The AMPK-TORC1 signalling axis regulates caffeine-mediated DNA damage checkpoint override and cell cycle effects in fission yeast

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Abstract

Caffeine, a widely consumed neuroactive compound, induces DNA damage checkpoint signalling override, and enhances sensitivity to DNA damaging agents. However, the precise underlying mechanisms have remained elusive. In fission yeast *S. pombe*, the Ataxia Telangiectasia Mutated (ATM) and Ataxia Telangiectasia mutated Related (ATR) orthologue Rad3 has been proposed as the cellular target of caffeine. Nevertheless, recent studies suggest that the Target of Rapamycin Complex 1 (TORC1) might be the main target. Caffeine mimics the effects of activating the Sty1-regulated stress response and the AMP-Activated Protein Kinase (AMPK) homologue Ssp1-Ssp2 pathways on cell cycle progression. Direct inhibition of TORC1 with the ATP-competitive inhibitor torin1, is sufficient to override DNA damage checkpoint signalling. It is, therefore, plausible, that caffeine modulates cell cycle kinetics by indirectly suppressing TORC1 through Ssp2 activation. S*sp1* and *ssp2* deletion suppresses the effects of caffeine on cell cycle progression. In contrast, direct inhibition of TORC1 advances cell division in these mutants. These observations suggest that caffeine overrides DNA damage signalling, in part, via the indirect inhibition of TORC1 through Ssp2 activation. Alternatively, Ssp1 and Ssp2 may potentiate the effect of caffeine on Cdc25 activity. The AMPK-mTORC1 signalling axis plays an important role in aging and disease and presents a potential target for chemo- and radio-sensitization. Our results provide further insights of the underlying mechanisms by which caffeine modulates cell cycle progression in the context of Ssp1-AMPKα^{Ssp2}-TORC1 signalling activities and can potentially aid in the development of novel dietary regimens, therapeutics, and chemo-sensitizing agents.

Key words: *S. pombe*, cell cycle, DNA damage response, environmental stress, ATP, caffeine, phleomycin, torin1, AMPK, Cek1, Igo1, Ppk18, Rad3, Ssp1, Ssp2

Introduction

The widely consumed neuroactive methylxanthine compound caffeine, has been linked to increased chronological lifespan (CLS), protective effects against diseases such as cancer and improved responses to clinical therapies [1–5]. Of particular interest, has been the deciphering of the mechanisms by which caffeine overrides DNA damage checkpoint signalling [1]. While extensively studied over the last three decades, the precise mechanisms whereby caffeine exerts its activity on cell cycle regulation have remained unclear [1,6–9].

Eukaryotic cells are subject of continuous DNA damage, resulting from the effects of both normal metabolism and exposure to environmental agents such as ionizing radiation, UV radiation, cigarette smoke as well as chemotherapeutic agents [10–12]. The fission yeast phosphatidylinositol 3-kinase-related serine/threonine kinase (PIKK) Rad3 (a homologue of mammalian ataxia telangiectasia mutated (ATM) and ATM related kinase (ATR)), is activated in response to DNA damage or replication stress. Rad3 activation and phosphorylation creates binding sites for adapter proteins that facilitate the activation of downstream kinases Cds1 and Chk1 in the S and G2 phases of the cell cycle, respectively. During S phase, Cds1 phosphorylates Cdc25 on serine and threonine residues resulting in its activation and sequestration within the cytoplasm following binding of the 14-3-3-related, Rad24 protein. Rad3 and Cds1 also activate the Mik1 kinase during S phase which directly inhibits Cdc2 [6,13]. In *S. pombe*, the reciprocal activity of the Cdc25 phosphatase and Wee1 kinase on Cdc2 activity, determines the actual timing of mitosis. Additionally, environmental cues such as nutrient limitation and environmental stress can influence the timing of mitosis through the target of rapamycin complex 1 (TORC1) signalling [14,15]. The inhibition of TORC1 activity results in the activation of the Greatwall-related kinases Ppk18 and Cek1. Ppk18 and Cek1, in turn, activate the endosulphine Igo1 which inhibits $PP2A^{pab1}$ to induce mitosis (Pab1 being the regulatory subunit of $PP2A^{pab1}$ in *S. pombe*) [16,17].

Initial studies have suggested that caffeine inhibits Rad3 and its homologues to override DNA damage checkpoint signalling in both yeast and mammalian cells [18–20]. These findings, based on *in vitro* experiments demonstrating that caffeine can inhibit Rad3 and ATM, have been proved controversial. Firstly, caffeine has been shown to override DNA damage checkpoint signalling without blocking ATM and Rad3 signalling [9,21,22]. Furthermore, caffeine can also inhibit other members of the PIKK family [23]. More recent studies indicate that caffeine inhibits TORC1 signalling in yeast and mammalian cells. Indeed, TORC1 inhibition in *S. pombe* is sufficient to override DNA damage checkpoint signalling [8,15]. It

remains unclear, if caffeine directly inhibits TORC1 by acting as a low intensity ATP competitor or indirectly via the *S. pombe* AMP-Activated Protein Kinase(AMPK) Ssp2 [24,25].

In the current study, we have identified novel roles for the Ssp1 and Ssp2 kinases in facilitating the indirect inhibition of TORC1 by caffeine. Caffeine failed to override DNA damage checkpoint signalling in *ssp1*Δ and *ssp2*Δ mutants and was less effective at sensitizing cells to DNA damage. Furthermore, co-exposure to ATP blocked the effects of caffeine on DNA damage sensitivity. Downstream of TORC1, caffeine accelerated *ppk18*Δ and *cek1*Δ *ppk18*Δ but not *igo1*Δ mutants into mitosis, but this activity did not correlate with its effect on DNA damage sensitivity. Caffeine may thus enhance DNA damage sensitivity independently of mitosis. TORC1 inhibition through the ATP-competitive inhibitor torin1 also potently sensitized *ppk18*Δ, *cek1*Δ *ppk18*Δ and *igo1*Δ mutants to DNA damage. These observations suggest roles for the Ppk18, the related kinase Cek1, an unknown kinase and possibly an unidentified probable role for Igo1 in DNA damage repair and/or checkpoint recovery [26–28].

Materials and methods

Strains, media, and reagents

Cells were grown in yeast extract plus supplements medium (YES) (Formedium, Hunstanton, United Kingdom). 100 mM stock solutions of caffeine (Sigma Aldrich, Gillingham, United Kingdom) were prepared in water and stored at -20°C. Phleomycin was purchased from Fisher scientific (Loughborough, United Kingdom) or Sigma Aldrich as a 20 mg/ mL solution and aliquots stored at -20°C. Torin1 (Tocris, Abingdon, United Kingdom) was dissolved in DMSO (3.3 mM) and stored at -20°C. For treatment with potassium chloride (KCl), media containing 0.6 M KCl were prepared in YES media. ATP (Alfa Aesar, Fisher Scientific) was dissolved in water (100 mM) and aliquots were stored at -20°C. Strains used in the study are listed in Table 1. Deletion strains made for this study were generated through an established PCR-based genomic targeting [29]. Disruptions were verified by PCR using genomic DNA extracted from mutants.

Microscopy

Calcofluor white (Sigma-Aldrich) staining and septation index assays were carried out as previously described [9,30]. Images were obtained with a Zeiss AxioCam on a Zeiss Axioplan 2 microscope with a 100× objective using a 4,6-diamidino-2-phenylindole (DAPI) filter set.

Alternatively, studies were performed using an Evos M5000 imaging platform (ThermoFisher). Statistical significance was determined using Wilcoxon test (see main text).

Immunoblotting

Antibodies directed against phospho-eIF2a (#9721), eIF2a (#9722), phospho-Ssp2 (#50081) and Ssp2 (#5832) were purchased from Cell Signaling Technologies (Leiden, The Netherlands). Antibodies directed against actin (A2066) were purchased from Sigma- Aldrich (Gillingham, United Kingdom). Antibodies directed against V5 (sc-81594) and HA (F7) were from Santa Cruz Biotechnology (Heidelberg, Germany). Secondary antibodies Goat Anti-Mouse IgG H&L (HRP) (ab205719) and Goat Anti-Rabbit IgG H&L (HRP) (ab205718) were purchased from Abcam (Abcam, Cambridge, United Kingdom). All antibodies were used in dilutions according to the manufacturers' instructions. Cell pellets were prepared for SDS-PAGE and treated as previously reported [5,9,31].

Results

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

As previously reported, both caffeine (10 mM) and torin1 (5 µM) override the cell cycle arrest induced by 5 µg/mL phleomycin in *wild type S. pombe* cells. In contrast, cells exposed to phleomycin remained arrested while control cultures proliferated with steady state kinetics (Figure 1A) [8]. Based on these observations, we hypothesized that caffeine overrides DNA damage checkpoint signalling via inhibition of TORC1 activity [8]. As direct inhibition of TORC1 activity through torin1 is more effective than exposure to caffeine, we investigated the possible role of Ssp1 and Ssp2 in modulating the mitotic effects of caffeine. Nutrient deprivation or exposure to environmental stress indirectly, inhibits TORC1 activity, via activation of the Ssp1-Ssp2 (AMPK catalytic (α) subunit) pathway. Deletion of *ssp1* or *ssp2* inhibited the effect of caffeine on DNA damage-induced cell cycle arrest in cells exposed to phleomycin (Figure 1B, C). In contrast, the effect of torin1 under similar conditions was partially suppressed but not inhibited. Thus, Ssp1 and Ssp2 may facilitate the mitotic effects of caffeine in *S. pombe* cells previously exposed to phleomycin. TORC1 inhibition leads to activation of the Greatwall homologue Ppk18 which, then, activates Igo1. Igo1 in turn, inhibits $PP2A^{Pab1}$ activity causing cells to enter mitosis prematurely with a shortened cell size [15,16,32,33]. Deletion of *ppk18*

did not suppress the ability of either caffeine or torin1 to override DNA damage checkpoints. We noted however, that the *ppk18*Δ mutant reached the maximum level of septation more slowly than wild type cells exposed to phleomycin and caffeine or torin1 (90 min versus 60 min in *ppk18*Δ and *wt* strains, respectively) (Figure 1D). Surprisingly, deletion of co-deletion *cek1* with *ppk18* (which regulates Igo1 together with Ppk18 [16] inhibited the effects of torin1 but not caffeine on phleomycin-induced cell cycle arrest (Figure 1E). In contrast, deletion of *igo1* markedly reduced the ability of both caffeine and torin1 to override DNA damage checkpoint signalling (Figure 1F). We and others have previously demonstrated that caffeine activates both Sty1 and Srk1 [9,34]. Furthermore, Ssp1 is required for TORC1 inhibition and cell cycle re-entry following exposure to environmental stresses [35–37]. Our findings suggest that caffeine-induced activation of Ssp1 and Ssp2 may mediate the mitotic effects of caffeine via Igo1 in *S. pombe*.

Ssp1 and Ssp2 are required for DNA damage checkpoint override in *S. pombe*

Inhibition of DNA damage checkpoint signalling by caffeine and torin1 enhanced phleomycin sensitivity in *wt* as previously reported [8] (Figure 2A). Deletion of *ssp1* and *ssp2* suppressed the ability of both caffeine and torin1 to enhance phleomycin sensitivity relative to wild type cells (Figure 2B and 2C). Indirect TORC1 inhibition may thus be sufficient to facilitate the override of the G2 DNA damage checkpoint. It has been reported that *ssp2*Δ (and presumably *ssp1*Δ) deletion increases TORC1 activity [38]. These observations may explain the increased resistance of these mutants to the effects of torin1. We similarly observed that deletion of *amk2* (The regulatory subunit β of the AMPK kinase) suppressed the ability of caffeine to enhance sensitivity to phleomycin (Figure 2D). Deletion of *ppk18* suppressed the ability of caffeine but not torin1 to enhance sensitivity to phleomycin (Figure 2E). Similar results were observed in a *ppk18*Δ *cek1*Δ double mutant. These findings are in accordance with our previous observations that a strict link between drug-induced mitosis and phleomycin sensitivity does not exist [8] (Figure 2F). Deletion of *igo1* suppressed the ability of caffeine but not that of torin1 to enhance sensitivity to phleomycin, despite suppressing the ability of both compounds to override DNA damage checkpoint signalling (Figure 1F, 2G, and 2H). We previously reported that the deletion of *pab1* exerts a similar effect on phleomycin sensitivity in this context [8]. Hence, the link between DNA damage checkpoint override and enhanced DNA damage sensitivity is not necessarily a direct one. Furthermore, the proper regulation of PP2A activity is required for DNA repair and checkpoint recovery. Thus, the sensitizing effect of torin1 may result from perturbations to DNA damage repair pathways [26,39]. Our results

nevertheless indicate that caffeine may induce the activation of Ssp1 and Ssp2. Ssp2 in turn, inhibits TORC1 signalling to advance mitosis. Ssp1 additionally, positively regulates Cdc25 activity and is required for cell cycle re-entry under environmental stress conditions [35].

ATP blocks the effects of caffeine and torin1 on mitotic progression

Suppression of intracellular ATP levels by environmental stress conditions such as nitrogen withdrawal, glucose deprivation and potassium chloride (KCl), has been linked to the activation of the Ssp1-Ssp2 signalling pathway in *S. pombe* [37,38]. As the addition of extracellular ATP can block Ssp2-TORC1-mediated mitotic progression, we investigated whether ATP could similarly inhibit the effects of caffeine on cell cycle progression under genotoxic conditions. Co-addition of 10 mM ATP clearly blocked the ability of caffeine to enhance sensitivity to phleomycin (Figure 2I). Microscopic analyses indicated that cells exposed to phleomycin were elongated compared to untreated cells (Figure 3A and 3B, average sizes 12 µm versus 29.2 µm, Wilcoxon $p<0.01$). When phleomycin-treated cells were co-exposed to ATP in the presence of caffeine, they remained significantly more elongated compared to cells exposed to caffeine alone (Figure 3A). Interestingly, ATP also suppressed the ability of torin1 to enhance sensitivity to phleomycin albeit to a lesser degree (Figure 2I). In contrast, to cells co-exposed to ATP and caffeine in the presence of phleomycin however, cells exposed to ATP and torin1 were not significantly different in size (Figure 3A, 3B). The average cell length in cultures exposed to phleomycin and caffeine was shorter, than that of cells exposed to phleomycin and caffeine in the presence of ATP (17.22 µm versus 21.7 µm, Wilcoxon $p<0.01$ between the two conditions, Figure 3B). In contrast, the average length of cells exposed to phleomycin and torin1 was similar to those co-cultured in the presence of ATP (average sizes for phleomycin and torin1 treatment was 13.3 µm while for Phleomycin, torin1 and ATP was 14.2 µm, Wilcoxon $p>0.05$). We did not however, note a significant effect of ATP on the ability of either caffeine or torin1 to advance mitosis (Data not shown). ATP may thus attenuate the sensitising effect of caffeine and torin1 to DNA damage via different mechanisms. Alternatively, ATP may increase TORC1 activity by suppressing ssp2 or as the physiological substrate, outcompete the two compounds.

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

Mutants lacking *ssp1* respond weakly to the mitotic effects of caffeine (Figure 1B and Figure 2B). Microscopic analyses demonstrated, that *ssp1*Δ mutants are longer that wt cells following exposure to phleomycin (29.2 µm versus 35.4 µm, Wilcoxon p<0.01). In addition, *ssp1*Δ mutants were longer than wt cells when exposed to phleomycin in the presence of caffeine (17.2 µm versus 26.1 µm, Wilcoxon p<0.01) or torin1 (13.3 µm versus 25.9 µm, Wilcoxon p<0.01) (Figure 3C and 3D). Ssp1 has been reported to regulate cell cycle re-entry, following Sty1-mediated Cdc25 inhibition, by suppressing Srk1 expression [37,40]. As Srk1 attenuates the effect of caffeine on cell cycle progression in a Sty1-dependent manner [9], we investigated its effect on the *ssp1*Δ mutant strain under heat and osmotic stress conditions. Caffeine exacerbated this phenotype of *ssp1*Δ mutants exposed to heat stress (Figure 4A and 4B). At 35° C *ssp1*Δ mutants are delay progression through mitosis [41]. This effect was clearly exacerbated by co-exposure to 10 mM caffeine (15.1 µm versus 18.7 µm after 4 h and 19.5 µm versus 37.8 µm after 24 h, in all comparisons Wilcoxon p<0.01) (Figure 4A and 4B). In contrast, both rapamycin (100 ng/ mL) and torin1 (5 µM) were sufficient to overcome the *ssp1*Δ phenotype under these conditions (13.4 µm versus 15.4 µm and 8.9 µm versus 21.8 µm at 4h and 24h for rapamycin and torin1 respectively) (Figure 4A and 4B). *ssp1*Δ mutants exposed to 1.0 M KCl for 4 h and 46 h became elongated from 14.3 µm and 13.4 µm to 16 µm and 25 µm at 4 and 46 h respectively (Wilcoxon p<0.01, Figure 4C). Exposure to 10 mM caffeine alone for 4h and 46 h also increased the average cell length from 14.3 µm and 13.4 µm to 18.1 µm and 27.2 µm respectively (Wilcoxon p<0.01, Figure 4C). Together, these observations further suggest a role for the Ssp1-Ssp2-TORC1 pathway in mediating the effects of caffeine on cell cycle progression under normal and environmental stress conditions.

Ssp2 mediates the effect of caffeine on cell cycle progression under normal conditions

We next investigated the role of Ssp2 in mediating the cell cycle effects of caffeine under normal growth conditions. Exposure to 10 mM caffeine or 5 µM torin1 advanced wild type 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). It has previously been reported, that *ssp2* mutants display increased resistance to torin1 because of increased TORC1 activity [15]. As *gsk3* genetically interacts with *ssp2* [42,43], we investigated its role in mediating the activity of caffeine and torin1. Surprisingly, deletion of gsk3 partially suppressed the cell cycle effects of torin1 but not caffeine (Figure 5C). Co-deletion of *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 reported to be higher compared to wild type cells [15,43]. Hence both genes are involved in advancing mitosis in *S. pombe* but with differential effects on cell cycle dynamics.

We compared the effects of caffeine and torin1 on cell cycle progression in wild type and *gsk3*Δ mutants exposed to phleomycin. As previously shown, torin1 was more effective than caffeine at advancing cells exposed to phleomycin into mitosis (Figure 1A and 6A). In contrast, deletion of *gsk3*Δ strongly suppressed the ability of both caffeine and torin1 to drive cells into mitosis (Figure 6B). This differential effect on cell cycle progression was reflected in the ability of both compounds to enhance DNA damage sensitivity. The effect of caffeine and torin1 on DNA damage sensitivity was partially supressed in *gsk3*Δ mutants (Figure 6C and 6D). It is unclear, why Gsk3 mediates the effects of caffeine in cells previously arrested with phleomycin but not under normal conditions (Figure 5A, 5C, 6A and 6B). Gsk3 positively regulates Pub1 stability which in turn regulates Cdc25 stability [44]. Increased TORC1 activity in *gsk3*Δ mutants [42] and strong inhibition of Cdc2 in the presence of phleomycin may attenuate the ability of caffeine to advance mitosis. These observations further demonstrate differential mechanisms control the respective effects of caffeine and torin1 on cell cycle progression.

Caffeine activates Ssp2 and partially inhibits TORC1 signalling

Given the observed effects on cell cycle progression in the different genetic backgrounds, we examined whether caffeine affects phosphorylation events that govern activities of Ssp2 and others that demonstrate modulation of mTOR activity.

Exposure to 10 mM Caffeine but not torin1 (5 µM) induced Ssp2 phosphorylation in *wt S. pombe* cells (Figure 7A). The effect of caffeine on Ssp2 phosphorylation was weaker than that observed following exposure to 0.03 % glucose [45]. Caffeine weakly induced eIF2α phosphorylation in contrast to direct TORC1 inhibition by torin1 (Figure 7B). As TORC1 inhibition downstream effects may vary with stress type, we examined the effect of caffeine on Sck1 which regulates mitosis together with Sck2 in *S. pombe* [32,46]. Caffeine suppressed Sck1-HA expression to a similar degree as caffeine with rapamycin and torin1 (Figure 7C and data not shown). Similarly, exposure to caffeine only moderately suppressed Maf1 phosphorylation (an indicator of TORC1 activity [15]). This effect was enhanced by coexposure to rapamycin. In contrast, torin1 completely abolished Maf1 phosphorylation

(Figure 7D). Caffeine also induced Ssp2 phosphorylation in the presence of phleomycin independently of ATP (Figure 7E). As expected, caffeine-induced Ssp2 phosphorylation was abolished in mutants (Figure 7F). In contrast to its effect on proliferating cells, caffeine unlike torin1 did not induce eIF2α phosphorylation in cultures pre-treated with phleomycin (Figure 6G). We also observed that 10 mM alone or in combination with 100 ng/mL rapamycin suppressed Sck1-HA expression in cells previously exposed to phleomycin. In contrast, 100 ng/ mL of rapamycin alone did not suppress Sck1-HA expression in agreement with previous reports [46] (Figure 7H). Thus, caffeine partially inhibits TORC1 activity by activating Ssp2 in an Ssp1-dependent manner. These observations suggest that caffeine only weakly inhibits TORC1 activity and in contrast to torin1, does not inhibit cell cycle progression [47].

Discussion

We have identified a role for the *S. pombe* Ssp1 and AMPK homologue Ssp2, in mediating the cell cycle effects of caffeine. Caffeine has generated much interest, by virtue of its ability to override DNA damage signalling and extend chronological life span (CLS) in various organisms [5,23,31,48–50]. A clearer understanding of the mechanisms by which caffeine exerts this activity, can lead to the development of novel therapeutic strategies [7]. In *S. pombe*, the ATM related kinase Rad3 is activated in response to DNA damage and co-ordinates cell cycle arrest with DNA damage repair. Caffeine was initially reported to override DNA damage checkpoint signalling through Rad3 inhibition and its homologues *in vitro* [19]. Recent studies have suggested that caffeine overrides DNA-damage checkpoint signalling by inhibiting TORC1 activity in *S. pombe* [5,25]. Under conditions of nutrient deprivation or environmental stress, Ssp2 inhibits TORC1 leading to the activation of Ppk18/Cek1-Igo1 signalling, $PP2A^{Pab1}$ inhibition and accelerated progression through mitosis [15,51]. Additionally, Gsk3 acts downstream of TORC2 to regulate cell division and genetically interacts with Ssp2 [42,43]. As $PP2A^{Pab1}$ regulates Cdc25 and Wee1 activity, TORC1 regulates the timing of cell division via modulation of Cdc2 activity in response to nutritional and environmental cues [16,44,52]. Direct TORC1 inhibition with torin1 mimics the effects of caffeine on cells exposed to phleomycin [8].

To shed light into the exact mechanism underlying the caffeine-dependent modulation of cell cycle progression and the interplays with TORC1 function, we have investigated the potential role of the Ssp1-Ssp2 signalling in mediating its effects on DNA damage checkpoints. Deletion of the *ssp1* and *ssp2* genes suppressed the ability of caffeine to override DNA damage checkpoint signalling in *S. pombe* cells exposed to phleomycin. In contrast, co-exposure to torin1 (a direct TORC1 and TORC2 inhibitor) was able to advance cells into mitosis.

Accordingly, deletion of *ssp1* and *ssp2* also attenuated the ability of caffeine to enhance sensitivity to DNA damage. These findings suggest that caffeine modulates cell cycle progression by indirectly inhibiting TORC1 via the Ssp1-Ssp2 signalling pathway. Caffeine activates Sty1 signalling, which is coupled to Ssp1, Ssp2, Rad24, Cdc25 and Wee1 activity under environmental stress conditions [9,34,36,53–55]. While Ssp2 responds mainly to a drop in cellular ATP concentration levels, it has also been shown to, specifically, mediate the response to specific environmental cues such as nitrogen limitation and oxygen stress [15,37,38,52]. We have noted that co-exposure to ATP suppressed the cell cycle effects of caffeine on cells previously treated with phleomycin. These observations suggest that caffeine modulates cell cycle kinetics by indirectly inhibiting TORC1 (Figure 8). Interestingly, Gsk3 mediated the effects of torin1 but not caffeine on cell cycle dynamics under normal conditions but not in the presence of phleomycin. Higher TORC1 activity in *gsk3*Δ mutants may attenuate the ability of caffeine to modulated cell cycle dynamics under genotoxic conditions [5,31,43]. These findings provided further evidence to support the notion, that caffeine and torin1 modulate cell cycle progression by distinct but overlapping mechanisms.

Ssp1 regulates cellular responses to various environmental stresses in *S. pombe*. In particular, its negative effect on Srk1 regulates cell cycle progression under these conditions. In addition, Ssp1 also regulates cellular morphology when cells are exposed to potassium chloride (KCl) [37,40,41,54,56,57]. We have, previously, demonstrated that caffeine induces Sty1-dependent Srk1 activation [9]. In the current study, caffeine enhanced the phenotypes of *ssp1*Δ mutants exposed to osmotic or heat stress. Deletion of *ssp1* also abolished caffeine-induced Ssp2 and eIF2α phosphorylation. Ssp1 is thus required to facilitate cell cycle progression in the presence of caffeine but not rapamycin and torin1. Exposure to caffeine mimics the effects of other environmental stresses on cell cycle progression in S. *pombe* [9,34]. Thus, caffeine appears to activate Ssp2 through the activation of Ssp1. Ssp1-mediated Ssp2 activation leads, in turn, to the partial inhibition of TORC1. Rapamycin enhances the activity of caffeine in *S. pombe* suggesting they might inhibit TORC1 activity via different mechanisms [5,31]. Our findings clearly demonstrate that caffeine inhibits TORC1 activity in a manner different to torin1.

We have observed that *ssp2* deletion did not completely suppress the effects of caffeine under normal cell cycle conditions, for example, in the absence of genotoxic agents. We have previously shown that caffeine induces the stabilisation of Cdc25 and Wee1 degradation [8,9]. The deletion of *igo1* abolished the effects of caffeine and torin1 on cell cycle dynamics. Exposure to caffeine alone or in the presence of phleomycin, suppressed Sck1 expression consistent with the role of this kinase in regulating mitotic progression via the phosphorylation of Ppk18 [33]. Surprisingly, the co-deletion of *ppk18* and *cek1* suppressed the effect of torin1

but not caffeine on mitosis. Since only Ppk18 and Cek1 have been identified as regulators of Igo1, additional mechanisms may exist to regulate its activity [16,32]. These observations further highlight mechanistic differences between caffeine and torin1-induced mitotic effects. TORC1 inhibition and downstream $PP2A^{Pab1}$ inhibition may account for the effect of caffeine on Wee1 stability [14]. We are currently investigating how caffeine mediates the stabilisation of Cdc25. The TORC2 complex regulates the stability of nutrient receptors in response to nutrient signalling via the ubiquitylation pathway [58,59]. It has recently been shown that TORC2 activation leads to the Gad8-dependent phosphorylation of Gsk3. This prevents Gsk3-mediated stabilization of the Pub1 E3 ligase and increases the levels of its substrates during nutrient stress. Interestingly, Pub1 is also the E3 ligase for Cdc25, suggesting that TORC2 regulates the expression of these proteins. Conversely, TORC2 inhibition with torin1 elevates Pub1 expression [44]. It is currently unclear whether caffeine activates or inhibits TORC2. In *S. pombe*, TORC2 is required for cellular resistance to various environmental stresses, replication stress and DNA damage [60,61]. Given that its effects on cell cycle progression mimic environmental stress signalling, it is probable that caffeine activates TORC2 signalling [62]. This would in theory, result in suppressed Pub1 E3 ligase activity and increased Cdc25 expression [44,63]. Future studies will investigate the effects of caffeine on TORC2 and its downstream targets such as Gsk3 and Pub1.

Caffeine can also exert lethal effects on certain *S. pombe* TORC1 pathway mutants without advancing cell cycle progression such as $PP2A^{Pab1}$ [8]. In fact, the Rad3-regulated DNA damage response pathway is required for resistance to caffeine [34]. Similarly, *igo1* deletion largely abolished the effect of torin1 on cell cycle kinetics but failed to suppress DNA damage sensitivity. As protein phosphatases also play in the DNA damage response, the deregulation of their activity by caffeine or torin1 could enhance DNA damage sensitivity without modulating cell cycle progression [27,39,64]. It has also been reported, that S6K activity plays a role in regulating DNA damage repair pathways [28]. These observations suggest that downstream components of the TORC1 signalling pathway are required for resistance to caffeine under genotoxic conditions.

In summary, our findings demonstrate that caffeine potentially modulates cell cycle progression by inhibiting TORC1 activity in *S. pombe* in an indirect manner. Exposure to caffeine activates the environmental stress response, leading to the Ssp1 and Ssp2 dependent inhibition of TORC1. The inhibition of TORC1 activity, results in the downstream inhibition of $PP2A^{Pab1}$ activity and accelerated entry into mitosis [33]. Perplexingly, the previously reported regulators of *S. pombe* $PP2A^{Pab1}$ activity, Ppk18 and Cek1, appear not to be required for the mitotic effects of caffeine. Our studies also suggest that caffeine may

enhance DNA damage sensitivity independently of mitosis by interfering with DNA damage repair mechanisms. Increasing the sensitivity of tumour cells to chemotherapy and radiotherapy remains an attractive approach in battling cancer. Recent studies suggest that mTOR inhibition may serve such a purpose. A clearer understanding of how caffeine and other mTOR inhibitors enhance DNA damage sensitivity, particularly in the context of AMPK signalling can lead to the development of novel chemo- and radio-sensitisation agents as well as dietary strategies to promote healthy aging [7,65].

Competing interests

The authors declare that they have no competing interests.

Author's contributions

J.P.A., C.R. conceived and designed the study. J.P.A. carried out experiments and analysed the data. J.P.A. and C.R. wrote the manuscript. The authors read, revised and approved the final manuscript.

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Figure and Table legends

Table 1: List of strains used in this study.

Figure 1: Ssp1 and Ssp2 mediate the mitotic effects of caffeine. A-F. The indicated strains were grown to log phase and incubated with 5 μg/ mL of phleomycin for 2 h. Cultures were then exposed to 10 mM caffeine or 5 μM torin1 and harvested at the indicated time points. Cells were stained with calcofluor and the septation index determined by microscopy (n >200)**.** Error bars represent the mean S.E. from 3 independent experiments.

Figure 2: Ssp1 and Ssp2 mediate the mitotic effects of caffeine. A-H. *wt S. pombe* cells were exposed to 5 μg/ mL phleomycin as indicated for 2 h. Cell were then incubated for a further 2 h with 10 mM caffeine or 5 μM torin1, adjusted for cell number (OD_{600} 0.5), serially diluted, and plated on YES agar plates for 3-5 days. I. *wt* cells were exposed to 5 μg/ mL phleomycin as indicated for 2 h. Cells were then incubated for a further 2 h with 10 mM caffeine or 5 μM torin1 alone or in combination with 10 mM ATP, adjusted for cell number, serially diluted and plated on YES agar plates for 3- 5 days.

Figure 3. Caffeine activity is suppressed by ATP and enhances the *ssp1*Δ phenotype. A. *wt* cells were exposed to 5 μg/ mL phleomycin as indicated for 2 h. Cells were then incubated for a further 2 h with 10 mM caffeine alone or with 10 mM ATP as indicated. B.

*ssp1*Δ mutants were incubated with 10 mM caffeine and or 0.6 M KCl for 4h. b. Cells in A were stained with calcofluor and cell length at division measured for each sample (n= 50). C. *ssp1*Δ mutants were exposed to 5 µg/ mL phleomycin and then treated as in A. Cell length was determined by calcofluor staining and microscopy for each sample (n=50).

Figure 4. Ssp1 mediates the effects of caffeine on cell division. A. *ssp1Δ* mutant cells were incubated at 35 °C in the presence of 10 nM caffeine, 100 ng/ mL rapamycin or 5 µg/ mL torin1 for 4h and 24h as indicated. B. Cells were treated as in A, stained with calcofluor and cell length determined by microscopy (n=50). C. *ssp1*Δ mutants were incubated at 30 °C in the presence of 1M KCl, 1M KCl with 10 mM caffeine or 10 mM caffeine alone for 4 h and 46h as indicated. Cells were stained with calcofluor, and cell length determined by microscopy (n= 50).

Figure 5. Caffeine modulates cell cycle progression independently of Gsk3. A-E. Log phase *wt*, *gsk3*Δ, *ssp2*Δ, *gsk3*Δ *ssp2*Δ gskΔ ssp1Δ and cells were cultured with 10 mM caffeine or 5 µM torin1 and harvested at the indicated time points. Cells were stained with calcofluor and the septation index determined by microscopy. (n >200). Error bars represent the mean S.E. from 3 independent experiments.

Figure 6. Differential roles for Gsk3 in mediating the cell cycle effects of caffeine and torin1. A. *wt* cells were exposed to 5 µM phleomycin for 2 h and then exposed to 10 mM caffeine or 5 µ M torin1 for a further 2 h. Cells were harvested at the indicated time points. Cells were stained with calcofluor and the septation index determined by microscopy (n >200). B. *gsk3* mutants were treated as in A. C. *wt S. pombe* cells were exposed to 5 µg/ mL phleomycin as indicated for 2 h. Cell were then incubated for a further 2 h with 10 mM caffeine or 5 µM torin., adjusted for cell number, serially diluted and plated on YES agar plates for 3-5 days. D. *gsk3* mutants were treated as in C.

Figure 7. Caffeine activates Ssp2. A. *wt* cells were grown to log phase and incubated with 10 mM caffeine or 5 µM torin1 for 1 hour. Cell lysates were resolved by SDS-PAGE and probed with antibodies specific for phospho-Ssp2 and total Ssp2. Ponceau S was used to monitor gel loading. B. Caffeine moderately inhibits eIF2 activity. Cells were treated as in a. and probed with antibodies specific for phospho- and total eIF2α. Ponceau S was used to monitor gel loading. Arrow indicate total eIF2α band. C. Caffeine suppresses Sck1 expression. Cells expressing Sck1- HA were exposed to 10 mM caffeine with or without 100 ng/ mL rapamycin or 5 µM torin1 for 90 mins and treated as in a and probed with antibodies directed against HA. D. Caffeine modestly inhibits TORC1 activity. Cells were treated as in a and probed with an anti-V-5 antibody to detect Maf1 phosphorylation. Arrows indicate phosphorylated Maf1

species. E. *wt* cells were pre-treated with 5 µg/ mL phleomycin for 2 h and then exposed caffeine and torin1 with or without 10 mM ATP as indicated for 60 minutes. Cell lysates were treated as in A. F. *ssp1Δ* cells were pre-treated with 5 µg/ mL phleomycin for 2 h and then exposed caffeine and torin1 or caffeine and torin1 alone as indicated. Cell lysates were treated as in A. G. wt cells were treated as in f. Cell lysates were analysed as in B. H. The Sck1- HA strain was incubated for 2h with 5 µg/ mL phleomycin, followed by incubation with 10 mM caffeine and 100 ng/ mL rapamycin alone and in combination, or with 5 µM torin1 alone for another 60 minutes. Cell lysates were treated as in C.

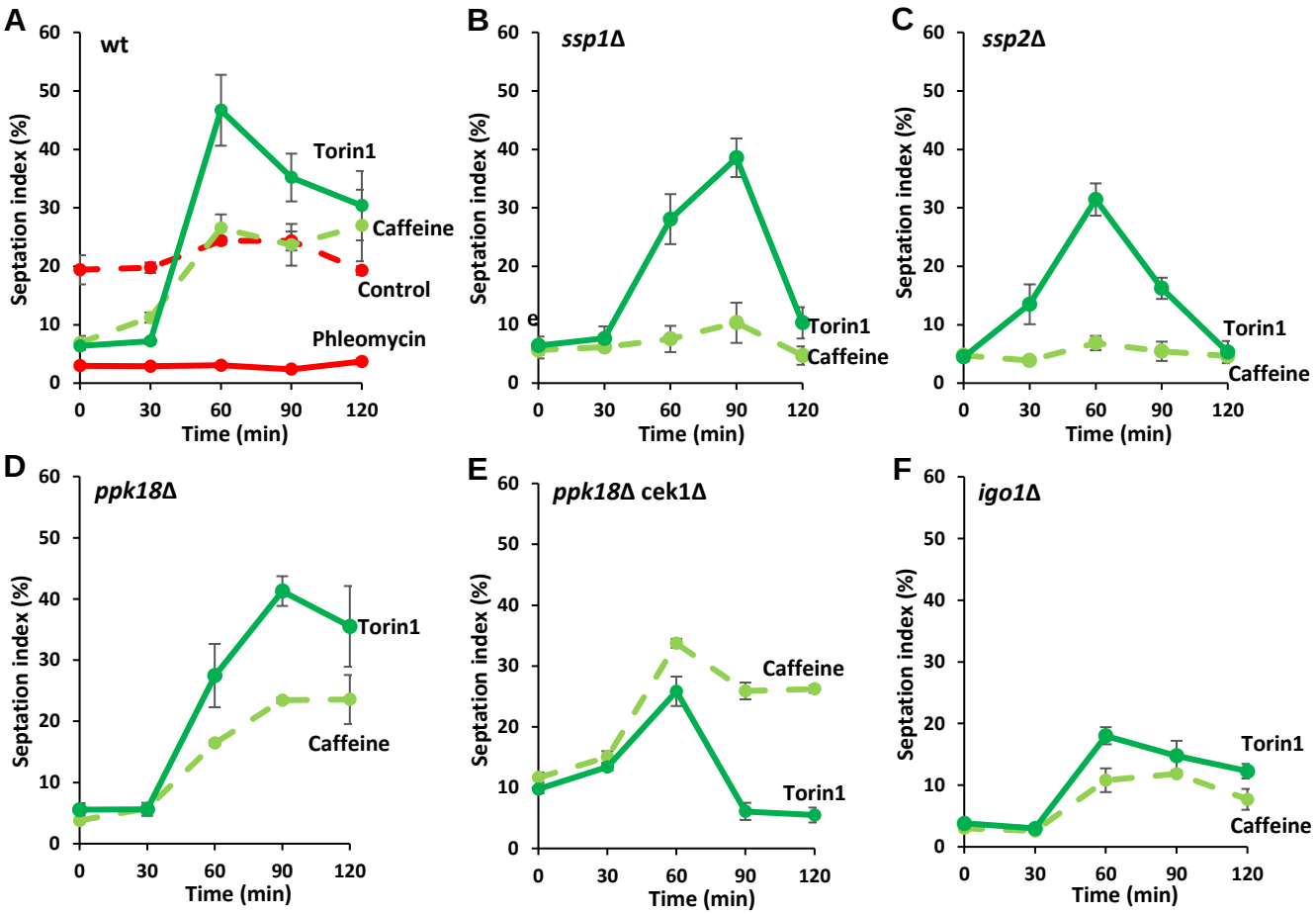
Figure 8. Molecular mechanisms of caffeine mediated cell cycle effects. Caffeine and other environmental stresses activate Sty1 which inhibits Cdc25 activity via Srk1 to transiently delay progression into mitosis. Similarly, environmental stresses activate Ssp1 which is required to suppress Srk1 activity to permit entry into mitosis. Ssp1 also activates Ssp2, which in turn inhibits TORC1 activity. Environmental stressors including caffeine thus inhibit TORC1 indirectly, in contrast to torin1 which directly inhibits the kinase complex. TORC1 inhibition facilitates inhibition of $PP2A^{Pab1}$ via the Ppk18 and Cek1 kinases and the endosulfine Igo1. The inhibition of $PP2A^{Pab1}$ leads to the activation of Cdc25 and inhibition of Wee1 to drive cells into mitosis. Under genotoxic conditions, the Rad3 kinase inhibits Cdc2 activity by inhibiting Cdc25 and activating Wee1 activity.

Table 1

Strain	Genotype	Source/Reference
h⁻ L972	*h-*	Laboratory stocks
tor1Δ	*h- tor1::kanMX6*	Laboratory stocks/[5]
ssp1Δ	*h - ssp1::kanMX6*	Moseley laboratory/[37,57]
pom1- GFP ssp1Δ	*pom1-GFP-kanMX6 ssp1Δ::ura4 ura4-D18*	Bahler laboratory
ssp2Δ	*h - ssp2::kanMX6, h + ssp2::kanMX6*	Moseley laboratory and Laboratory stocks/[37,57]
amk2Δ	*h - amk2::kanMX6*	Moseley laboratory/[37,57]
gsk3Δ	*h− gsk3::kanMX6*	Laboratory stocks/[43]
gsk3Δ ssp1Δ	*h− ssp1::kanMX6 gsk3::hphMX6*	Laboratory stocks/[43]
gsk3Δ ssp2Δ	*h− ssp2::kanMX6 gsk3::hphMX6*	Laboratory stocks/[43]
gsk3Δ amk2Δ	*h− amk2::natMX6 gsk3::hphMX6*	Laboratory stocks/[43]
ppk18Δ	*ppk18Δ::KanMX6 h+*	Moseley laboratory
cek1Δ ppk18Δ	*h+ ppk18::KanMX6 cek1::ura4+ ura4-D18*	Moreno laboratory
igo1Δ	*h- igo1Δ::KanMX6*	Moseley laboratory/[37,57]
sck1-3HA	*h+ sck1-3HA:natMX6*	YGRC*
Maf1.pk	*maf1.pk::KanMX6*	Petersen laboratory/[15]

* Yeast Genetic Resource Center (YGRC) Japan

Figure 1


A
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Time (min)
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Caffeine
Control
Phleomycin
B
ssp1Δ
Septation index (%)
Time (min)
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Caffeine
C
ssp2Δ
Septation index (%)
Time (min)
Torin1
Caffeine
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ppk18Δ
Septation index (%)
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Caffeine
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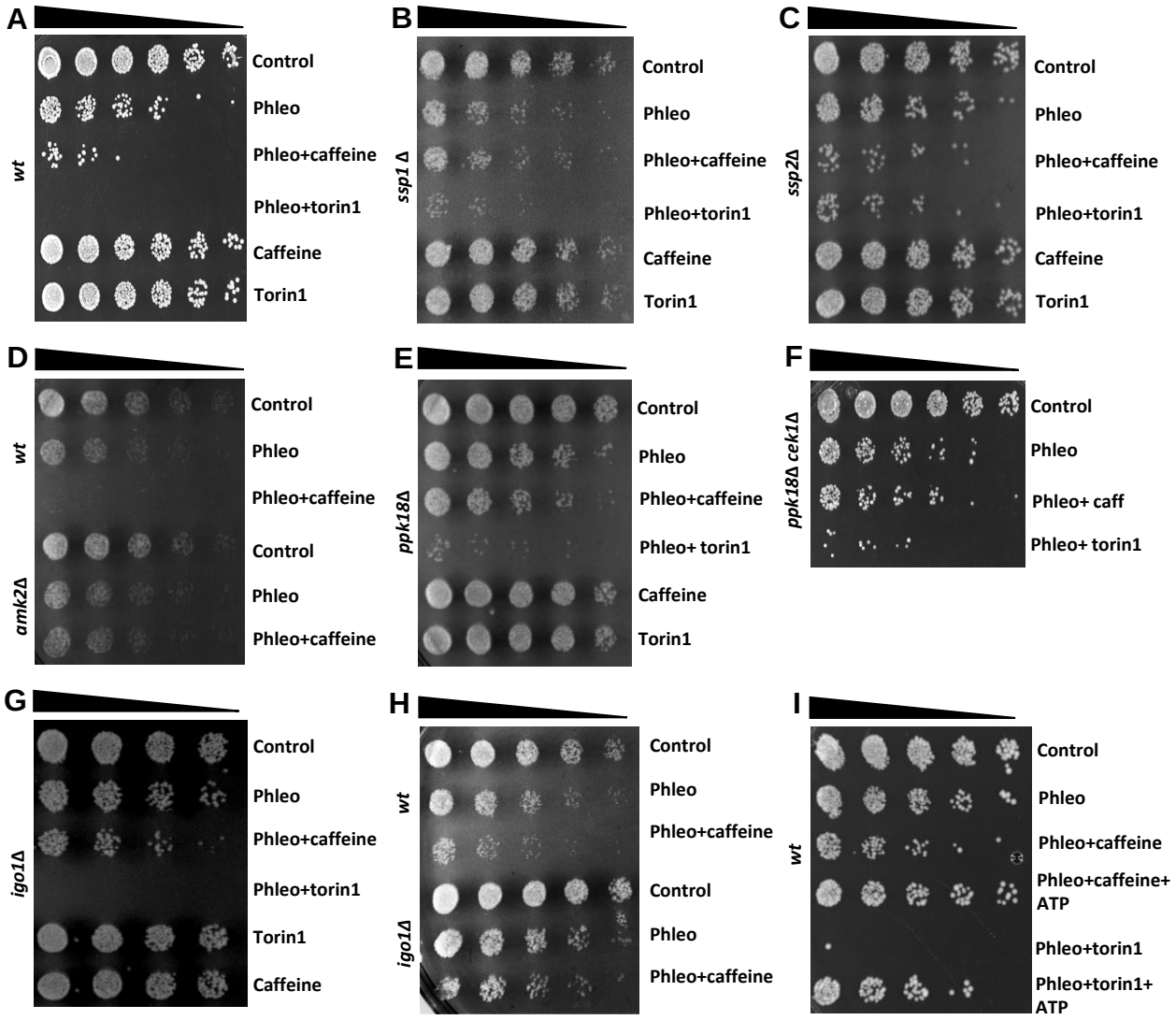
Septation index (%)
Time (min)
Torin1
Caffeine

Figure 2


A
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Phleo+torin1
Caffeine
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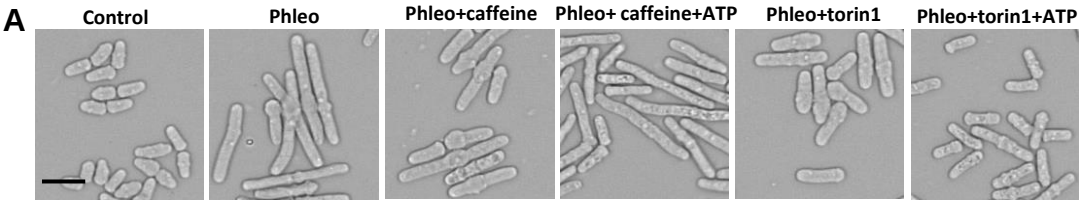
Phleo+torin1+ ATP

Figure 3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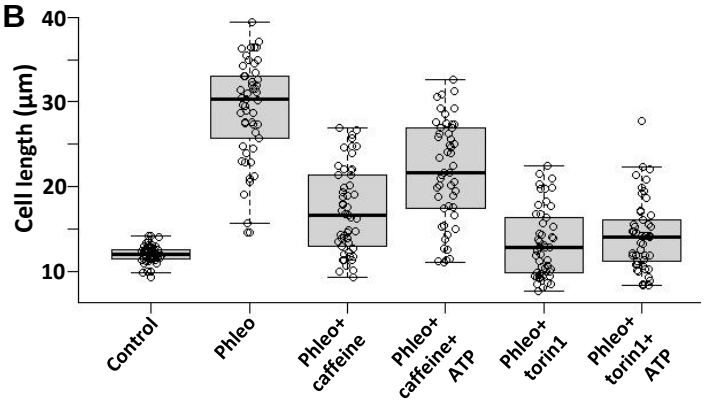
Phleo+torin1+ATP


B
Cell length (µm)
40
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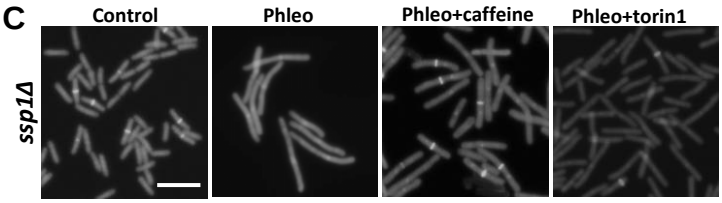
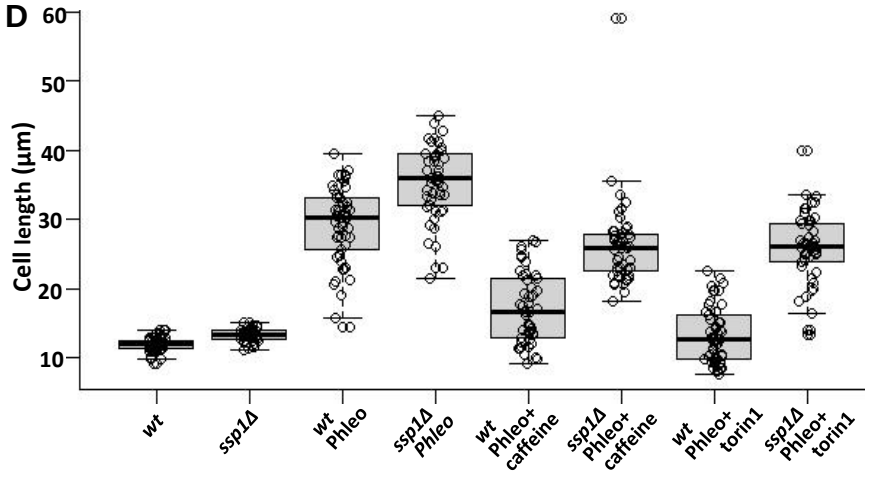
Phleo
Phleo+ caffeine
Phleo+ caffeine+ ATP
Phleo+ torin1
Phleo+ torin1+ ATP


C
Control
Phleo
Phleo+caffeine
Phleo+torin1
ssp1Δ


D
Cell length (µm)
60
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wt Phleo+ caffeine
ssp1Δ Phleo+ caffeine
wt Phleo+ torin1
ssp1Δ Phleo+ torin1

Figure 4

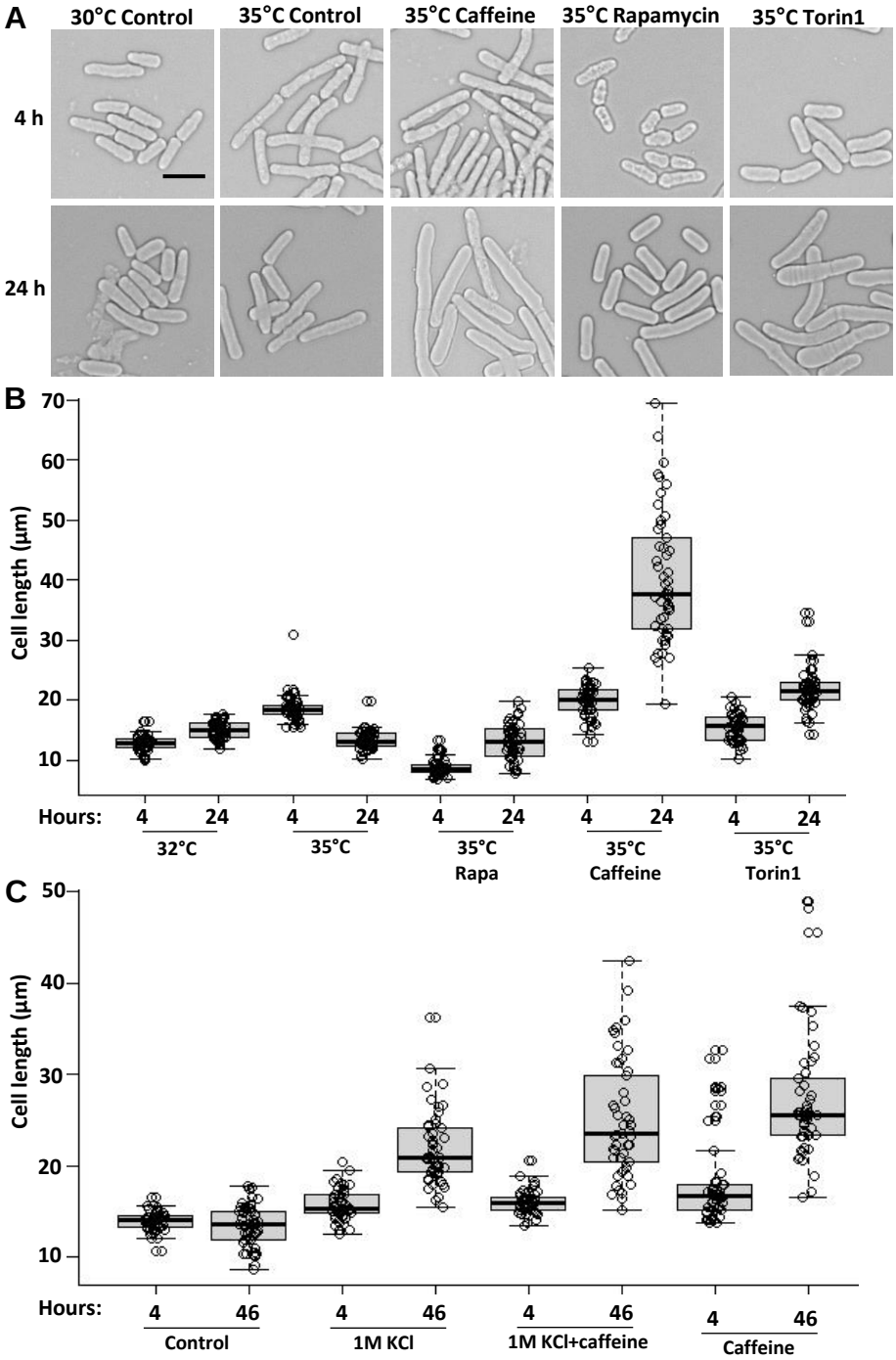

A
30°C Control
35°C Control
35°C Caffeine
35°C Rapamycin
35°C Torin1
4 h
24 h
B
Cell length (µm)
70
60
50
40
30
20
10
Hours:
4
24
32°C
35°C
35°C Rapa
35°C Caffeine
35°C Torin1
C
Cell length (µm)
50
40
30
20
10
Hours:
4
46
Control
1M KCl
1M KCl+caffeine
Caffeine

Figure 5

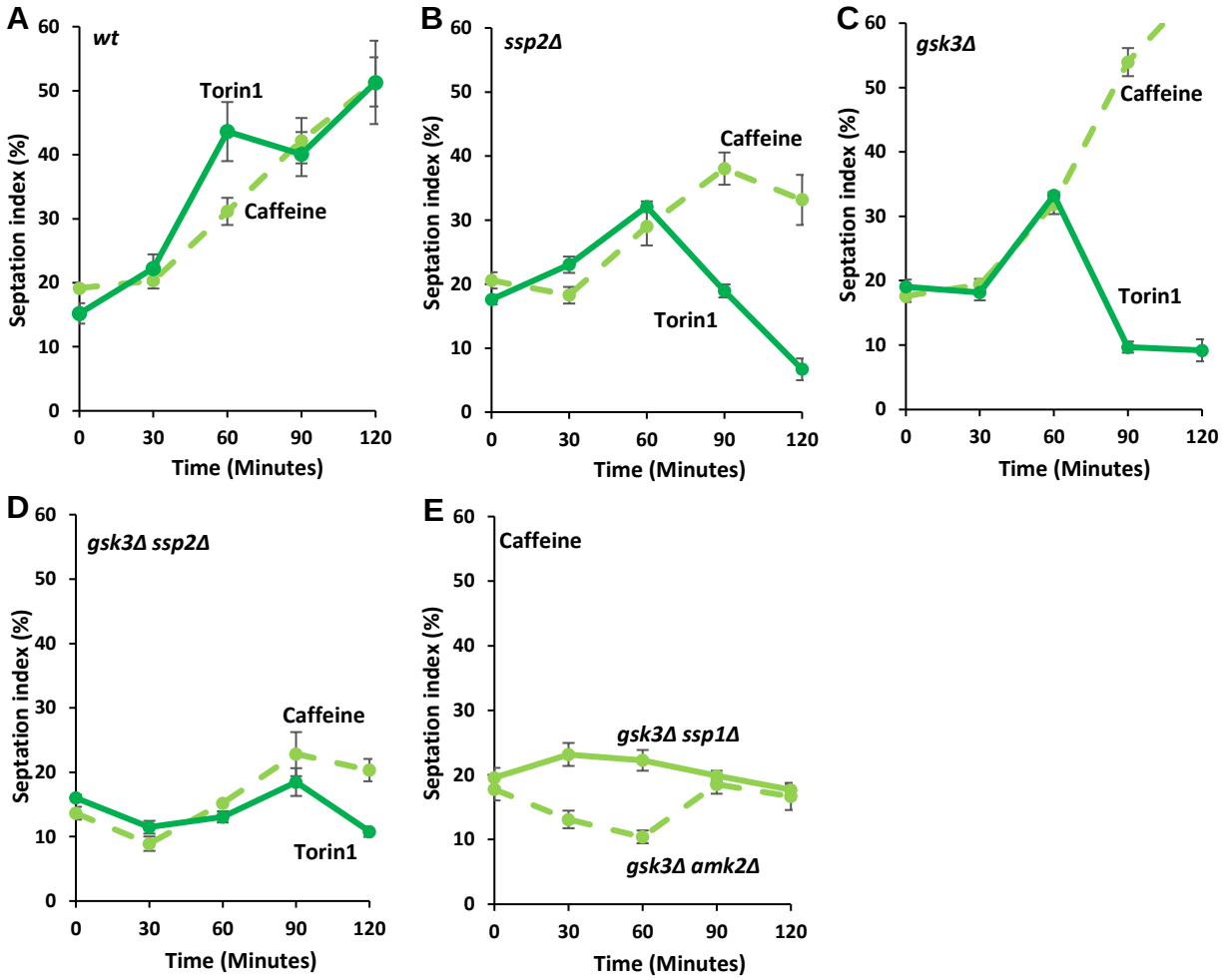

A
wt
Torin1
Caffeine
Septation index (%)
Time (Minutes)
B
ssp2Δ
Caffeine
Torin1
Septation index (%)
Time (Minutes)
C
gsk3Δ
Caffeine
Torin1
Septation index (%)
Time (Minutes)
D
gsk3Δ ssp2Δ
Caffeine
Torin1
Septation index (%)
Time (Minutes)
E
Caffeine
gsk3Δ ssp1Δ
gsk3Δ amk2Δ
Septation index (%)
Time (Minutes)

Figure 6

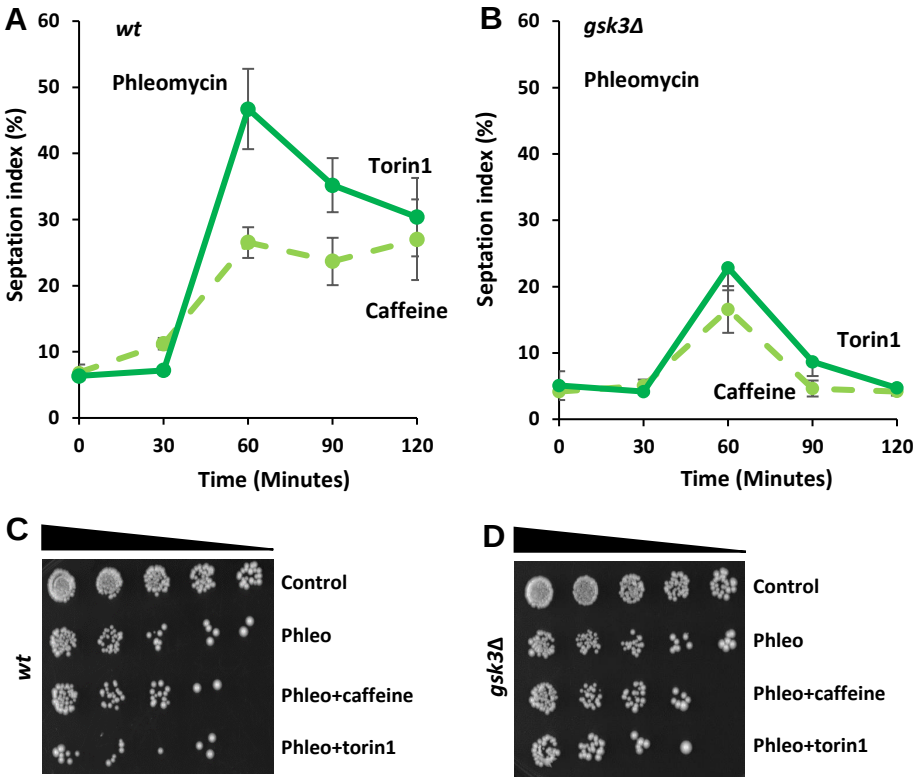

A
wt
Phleomycin
Torin1
Caffeine
Septation index (%)
Time (Minutes)
B
gsk3Δ
Phleomycin
Torin1
Caffeine
Septation index (%)
Time (Minutes)
C
wt
Control
Phleo
Phleo+caffeine
Phleo+torin1
D
gsk3Δ
Control
Phleo
Phleo+caffeine
Phleo+torin1

Figure 7

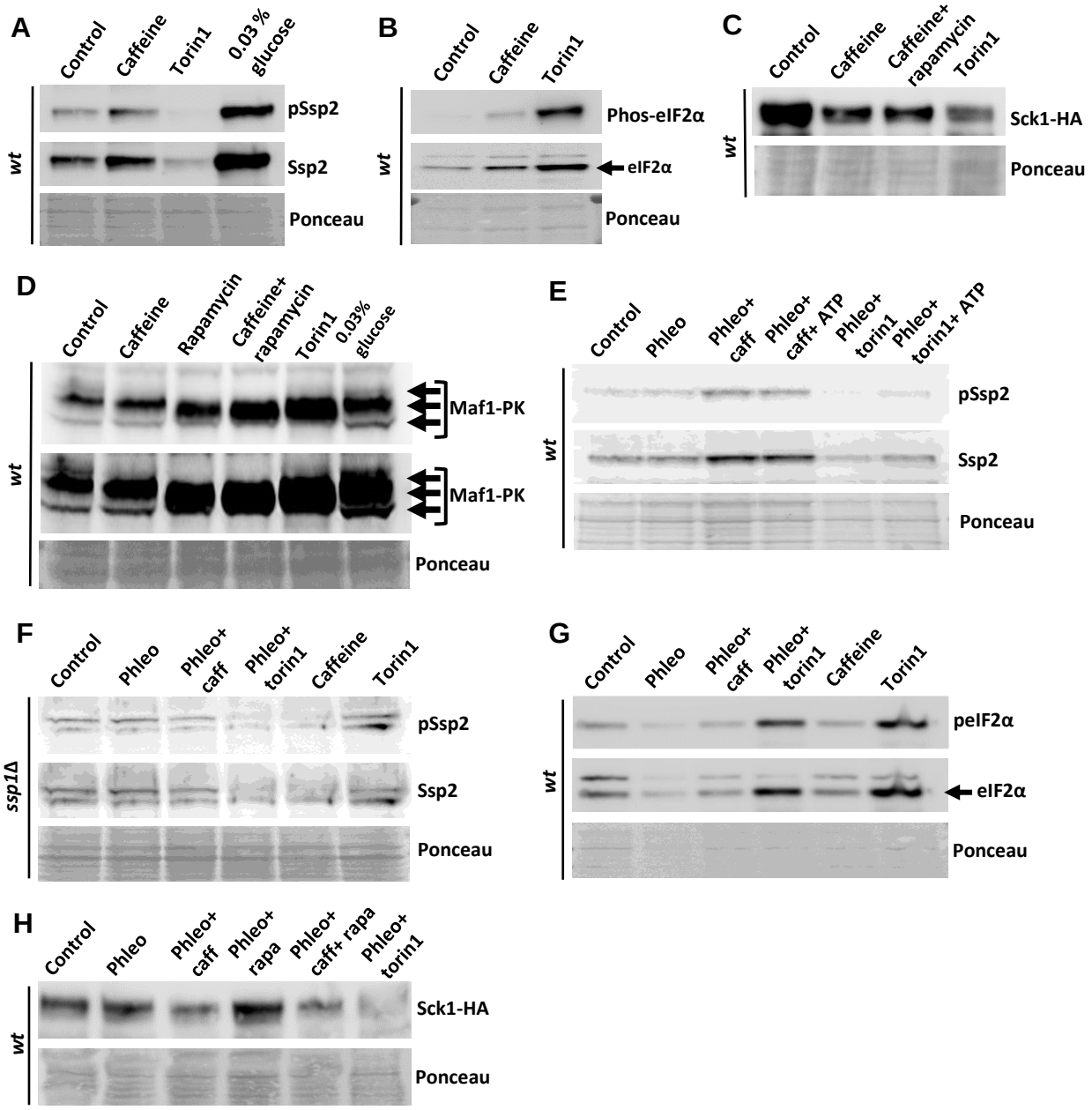
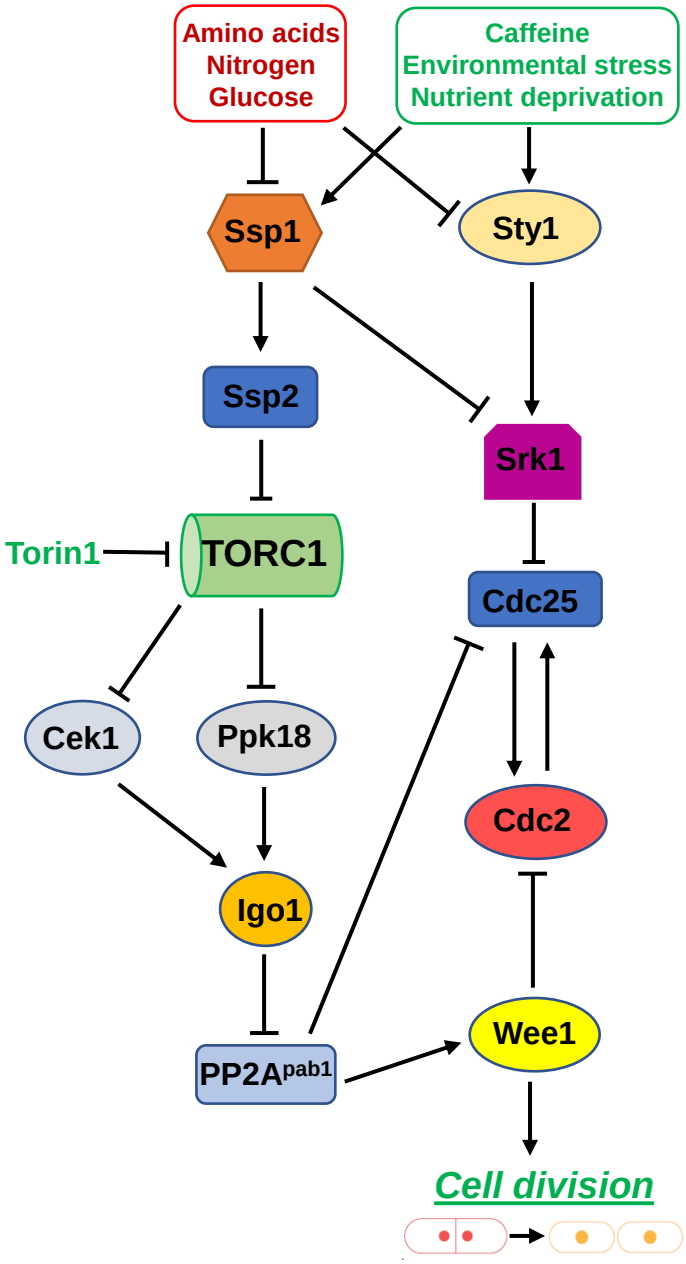

A
Control
Caffeine
Torin1
0.03 % glucose
wt
pSsp2
Ssp2
Ponceau
B
Control
Caffeine
Torin1
wt
Phos-eIF2α
eIF2α
Ponceau
C
Control
Caffeine
Caffeine+ rapamycin
Torin1
wt
Sck1-HA
Ponceau
D
Control
Caffeine
Rapamycin
Caffeine+ rapamycin
Torin1
0.03% glucose
wt
Maf1-PK
Maf1-PK
Ponceau
E
Control
Phleo
Phleo+ caff
Phleo+ caff+ ATP
Phleo+ torin1
Phleo+ torin1+ ATP
wt
pSsp2
Ssp2
Ponceau
F
Control
Phleo
Phleo+ caff
Phleo+ torin1
Caffeine
Torin1
ssp1Δ
pSsp2
Ssp2
Ponceau
G
Control
Phleo
Phleo+ caff
Phleo+ torin1
Caffeine
Torin1
wt
peIF2α
eIF2α
Ponceau
H
Control
Phleo
Phleo+ caff
Phleo+ rapa
Phleo+ caff+ rapa
Phleo+ torin1
wt
Sck1-HA
Ponceau

Figure 8


Amino acids
Nitrogen
Glucose
Caffeine
Environmental stress
Nutrient deprivation
Ssp1
Sty1
Ssp2
Srk1
Torin1
TORC1
Cdc25
Cek1
Ppk18
Cdc2
Igo1
Wee1
PP2A^pab1
Cell division