Dissecting the cell cycle regulation, DNA damage sensitivity and lifespan effects of caffeine in fission yeast

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Abstract

Caffeine is a widely consumed neuroactive substance. It can modulate cell cycle progression, override DNA damage checkpoint signalling and increase chronological lifespan (CLS) in various model systems. Early studies suggested that caffeine inhibits the phosphatidylinositol 3-kinase-related kinase (PIKK) Rad3 to override DNA damage-induced cell cycle arrest in fission yeast. We have previously suggested that caffeine modulates cell cycle progression and lifespan by inhibiting the Target of Rapamycin Complex 1 (TORC1). Nevertheless, whether this inhibition is direct or not, has remained elusive. TORC1 controls metabolism and mitosis timing by integrating nutrients and environmental stress response (ESR) signalling. Nutritional or other stresses activate the Sty1-Ssp1-Ssp2 (AMP-activated protein kinase complex, AMPK) pathway, which inhibits TORC1 and accelerates mitosis through Sck2 inhibition. Additionally, activation of the ESR pathway can extend lifespan in fission yeast. Here, we demonstrate that caffeine activates Ssp1, Ssp2 and the AMPKβ regulatory subunit Amk2 to advance mitosis. Ssp2 is phosphorylated in an Ssp1-dependent manner following exposure to caffeine. Furthermore, Ssp1 and Amk2, are required for resistance to caffeine under conditions of prolonged genotoxic stress. The effects of caffeine on DNA damage sensitivity are uncoupled from mitosis in AMPK pathway mutants. We propose that caffeine interacts synergistically with other genotoxic agents to increase DNA damage sensitivity. Our findings suggest that caffeine accelerates mitotic division and is beneficial for CLS through AMPK. Direct pharmacological targeting of AMPK may serve towards health span and lifespan benefits beyond yeasts, given the highly conserved nature of this key regulatory cellular energy sensor.

74 **Introduction**

75 The widely consumed neuroactive methylxanthine compound caffeine, has been linked to
76 increased chronological lifespan (CLS), protective effects against diseases such as cancer
77 and improved responses to clinical therapies (Alao and Sunnerhagen, 2020; Bode and Dong,
78 2007; Cross and Gunter, 2018; Löf et al., 2015; Rallis et al., 2013). Deciphering the
79 mechanisms by which caffeine overrides DNA damage checkpoint signalling has been of
80 particular interest (Alao and Sunnerhagen, 2020). While extensively studied over the last three
81 decades, the precise mechanisms whereby caffeine exerts its activity on cell cycle regulation
82 have remained unclear (Alao et al., 2021, 2020, 2014; Alao and Sunnerhagen, 2020; Bentley
83 et al., 1996).

84 Eukaryotic cells are subject to continuous DNA damage, resulting from the effects of metabolic
85 processes as well as exposure to environmental agents such as ionizing radiation, UV
86 radiation, cigarette smoke or chemotherapeutic agents (Carusillo and Mussolino, 2020; Ciccia
87 and Elledge, 2010; Paviolo et al., 2019). The *S. pombe* phosphatidylinositol 3-kinase-related
88 serine/threonine kinase (PIKK) Rad3 (a homologue of mammalian ataxia telangiectasia
89 mutated (ATM) and ATM related kinase (ATR)), is activated in response to DNA damage or
90 replication stress. Rad3 activation and phosphorylation creates binding sites for adapter
91 proteins that facilitate the activation of downstream kinases Cds1 and Chk1 in the S phase
92 and G2 phases of the cell cycle, respectively. During S phase Cds1 phosphorylates Cdc25 on
93 serine and threonine residues resulting in its activation and sequestration within the cytoplasm
94 following binding of the 14-3-3-related, Rad24 protein. Rad3 and Cds1 also activate the Mik1
95 kinase during S phase which directly inhibits Cdc2 (Bentley et al., 1996; Christensen et al.,
96 2000). In *S. pombe*, the reciprocal activity of the Cdc25 phosphatase and Wee1 kinase on
97 Cdc2 activity, determines the actual timing of mitosis. Additionally, environmental cues such
98 as nutrient limitation and environmental stress can influence the timing of mitosis through
99 target of Rapamycin complex 1 (TORC1) signalling (Atkin et al., 2014; Davie et al., 2015). The
100 inhibition of TORC1 activity results in the activation of the Greatwall related- kinases Ppk18
101 and Cek1. Ppk18 and Cek1 in turn activate the endosulphine Igo1 which inhibits $PP2A^{pab1}$ to
102 induce mitosis (Pab1 being the regulatory subunit in *S. pombe*) (Aono et al., 2019; Chica et
103 al., 2016a).

104 Initial studies suggested that caffeine inhibits Rad3 and its homologues to override DNA
105 damage checkpoint signalling in both yeast and mammalian cells (Moser et al., 2000; Osman
106 and McCready, 1998; Wang et al., 1999). These findings, based on *in vitro* experiments
107 demonstrating that caffeine can inhibit Rad3 and ATM, have been proven controversial. Firstly,
108 caffeine has been shown to override DNA damage checkpoint signalling without blocking ATM
109 and Rad3 signalling (Alao et al., 2014; Cortez, 2003; Kunoh et al., 2008). Furthermore,
110 caffeine can also inhibit other members of the PIKK family (Sarkaria et al., 1999). More recent

111 studies indicate that caffeine inhibits TORC1 signalling in yeast and mammalian cells. Indeed,
112 TORC1 inhibition in *S. pombe* is sufficient to override DNA damage checkpoint signalling (Alao
113 et al., 2020; Davie et al., 2015). It remains unclear, if caffeine directly inhibits TORC1 by acting
114 as a low intensity ATP competitor or indirectly via the *S. pombe* AMP-Activated Protein Kinase
115 (AMPK) Ssp2 (Reinke et al., 2006; Wanke et al., 2008). Caffeine has also been shown to
116 extend CLS in *S. pombe* and induce in combination with Rapamycin, gene expression patterns
117 similar to nitrogen-starved cells or cells in which *tor2* is conditionally deleted (through a
118 temperature sensitive (TS) mutation). Mutants lacking *ssp2* display a shortened CLS. It
119 remains unclear if caffeine alters gene expression or extends CLS directly, or through Ssp2
120 activation (Rallis et al., 2017, 2014).
121 In the current study, we have identified novel roles for the Ssp1 and Ssp2 kinases in facilitating
122 the indirect inhibition of TORC1 by caffeine. Caffeine failed to override DNA damage
123 checkpoint signalling in *ssp1*Δ and *ssp2*Δ mutants. We demonstrate that caffeine interacts with
124 Ssp1 signalling and induces Ssp2 phosphorylation in an Ssp1-dependent manner. We also
125 identify a specific role for Amk2, the β-regulatory subunit of the AMPK complex, in mediating
126 resistance to caffeine, possibly through chromatin remodelling modulation and DNA damage
127 repair. Downstream of TORC1, caffeine induced mitosis in *ppk18*Δ and *cek1*Δ *ppk18*Δ but not
128 *igo1*Δ mutants, but this activity did not correlate with its effect on DNA damage sensitivity.
129 Caffeine may, thus, enhance DNA damage sensitivity independently of mitosis by interfering
130 with DNA damage repair. TORC1 inhibition with Torin1 also potently sensitized *ppk18*Δ, *cek1*Δ
131 *ppk18*Δ and *igo1*Δ mutants to DNA damage. These observations suggest roles for Ppk18, the
132 related kinase Cek1, an unknown kinase and possibly an unidentified probable role for Igo1 in
133 DNA damage repair and/or checkpoint recovery (Amar-Schwartz et al., 2022; Campos and
134 Clemente-Blanco, 2020; Ramos et al., 2019). Exposure to caffeine also suppressed Sck2
135 expression. Sck2 functions downstream of TORC1 to regulate the timing of mitosis (Chica et
136 al., 2016b) and plays a role in regulating CLS (Roux et al., 2006). We previously demonstrated
137 that caffeine prolongs CLS in *S. pombe* (Rallis et al., 2013). Nevertheless, whether this is due
138 to direct TORC1 inhibition or through stress response induction has remained elusive. In this
139 study, we demonstrate that the caffeine-mediated CLS extension is Ssp1, Ssp2 and Amk2-
140 dependent. Together these findings provide evidence for the Ssp1-AMKP-TORC1 signalling
141 axis in mediating the effects of caffeine on both cell cycle progression and chronological ageing
142 in *S. pombe*.
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148 **Results**

149 ***S. pombe* Ssp1 and Ssp2 mediate the effect of caffeine on the G2 DNA damage**
150 **checkpoint**

151 As previously reported, both caffeine (10mM) and Torin1 (5μM) override the cell cycle arrest
152 induced by 5μg/mL phleomycin in wild type *S. pombe* cells. In contrast, cells exposed to
153 phleomycin or left untreated remained arrested while control cultures proliferated with steady
154 state kinetics (Figure 1A) (Alao et al., 2020). We hypothesized that caffeine overrides DNA
155 damage checkpoint signalling at least partially, via inhibition of TORC1 activity (Alao et al.,
156 2020). Direct inhibition of TORC1 activity with Torin1 is more effective than exposure to
157 caffeine raising the possibility that the latter may act indirectly. We, therefore, wondered about
158 the implication of AMPK and investigated the possible role of Ssp1 and Ssp2 in modulating
159 the mitotic effects of caffeine. Nutrient deprivation or exposure to environmental stress
160 indirectly inhibits TORC1 activity, via activation of the Ssp1-Ssp2 (AMPK catalytic (α) subunit)
161 pathway. Deletion of *ssp1* or *ssp2* inhibited the effect of caffeine on DNA damage induced cell
162 cycle arrest in cells exposed to phleomycin (Figure 1B, C). It is likely, that deletion of *ssp1* or
163 *ssp2* affects TORC1 activity and hence the kinetics of Torin1- induced mitosis relative to *wt*
164 cells. In contrast, the effect of Torin1 under similar conditions was partially suppressed (or
165 dampened) but not inhibited until after 120 min. TORC1 inhibition leads to activation of the
166 Greatwall homologue Ppk18 which activates Igo1. Igo1 in turn, inhibits $PP2A^{Pab1}$ activity
167 causing cells to enter mitosis prematurely with a shortened cell size (Aono et al., 2019; Chica
168 et al., 2016b; Davie et al., 2015; Pérez-Hidalgo and Moreno, 2017). Deletion of *ppk18* did not
169 suppress the ability of either caffeine or Torin1 to override DNA damage checkpoints. We
170 noted however, that the *ppk18*Δ mutant reached the maximum level of septation more slowly
171 than wild type cells exposed to phleomycin and caffeine or Torin1 (90 min versus 60 min in
172 *ppk18*Δ and *wt* strains respectively) (Figure 1D). Surprisingly, absence of both *cek1* with
173 *ppk18* (regulating Igo1, (Aono et al., 2019)) suppressed the effects of Torin1 but not caffeine
174 on phleomycin-induced cell cycle arrest (Figure 1E). It should be noted, that Torin1 induces
175 cell cycle arrest while caffeine does not. It is possible that these differential results are due to
176 TORC2 inhibition by Torin1 but not caffeine. In contrast, deletion of *igo1* markedly reduced
177 the ability of both caffeine and Torin1 to override DNA damage checkpoint signalling (Figure
178 1F).

179 Wild type and *ssp1*Δ strains were sequentially exposed to phleomycin and caffeine or Torin1
180 and subjected to FACS analysis. No changes in wild type DAPI intensity were observed when
181 cells were exposed to phleomycin alone (1.03% vs 0,83% of cells passing a 40K DAPI
182 intensity arbitrary cutoff in time 0 and 120min respectively and with geometric means of
183 fluorescent intensity 22,755 and 23,329, respectively). An increase in the case of *ssp1*Δ was
184 observed with phleomycin exposure (1.09% vs 6.34% of cells passing a 40K DAPI intensity

185 arbitrary cutoff in time 0 and 120min respectively and with geometric means of 24,581 and
186 28986 respectively). Exposure of *wt* cells to caffeine in the presence of phleomycin clearly
187 resulted in an increase in DAPI intensity above 40K point (0.69% vs 21.4% of cells above 40K
188 respectively and geometric means 22,611 vs 31,766). This effect was suppressed in *ssp1*Δ
189 mutants where the increase above 40K was from 0.69% to 9.49% (geometric means from
190 22,897 to 30212). Given that phleomycin alone, induces an increase of DAPI intensity in
191 *ssp1*Δ, the change attributable to caffeine is even lower compared to wild type cells
192 (Supplementary figure 1). No notable changes can be observed for wild type or *ssp1*Δ cells
193 treated with phleomycin and Torin1. Taken together (see also relevant septation index
194 measurements in Figure 1A and 1B), our results may indicate that following phleomycin
195 treatment, caffeine can drive DNA replication and mitosis in an Ssp1-dependent manner.
196 Interestingly, we could not detect differences in DNA damage sensitivity in *wt*, *ssp1*Δ, *ssp2*Δ,
197 *cek1*Δ *ppk18*Δ or *igo1*Δ mutants co-exposed to phleomycin and caffeine or Torin1 (Figure 2,
198 Supplementary Figure 2).

199 As previously reported, both caffeine and Torin1 increase sensitivity to phleomycin in
200 liquid culture (Alao et al., 2014) (Figure 2A). We have consistently observed, that Torin1 is
201 more effective at enhancing DNA damage sensitivity. TORC2 is required resistance to DNA
202 damage, hence its inhibition by Torin1 might exert different effects on viability. Although
203 deletion of *ssp1*, *ssp2* and *igo1* abrogated the effect of caffeine on mitosis, we did not detect
204 a reduction in DNA damage sensitivity relative to *wt* cells under these conditions (Figure 1,
205 Figure 2B, Supplementary Figure 2). Both drugs clearly enhanced the sensitivity of *ssp2*Δ,
206 *cek1*Δ *ppk18*Δ, *igo1*Δ mutants to phleomycin, despite the lack of mitotic progression (Figure
207 1, Figure 2B). While Ssp1 cells may display slightly higher resistance to phleomycin with
208 caffeine, we did not detect a clear relationship between mitosis and viability (Supplementary
209 figure 2A to 2C). Similarly, both caffeine and Torin1 sensitized *igo1*Δ mutants to phleomycin
210 like *wt* cells, despite the marked difference in mitosis (Figure 1F, 2B and supplementary figure
211 2D and 2E). Caffeine also slightly enhanced the DNA damage sensitivity of *wt* cells exposed
212 to phleomycin in cultures treated in stationary phase (Figure 2C). Thus, caffeine and Torin1
213 exert differential effects on DNA damage sensitivity in a context dependent manner. As
214 caffeine can increase HU sensitivity in a Cdc25-dependent manner (2D) (Alao et al., 2014),
215 we next examined the effect of caffeine on *wt* and mutant cells under chronic genotoxic
216 conditions. Under these conditions, *ssp2* and *amk2* mutants showed increased sensitivity to
217 HU combined with caffeine. Furthermore, *ssp2* and *amk2* mutants showed increased
218 sensitivity to phleomycin alone while co-exposure to caffeine was lethal to all strains (Figure
219 2E). Caffeine has been reported to be genotoxic and can enhance DNA damage sensitivity
220 without advancing mitosis. These observations suggest that caffeine may induce a specific

221 form of DNA damage when combined with genotoxic agents (Calvo et al., 2009; Rowley and
222 Zhang, 1999).
223
224 **Caffeine induces Ssp1-dependent Ssp2 phosphorylation on Thr169**
225 To further investigate the effect of caffeine on Ssp2 signalling, we used a previously reported
226 phospho-specific antibody (Schutt and Moseley, 2017). Ssp2 phosphorylation was clearly
227 induced in *wt* cells expressing Myc-tagged Ssp2 when exposed to 10mM caffeine for 2h, albeit
228 to a lesser degree than glucose deprivation. Ssp2 phosphorylation was suppressed in cells
229 exposed to 5μM Torin1 under similar conditions (Figure 3A, 3B and 3C). Environmental
230 stresses induce Ssp1-mediated phosphorylation of Ssp2. In contrast to the previously reported
231 transient effect of 1M KCl on Ssp2 phosphorylation (Schutt and Moseley, 2017), this effect
232 was sustained in cells exposed to caffeine over a 2h period (Figure 3C). Caffeine also
233 increased the amount of Ssp2 phosphorylation in cells previously exposed to phleomycin,
234 while co-exposure to Torin1 exhibited the opposite effect (Figure 3D). Although Ssp2 is
235 activated by changes in the ratio of AMP to ATP, it can also be activated by cellular stresses.
236 Exposure to 1M KCl, has previously been shown to suppress cellular ATP levels (Schutt and
237 Moseley, 2017). This suggests that caffeine indirectly inhibits TORC1 via Ssp2 in contrast to
238 Torin1. It is unclear why ATP suppresses sensitivity to phleomycin but supports our contention
239 that viability is independent of mitosis in this context. The effect of caffeine on Ssp2
240 phosphorylation was largely independent of ATP in cells previously exposed to phleomycin,
241 while Torin1 suppressed phospho-Ssp2 levels. ATP slightly suppressed the caffeine- induced
242 phosphorylation of Ssp2. Additionally, ATP also increased phospho- Ssp2 levels in cells
243 exposed to phleomycin and Torin1 (Figure 3E). Together, these finding suggest that caffeine
244 activates Ssp2 independently of ATP although it may suppress Torin1 activity. ATP did not
245 affect the ability of caffeine or Torin1 to drive phleomycin-arrested cells into mitosis (Figure
246 3F). We noted however, that septated cells exposed to ATP in the presence of phleomycin
247 and caffeine were longer than cells exposed to the latter alone (Figure 3G). Additionally, ATP
248 suppressed the effect of caffeine and Torin1 on phleomycin sensitivity independently of its
249 effects on cell cycle progression (Figure 3H). Caffeine in contrast to 1M KCl, thus, appears to
250 induce Ssp2 phosphorylation and cell cycle progression independently of ATP.
251
252 **Caffeine modulates TORC1- regulated mitotic signalling**
253 Indirect inhibition of TORC1 by Ssp2 results in the suppression of Sck2 activity, Ppk18 and
254 Cek1 activation, Igo1 activation and downstream inhibition of PP2A activity leading to mitotic
255 entry (Chica et al., 2016b). We, thus, examined the effect of caffeine on TORC1-regulated cell
256 signalling. Caffeine clearly suppressed Sck2 but not Sck1 (an Sck2 homologue that plays a
257 minor role in regulating the timing of mitosis) expression in a time-dependent manner (Figure

4A and 4B). A similar effect was observed in cells previously exposed to phleomycin, where caffeine may be able to induce the accumulation of Sck1 (Figure 4C) but suppresses Sck2 expression in a time-dependent manner (Figure 4D). Caffeine may thus indirectly induce Cdc25 activation, Wee1 inhibition and consequently, mitosis via suppression of Sck2 expression and, consequently, reduction of its corresponding enzymatic activity (Figure 4B). We did not detect an appreciable level of reduced Maf1 phosphorylation in cells exposed to caffeine alone (Figure 4E). The output of caffeine-mediated TORC1 inhibition, may thus differ from those of Rapamycin and Torin1. Caffeine may affect Cdc25 activity independently of TORC1. We previously reported that caffeine stabilizes Cdc25 in *S. pombe* (Alao and Sunnerhagen, 2020). We note that HU but not phleomycin induces degradation of the Cdc25(12A)-GFPint (Figure 4F and 4G). Inappropriate Cdc25 activity during S- phase may interfere with DNA replication and repair. Caffeine likely drives mitosis via TORC1 inhibition to activate PP2A while simultaneously stabilizing Cdc25 via activation of TORC2 (See below).

Ssp2 mediates the effect of caffeine on cell cycle progression in the absence of checkpoint signalling

We next investigated the role of Ssp2 in mediating the cell cycle effects of caffeine under normal growth conditions. Exposure to 10mM caffeine or 5μM Torin1 advanced *wt* cells into mitosis over a 2 h period (Figure 5A). Deletion of *ssp2* partially suppressed the effect of both caffeine and Torin1 on cell cycle progression (Figure 5B, for caffeine evident on the 120min timepoint). It has previously been reported, that *ssp2* mutants display increased resistance to Torin1 because of increased TORC1 activity (Atkin et al., 2014). As *gsk3* genetically interacts with *ssp2* (Qingyun et al., 2016; Rallis et al., 2017), we investigated its role in mediating the activity of caffeine and Torin1 in relation to AMPK-related genes *ssp1, ssp2 and amk2*. Surprisingly, deletion of *gsk3* partially suppressed the cell cycle effects of Torin1 but not caffeine (Figure 5C). Deletion of both *gsk3* and *ssp2* strongly suppressed the effects of both caffeine and Torin1 on cell cycle progression (Figure 5D). Additionally, caffeine failed to advance cell cycle progression in *gsk3*Δ *ssp1*Δ and *gsk3*Δ *amk2*Δ double mutants (Figure 5E). We conclude that Ssp2 signalling mediates the effects of caffeine on cell cycle progression. In contrast, both Gsk3 and Ssp2 are required to mediate the effects of Torin1 on cell cycle progression. The intrinsic levels of TORC1 activity within *gsk3* and *ssp2* mutant cells have been presumed to be higher compared to *wt* cells (Rallis et al., 2017). Hence, both genes are involved in advancing mitosis in *S. pombe* but with differential effects on cell cycle dynamics. Mutants lacking Tor1 have been reported to be sensitive to caffeine (Rodríguez-López et al., 2023). We also noted that *tor1*Δ and *gad8*Δ mutants were sensitive to caffeine, suggesting this pathway confers resistance to the drug (Figure 5F, Supplementary figure 1F) Caffeine may thus impact on Gsk3 function via activation of TORC2 signalling albeit indirectly

295 (Du et al., 2016). Indeed, TORC1 and TORC2 exert opposing roles on mitosis with the latter
296 playing a role in cell cycle re- entry following DNA damage repair (Ikai et al., 2011).
297 We compared the effects of caffeine and Torin1 on cell cycle progression in *wt* and *gsk3*Δ
298 mutants exposed to phleomycin. The effect of caffeine and Torin1 on DNA damage sensitivity
299 was partially supressed in *gsk3*Δ mutants (Figure 5I and 5J, more evidently in the case of Torin1
300 in the shown conditions). It is unclear, why Gsk3 mediates the effects of caffeine in cells
301 previously arrested with phleomycin but not under normal conditions. Gsk3 positively regulates
302 the stability of the E3 ligase Pub1 which in turn regulates Cdc25 stability (Nefsky and Beach,
303 1996). Increased TORC1 activity in *gsk3*Δ mutants (Qingyun et al., 2016; Rallis et al., 2017)
304 and strong inhibition of Cdc2 in the presence of phleomycin may attenuate the ability of caffeine
305 to advance mitosis. These observations further demonstrate that differential mechanisms
306 control the respective effects of caffeine and Torin1 on cell cycle progression via direct and
307 indirect activation/ inhibition of TORC1 and 2.
308

309 **Caffeine exacerbates the *ssp1*Δ phenotype under environmental stress conditions**

310 Mutants lacking *ssp1* respond weakly to the mitotic effects of caffeine (Figure 1B). Ssp1 has
311 been reported to regulate cell cycle re-entry, following Sty1-mediated Cdc25 inhibition, by
312 suppressing Srk1 expression (Gómez-Hierro et al., 2015). As Srk1 attenuates the effect of
313 caffeine on cell cycle progression in a Sty1-dependent manner (Alao et al., 2014) we
314 investigated its effect on the *ssp1*Δ mutant strain under heat and osmotic stress conditions.
315 *ssp1*Δ mutant cells exposed to 1M KCl for 4h and 46h became elongated (cell size at division)
316 from 14.3μm and 13.4μm to 16μm and 25μm at 4 and 46h respectively. Exposure to 10mM
317 caffeine alone for 4h and 46 h also increased the average cell length from 14.3μm and 13.4μm
318 to 18.1μm and 27.2μm respectively (Figure 6A). Microscopic analyses demonstrated, that
319 *ssp1*Δ mutants are longer than *wt* cells following exposure to phleomycin (29.2μm versus
320 35.4μm) (data not shown). *ssp1*Δ mutants exhibited slight sensitivity when stationary phase
321 cells were plated on YES media containing 10mM caffeine. In contrast, *amk2* Δ mutants were
322 unable to regrow showing that caffeine exacerbated the phenotype of *ssp1*Δ mutants exposed
323 to environmental stress (Figure 6B). At 35°C *ssp1*Δ mutants delay progression through mitosis
324 (Gómez-Hierro et al., 2015). This effect was clearly exacerbated by co-exposure to 10mM
325 caffeine (19.5μm versus 37.8μm after 24h). In contrast, both Rapamycin (100ng/mL) and
326 Torin1 (5μM) were sufficient to overcome the *ssp1*Δ phenotype under these conditions
327 (15.4μm and 21.8μm at 24h for Rapamycin and Torin1 respectively) (Figure 6C). Inhibition of
328 TORC1 with Rapamycin or deletion of *sck1*, has been shown to permit growth under restrictive
329 temperatures (Morozumi et al., 2024). The Ssp1-Ssp2-TORC1 pathway is required for
330 proliferation in the presence of KCl. Caffeine enhanced the sensitivity *ssp1*Δ and *amk2*Δ
331 mutants to KCl (Figure 6D). Caffeine at 10 mM and KCl at 0.6 M induced mitochondrial fission

332 as seen through patterns of Cox4-GFP (Figure 6E), in a way like the reported effect of 1M KCl
333 treatment (Schutt and Moseley, 2017). Together, these observations further suggest a role for
334 the Ssp1-Ssp2-TORC1 pathway in mediating the effects of caffeine on cell cycle progression
335 under normal and environmental stress conditions.
336 Amk2 has been reported to play a role in DNA damage repair and chromatin remodelling in
337 *S. pombe* and mammalian cells (Ait-Saada et al., 2019; Lee et al., 2017). As caffeine is itself
338 a genotoxin (Calvo et al., 2009; Rowley and Zhang, 1999), we further investigated the role of
339 Amk2 in mediating resistance to caffeine. Amk2 specifically mediated the resistance of
340 stationary phase cells plated on caffeine to the drug (Figure 6B and Supplementary figure 4A).
341 When stationary phase cells were inoculated into YES media containing caffeine, *amk2*
342 mutants showed a significantly reduced O.D. relative to *wt* cells over the first 24h. At 48h the
343 *amk2*Δ and *wt* cultures had similar O.D.s suggesting *Amk2* regulates cell cycle re-entry or
344 proliferation rate in the presence of caffeine (Supplementary figure 4B). This role is specific
345 for Amk2, as we did not observe delayed proliferation in *ssp1*Δ, *ssp2*Δ, *cek1*Δ *ppk18*Δ and
346 *igo1*Δ mutants. Given the specific sensitivity of *ssp2*Δ and *amk2*Δ mutants to phleomycin and
347 their enhanced sensitivity to HU in the presence of caffeine (Figure 2J), Amk2 may mediate
348 resistance to the DNA damaging effects of caffeine in stationary phase cells. When inoculated
349 into media containing caffeine *wt* cells had approximately a 3- fold higher O.D. relative to amk2
350 mutants after 24h (Supplementary figure 4B and 4C).
351
352 **Caffeine enhances DNA damage sensitivity independently of its cell cycle effects**
353 The E2 ubiquitin-conjugating enzyme Rhp6 (belonging to the histone H2B-K119 ubiquitin
354 ligase complex HULC) cooperates with Cut8, a tethering factor of the nuclear proteasome,
355 towards mediating DNA repair in *S. pombe*. Furthermore, Rhp6 has been shown to mediate
356 the cell cycle effects of caffeine (Rowley and Zhang, 1999). Caffeine may thus interfere with
357 Rhp6- and Cut8-mediated DNA damage repair (Kearsey et al., 2007; Rowley and Zhang,
358 1999; Takeda and Yanagida, 2005). We have confirmed that caffeine even at 10mM did not
359 drive *rhp6*Δ mutants, previously exposed to HU, into mitosis (Figure 7A). In contrast, caffeine
360 blocked the HU- induced cell cycle arrest in *cut8*Δ mutants (Figure 7B). While *rhp6*Δ mutants
361 are checkpoint competent they were still sensitised to HU by caffeine (Figure 7C and 7E). We
362 observed that *rhp6*Δ mutants became elongated indicating checkpoint arrest but remained
363 sensitive to HU. Caffeine similarly enhanced the sensitivity of *cut8*Δ mutants to HU (Figure 7B
364 and 7D). Caffeine may induce a specific form of DNA damage that is repaired by Rhp6 and
365 enhances HU sensitivity (Calvo et al., 2009; Rowley and Zhang, 1999). Both *rhp6*Δ and *cut8*Δ
366 mutants were highly sensitive to phleomycin with the latter losing viability rapidly (as noted by
367 the lack of cell growth in the presence of phleomycin compared to *rhp6*Δ mutants) (Figure 7C
368 to 7E). Caffeine alone or in combination with Rapamycin as well as Torin1 suppressed Cut8

369 expression in cells previously exposed to phleomycin but not under normal growth conditions
370 (Figure 7F). These findings provide further evidence for a cell cycle-independent effect of
371 caffeine on DNA damage sensitivity.
372

373 **The AMPK pathway is required for caffeine-mediated lifespan extension in *S. pombe***

374 We previously demonstrated that caffeine extends chronological lifespan (CLS) in *S. pombe*
375 (Rallis et al., 2013). To determine if the Ssp1-Ssp2 pathway is required for caffeine-induced
376 lifespan extension, we compared the effect of caffeine on CLS of *wt*, *ssp1Δ*, *ssp2Δ* and *amk2Δ*
377 postmitotic (stationary phase) cell populations. Caffeine clearly extended CLS in *wt* cells
378 (Figure 8A, log rank $p=4.3x10^{-4}$). Caffeine failed to extend CLS in *ssp1Δ* (Figure 8B, log rank
379 p=0.08), *amk2Δ* (Figure 9C, log rank p=0.85) and *ssp2Δ* (Figure 8D, log rank p=0.22). This
380 result might be related with our earlier findings that these genes mediate resistance to caffeine
381 (Figure 6). Although we cannot rule out, that caffeine is toxic to mutant cells under
382 investigation, caffeine treatment is not detrimental to their chronological ageing (although a
383 very mild, albeit not statistically significant effect, is observed for *ssp1*Δ).
384 Semi-quantitative lifespan analyses using growing cells confirmed that *ssp1*Δ, *ssp2*Δ and
385 *amk2*Δ mutants are short-lived relative to *wt* type cells. We performed these experiments to
386 complement our standard CLS assays (Matecic et al., 2010). Cells were harvested at the
387 indicated time points over a 4-day period to match our CLS assays, adjusted for cell numbers
388 and plated on YES media (Figure 8E). Caffeine clearly extended the CLS of only *wt* cells
389 consistent with a role for *ssp1*Δ, *ssp2*Δ and *amk2*Δ mutants in mediating resistance to caffeine
390 (Figure 8A-8E). Together our findings suggest that all components of the *S. pombe* AMPK
391 heterotrimer are required for caffeine-mediated extension of CLS.
392

393 **Discussion**

394 In the present study, we sought to investigate if caffeine mediates its effects on cell cycle
395 progression via the Ssp1 activated Ssp2-TORC1 signalling axis. In *S. pombe*, environmental
396 stress and nutrient availability signalling converge via the TORC1 and TORC2 kinase
397 complexes to regulate Cdc25 activity and cell cycle fate (Alao et al., 2023, 2021). We have
398 previously demonstrated that caffeine drives mitosis by modulating Cdc25 and Wee1 activity
399 in a manner reminiscent of TORC1 inhibition (Alao et al., 2020, 2014). Furthermore, caffeine
400 modulates mitosis in *S. pombe*, by temporarily inhibiting Cdc25 activity via the Sty1-dependent
401 activation of Srk1 (Alao et al., 2014; Calvo et al., 2009). However, caffeine stabilises Cdc25
402 independently of inhibitory phosphorylation sites (Alao et al., 2014) (Figure 4F and 4G).
403 Ssp1 is required for TORC1 inhibition and cell cycle re-entry following exposure to
404 environmental stresses (Freitag et al., 2014; Gómez-Hierro et al., 2015; Schutt and Moseley,
405 2017). Our findings indicated that caffeine strongly interacts with Ssp1 to regulate mitosis and

406 resistance to environmental stress. We also demonstrated that caffeine activates Ssp2 in an
407 Ssp1-dependent manner. Ssp2 activation is required for the mitotic effects of caffeine in both
408 log phase and DNA damage checkpoint-activated cell populations. Ssp2-mediated TORC1
409 inhibition relieves the inhibition of Sck2 on Ppk18 and Cek1, to drive cells into mitosis due to
410 the downstream inhibition of $PP2A^{Pab1}$ (Aono et al., 2019). Surprisingly *ppk18 cek1* co-deletion
411 suppressed the mitotic effects of Torin1 but not those of caffeine. Thus, an additional kinase
412 such as Ppk31 may mediate the effects of caffeine on mitotic progression. In contrast, *igo1*
413 deletion suppressed the mitotic effects of both caffeine and Torin1. Igo1 is indispensable for
414 rapid mitotic progression and proper cell cycle exit following TORC1 inhibition (Aono et al.,
415 2019). Our findings suggest that caffeine mediates cell cycle progression in an Igo1-
416 dependent manner, but this may be due to increased intrinsic $PP2A^{Pab1}$ activity in *igo1*Δ
417 mutants independently of Cek1/ Ppk18 (Figure 8F). It should be noted that caffeine differs
418 from other stresses such as glucose deprivation and ionic stress by driving cells into mitosis,
419 rather than delaying mitosis (Masuda et al., 2016), partially mimicking the effects of
420 Rapamycin-induced TORC1 inhibition on cell cycle progression. However, signalling outputs
421 downstream of TORC1 may differ between caffeine and direct TORC1 and/or TORC2
422 inhibitors. We noted for instance, that caffeine does not inhibit Maf1 phosphorylation but
423 clearly suppresses Sck2 expression. 10mM caffeine has only a modest effect on cell growth
424 in *S. pombe* compared to caffeine in combination with 100ng/mL Rapamycin or 5μM Torin1
425 exposure (Rallis et al., 2013; Rodríguez-López et al., 2020). Furthermore, ATP did not block
426 the effects of caffeine on Ssp2 phosphorylation or mitosis. In contrast, ATP counteracted the
427 negative effect of Torin1 on Ssp2 phosphorylation and mimicked the effect of *ssp1* or *ssp2*
428 deletion on mitosis. In fact, addition of ATP mimicked the effect of ssp2 deletion on Torin1-
429 induced mitosis. These observations suggest that caffeine activates Ssp2 independently of
430 intracellular ATP levels. Caffeine induced mitochondrial fission in a manner similar to KCl as
431 previously reported with the effect of KCl occurring independently of Ssp2 (Schutt and
432 Moseley, 2017).
433 FACS analyses suggest that while caffeine drives cells into mitosis in an Ssp1-dependent
434 manner, DNA replication may not be completely inhibited in *ssp1* mutants co-exposed to
435 phleomycin. Deletion of *ssp1*, thus, may uncouple cytokinesis from DNA replication when
436 these mutants are exposed to caffeine in the presence of phleomycin. Aside from *gsk3*
437 mutants, we did not detect a differential decrease in DNA damage sensitivity in any of the
438 Ssp1-Ssp2-TORC1 axis mutants. Interestingly, *ssp2*Δ and *amk2*Δ mutants showed enhanced
439 sensitivity to phleomycin and were more sensitive to the effects of caffeine in the presence of
440 HU. Caffeine-mediated activation of Ssp2 and Amk2 may thus have a protective effect during
441 activation of the DNA damage response. Caffeine is clearly genotoxic in *S. pombe* at 10mM.
442 Resistance to caffeine requires Rad3 and the Homologous Recombination (HR) DNA Damage

443 Repair proteins Rad51 and Rhp54 as well as the 26S proteasome nuclear tether Cut8 required
444 for DNA damage repair (Calvo et al., 2009; Rowley and Zhang, 1999). Furthermore, caffeine
445 appears to induce Cds1 and Chk1 signalling and clearly enhances phleomycin sensitivity even
446 in stationary phase cells (Alao et al., 2014) (Figure 2h). Early studies have, indeed, identified
447 caffeine as a genotoxic agent (Lehmann, 1972; Prakash, 1981; Rowley and Zhang, 1999; Ts'o
448 and Lu, 1964). Our studies suggest that caffeine may be inducing a specific type of DNA
449 damage in HU-exposed cells. We have confirmed that the *S. cerevisiae* RAD6 homologue
450 Rhp6 is required for the effects of caffeine on the HU-induced DNA damage checkpoint. Rhp6
451 is a ubiquitin conjugating enzyme required for post replication repair (PRR), while also
452 mediating mitotic progression (Rowley and Zhang, 1999). Crucially, caffeine enhanced the
453 sensitivity of *rhp6*Δ mutants to HU despite its inability to advance mitosis under these
454 conditions. Rhp6 regulates the abundance of the 26S proteasome within the nucleus by
455 regulating the stability of its nuclear tether Cut8 (Takeda and Yanagida, 2005). We also noted
456 that caffeine suppresses Cut8 expression in the presence of phleomycin. Caffeine may, thus,
457 interfere with ubiquitin-dependent degradation within the nucleus in *S. pombe*. These findings
458 are consistent with our previous finding that caffeine advances mitosis by stabilising Cdc25
459 within the nucleus. Our observation, that this activity is more evident in HU-treated than in
460 phleomycin-treated cells hints at cell cycle phase specific effects. When Cdc25 activity is high
461 (High Cdc2 activity), caffeine is readily able to drive cells into mitosis in a manner partially
462 dependent on Ssp2. When Cdc25 activity is low, however, (low Cdc2 activity), caffeine
463 requires the Ssp1-dependent activation of Ssp2 to drive cells into mitosis via inactivation of
464 Srk1 and $PP2A^{Pab1}$ (Chica et al., 2016b; Gómez-Hierro et al., 2015; Schonbrun et al., 2013)
465 (Figure 8F). Enhanced Cdc2 activity in cells exposed to caffeine may be sufficient to drive
466 DNA replication in the absence of mitosis in *ssp1*Δ and *ssp2*Δ mutants. The ability of caffeine
467 to enhance phleomycin sensitivity even in stationary phase cells, suggests it might interfere
468 with DNA damage repair. Additionally, $PP2A^{Pab1}$ and other phosphatases in this family
469 regulate DNA damage repair (Kim et al., 2019; Ramos et al., 2019). Caffeine and other TORC1
470 inhibitors may, therefore, drive cells into mitosis, while inhibiting their ability to mediate DNA
471 damage repair and TORC2 crosstalk. TORC2 has been reported to regulate stress and DNA
472 damage resistance by modulating chromatin remodelling, gene expression particularly in S-
473 phase and exit from DNA damage cell cycle checkpoints. Caffeine may, thus, also impact on
474 DNA damage sensitivity due to its effects on TORC2 signalling (Schonbrun et al., 2009).
475 In this study, we identified a role for the AMPK β-subunit Amk2 in specifically mediating the
476 resistance of stationary phase *S. pombe* cells when plated on YES media that contain caffeine.
477 The AMPK β-subunit Amk2 is required for heterotrimer formation and its subcellular
478 localization (Valbuena and Moreno, 2012). Ssp2 regulates the localisation of transcription
479 factors and transcriptional repressors in fission yeast. When activated, Ssp2 also suppresses

480 the expression of genes involved in ATP-dependent processes such as DNA replication, DNA
481 repair, translation and the expression of genes required for cell cycle exit and sexual
482 development (Valbuena and Moreno, 2012). Ssp2 is not strictly required for progression
483 through mitosis, however, it may enhance it. These reports are consistent with our finding, that
484 caffeine can induce mitosis in *ssp2* mutants exposed to phleomycin when Gsk3 is expressed.
485 A strong genetic interaction has been reported for *amk2* and *gsk3*, suggesting the latter may
486 suppress TORC1 in a TORC2-dependent manner (Rallis et al., 2017). The AMPK β and γ
487 subunits are not required for the induction of mitosis following nitrogen stress. In contrast, all
488 three subunits appear to be required for cell cycle resumption following exposure to osmotic
489 stress through KCl. This suggests that Ssp2 differentially modulates mitosis in a context-
490 dependent manner (Davie et al., 2015; Forte et al., 2019; Rallis et al., 2013; Schutt and
491 Moseley, 2017; Valbuena and Moreno, 2012). Its major function may be to regulate
492 transcription in response to a variety of environmental stresses (Saitoh et al., 2015; Valbuena
493 and Moreno, 2012). In contrast to its orthologue in *S. cerevisiae* (SNF1), Ssp2 appears not to
494 be required for the induction of autophagy in response to glucose deprivation in *S. pombe*
495 (Pérez-Díaz et al., 2023). Rather, AMPK/Ssp2 migrates to the nucleus, where it
496 phosphorylates the Scr1 transcriptional repressor to induce its nuclear export (Saitoh et al.,
497 2015). This facilitates the increased expression of high affinity hexose transporters in response
498 to glucose deprivation (Saitoh et al., 2015). We did not observe any effect of caffeine on Ght5
499 expression (data not shown, separate manuscript in preparation). Conversely, ATP did not
500 affect caffeine-induced Ssp2 phosphorylation or cell cycle progression. Caffeine did induce
501 mitochondrial fission similarly to KCl. Indirect caffeine-induced Ssp2 activation may sufficiently
502 inhibit TORC1 activity, to drive mitosis via the inhibition of Sck2 and Igo1. This would lead to
503 caffeine stabilised Cdc25 activation and its subsequent degradation, to permit activation of the
504 Septation Initiation Network (SIN) and exit from or further rounds of cell cycle progression
505 (Chica et al., 2016b; Esteban et al., 2008; García-Blanco et al., 2019).
506 We have previously demonstrated, that caffeine-induces Cdc25 nuclear accumulation
507 independently of DNA damage checkpoint-induced phosphorylation (Alao et al., 2014).
508 Furthermore, we demonstrated in this study, that TORC2 is required for caffeine resistance.
509 TORC2 regulates protein stability by regulating Gsk3-mediated stabilization of the Pub1 E3-
510 ligase, which in turn regulates Cdc25 stability. Torin1-mediated TORC2 inhibition, has been
511 shown to induce Pub1 stabilisation in a Gsk3-dependent manner. Caffeine-induced TORC2
512 activation would thus induce Cdc25 stability by suppressing Pub1 expression (Nefsky and
513 Beach, 1996; Wang et al., 2022). Indeed, a major difference between caffeine and Torin1, is
514 that the latter induces Cdc25 degradation in a manner like nitrogen deprivation (Figure 4F and
515 4G) (Ducommun et al., 1990; Nefsky and Beach, 1996). The present study also demonstrated
516 that Gsk3, mediates the mitotic effects of caffeine in a *ssp2* mutant background. As noted

517 above, we previously demonstrated a strong genetic interaction between *gsk3* and *ssp2* (Rallis
518 et al., 2017). Caffeine-induced Ssp2 activation, may thus lead to the activation of accumulated
519 nuclear Cdc25 via Igo1-dependent $PP2A^{Pab1}$ inhibition. This would explain the inability of
520 caffeine to induce mitosis in *igo1*Δ mutants. In addition, $PP2A^{Pab1}$ inhibition could result in
521 Wee1 inactivation and degradation (Alao et al., 2020; Lucena et al., 2017; Qiu et al., 2018;
522 Smith et al., 2007). Our previous studies demonstrated that caffeine influences the stability of
523 both Cdc25 and Wee1.
524 Importantly, we noted that the cell cycle effects of caffeine are distinct from its ability to
525 enhance DNA damage sensitivity. Several studies have suggested that caffeine is a genotoxic
526 agent (Lehmann and Kirk-Bell, 1972; Prakash, 1981; Ts'o and Lu, 1964). Caffeine can clearly
527 enhance DNA damage sensitivity independently of its effects on mitosis. This is clearly
528 observable in *rhp6* mutants which are defective in DNA damage repair (Figure 7). It has been
529 suggested, that caffeine may interfere with DNA damage repair (Rowley and Zhang, 1999).
530 Alternatively, caffeine could synergistically induce DNA damage in the presence of other
531 genotoxins. This could lead to the activation of DNA damage response pathways that are
532 incompatible with HU for instance (Bakuradze et al., 2016). We also noted that caffeine
533 suppresses Cut8 expression in the presence of phleomycin. This suggests caffeine interferes
534 with DNA damage repair, as *cut8*Δ mutants are extremely sensitive to phleomycin. *cut8*Δ
535 mutants are also sensitive to caffeine, providing further evidence for its genotoxic activity
536 (Calvo et al., 2009). The enhanced sensitivity of *ssp2*Δ and *amk2*Δ mutants to phleomycin
537 suggests a role for these genes in the DNA damage response. of *S. pombe* AMPK is,
538 therefore, required for resistance to caffeine and this is illustrated by the inability of the drug
539 to extend CLS in this organism.
540

541 **Conclusion**

542 The precise mechanisms by which caffeine exerts its effects on cell cycle progression, DNA
543 damage sensitivity and CLS in *S. pombe* have remained obscure for many decades (Alao
544 and Sunnerhagen, 2020; Moser et al., 2000; Rallis et al., 2013). A clearer understanding of
545 the molecular pharmacology of caffeine, can draw links between genes that are important in
546 cellular physiology and pathology (Alao et al., 2014; Alao and Sunnerhagen, 2020; Osman
547 and McCready, 1998). We have demonstrated that caffeine accelerates mitosis in a manner
548 partially dependent on Ssp2-mediated TORC1 inhibition. Deletion of *ssp2* prevents caffeine-
549 mediated DNA damage checkpoint override but not DNA replication. In contrast, deletion of
550 *ssp1* which positively regulates Cdc25 activity blocked both effects of caffeine in the
551 presence of phleomycin. This activity is dependent on Igo1, which lies downstream of
552 TORC1 and Gsk3. The latter, functions downstream of TORC2 to regulate protein stability
553 and TORC1 activity (Pérez-Hidalgo and Moreno, 2017; Wang et al., 2022). Caffeine actions

554 depend on TORC1-TORC2 crosstalk to drive DNA replication and mitosis via Cdc25. We
555 also demonstrate that Ssp1 and Amk2 mediate the ability of caffeine to extend CLS.
556 Paradoxically, while Ssp2 appears to mediate the ability of caffeine to override DNA damage
557 checkpoint signalling, it is also required for DNA damage resistance. In the presence of other
558 genotoxins such as hydroxyurea, caffeine may act synergistically to induce DNA damage or
559 interfere with its repair. At the same time, activation of Ssp2 may exert a protective effect
560 masking the effects of caffeine on DNA damage sensitivity. Our findings suggest that
561 caffeine enhances DNA damage sensitivity independently of its effects on mitosis. It would
562 be interesting to further compare the effects of direct Ssp2 activators and caffeine on
563 mitosis, DNA damage sensitivity and ageing in *S. pombe*.
564

565 **Materials and methods**

566 **Strains, media, and reagents**

567 Strains are listed in Supplementary Table 1. Cells were grown in yeast extract plus
568 supplements medium (YES) (Formedium, Hunstanton, United Kingdom). Stock solutions of
569 caffeine and hydroxyurea (HU) (Sigma Aldrich, Gillingham, United Kingdom) (100 and
570 200mM respectively) were prepared in water stored at -20°C. Phleomycin was purchased
571 from Fisher scientific (Loughborough, United Kingdom) or Sigma Aldrich as a 20mg/mL
572 solution and aliquots stored at -20°C. Torin1 (Tocris, Abingdon, United Kingdom) was
573 dissolved in DMSO (3.3mM) and stored at -20°C. For treatment with potassium chloride
574 (KCl), 0.6M KCl was prepared in YES media with or without 10mM caffeine. ATP was
575 obtained from Alfa Aesar, Fisher Scientific, and dissolved in water (100mM). Aliquots were
576 stored at -20°C. Experiments on the effect of caffeine on HU sensitivity, we carried out as
577 previously reported (Khan et al., 2020).
578

579 **Microscopy**

580 Calcofluor white (Sigma-Aldrich), 4,6-diamidino-2-phenylindole (DAPI) staining and septation
581 index assays were carried out as previously described (Alao et al., 2014). Images were
582 obtained using a LEICA DMRA2 microscope using a 100x objective using the appropriate
583 filter set. Alternatively, studies were performed using an Evos M5000 imaging platform
584 (Thermofisher Scientific, Dartford, United Kingdom).
585

586 **Immunoblotting**

587 Antibodies directed against phospho-Ssp2 (#50081) were purchased from Cell Signalling
588 Technologies (Leiden, The Netherlands). Antibodies directed against GFP (11814460001
589 Roche) were purchased from Sigma-Aldrich (Gillingham, United Kingdom). Antibodies
590 directed against V5 (sc-81594), HA (F7) (sc-7392) and Myc (9E10) (sc-40) were from Santa

591 Cruz Biotechnology (Heidelberg, Germany). Secondary antibodies directed against mouse
592 (ab205719) and rabbit (ab205718) IgG were purchased from Abcam (Abcam, Cambridge,
593 United Kingdom). Cell pellets were prepared for SDS-PAGE and treated as previously
594 reported (Alao et al., 2014). Where deemed appropriate, images were quantified using
595 Image J software.
596
597 **Flow cytometry**
598 Cells were harvested and stored in 70% ethanol at -20° C until analysis. After rehydration
599 with PBS cells were permeabilised with 0.1% Triton, 1%BSA in PBS and stained with1ug/ml
600 FxCycle violet stain (ThermoFisher, catalogue number F10347). The samples were
601 processed on a Biorad ZE5 Analyser using the violet laser to detect FxCycle violet stain.
602 Flow rate was slow and linear range of fluorescent intensity was used to capture changes in
603 DNA content. The data were subsequently analysed using FlowJo 10.7.2.
604
605 **Chronological Lifespan Assays**
606 CLS was performed as previously described (Rallis et al., 2013; Roux et al., 2006).
607 Stationary phase cells (~16 h) in YES media, were diluted into fresh media at an OD_{600} 0.2
608 and grown to an OD_{600} 0.35-0.5. Cultures were treated with water or 10mM caffeine for a
609 further 24h. Appropriate serial dilutions were plated on YES media daily for 7 days and
610 Colony Forming Units (CFUs) were counted. Survival curves were statistically analysed with
611 Kaplan-Meier survival plots and log rank tests from at least 3 independent experiments.
612 Semi-quantitative CLS assays were also carried out as previously described (Matecic et al.,
613 2010).
614
615 **Author's contributions**
616 Conceptualisation and design of study: J.P.A., C.R. Experiments and data analysis: J.P.A.,
617 J.K., D.S. and C.R. Manuscript writing: J.P.A., J.B., D.S. and C.R. Funding acquisition: C.R.,
618 J.B. All authors have read, revised and approved the final manuscript.
619
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881 **Figure legends**

882 **Figure 1. *S. pombe* AMPK mediates the effects of caffeine on cell cycle progression. (A-**
883 **F).** Wild type (*wt*) and mutant cells were treated as indicated and harvested at every 30
884 minutes over a 2h period. Cells were stained with calcofluor and the septation indices
885 determined by fluorescent microscopy
886

887 **Figure 2**. **Caffeine enhances DNA damage sensitivity independently of its cell cycle**
888 **effects.** (**A).** The indicated wild type cells were grown to log phase and left untreated or
889 incubated with 5 µg/ mL phleomycin for 2h. Cultures were then incubated for a further 2h with
890 or without 10mM caffeine or 5µM Torin1 in 10mL medium for a further 2h as indicated. Cultures
891 were adjusted for cell number, serially diluted 3-fold, plated on YES agar and incubated for 3-
892 5 days at 32°C. (**B).** Log phase cultures of the indicated strains were treated as in A. **(C).** Wild
893 type cells were grown to stationary phase and exposed to phleomycin for 2h and then cultured
894 with and without caffeine for a further 3 h. Samples were adjusted for cell number and treated
895 as in A. **(D).** Wildtype cells expressing Cdc25-GFP and Cdc25 (12A)-GFP were exposed to
896 20mM HU for 2h and then incubated with 10mM caffeine for a further 2h and treated as in A.
897 **(E).** Log phase cultures of the indicated strains were plated on YES agar containing 5 mM HU
898 or 5 µM phleomycin with or without 10 mM caffeine.
899

900 **Figure 3. Caffeine induces Ssp2 phosphorylation independently of ATP. (A).** Log phase
901 wild type cells were exposed to 10 mM caffeine, 5 µM Torin1 or grown in media containing
902 0.03% glucose for 2 hrs. Samples were resolved by SDS-PAGE and probed with antibodies
903 directed against phospho-Ssp2. Membranes were subsequently stained with ponceau S. **(B).**
904 Blots were analysed using imageJ software. Error bars represent the mean ±S.E. of 3-
905 independent experiments. **(C).** Log phase cells expressing Ssp2-myc, were exposed to 10mM
906 caffeine and harvested at the indicated time points. Samples were resolved by SDS- PAGE
907 and probed with antibodies directed against phospho-Ssp2 and Myc. **(D).** Log phase cells
908 expressing Ssp2-myc, were exposed to 5µg/mL phleomycin for 2 hrs and then exposed further
909 with 10mM caffeine or 5µM Torin1 as indicated. Samples were treated as in A. **(E).** Log phase
910 wild type cells were treated as in D. Cells were pretreated with 10mM ATP for 30 minutes. (**F).**
911 Log phase cells were diluted into fresh media and exposed to 5µg/mL phleomycin for 2 hrs.
912 Cultures were then left untreated or exposed to 10mM caffeine or 5 µM Torin1 with or without
913 10mM ATP and harvested at the indicated time points. Cells were stained with calcofluor and
914 the septation index determined using fluorescent microscopy. **(G).** Cells were treated as in F
915 and cell length (n= 50) determined. Results were analysed using a student's t- test with
916 unequal distribution. **(H).** Cells were treated as in F, serially diluted 3- fold and plated on YES
917 agar.

918 **Figure 4. Caffeine indirectly inhibits TORC1 signalling in *S. pombe*. (A).** Cells expressing
919 Sck1-HA were grown to log phase, resuspended in fresh YES media and exposed to 10mM
920 caffeine. Samples were harvested at the indicated at the indicated timepoints, resolved by
921 SDS-PAGE and membranes probed with anti-HA antibodies. Ponceau S was used to monitor
922 gel loading. **(B).** Cells expressing Sck2-HA were treated as in A. **(C).** Cells expressing Sck1-
923 HA were exposed to 5µg/mL phleomycin for 2h and then further incubated for 2h with or
924 without 10mM caffeine and treated as in A. **(D).** Cells expressing Sck2-HA were treated as in
925 C. **(E).** Cells expressing Maf1-pk were treated with 10 mM caffeine, 100 ng/mL Rapamycin,
926 caffeine and Rapamycin or 5µM Torin1 for 90 minutes and analysed by SDS-PAGE.
927 Membranes were probed with antibodies directed against V5. **(F).** Cells expressing Cdc25-
928 GFP were exposed to 20mM HU for 2h and then for a further 2h with or without 10mM caffeine
929 or 5µM Torin1. **(G).** Cells expressing Cdc25(12A)-GFP were exposed to 5ug/ mL phleomycin
930 for 2h and then for a further 2h with or without 10mM caffeine or 5µM Torin1. Membranes were
931 probed with antibodies directed against GFP.

932

933 **Figure 5. Caffeine modulates cell cycle kinetics via TORC1 and TORC2 complexes in *S.***
934 ***pombe*. (A- E).** Cultures were incubated for 2h with or without 10mM caffeine or 5µM Torin1
935 in 10mL medium as indicated. Fixed cells were stained with calcofluor and the septation index
936 determined using fluorescent microscopy. **(F).** The indicated wildtype and mutant strains were
937 grown to log phase and resuspended in fresh YES media. Cultures were adjusted for cell
938 number, serially diluted 3-fold and plated on YES agar containing 10mM caffeine. **(G-H).**
939 Wildtype and *gsk3*Δ cultures were grown to log phase, left untreated or exposed to 5 µg/mL
940 of phleomycin for 2h. Cultures were then exposed to 10mM caffeine or 5µM Torin1, harvested
941 at the indicated time points and septation indices monitored. **(I-J).** Wild type and *gsk3*Δ
942 cultures were grown to log phase, left untreated or exposed to 5µg/mL of phleomycin for 2h.
943 Cultures were then exposed to 10mM caffeine or 5µM Torin1 for 2h, adjusted for cell number,
944 serially diluted 3-fold and plated on YES agar.

945

946 **Figure 6. Caffeine signals via the Ssp1-dependent stress response pathway. (A).**
947 *ssp1*Δ mutants were grown to log phase at 32°C, resuspended in fresh YES media with or
948 without 1M KCl or 10mM caffeine alone or in combination for 4h or 46 h. Cells were stained
949 with calcofluor and the cell length determined using fluorescent microscopy (*n*=50). Under
950 conditions where septate cells were absent, the average cell length of non-septate cells in at
951 least 5 different fields was determined. **(B).** The indicated wildtype and mutant strains were
952 grown to stationary phase, adjusted for cell number, serially diluted 3-fold and plated on YES
953 agar containing 10mM caffeine. **(C).** *ssp1*Δ mutants were grown for 24h under the indicated

954 conditions, harvested and treated as in A. **(D).** The indicated wild type and mutant strains
955 were grown to stationary phase at 32°C. Cultures were adjusted for cell number, serially
956 diluted 3-fold and plated on YES agar containing 0.6M KCl alone or in combination with
957 10mM caffeine. Alternatively, stationary phase cultures were adjusted to an OD_{600} of 0.3,
958 grown to log phase and treated as in the stationary phase panel. **(E).** Cells expressing Cox4-
959 GFP were exposed to 10mM caffeine or 0.6M KCl and mitochondria visualised by
960 fluorescent microscopy. Asterisks indicate fragmentated mitochondria.
961
962 **Figure 7. Caffeine enhances DNA damage sensitivity independently of checkpoint**
963 **signalling and interferes with DNA damage repair. (A and B).** *rhp6*Δ and *cut8*Δ strains
964 were grown to log phase overnight. Cells were incubated with 20mM hydroxyurea (HU) with
965 or without 10mM caffeine. Cells were stained with calcofluor and the septation index
966 determined using fluorescent microscopy. **(C and D).** *rhp6*Δ and *cut8*Δ strains were grown to
967 log phase and exposed to 20mM HU or 5µg/ mL phleomycin for 2h. Cells were then incubated
968 for a further 2h following the addition of 10mM caffeine as indicated. Samples were adjusted
969 for cell number, serially diluted and plated on YES media. **(E).** Samples of *rhp6*Δ and *cut8*Δ
970 strains in C and D were examined by DIC microscopy. **(F).** Cells expressing Cut8-myc were
971 left untreated or incubated with 5µg/ mL phleomycin for 2h. Cells were then incubated for a
972 further 2h following the addition of 10mM caffeine, caffeine with 100ng/ mL Rapamycin or 5µM
973 Torin1 as indicated. Samples were harvested, resolved by SDS-PAGE and Cut8 detected
974 using antibodies directed against myc. Gel loading was monitored using ponceau S.
975
976 **Figure 8. Caffeine extends lifespan in an Ssp1, Ssp2 and Amk2- dependent manner in**
977 **fission yeast and summary of this work. (A- D).** Wild type *ssp1*Δ, *ssp2*Δ and *amk2*Δ strains
978 were grown to stationary phase with or without 10 mM caffeine as described in materials and
979 methods. Statistical analyses represent the mean from at least 3 biological repeats (n= 3). **(E).**
980 Semi- quantitative analysis of the effect of caffeine on CLS in fission yeast. The indicated
981 strains were grown to log phase and then grown to stationary phase with or without 10 mM
982 caffeine. Samples were harvested on the indicated days, adjusted for cell number (OD_{600} 0.3),
983 serially diluted 3- fold, plated on YES agar. **(F).** Schematic model summarising the results of
984 this study together with relationships from existing literature (see main text for details).
985 Caffeine activates environmental stress response signalling and indirectly inhibits TORC1
986 advancing mitosis and modulating DNA damage repair signalling in fission yeast.
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Figure 1

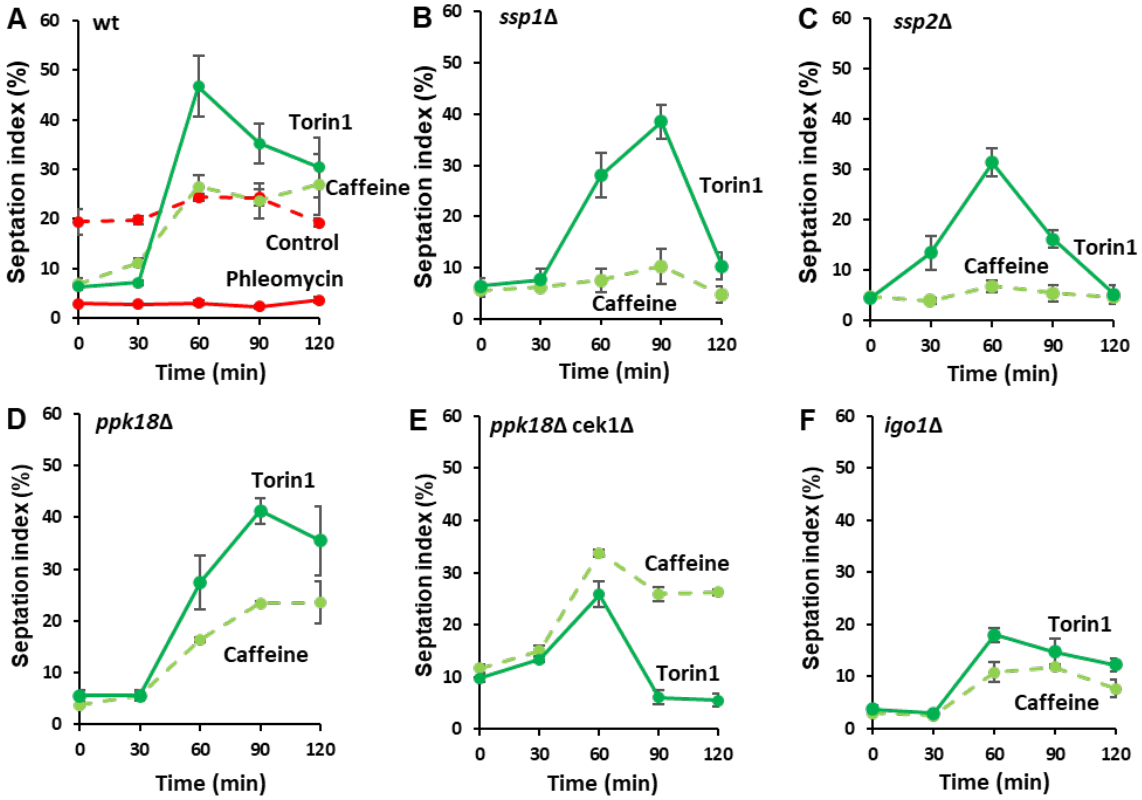

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Figure 2

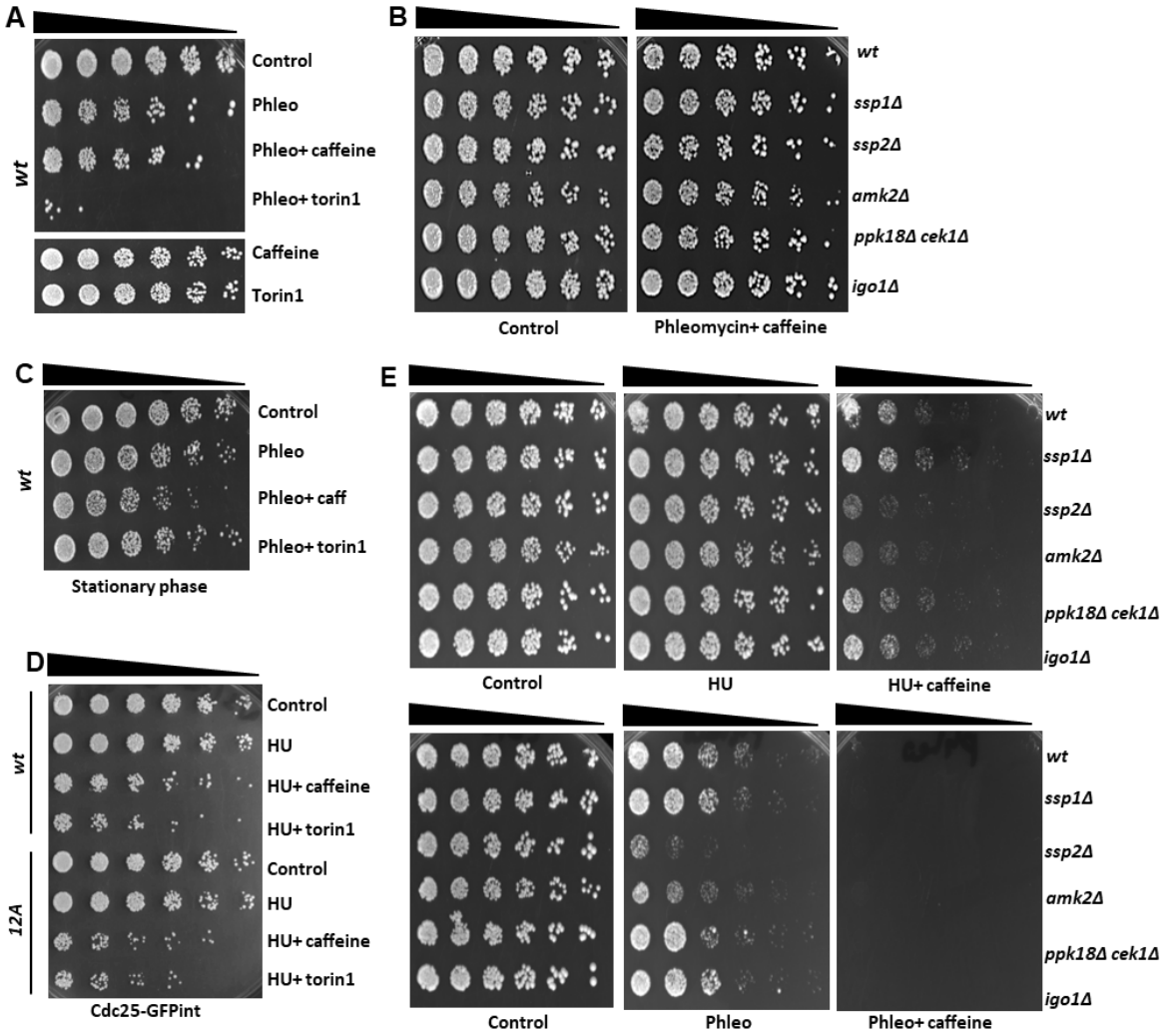

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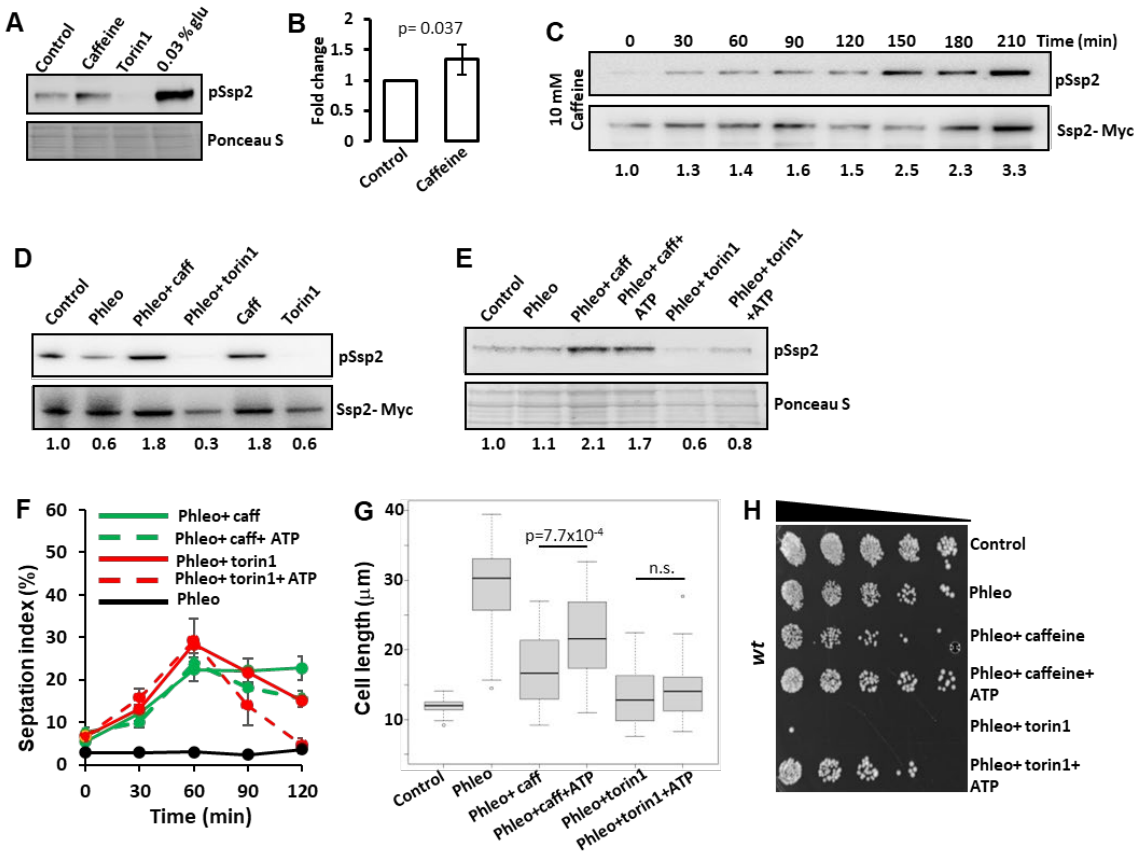
Figure 3


A
Control
Caffeine
Torin1
0.03 % glu
pSsp2
Ponceau S
B
p= 0.037
Fold change
Control
Caffeine
C
0 30 60 90 120 150 180 210 Time (min)
10 mM Caffeine
pSsp2
Ssp2- Myc
1.0 1.3 1.4 1.6 1.5 2.5 2.3 3.3
D
Control
Phleo
Phleo+ caff
Phleo+ torin1
Caff
Torin1
pSsp2
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1.0 0.6 1.8 0.3 1.8 0.6
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Phleo+ torin1
Phleo+ torin1 +ATP
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Phleo+ caff+ ATP
Phleo+ torin1
Phleo+ torin1+ ATP
Phleo
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n.s.
Cell length (µm)
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Phleo+ caff
Phleo+caff+ATP
Phleo+torin1
Phleo+torin1+ATP
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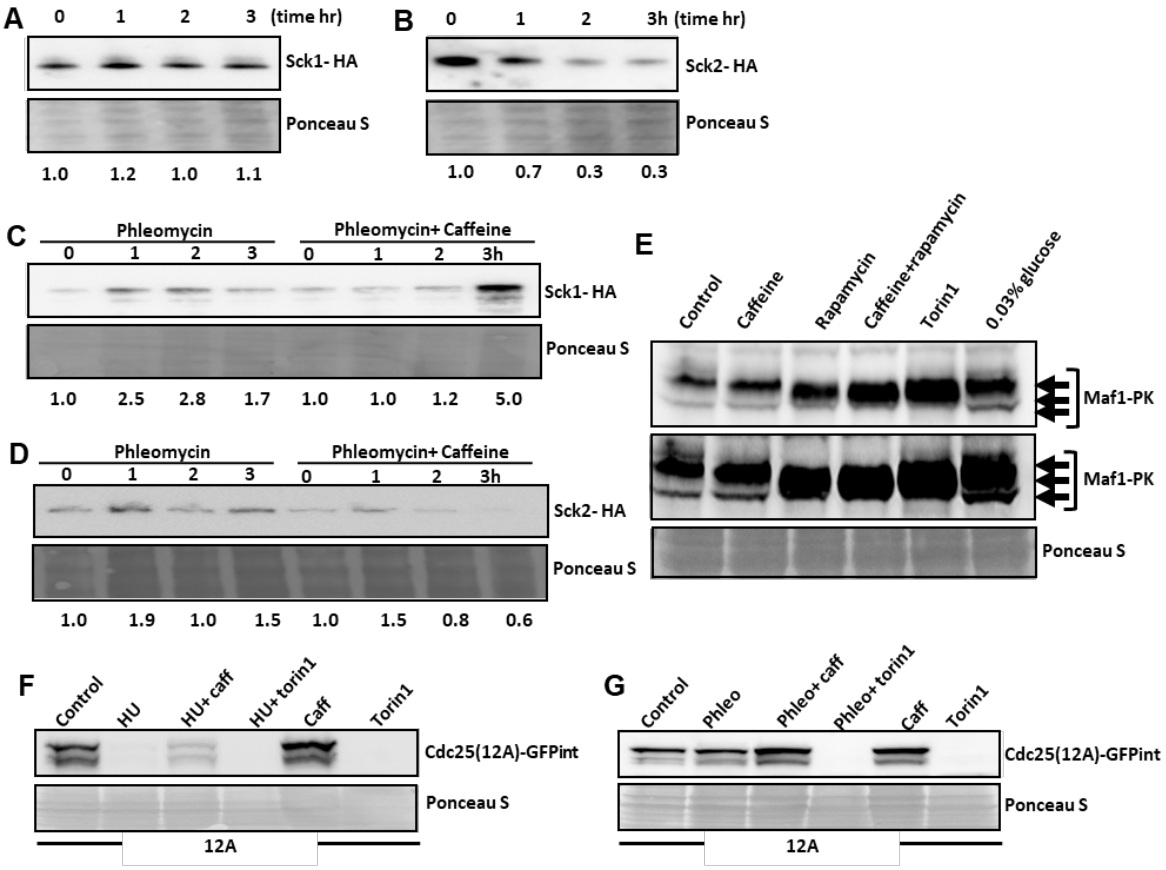
Phleo+ torin1
Phleo+ torin1+ ATP

Figure 4


A
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Sck1- HA
Ponceau S
1.0 1.2 1.0 1.1
B
0 1 2 3h (time hr)
Sck2- HA
Ponceau S
1.0 0.7 0.3 0.3
C
Phleomycin
Phleomycin+ Caffeine
0 1 2 3 0 1 2 3h
Sck1- HA
Ponceau S
1.0 2.5 2.8 1.7 1.0 1.0 1.2 5.0
D
Phleomycin
Phleomycin+ Caffeine
0 1 2 3 0 1 2 3h
Sck2- HA
Ponceau S
1.0 1.9 1.0 1.5 1.0 1.5 0.8 0.6
E
Control
Caffeine
Rapamycin
Caffeine+rapamycin
Torin1
0.03% glucose
Maf1-PK
Maf1-PK
Ponceau S
F
Control
HU
HU+ caff
HU+ torin1
Caff
Torin1
Cdc25(12A)-GFPint
Ponceau S
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Phleo+ torin1
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Ponceau S
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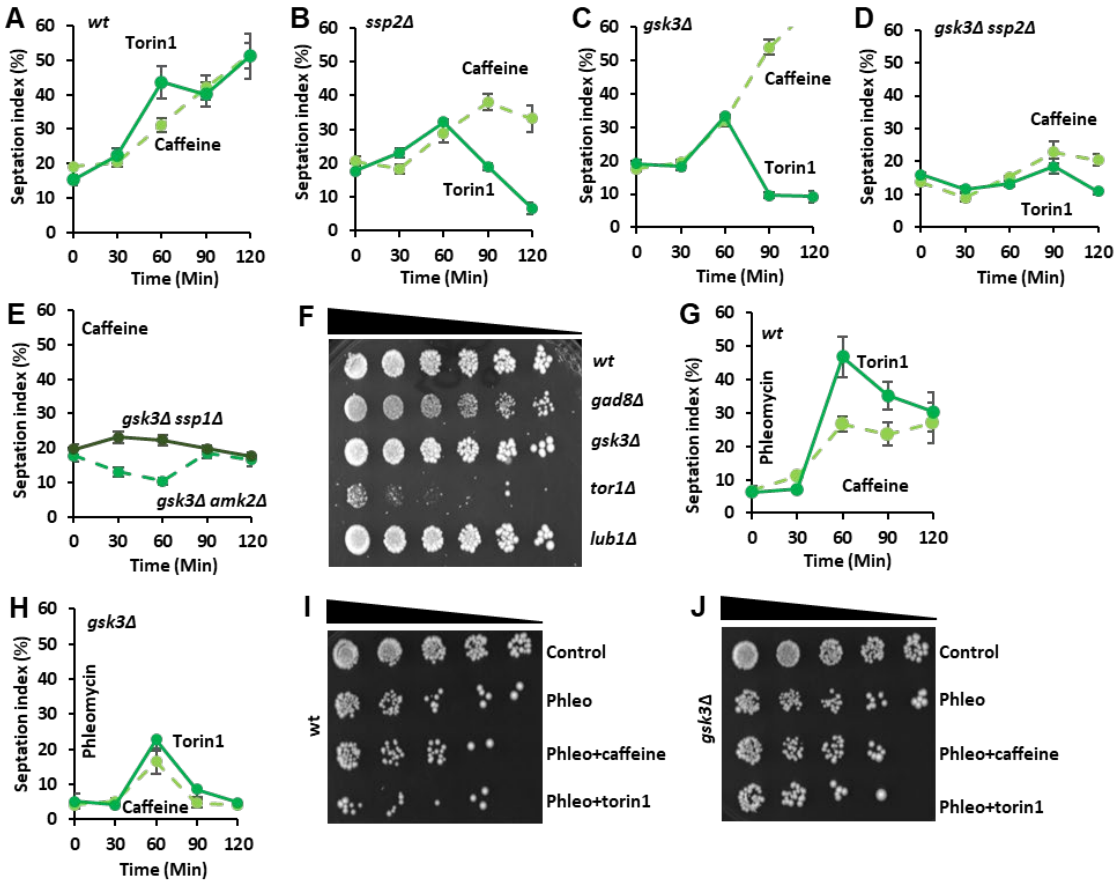
Figure 5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gsk3Δ amk2Δ
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gad8Δ
gsk3Δ
tor1Δ
lub1Δ
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Torin1
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Phleo+torin1

Figure 6

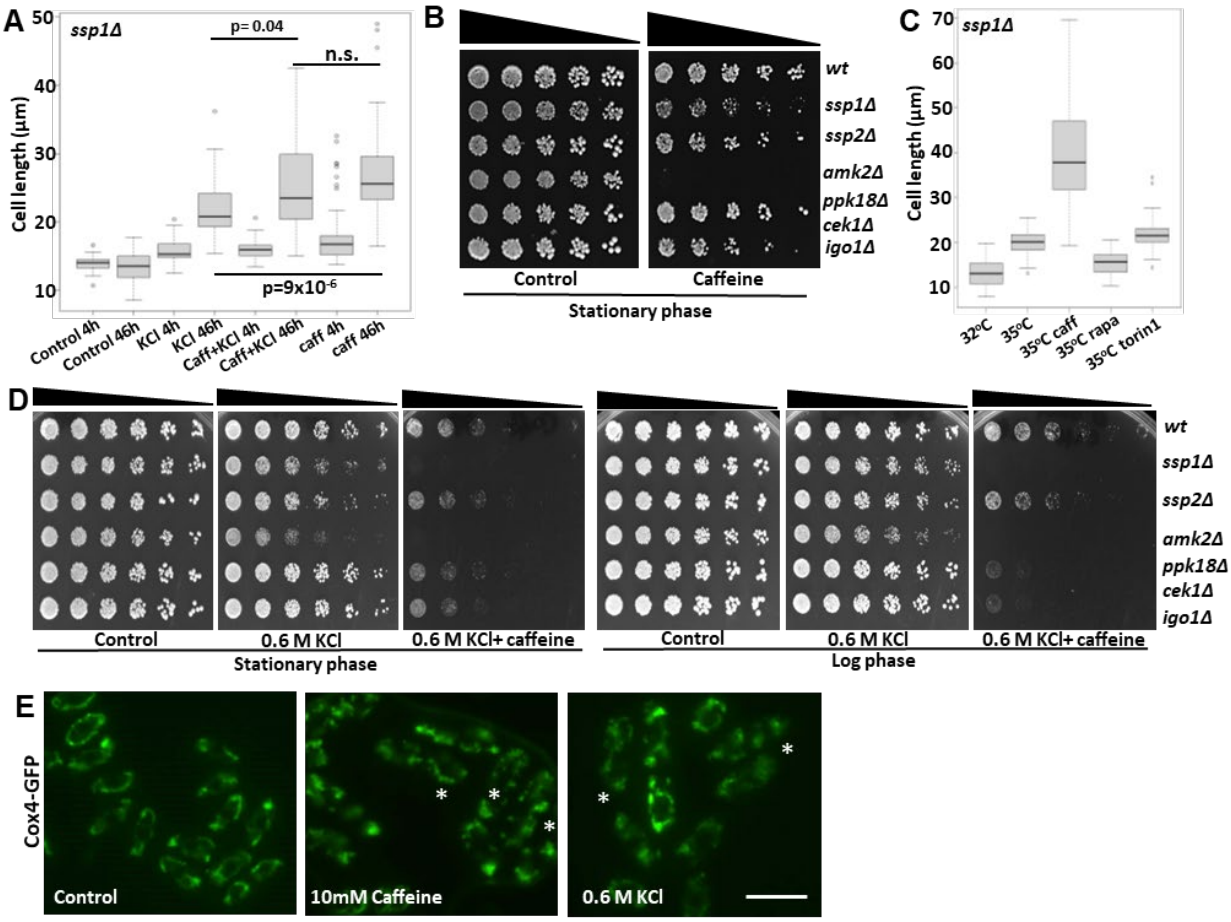

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Figure 7

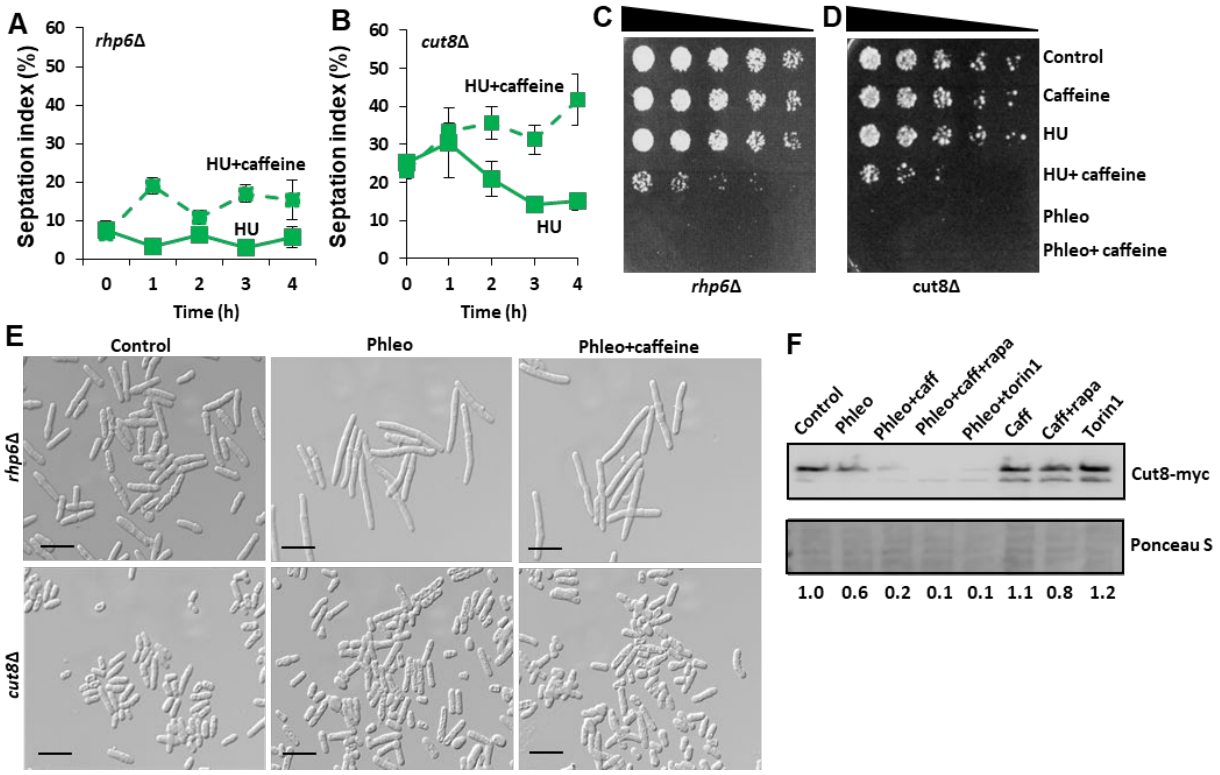

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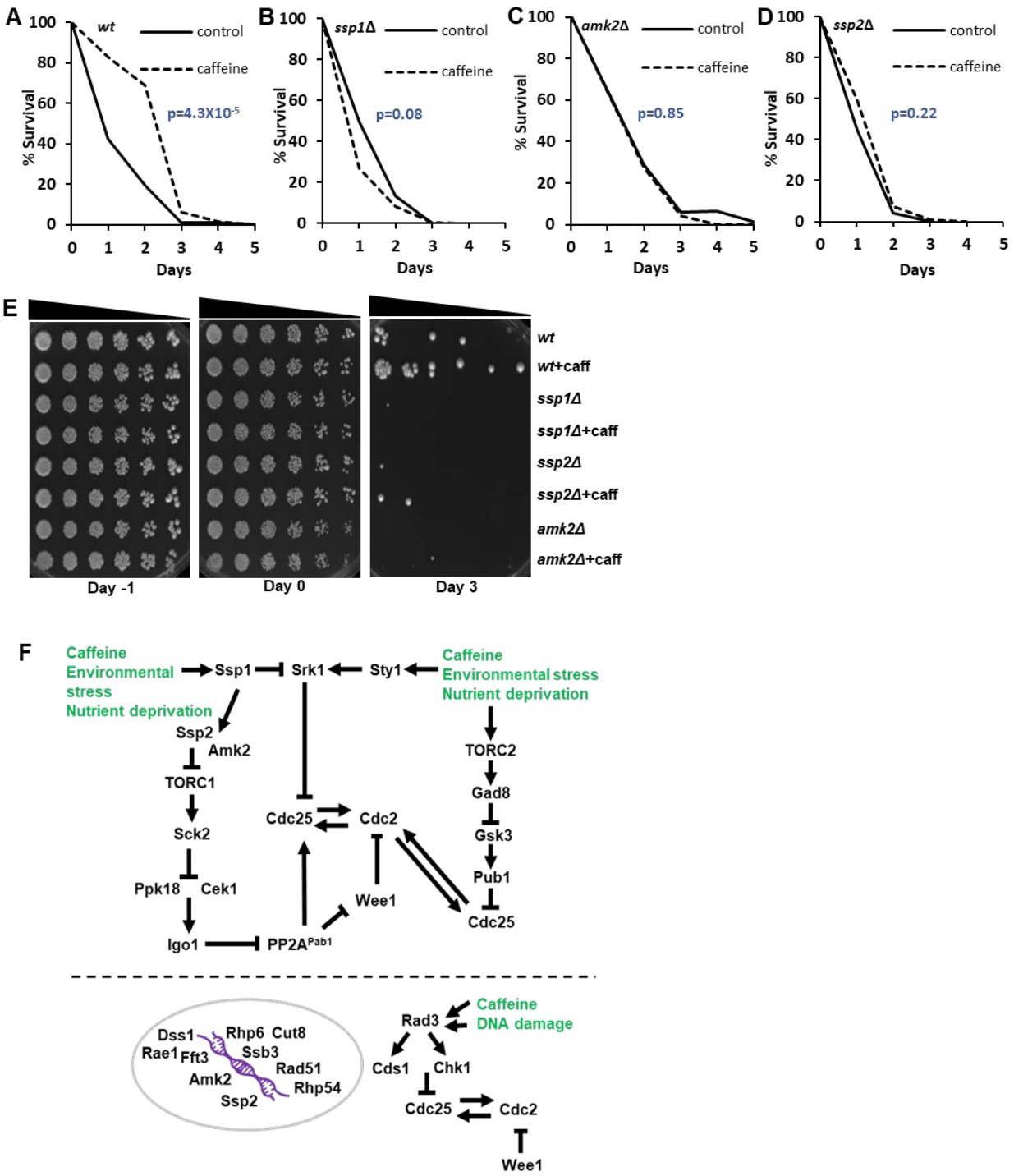
Figure 8



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