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. However, exposure to rapamycin induced Ght5-GFP accumulation.
142 Exposure to caffeine and rapamycin induced Ght5-GFP degradation to an extent like that of
143 Torin1. It is unclear if this effect is due to the induction of autophagy rather than Tor1 inhibition
144 and degradation *via* ESCRT signalling. Caffeine also counteracted the effect of rapamycin on
145 Ght5-GFP accumulation (Fig. 1E). While we identified *atg6* as the only autophagy-regulating
146 gene in our screen for Torin1 resistance, exposure to the drug also induces autophagy as
147 detected by GFP-Atg8 cleavage (Additional file 1A). In contrast to glucose deprivation,
148 exposure to Torin1 thus mimics the effects of nitrogen withdrawal on Ght5-GFP (Fig. 1F).
149 Microscopic analyses demonstrated that exposure to 5 μM Torin1 induced Ght5-GFP vacuolar
150 accumulation after 1h, followed by degradation (1h vs 3h) (Fig. 1C and 1G). Similar results
151 were observed only when caffeine was combined with rapamycin. Caffeine and rapamycin
152 also exerted differential effects on Ght5 localization, which was more diffuse than the latter.
153 This is consistent with their mechanisms of TORC1 inhibition. We also noted that in contrast
154 to cells exposed to Torin1, cells exposed to caffeine with or without rapamycin were still
155 undergoing division after 3h (Fig. 1G). Since TORC2 prevents Ght5-GFP ubiquitylation, we
156 conclude that caffeine combined with rapamycin induces its degradation independently of the
157 ESCRT system. In contrast, Torin1 induces the degradation of Ght5-GFP via the ESCRT
158 pathways by inhibiting TORC2. Pub1 and Pub3 have been identified as Ght5 E3 ligases (10).

159 The deletion of *pub1* clearly suppressed the degradation of Ght5-GFP, confirming our previous
160 findings. Ght5-GFP degradation was not abolished in this mutant, likely due to residual Pub3
161 activity (Additional file 1C). Torin1 thus arrests cell division in G2, in part by preventing glucose
162 import via the Ght5 transporter at the PM (10,17).

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

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

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

179 We next compared the expression of Ght5-GFP in wt and ESCRT pathway mutants following
180 exposure to 5 μM Torin1. In untreated cells, Ght5-GFP was expressed at the PM in bright
181 patches with little expression in the cytoplasm. Exposure to Torin1 induced the rapid import
182 of Ght5-GFP in a speckled pattern and subsequent degradation within the vacuole after 3h
183 (Fig. 1 and 3A). ESCRT mutants also expressed Ght5 at the PM, but the expression pattern
184 differed in different mutants. Ght5-GFP was visible in a granular cytoplasmic pattern even in

185 untreated *hse1*Δ, *vps25*Δ mutants and strongly at the cell membrane in *sst4*Δ, and *sst6*Δ cells
186 (Fig. 3B to 3E). Defective TORC2 and ESCRT-mediated protein degradation thus result in the
187 accumulation of Ght5 (ref). We noted by immunoblotting that basal Ght5-GFP levels were not
188 identical in these mutants (not shown). Following exposure to Torin1, Ght5-GFP was still
189 expressed at the PM in *hse1*Δ, *vps25*Δ, *sst4*Δ, and *sst6*Δ mutants. Torin1 exposure did not
190 affect the expression of Ght5 in *sst4*Δ mutants (Fig. 3D). Exposure to Torin1 appeared to
191 increase the expression of Ght5 at the PM in *sst6*Δ mutants (Fig. 3E). We concluded that,
192 unlike wt cells, *hse1*Δ, *vps25*Δ, *sst4*Δ, and *sst6*Δ mutants continue to express Ght5-GFP at
193 the PM following exposure to Torin1 (Fig. 3A to 3E). Glucose is required for cell division, and
194 low concentrations strongly induce the expression of Ght5 at the PM (Fig. 1F) (11). We
195 investigated whether growth on 0.03 % glucose induces resistance to Torin1. The Torin1
196 sensitivity of the *wt*, *tor1*Δ and ESCRT mutants rather increased under these conditions
197 (Additional file 1B). Thus, overexpression of Ght5 alone is insufficient to suppress Torin1
198 sensitivity. Torin1 induces Ght5 degradation, and its expression is maintained at the cell
199 membrane of ESCRT mutants. These findings partly explain the different effects of caffeine,
200 rapamycin and Torin1 on cell cycle progression in fission yeast (17,24).

201

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

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

212 rapamycin may induce *aat1* expression and accumulation of Aat1 in *pub1* mutants, as it cannot
213 be degraded. (Fig. 4B). These findings also highlight significant differences between caffeine
214 and rapamycin in terms of TORC1 inhibition (19,24). The deletion of *gsk3* did not abolish the
215 Torin1-induced degradation of Aat1-GFP. Our observations suggest, however, that it does
216 affect the dynamics of Aat1-GFP degradation following exposure to Torin1 (Fig. 4E and 4F).

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

233

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

235 As the PM amino acid transporter Cat1 is regulated like Aat1, we next investigated the effect
236 of Torin1 on its expression. Exposure to 5 μM Torin1 induced Cat1-GFP degradation as
237 observed in Aat1-GFP and Ght5-GFP expressing strains (Fig. 1, 2 and 5A). Cat1-GFP

238 appeared to be more sensitive to exposure to 10 mM caffeine alone or with 100 ng/ mL
239 rapamycin than Aat1-GFP (Fig. 5B). Deletion of *pub1*Δ abolished the effect of Torin1 on Cat1-
240 GFP expression. We noted, however, that GFP still accumulated, indicating Pub1-independent
241 alternate Cat1-GFP degradation mechanisms. Pub3 may thus substitute for Pub1 in mediating
242 Cat1 but not Aat1 degradation (Fig. 5C). As observed with Aat1-GFP, deletion of *gsk3*Δ
243 delayed but did not abolish Torin1-induced Cat1-GFP degradation in this strain (Fig. 5C and
244 5D). This observation resembles that observed with the *aat1-GFP* strain (Fig. 4E and 4F).
245 Fluorescence microscopy revealed that exposure to Torin1 induced the relocation of Cat1-
246 GFP from the Golgi/ endosomes to vacuoles. In untreated wt cells, Cat1 was localized to the
247 Golgi/ endosomes. Exposure to Torin1 resulted in a diffuse appearance in the cytoplasm with
248 bright speckles (Fig. 5Ea and 5Eb). Deletion of *pub1* resulted in the dispersion of Cat1-GFP
249 from the Golgi/ endosomes to vacuoles to the cytoplasm, PM and septum of dividing cells (Fig.
250 5Ec and Ed). Deletion of *pub1*Δ prevented the degradation of Cat1-GFP following exposure
251 to Torin1. While exposure to Torin1 in wt cells resulted in a speckled appearance, this was not
252 observed in *pub1*Δ mutants, which fail to degrade Cat1 (Fig. 5B). Furthermore, *pub1* mutants
253 continued to express Cat1-GFP at the PM following exposure to Torin1 (Fig. 5Ec and 5Ed).
254 We next examined the effect of deleting *gsk3* in the *aat1-GFP, cat1-GFP, and Ght5-GFP*
255 strains. The deletion of *gsk3* did not affect the localization of *cat1* -GFP as observed in pub1Δ
256 mutants (Figure 5E and 5F). The deletion of *gsk3*, similarly, did not affect the expression of
257 Ght5-GFP in untreated cells. Together, our findings suggest that while Pub1 regulates the PM
258 expression of both Aat1 and Cat1, Gsk3 regulates only the localization of the former (Fig. 5F).
259 This observation is consistent with previous reports linking Gsk3 to Pub1 activity and Aat1
260 expression. Pub1 is, however, required for the degradation of Aat1, Cat1 and Ght5 following
261 exposure to Torin1. In this model, the deletion of *gsk3* would limit the availability of Pub1 and
262 suppress sensitivity to Torin1 by maintaining nutrient transporter expression (13). Fission yeast
263 cells can thus selectively fine-tune the expression of nutrient transporters under various
264 conditions in a TORC1 and TORC2-dependent manner.

265

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

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

292

293 **Discussion**

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

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

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

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

369 **Conclusions**

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

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

376

377 **List of abbreviations**

378 EGFR- Epidermal Growth Factor Receptor

379 ESCRT- Endosomal Sorting Complex Required for Transport

380 GFP- Green Fluorescent Protein

381 PM- Plasma Membrane

382 TORC1- Target of Rapamycin Complex 1

383 TORC2- Target of Rapamycin Complex 2

384 **Declarations**

385 **Consent for publication**

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

387 **Availability of data and materials**

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

389 **Competing interests**

390 The authors declare no competing interests.

391 **Funding**

392 C.R. acknowledges funding from the Royal Society (Research Award grant number
393 RGS\R1\201348), BBSRC (grant numbers BB/V006916/1, BB/V006916/2) and MRC (grant
394 number MR/W001462/1). Y.T. acknowledges funding from Grants-in-Aid for Scientific
395 Research (C) from the Japan Society for the Promotion of Science (23K05758).

396

397 **Authors' contributions**

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

401 **Acknowledgements**

402 We are grateful to the Matsumoto and Nakashima laboratories and the Yeast Genetic
403 Resource Centre (YGRC) Japan for mutant *S. pombe* strains.

404

405 **References**

406 1. Eyre TA, Collins GP, Goldstone AH, Cwynarski K. Time now to TORC the TORC?
407 New developments in mTOR pathway inhibition in lymphoid malignancies. Br J
408 Haematol. 2014 Aug;166(3):336–51.
409 2. Faes S, Demartines N, Dormond O. Resistance to mTORC1 Inhibitors in Cancer
410 Therapy: From Kinase Mutations to Intratumoral Heterogeneity of Kinase Activity. Cai
411 Z, editor. Oxid Med Cell Longev. 2017 Jan;2017(1):1726078.
412 3. Rodríguez-López M, Gonzalez S, Hillson O, Tunnacliffe E, Codlin S, Tallada VA, et al.
413 The GATA Transcription Factor Gaf1 Represses tRNAs, Inhibits Growth, and Extends
414 Chronological Lifespan Downstream of Fission Yeast TORC1. Cell Rep. 2020
415 Mar;30(10):3240-3249.e4.
416 4. Hurley JH, Coyne AN, Miączyńska M, Stenmark H. The expanding repertoire of
417 ESCRT functions in cell biology and disease. Nature. 2025 June 26;642(8069):877–
418 88.
419 5. Remec Pavlin M, Hurley JH. The ESCRTs – converging on mechanism. J Cell Sci.
420 2020 Sept 15;133(18):jcs240333.

421 6. Abdelrazik NM, Patel A, Conn A, Sutton CW, Kantamneni S, Shnyder SD. A Novel
422 Modulator of Resistance for Oxaliplatin-Based Therapy for Colorectal Cancer: The
423 ESCRT Family Member VPS4A. Cells. 2025 June 19;14(12):929.
424 7. Baldys A, Raymond JR. Critical Role of ESCRT Machinery in EGFR Recycling.
425 Biochemistry. 2009 Oct 13;48(40):9321–3.
426 8. Singh S, Yeat NY, Wang YT, Lin SY, Kuo IY, Wu KP, et al. PTPN23 ubiquitination by
427 WDR4 suppresses EGFR and c-MET degradation to define a lung cancer therapeutic
428 target. Cell Death Dis. 2023 Oct 11;14(10):671.
429 9. Nakashima A, Kamada S, Tamanoi F, Kikkawa U. Fission yeast arrestin-related
430 trafficking adaptor, Arn1/Any1, is ubiquitinated by Pub1 E3 ligase and regulates
431 endocytosis of Cat1 amino acid transporter. Biol Open. 2014 June 15;3(6):542–52.
432 10. Toyoda Y, Masuda F, Saitoh S. Phosphorylation of the Aly3 C-terminus impedes
433 aberrant endocytosis of *Schizosaccharomyces pombe* hexose transporter Ght5. J
434 Cell Sci. 2025 Apr 1;138(7):jcs263572.
435 11. Toyoda Y, Soejima S, Masuda F, Saitoh S. TORC2 inhibition of α-arrestin Aly3
436 mediates cell surface persistence of *S. pombe* Ght5 glucose transporter in low
437 glucose. J Cell Sci. 2021 May 15;134(10):jcs257485.
438 12. Laor D, Cohen A, Pasmanik-Chor M, Oron-Karni V, Kupiec M, Weisman R. Isp7 Is a
439 Novel Regulator of Amino Acid Uptake in the TOR Signaling Pathway. Mol Cell Biol.
440 2014 Mar 1;34(5):794–806.
441 13. Wang T, Woodman P, Humphrey SJ, Petersen J. Environmental control of Pub1
442 (NEDD4 family E3 ligase) in *Schizosaccharomyces pombe* is regulated by TORC2
443 and Gsk3. Life Sci Alliance. 2022 May;5(5):e202101082.
444 14. Nefsky B, Beach D. Pub1 acts as an E6-AP-like protein ubiquitiin ligase in the
445 degradation of cdc25. EMBO J. 1996 Mar 15;15(6):1301–12.
446 15. Esteban V, Sacristán M, Andrés S, Bueno A. The Flp1/Clp1 phosphatase cooperates
447 with HECT-type Pub1/2 protein-ubiquitin ligases in Schizosaccharomyces pombe.
448 Cell Cycle Georget Tex. 2008 May 1;7(9):1269–76.

449 16. Masuda F, Ishii M, Mori A, Uehara L, Yanagida M, Takeda K, et al. Glucose restriction
450 induces transient G2 cell cycle arrest extending cellular chronological lifespan. Sci
451 Rep. 2016 Jan 25;6(1):19629.
452 17. Atkin J, Halova L, Ferguson J, Hitchin JR, Lichawska-Cieslar A, Jordan AM, et al.
453 Torin1-mediated TOR kinase inhibition reduces Wee1 levels and advances mitotic
454 commitment in fission yeast and HeLa cells. J Cell Sci. 2014 Mar 15;127(Pt 6):1346–
455 56.
456 18. Ikai N, Nakazawa N, Hayashi T, Yanagida M. The reverse, but coordinated, roles of
457 Tor2 (TORC1) and Tor1 (TORC2) kinases for growth, cell cycle and separase-
458 mediated mitosis in *Schizosaccharomyces pombe*. Open Biol. 2011 Nov;1(3):110007.
459 19. Alao JP, Kumar J, Stamataki D, Rallis C. Dissecting the cell cycle regulation, DNA
460 damage sensitivity and lifespan effects of caffeine in fission yeast. Microb Cell. 2025
461 June 24;12:141–56.
462 20. Alao JP, Sjölander JJ, Baar J, Özbaki‐Yagan N, Kakoschky B, Sunnerhagen P.
463 Caffeine stabilizes Cdc 25 independently of Rad 3 in *S chizosaccharomyces pombe*
464 contributing to checkpoint override. Mol Microbiol. 2014 May;92(4):777–96.
465 21. Lie S, Banks P, Lawless C, Lydall D, Petersen J. The contribution of non-essential
466 *Schizosaccharomyces pombe* genes to fitness in response to altered nutrient supply
467 and target of rapamycin activity. Open Biol. 2018 May;8(5):180015.
468 22. Arines FM, Li M. An optimized protocol to analyze membrane protein degradation in
469 yeast using quantitative western blot and flow cytometry. STAR Protoc. 2022
470 June;3(2):101274.
471 23. Vijayasimha K, Tran MV, Leestemaker-Palmer AL, Dolan BP. Direct Conjugation of
472 NEDD8 to the N-Terminus of a Model Protein Can Induce Degradation. Cells. 2021
473 Apr 9;10(4):854.

474 24. Rallis C, Codlin S, Bähler J. TORC 1 signaling inhibition by rapamycin and caffeine
475 affect lifespan, global gene expression, and cell proliferation of fission yeast. Aging
476 Cell. 2013 Aug;12(4):563–73.
477 25. Nakase Y, Nakase M, Kashiwazaki J, Murai T, Otsubo Y, Mabuchi I, et al. Fission
478 yeast Any1, β-arrestin-like protein, is involved in TSC-Rheb signaling and the
479 regulation of amino acid transporters. J Cell Sci. 2013 Jan 1;jcs.128355.
480 26. Graml V, Studera X, Lawson JLD, Chessel A, Geymonat M, Bortfeld-Miller M, et al. A
481 Genomic Multiprocess Survey of Machineries that Control and Link Cell Shape,
482 Microtubule Organization, and Cell-Cycle Progression. Dev Cell. 2014
483 Oct;31(2):227–39.
484 27. Rodríguez-López M, Bordin N, Lees J, Scholes H, Hassan S, Saintain Q, et al. Broad
485 functional profiling of fission yeast proteins using phenomics and machine learning.
486 eLife. 2023 Oct 3;12:RP88229.
487 28. Ogiso Y, Sugiura R, Kamo T, Yanagiya S, Lu Y, Okazaki K, et al. Lub1 Participates in
488 Ubiquitin Homeostasis and Stress Response via Maintenance of Cellular Ubiquitin
489 Contents in Fission Yeast. Mol Cell Biol. 2004 Mar 1;24(6):2324–31.
490 29. Frazer C, Young PG. Carboxy-terminal phosphorylation sites in Cdc25 contribute to
491 enforcement of the DNA damage and replication checkpoints in fission yeast. Curr
492 Genet. 2012 Aug;58(4):217–34.
493 30. Nachmias D, Frohn BP, Sachse C, Mizrahi I, Elia N. ESCRTs – a multi-purpose
494 membrane remodeling device encoded in all life forms. Trends Microbiol. 2025
495 Feb;S0966842X25000083.
496 31. Hasegawa J, Jebri I, Yamamoto H, Tsujita K, Tokuda E, Shibata H, et al. SH3YL1
497 cooperates with ESCRT-I in the sorting and degradation of the EGF receptor. J Cell
498 Sci. 2019 Oct 1;132(19):jcs229179.
499 32. Nakase M, Nakase Y, Chardwiriyapreecha S, Kakinuma Y, Matsumoto T, Takegawa
500 K. Intracellular trafficking and ubiquitination of the Schizosaccharomyces pombe
501 amino acid permease Aat1p. Microbiology. 2012 Mar 1;158(3):659–73.

502 33. Aspuria PJ, Tamanoi F. The Tsc/Rheb signaling pathway controls basic amino acid
503 uptake via the Cat1 permease in fission yeast. Mol Genet Genomics. 2008
504 May;279(5):441–50.
505 34. Pérez-Díaz AJ, Vázquez-Marín B, Vicente-Soler J, Prieto-Ruiz F, Soto T, Franco A, et
506 al. cAMP-Protein kinase A and stress-activated MAP kinase signaling mediate
507 transcriptional control of autophagy in fission yeast during glucose limitation or
508 starvation. Autophagy. 2023 Apr 3;19(4):1311–31.
509 35. Kumar J, Ng K, Rallis C. Rapalink-1 reveals TOR-dependent genes and an
510 agmatinergic axis-based metabolic feedback regulating TOR activity and lifespan in
511 fission yeast. Commun Biol. 2025 Sept 29;8(1):1364.
512
513
514 **Figure legends**
515 **Fig. 1** Torin1 induces Ght5 degradation. **A** Mutations in ESCRT complex protein subunits
516 induce resistance to Torin1. Subunits in dark green were identified independently of our drug
517 screening. The resistance of *atg6* and *vps38*, but not *atg38* mutants links ESCRT signalling to
518 Torin1 sensitivity. **B** Wt, *hse1*Δ, *vps25*Δ and *vps28*Δ mutants were grown to stationary phase
519 and then cultured in fresh YES media for at least 2h. Cells were adjusted to an $O.D._{600}$ of 0.3
520 to 0.5 and plated on YES agar with or without 5μM Torin1 and incubated at 32°C for 2-3 days.
521 **C** Cells expressing Ght5-GFP were grown to log phase and exposed to 5μM Torin1 for 3h or
522 as indicated. Cell pellets were stored at -80° C and analysed by SDS-PAGE. GFP expression
523 was monitored by immunoblot analysis using monoclonal antibodies directed against GFP.
524 Full-length Ght5-GFP and cleaved GFP is indicated. Ponceau S was used to monitor gel
525 loading. **D** Quantification of Torin1-induced Ght5-GFP degradation. Experiments were carried
526 out as in **C** (*n*= 3). **E** Cells were grown to log phase and incubated with 10mM caffeine,
527 100ng/mL Rapamycin alone or in combination, 150mM NaCl or 5μM Torin1 for 3h. **F** Cells
528 were grown to log phase and transferred into fresh media with 3% glucose, 0.03% glucose or

529 EMM2-N for 3h. Samples were analysed as in D. **G** Cells expressing Ght5-GFP were grown
530 to log phase, exposed to 5μM Torin1 for 1 and 3h and examined by fluorescent microscopy.
531 White arrowheads indicate septa. Scale bars indicate 10μm.
532
533 **Fig. 2** Torin1 induces ESCRT-dependent Ght5 degradation. **A** *Wt* cells expressing Ght5-GFP
534 were exposed to 5μM Torin1 for 3h and samples analysed by immunoblot analysis. Numbers
535 indicate relative expression levels. **B** *hse1*Δ and *sst4*Δ mutants were exposed to 5μM Torin1
536 for 3h and analysed as in A. **C** Quantification of Torin1-induced Ght5 degradation in wt and
537 *hse1*Δ cells (*n*=3). **D** Wt and *vps25*Δ mutants were treated as in A. **E** Quantification of Torin1-
538 induced Ght5 degradation in wt and *vps*Δ cells (*n*=3). **F** Regulation of nutrient transporter
539 stability by TORC2. Torin1 inhibits TORC2, blocks Gad8 activity and induces Gsk3 activation.
540 Gsk3 stabilizes Pub1, which targets Cdc25 for ubiquitin-dependent proteasomal degradation.
541 Inhibition of Gad8 facilitates arrestin-related adaptor-mediated ubiquitylation of Aat1, Cat and
542 Ght5 by Pub1 and Pub3. TORC2 also regulates Aat1 and Cat1 expression by regulating the
543 activity of Isp7. **G** *Wt* and *gsk3*Δ mutants were exposed to 5μM Torin1 as indicated. Samples
544 were processed as in A. **H** Quantification of Torin1-induced Ght5 degradation in *wt* and *gsk3*Δ
545 mutants (*n*=1). **I** *Wt* and *gsk3*Δ mutants expressing Ght5-GFP were exposed to 5μM Torin1 for
546 3h and examined by fluorescent microscopy. Scale bars indicate 10μm.
547
548 **Fig 3.** ESCRT mutants maintain Ght5 expression at the PM following exposure to Torin1. **A**
549 *Wt* **B** *hse1*Δ, **C** *vps25*Δ, **D** *sst4*Δ and **E** *sst6*Δ strains were examined by fluorescent microscopy.
550 Cells were exposed to 5μM Torin1 for 3h. White arrowheads indicate Ght5 expression at the
551 PM. Scale bars indicate 10μm.
552
553 **Fig 4.** Torin1 induces Aat1 degradation in an ESCRT-dependent manner. **A** Cells expressing
554 Aat1-GFP were exposed to 10mM caffeine, 100ng/mL Rapamycin alone or in combination,
555 5μM Torin1 or EMM2-N for 3h. Samples were analysed by immunoblotting. Full-length Aat1-
556 GFP and cleaved GFP are indicated. **B** *pub1*Δ mutants expressing Aat1-GFP were treated as

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

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

Fig. 1

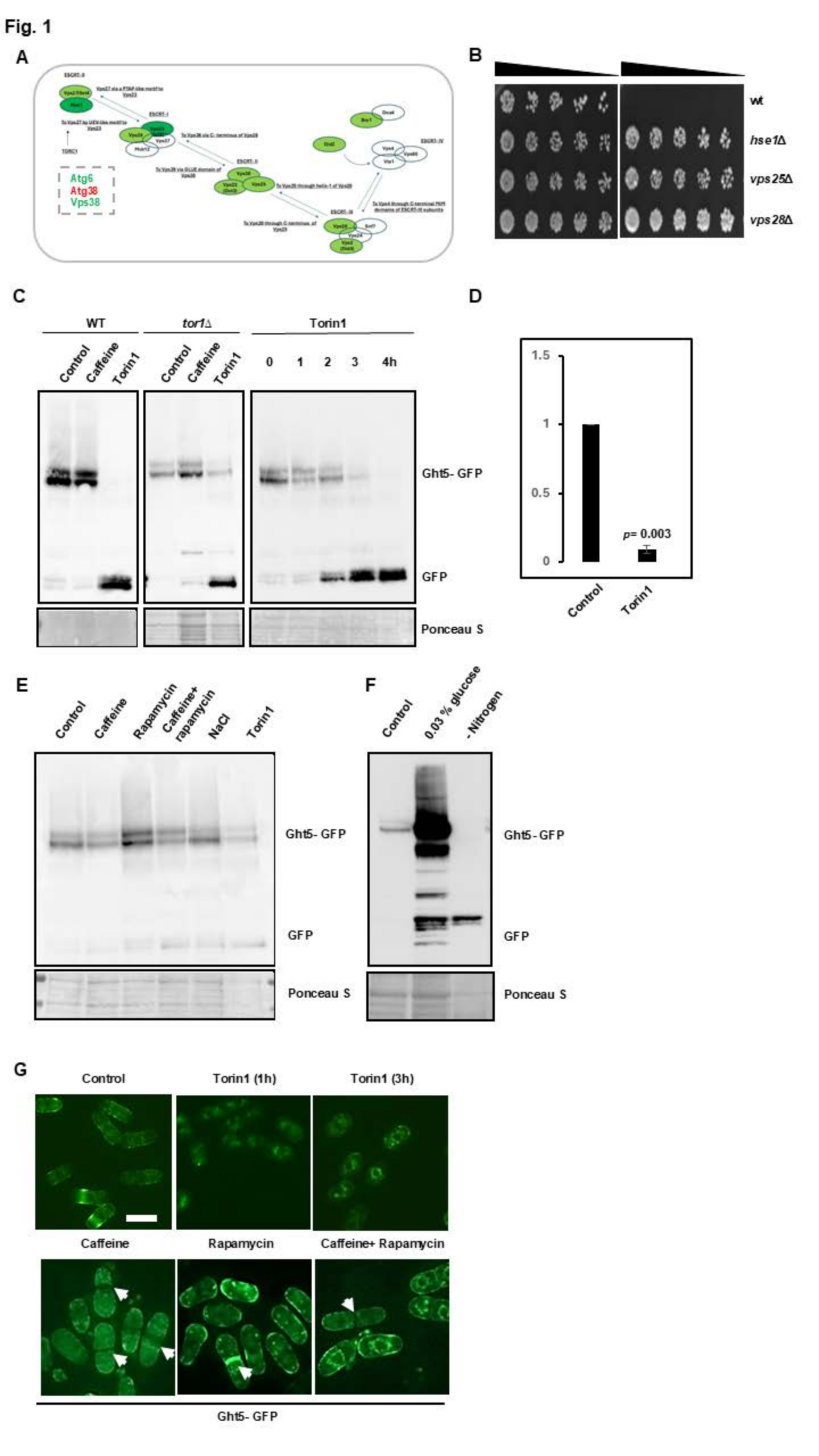

A
Atg6
Atg38
Vps38
TORC1
B
wt
hse1Δ
vps25Δ
vps28Δ
C
WT
tor1Δ
Torin1
Control
Caffeine
Torin1
Control
Caffeine
Torin1
0
1
2
3
4h
Ght5- GFP
GFP
Ponceau S
D
1.5
1
0.5
0
p= 0.003
Control
Torin1
E
Control
Caffeine
Rapamycin
Caffeine+ rapamycin
NaCl
Torin1
Ght5- GFP
GFP
Ponceau S
F
Control
0.03 % glucose
- Nitrogen
Ght5- GFP
GFP
Ponceau S
G
Control
Torin1 (1h)
Torin1 (3h)
Caffeine
Rapamycin
Caffeine+ Rapamycin
Ght5- GFP

Fig. 2

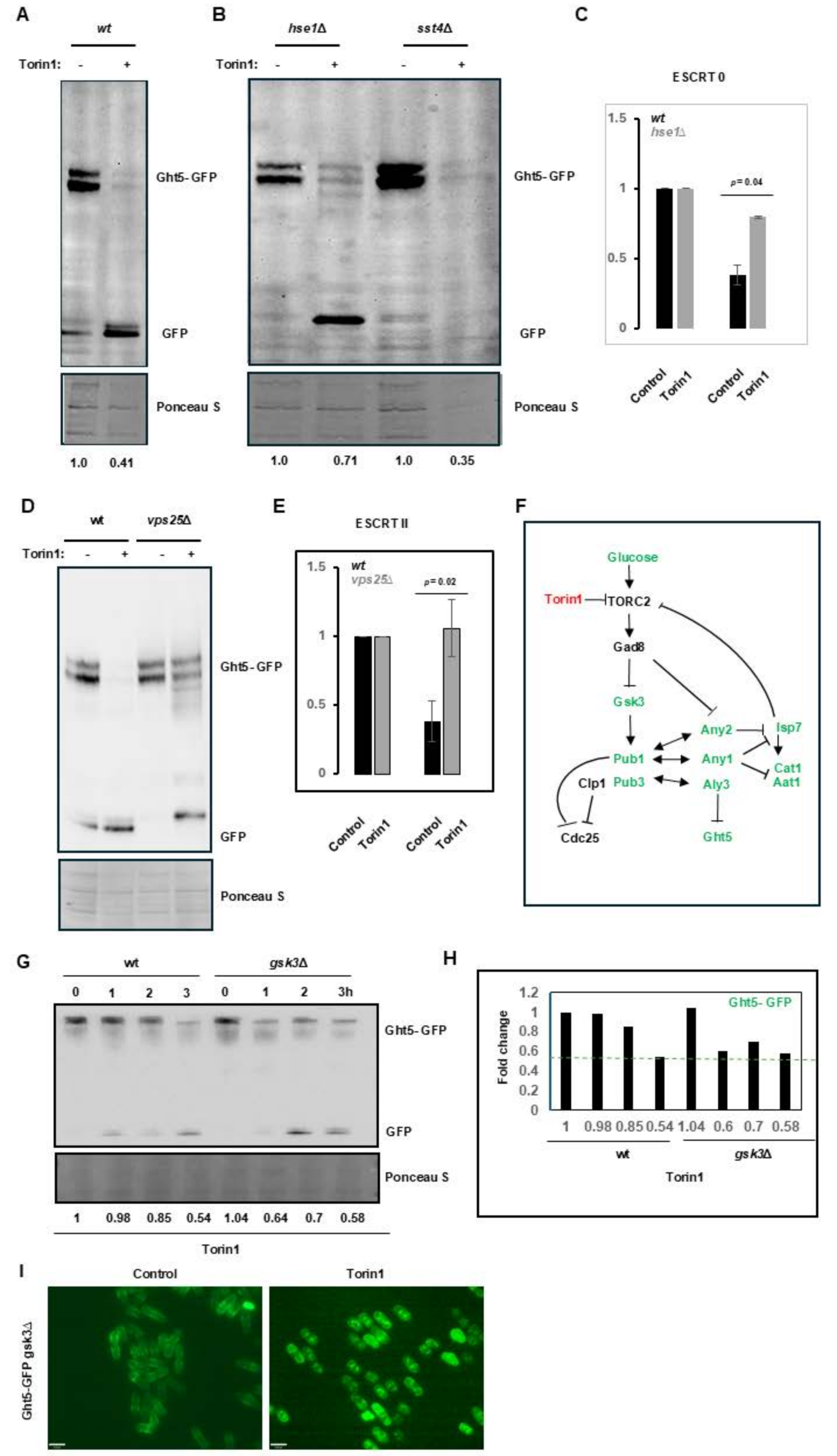

A
wt
Torin1: - +
Ght5-GFP
GFP
Ponceau S
1.0 0.41
B
hse1Δ
sst4Δ
Torin1: - + - +
Ght5-GFP
GFP
Ponceau S
1.0 0.71 1.0 0.35
C
ESCRT 0
wt
hse1Δ
p = 0.04
1.5
1
0.5
0
Control Torin1 Control Torin1
D
wt
vps25Δ
Torin1: - + - +
Ght5-GFP
GFP
Ponceau S
E
ESCRT II
wt
vps25Δ
p = 0.02
1.5
1
0.5
0
Control Torin1 Control 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

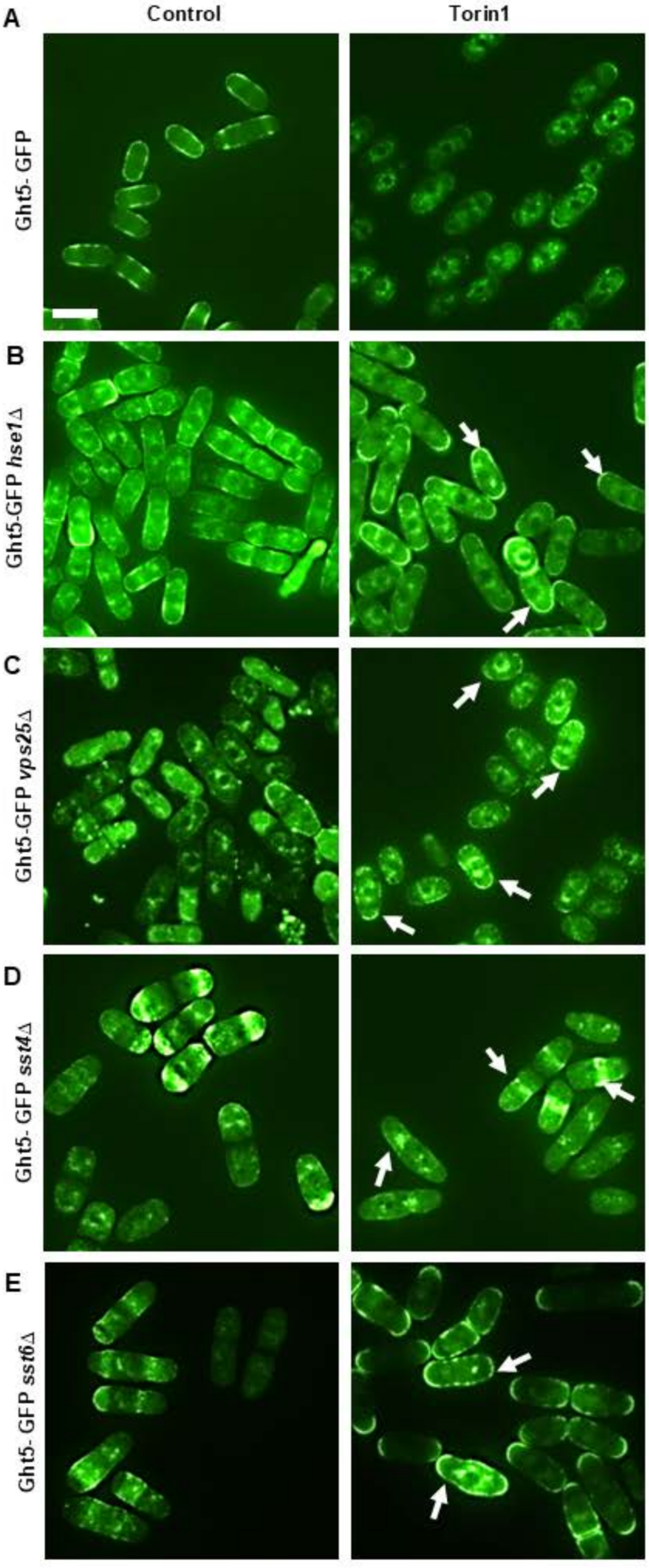

A
Control
Torin1
Ght5- GFP
B
Ght5-GFP hse1Δ
C
Ght5-GFP vps25Δ
D
Ght5- GFP sst4Δ
E
Ght5- GFP sst6Δ

Fig. 4

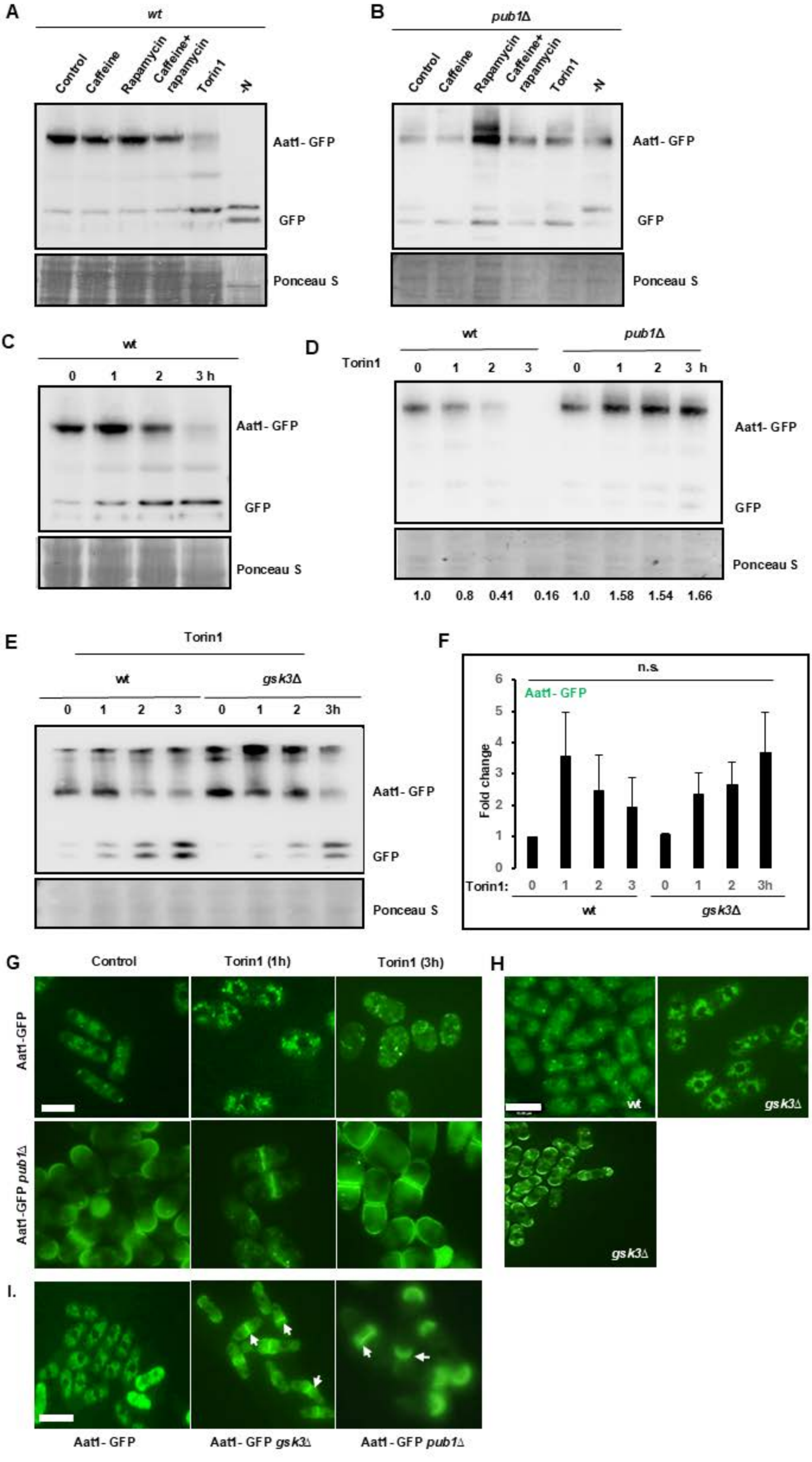
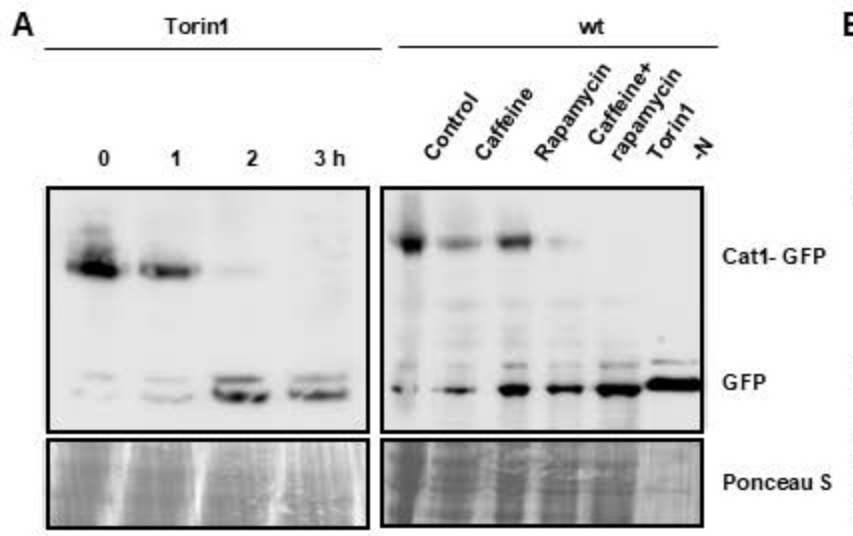
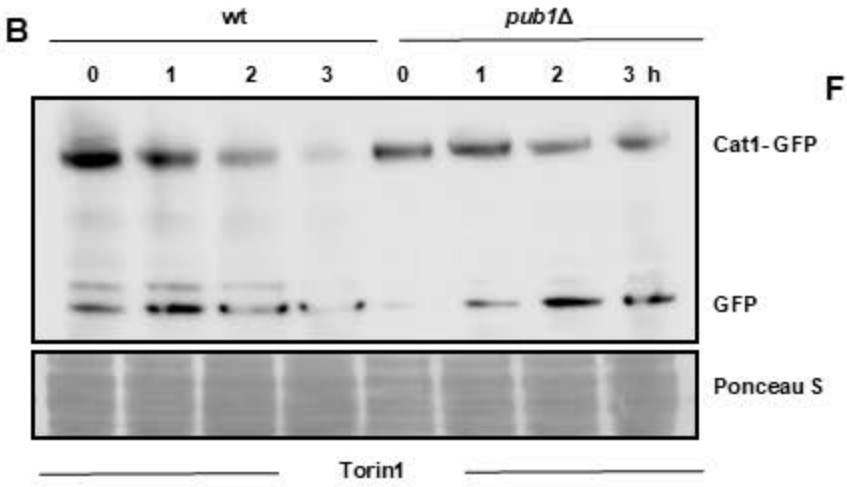
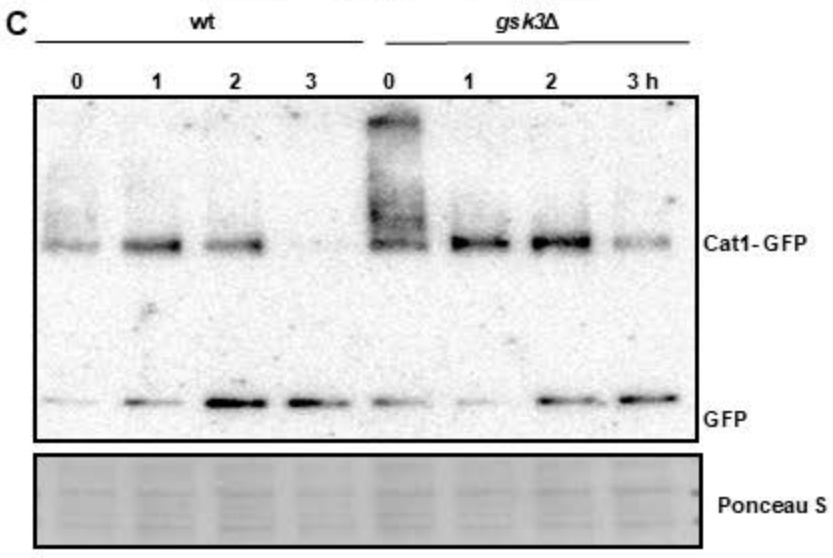
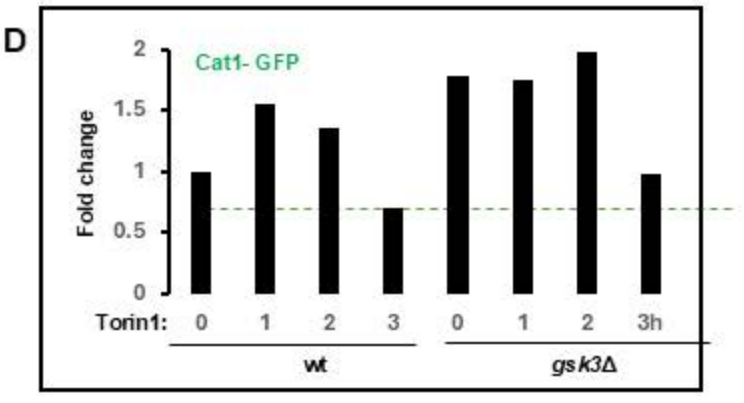

A
wt
Control
Caffeine
Rapamycin
Caffeine+ rapamycin
Torin1
-N
Aat1- GFP
GFP
Ponceau S
B
pub1Δ
Control
Caffeine
Rapamycin
Caffeine+ rapamycin
Torin1
-N
Aat1- GFP
GFP
Ponceau S
C
wt
0
1
2
3 h
Aat1- GFP
GFP
Ponceau S
D
Torin1
wt
pub1Δ
0
1
2
3
0
1
2
3 h
Aat1- GFP
GFP
Ponceau S
1.0
0.8
0.41
0.16
1.0
1.58
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

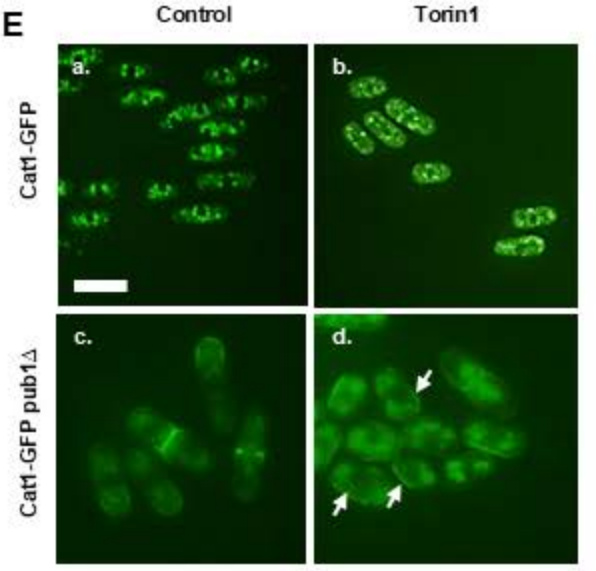
Fig. 5


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.

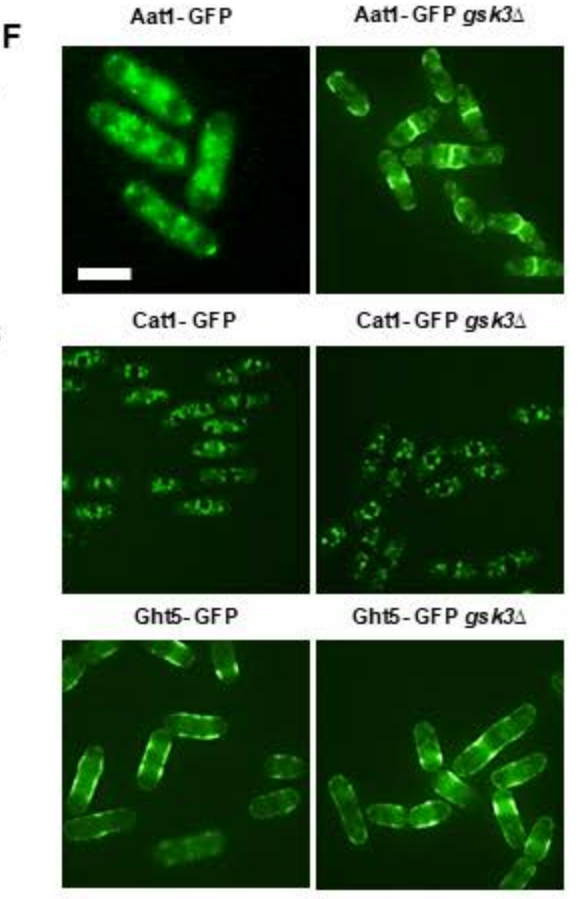
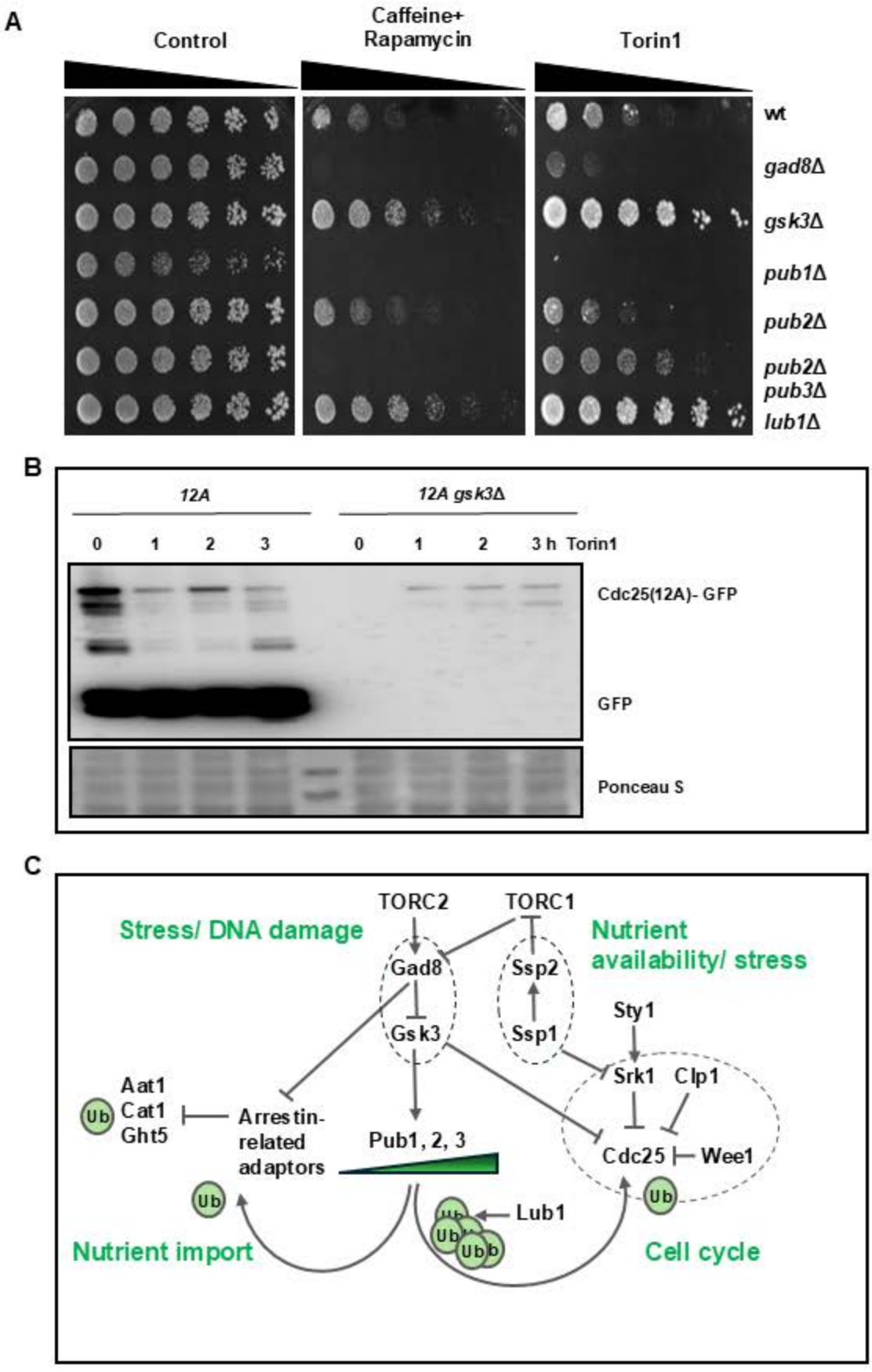
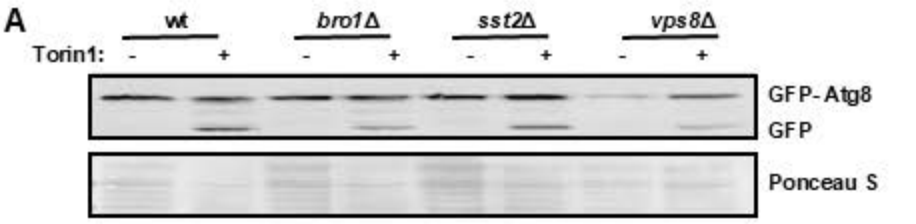
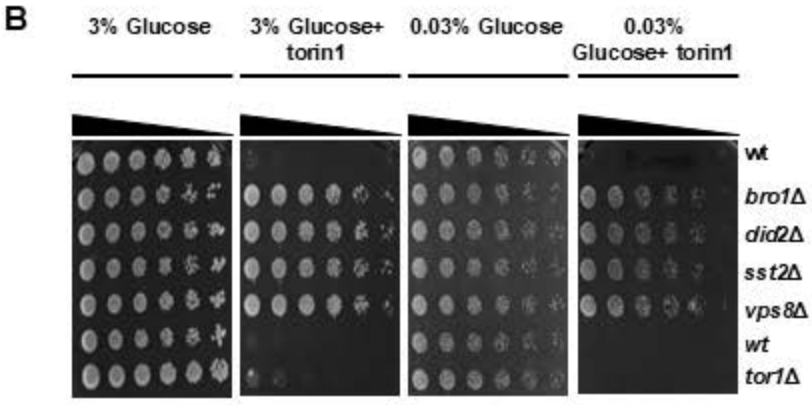

F
Aat1- GFP
Aat1- GFP gsk3Δ
Cat1- GFP
Cat1- GFP gsk3Δ
Ght5- GFP
Ght5- GFP gsk3Δ

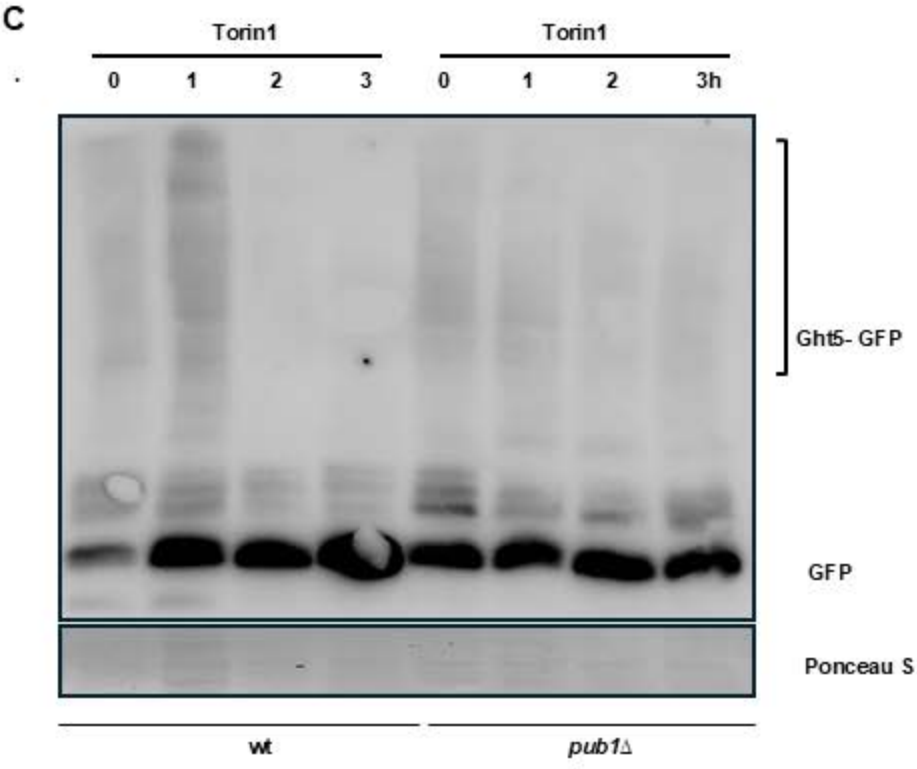
Fig. 6


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
Gsk3
Ssp2
Ssp1
Sty1
Srk1
Clp1
Cdc25
Wee1
Aat1
Cat1
Ght5
Ub
Arrestin-related adaptors
Pub1, 2, 3
Lub1
Nutrient import
Cell cycle

Additional file 1







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

*Yeast Genetic Resource Center (YGRC) Japan