593 **Additional file 1 A** Wt, *bro1*Δ, *sst2*Δ and *vps8*Δ strains expressing GFP-Atg8 were grown to
594 log phase and exposed to 5μM Torin1 for 2h. Cell pellets were resolved by SDS-PAGE and
595 blotted with monoclonal antibodies directed against GFP. **B** Wt, *bro1*Δ, *did2*Δ, *sst2*Δ and *vps8*Δ
596 strains were grown to stationary phase, diluted into fresh media and incubated for a further
597 2h. Cells were serially diluted and plated on YES media with 3% or 0.03% glucose with or
598 without 5μM Torin1 and incubated for 3-5 days.
599
600 **Additional Table 1** *S. pombe* strains used in this study.
601
602
603
604 **Additional Table 1.** List of fission yeast strains used in this study.

Strain	Genotype	Source/Reference
h⁻ L972	*h-*	Laboratory stocks
tor1Δ	*h- tor1::kanMX6*	Laboratory stocks/(Rallis *et al.*, 2013)
gad8Δ	*h- gad8:: ura4 ade6- M216 leu1 ura4- D18*	YGRC

gsk3Δ	*h− gsk3::kanMX6*	Laboratory stocks/ (Rallis *et al*., 2017)
lub1Δ	*h+ lub1::KanMX ade6-M216 ura4-D18 leu1-32*	Laboratory stocks
ght5-GFP	*h- gad8^{TS} ght5-GFP::KanR*	Laboratory stocks/ (Saitoh *et al*., 2015)
ght5-GFP gad8^{TS} gsk3Δ	*h- gad8^{TS} gsk3::natMX ght5-GFP::KanR*	Laboratory stocks/ (Toyoda *et al*., 2021)
ght5-GFP gad8^{TS} pub1Δ	*h- gad8^{TS} pub1::hphR ght5-GFP::KanR*	Laboratory stocks/ (Toyoda *et al*., 2025)
ght5-GFP gad8^{TS} hse1Δ	*h- gad8^{TS} hse1::hphR ght5-GFP::KanR*	Laboratory stocks/ (Toyoda *et al*., 2021)
ght5-GFP gad8^{TS} vps25Δ	*h- gad8^{TS} vps25::hphR ght5-GFP::KanR*	Laboratory stocks/ (Toyoda *et al*., 2021)
ght5-GFP gad8^{TS} vps28Δ	*h- gad8^{TS} vps28::hphR ght5-GFP::KanR*	Laboratory stocks/ (Toyoda *et al*., 2021)
ght5-GFP gad8^{TS} sst4Δ	*h- gad8^{TS} sst4::hphR ght5-GFP::KanR*	Laboratory stocks/ (Toyoda *et al*., 2021)
ght5-GFP gad8^{TS} sst6Δ	*h- gad8^{TS} sst6::hphR ght5-GFP::KanR*	Laboratory stocks/ (Toyoda *et al*., 2021)
aat1-GFP	*h- aat1-GFP::KanR*	Matsumoto Laboratory
aat1-GFP gsk3Δ	*h- aat1-GFP<LEU2 leu1-32 gsk3::KanMx6*	This study
aat1-GFP pub1Δ	*h- pub1:: ura4+ ura4-D18 aat1-GFP<<LEU2 leu1-32*	Matsumoto Laboratory
cat1-GFP	*h- cat1+-GFP::kanR*	Nakashima laboratory
cat1-GFP gsk3Δ	*h- gsk3::natMX cat1+-GFP-kanMX*	This study
cat1-GFP pub1Δ	*h- pub1::hphMX cat1+-GFP-kanMX*	Nakashima laboratory
cdc25 (12A)- GFP	*h- cdc25(12A)-GFPint cdc25:: kanMX6 ura4-D18 leu1-32*	Young laboratory
cdc25 (12A)- GFP *gsk3Δ*	*h- cdc25(12A)-GFPint cdc25:: kanMX6 gsk3::natMX6 ura4-D18 leu1-32*	This study
pub1Δ	*h- pub1::ura4+ leu1-32 ura4-D18*	YGRC

pub2Δ	*h+ pub2::ura4+ ura4-D18*	YGRC
pub2Δ pub3Δ	*h90 pub2::ura4+ pub3::ura4+ ade6-M216 leu1-32 ura4-D18*	YGRC
GFP-atg8		Laboratory stocks
GFP-atg8 bro1Δ		Laboratory stocks
GFP-atg8 sst2Δ		Laboratory stocks
GFP-atg8 vps8Δ		Laboratory stocks

605 *Yeast Genetic Resource Center (YGRC) Japan

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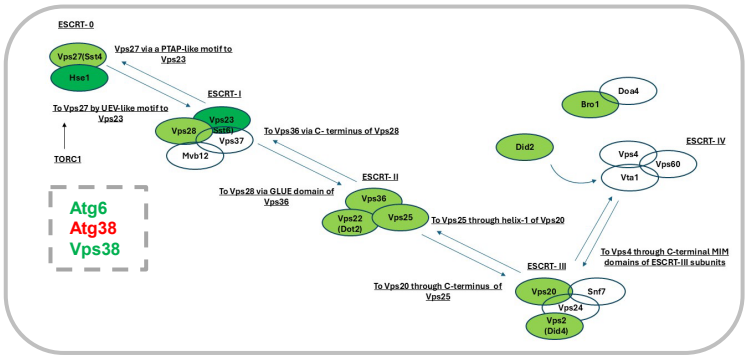
607

608

Fig. 1

A



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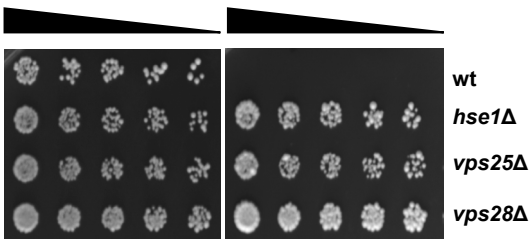

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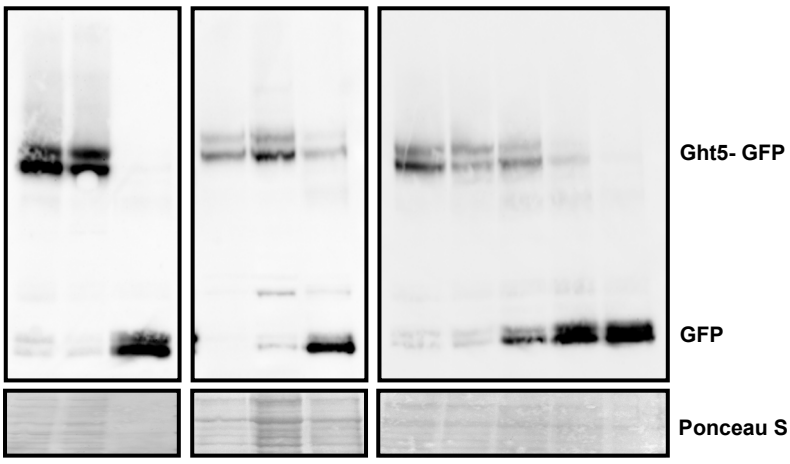

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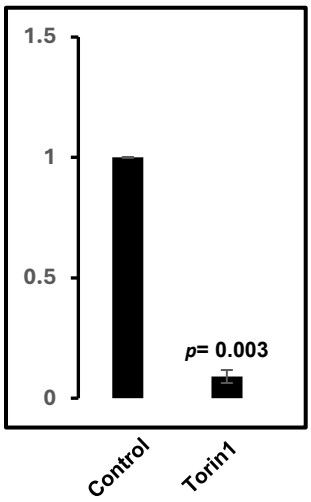

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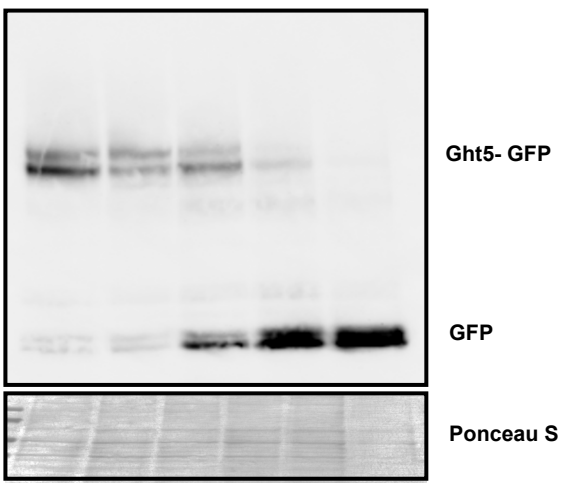

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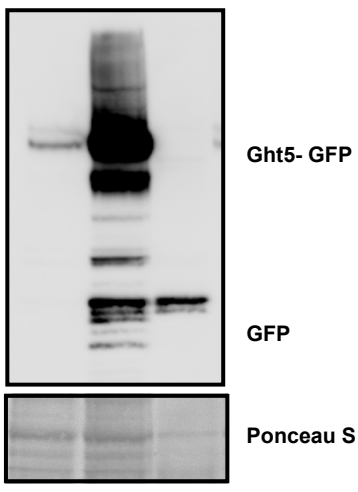

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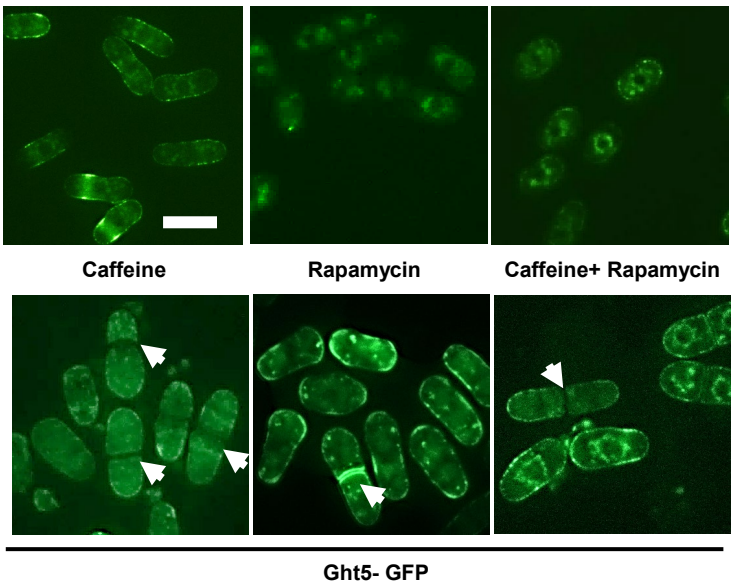


Fig. 2

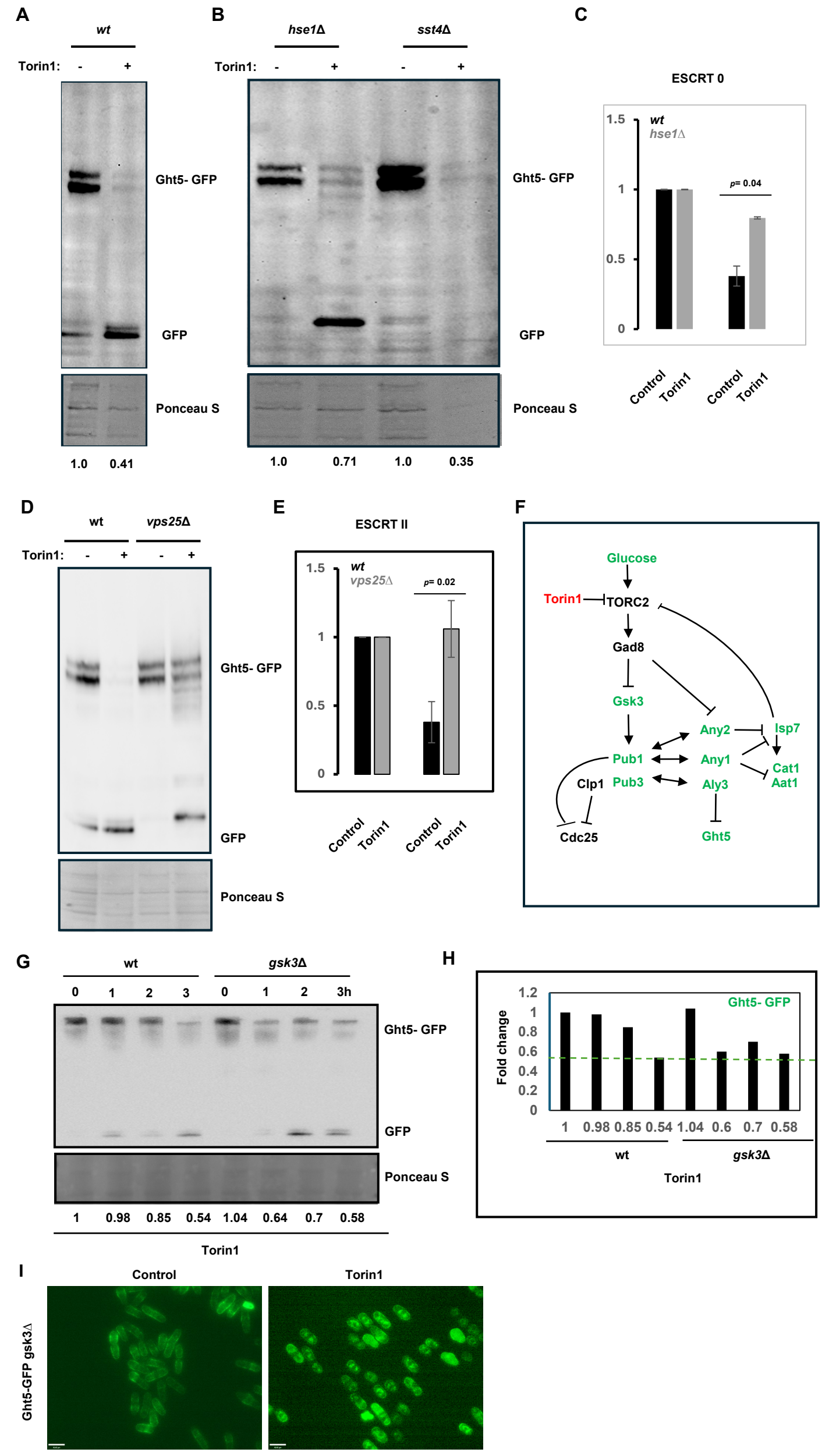
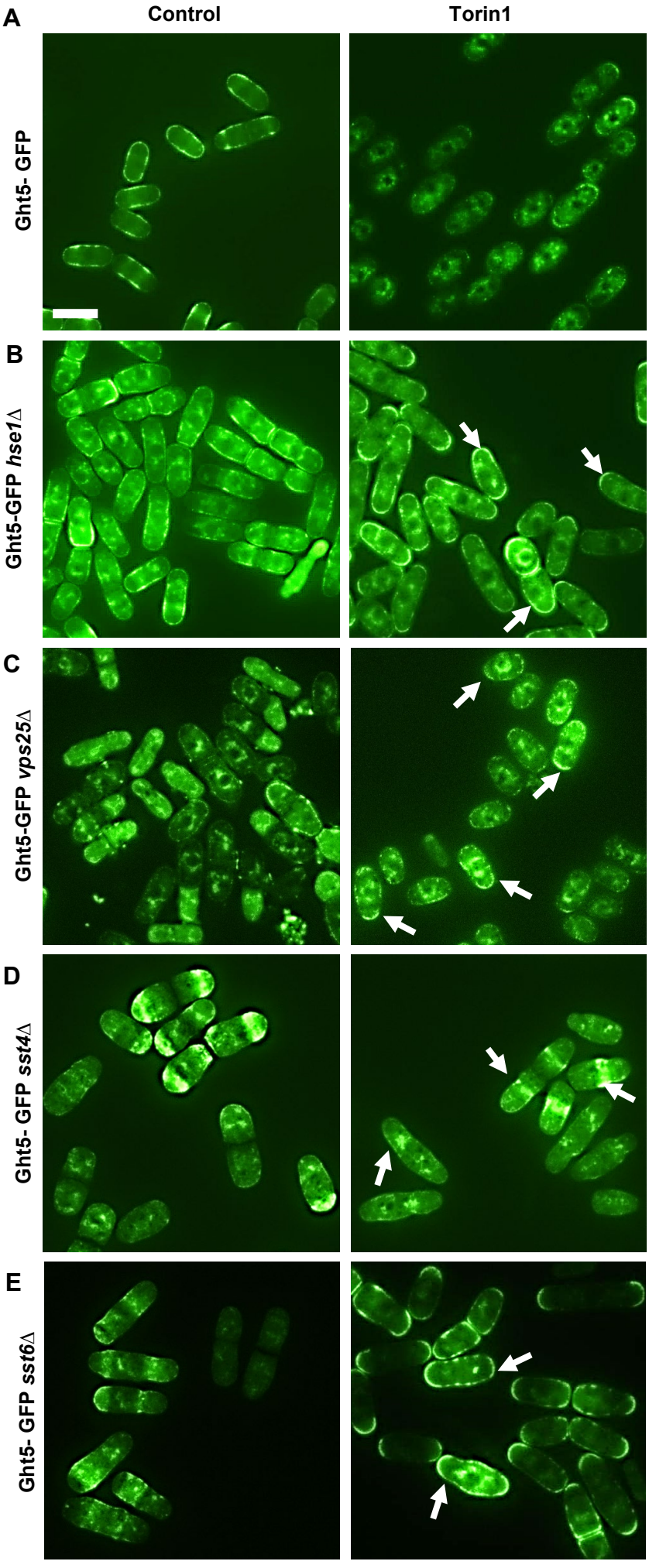
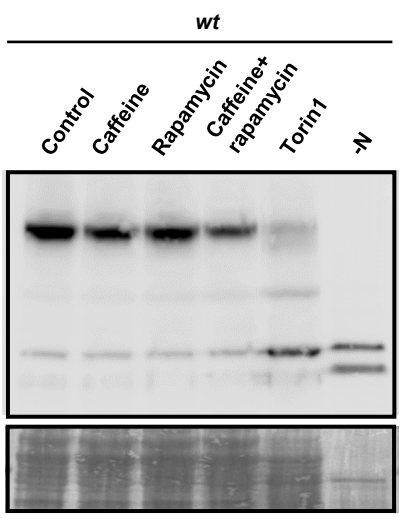
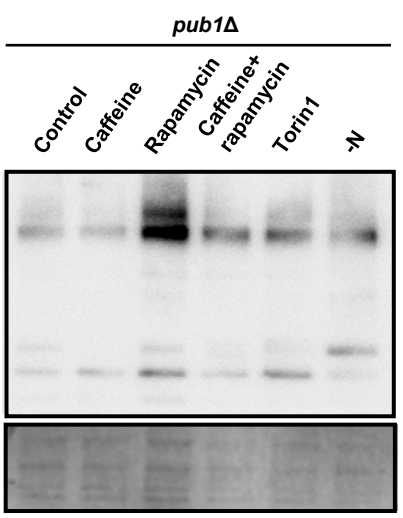

A
wt
Torin1: - +
Ght5- GFP
GFP
Ponceau S
1.0 0.41
B
hse1Δ
sst4Δ
Torin1: - + - +
Ght5- GFP
GFP
Ponceau S
1.0 0.71 1.0 0.35
C
ESCRT 0
wt
hse1Δ
p= 0.04
1.5
1
0.5
0
Control
Torin1
Control
Torin1
D
wt
vps25Δ
Torin1: - + - +
Ght5- GFP
GFP
Ponceau S
E
ESCRT II
wt
vps25Δ
p= 0.02
1.5
1
0.5
0
Control
Torin1
Control
Torin1
F
Glucose
Torin1
TORC2
Gad8
Gsk3
Any2
Isp7
Pub1
Any1
Cat1
Aat1
Clp1
Pub3
Aly3
Cdc25
Ght5
G
wt
gsk3Δ
0 1 2 3 0 1 2 3h
Ght5- GFP
GFP
Ponceau S
1 0.98 0.85 0.54 1.04 0.64 0.7 0.58
Torin1
H
Ght5- GFP
Fold change
1.2
1
0.8
0.6
0.4
0.2
0
1 0.98 0.85 0.54 1.04 0.6 0.7 0.58
wt
gsk3Δ
Torin1
I
Control
Torin1
Ght5-GFP gsk3Δ

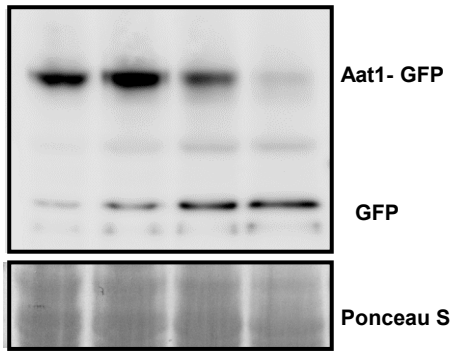
Fig. 3

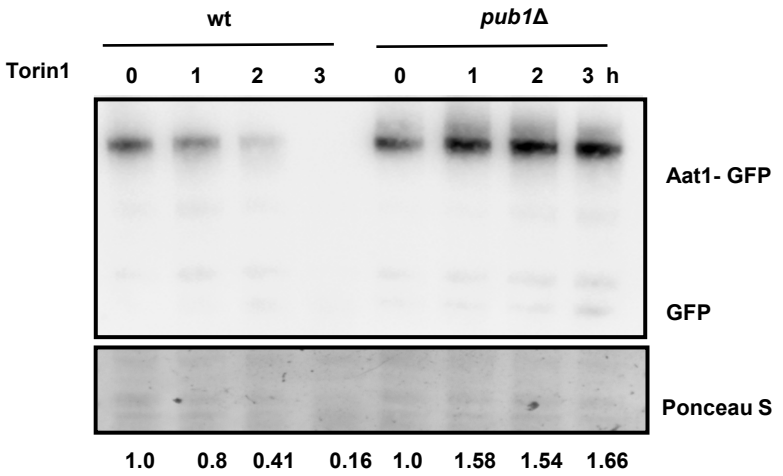

A
Control
Torin1
Ght5- GFP
B
Ght5-GFP hse1Δ
C
Ght5-GFP vps25Δ
D
Ght5- GFP sst4Δ
E
Ght5- GFP sst6Δ

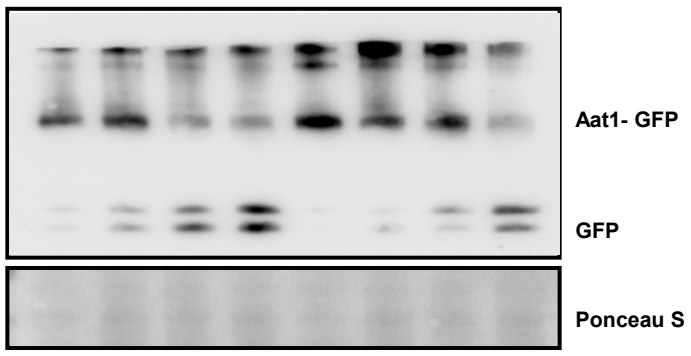
Fig, 4

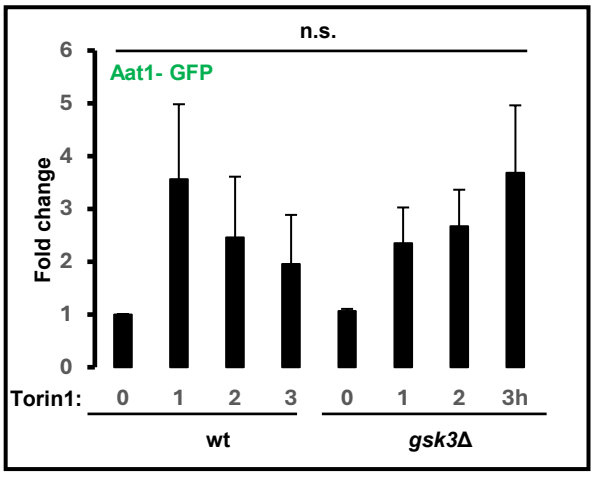

A
wt
Control
Caffeine
Rapamycin
Caffeine+ rapamycin
Torin1
-N
Aat1- GFP
GFP
Ponceau S

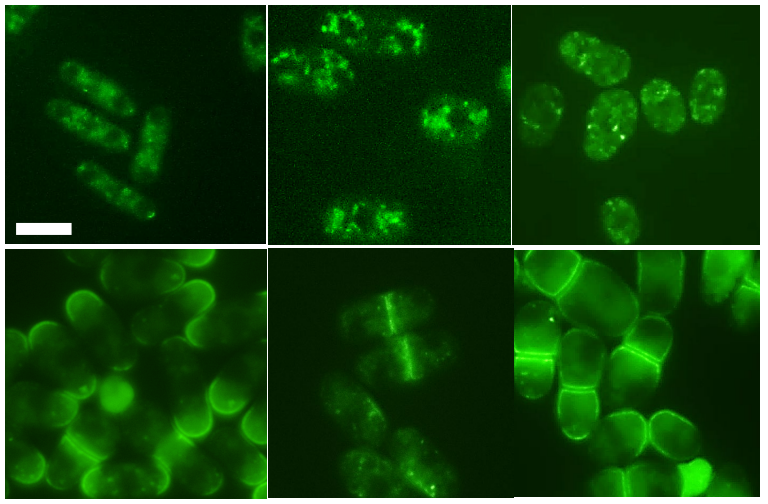

B
pub1Δ
Control
Caffeine
Rapamycin
Caffeine+ rapamycin
Torin1
-N
Aat1- GFP
GFP
Ponceau S

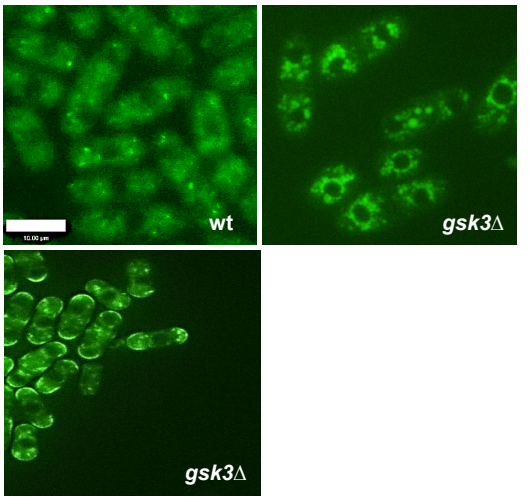

C
wt
0
1
2
3 h
Aat1- GFP
GFP
Ponceau S


D
wt
pub1Δ
Torin1
0
1
2
3
0
1
2
3 h
Aat1- GFP
GFP
Ponceau S
1.0
0.8
0.41
0.16
1.0
1.58
1.54
1.66


E
Torin1
wt
gsk3Δ
0
1
2
3
0
1
2
3h
Aat1- GFP
GFP
Ponceau S


F
n.s.
Aat1- GFP
Fold change
0
1
2
3
4
5
6
Torin1:
0
1
2
3
0
1
2
3h
wt
gsk3Δ


G
Control
Torin1 (1h)
Torin1 (3h)
Aat1-GFP
Aat1-GFP pub1Δ


H
wt
gsk3Δ
gsk3Δ

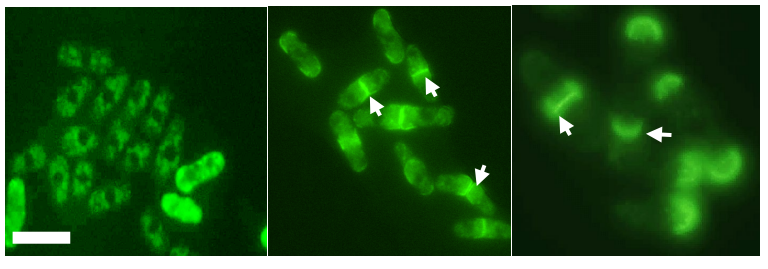
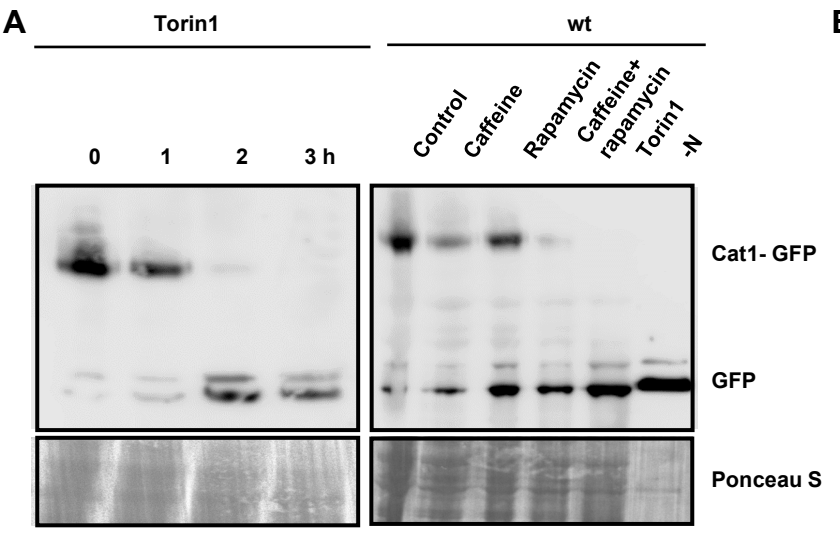
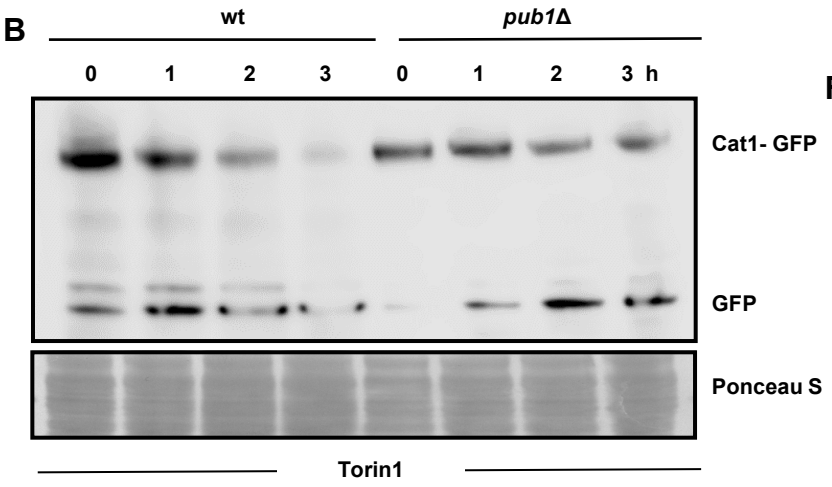
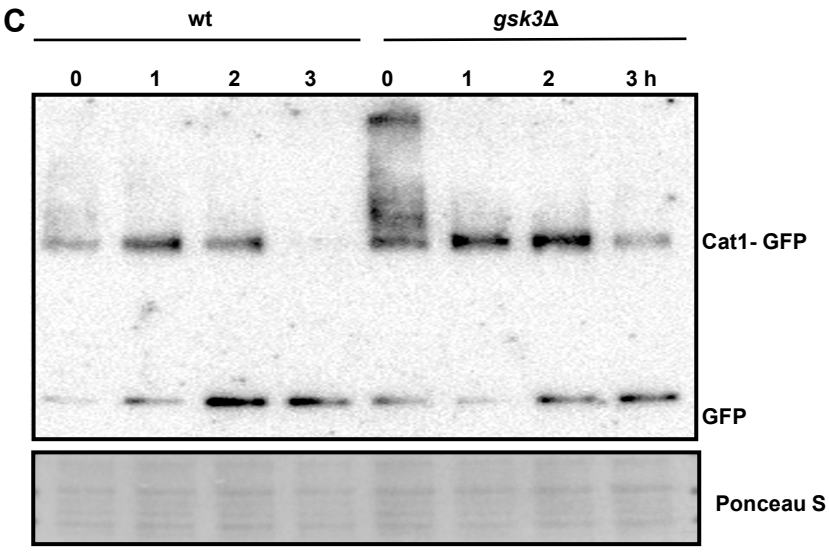
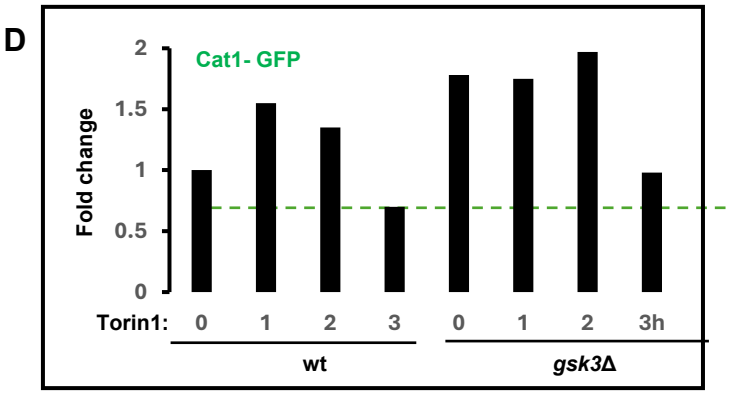

I.
Aat1- GFP
Aat1- GFP gsk3Δ
Aat1- GFP pub1Δ

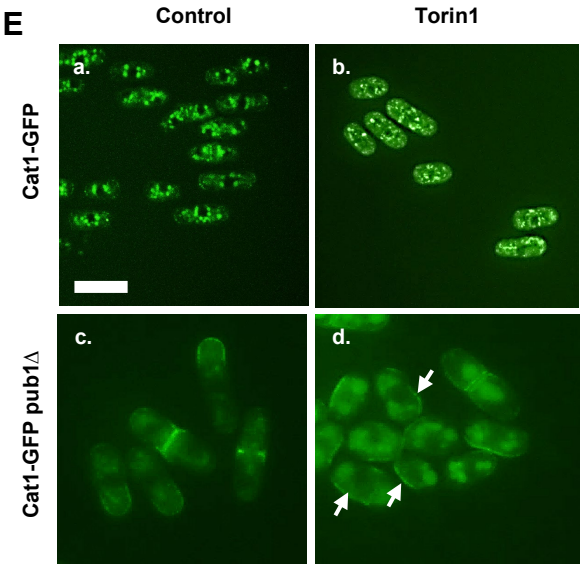
Fig. 5


A
Torin1
wt
0
1
2
3 h
Control
Caffeine
Rapamycin
Caffeine+ rapamycin
Torin1 -N
Cat1- GFP
GFP
Ponceau S


B
wt
pub1Δ
0
1
2
3
0
1
2
3 h
Cat1- GFP
GFP
Ponceau S
Torin1


C
wt
gsk3Δ
0
1
2
3
0
1
2
3 h
Cat1- GFP
GFP
Ponceau S


D
Cat1- GFP
Fold change
2
1.5
1
0.5
0
Torin1:
0
1
2
3
0
1
2
3h
wt
gsk3Δ


E
Control
Torin1
Cat1-GFP
Cat1-GFP pub1Δ
a.
b.
c.
d.

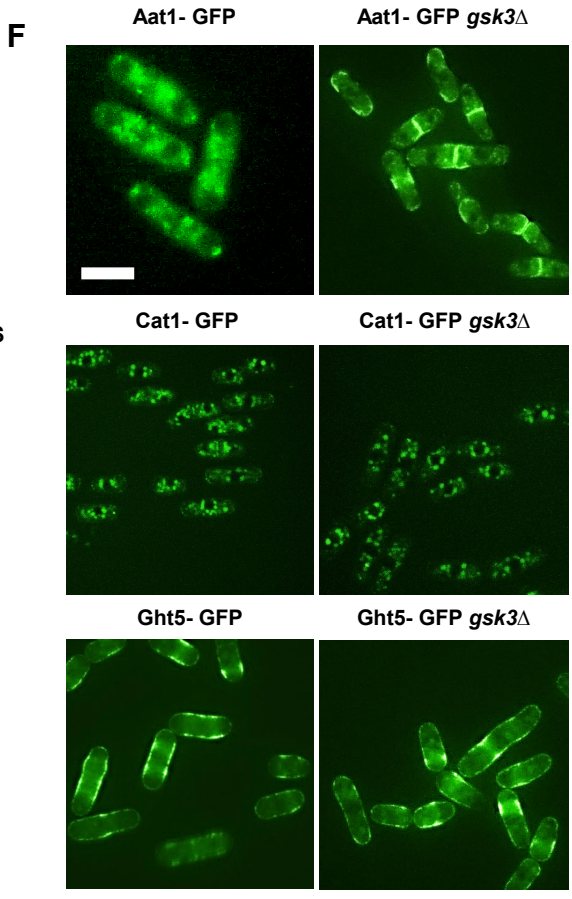
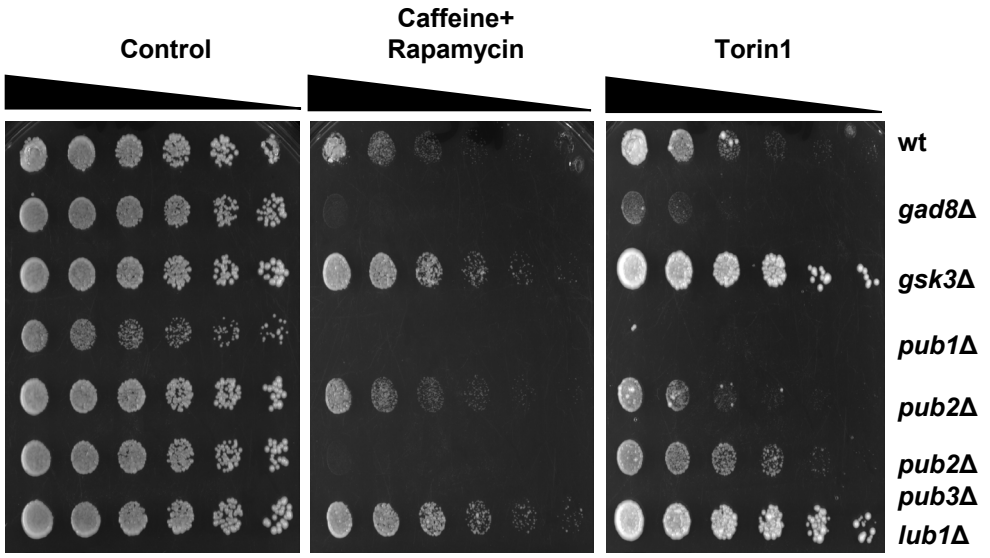
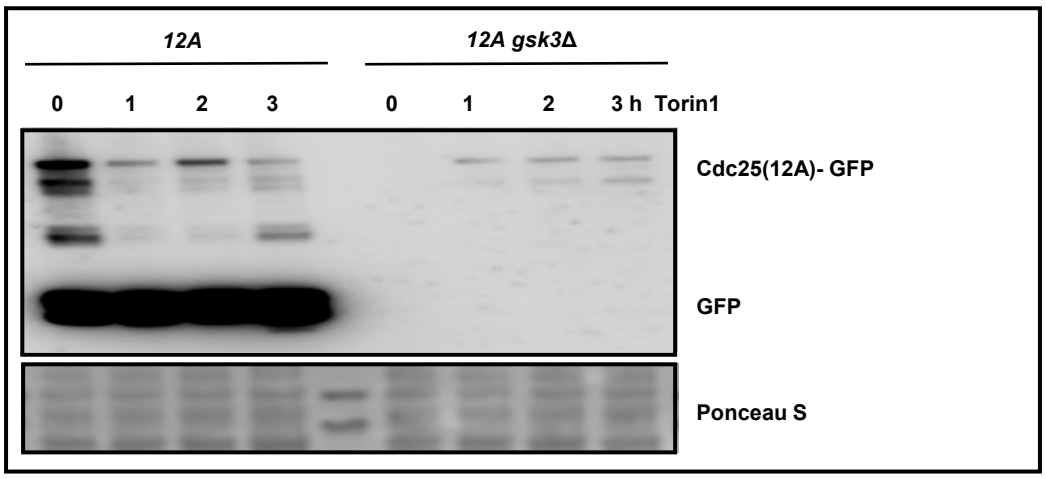

F
Aat1- GFP
Aat1- GFP gsk3Δ
Cat1- GFP
Cat1- GFP gsk3Δ
Ght5- GFP
Ght5- GFP gsk3Δ

Fig. 6

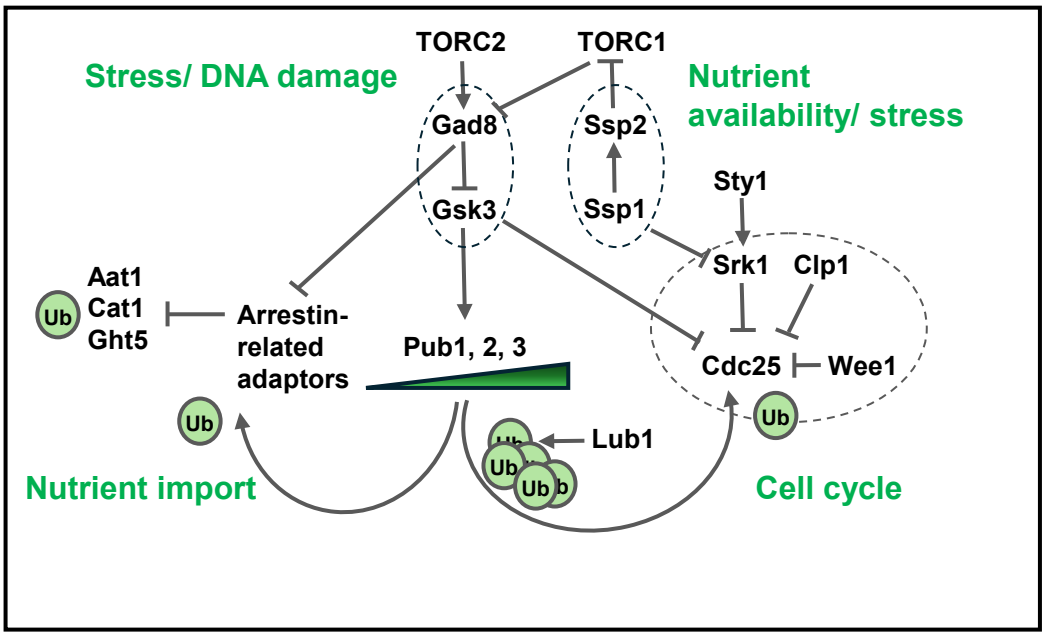
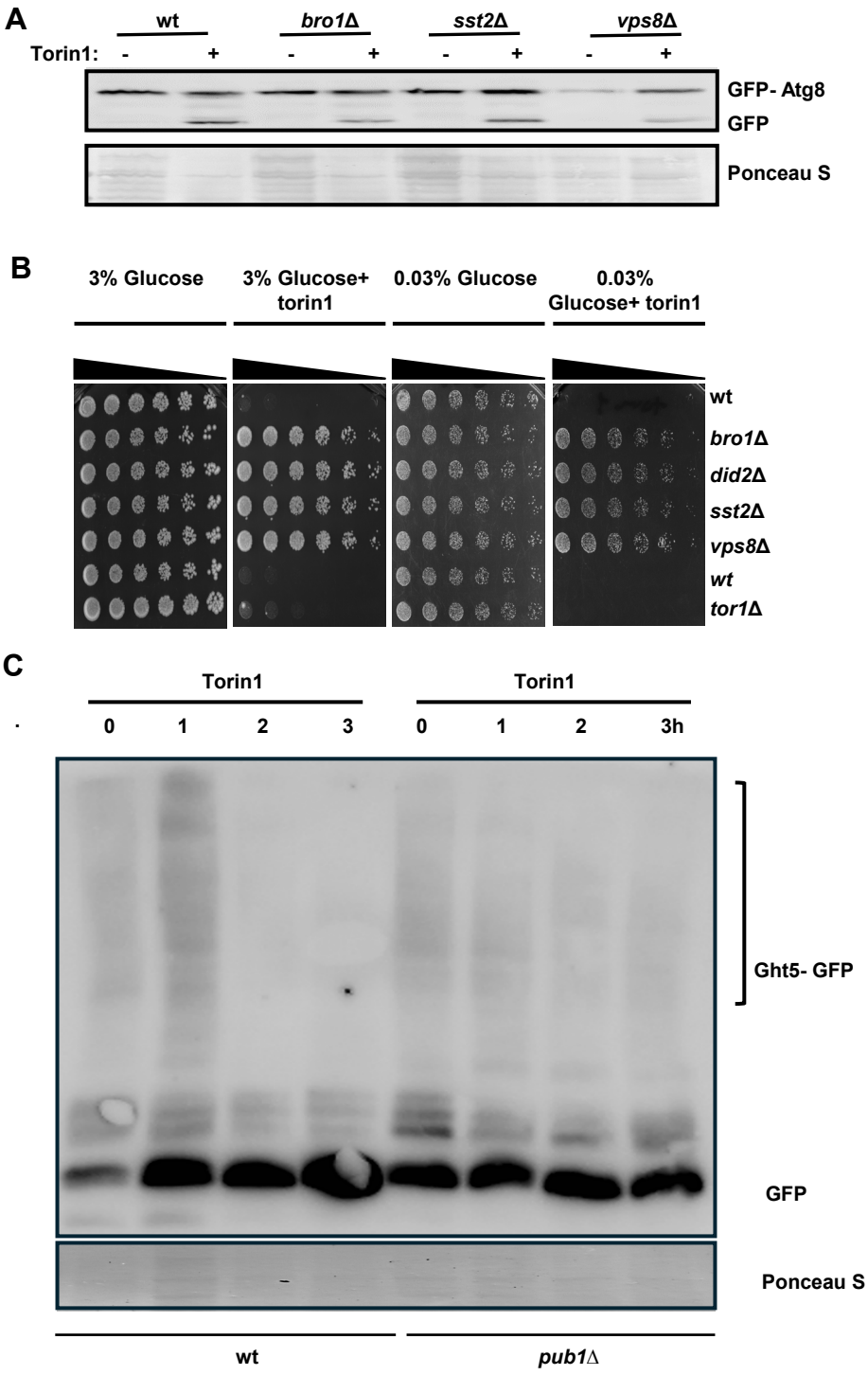
A


Control
Caffeine+
Rapamycin
Torin1
wt
gad8Δ
gsk3Δ
pub1Δ
pub2Δ
pub2Δ
pub3Δ
lub1Δ

B


12A
12A gsk3Δ
0
1
2
3
0
1
2
3 h Torin1
Cdc25(12A)- GFP
GFP
Ponceau S

C


TORC2
TORC1
Stress/ DNA damage
Nutrient
availability/ stress
Gad8
Ssp2
Gsk3
Ssp1
Sty1
Srk1
Clp1
Aat1
Cat1
Ght5
Ub
Arrestin-
related
adaptors
Pub1, 2, 3
Cdc25
Wee1
Ub
Ub
Lub1
Nutrient import
Cell cycle

Additional file 1