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 is among the most widely consumed neuroactive compounds in the world. It induces DNA damage checkpoint signalling override and enhances sensitivity to DNA damaging agents. However, the precise underlying mechanisms have remained elusive. The Ataxia Telangiectasia Mutated (ATM) 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. In the fission yeast *Schizosaccharomyces pombe* (*S. pombe*), 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 activation of Ssp2. Deletion of *ssp1* and *ssp2* suppresses the effects of caffeine on cell cycle progression. In contrast, direct inhibition of TORC1 enhances DNA damage sensitivity in these mutants. These observations suggest that caffeine overrides DNA damage signalling, in part, via the indirect inhibition of TORC1 through Ssp2 activation. 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 a clear understanding of the mechanism of how 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: DNA damage response, environmental stress, ATP, phleomycin, ATP, AMPK, Igo1, Rad3

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 and other gases as well as chemotherapeutic agents [10–12]. The phosphatidylinositol 3-kinase-related serine/threonine kinase (PIKK) Rad3 (a homologue of mammalian ataxia telangiectasia mutated or ATM), is activated in response to DNA damage. Rad3 activation and phosphorylation creates binding sites for adapter proteins that facilitate the activation of downstream kinases Cds1 and Chk1 in the S phase 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 [16,17].

Initial studies 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 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 checkpoint signalling. Downstream of TORC1, caffeine failed to accelerate *ppk18*Δ but not *igo1*Δ and partially overrode DNA damage checkpoint signalling. Surprisingly, TORC1 inhibition with torin1 potently sensitized *ppk18*Δ and *igo1*Δ mutants to DNA damage. These observations suggest roles for the Ppk18 related kinase Cek1 and possibly an unidentified probable role for Igo1 in DNA damage repair and/or checkpoint recovery [26,27].

Materials and methods

Strains, media, and reagents

Strains are listed in Table 1. Cells were grown in yeast extract plus supplements medium (YES) Formedium (Hunstanton, United Kingdom). Stock solutions of caffeine (Sigma Aldrich, Gillingham, United Kingdom) (100 mM) were prepared in water 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) were 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 was obtained from (Alfa Aesar, Fisher Scientific)) and dissolved in water (100 mM). Aliquots were stored at -20°C.

Molecular genetics

Deletion of the open reading frames was done by PCR-based genomic targeting using a KanMX6 construct [28]. 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 [9,29]. 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 5000 imaging platform.

Immunoblotting

Antibodies directed against phospho-eIF2a (#9721), eIF2a (#9722), phosphor- Ssp2 (#50081), Ssp2 (#5832) and the V5 (#80076) antibody were purchased from Cell Signaling Technologies (Leiden, The Netherlands). Antibodies directed against actin (A2066) were purchased from Sigma- Aldrich (Gillingham, United Kingdom). Secondary antibodies directed against mouse (ab205719) and rabbit (ab205718) were purchased from Abcam (Abcam, Cambridge, United Kingdom). Cell pellets were prepared for SDS-PAGE and treated as previously reported [5,9,30].

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 (Figure 1A) [8]. These observations suggest that caffeine overrides DNA damage checkpoint signalling via inhibition of TORC1 activity [8]. As direct inhibition of TORC1 activity with torin1 is more effective than exposure to caffeine, the latter may exert an indirect inhibitory effect on the kinase complex. 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, caffeine appears to indirectly inhibit TORC1 activity via Ssp1 and Ssp2 in *S. pombe* cells previously exposed to phleomycin. TORC1 inhibition leads to activation of the Greatwall homologue Ppk18 which in turn activates Igo1. Igo1 in turn, inhibits $PP2A^{Pab1}$ activity causing cells to enter mitosis prematurely with a shortened cell size [15,16,31,32]. Deletion of *ppk18* did not suppress the ability of either caffeine or torin1 to override DNA damage checkpoints, presumably due to the redundant activity of the Cek1 kinase. We noted however, that the *ppk18*Δ mutant displayed slower cell cycle kinetics than wild type cells exposed to phleomycin and caffeine or torin1 (Figure 1D). In contrast, deletion

of *igo1* markedly reduced the ability of both caffeine and torin1 to override DNA damage checkpoint signalling (Figure 1E). Our findings indicate that caffeine and torin1 indirectly and directly inhibit TORC1 activity respectively.

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* and *tor1*Δ mutants (Figure 2A, 2B). Deletion of *ssp1* and *ssp2* suppressed the ability of both caffeine and torin1 to enhance phleomycin sensitivity (Figure 2C and 2D). TORC1 inhibition is thus 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 [33]. 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 2E). Surprisingly, deletion of ppk18 suppressed the ability of caffeine to enhance sensitivity to phleomycin. In contrast, Ppk18 was not required to mediate the effects of torin1. (Figure 2F). Deletion of *igo1* suppressed the ability of caffeine but not torin1 to enhance sensitivity to phleomycin, despite suppressing the ability of both compounds to override DNA damage checkpoint signalling (Figure 1E, 2G, and 2H). We previously reported that the deletion of *pab1* exerts a similar effect on phleomycin sensitivity in this context (Alao et al., 2020). 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 [26,34]. Our results indicate that caffeine induces the activation of Ssp2. Ssp2 in turn, inhibits TORC1 signalling to advance mitosis.

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* [33,35]. 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 co-exposed to ATP in the presence of caffeine, remained elongated in contrast to cells exposed to caffeine in the presence of phleomycin alone (Figure 3A). Interestingly, ATP also suppressed the ability of torin1 to override DNA damage checkpoint signalling 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 elongated (Figure 3A). ATP may thus attenuate the sensitising effect of caffeine and torin1 to DNA damage via different mechanisms. Alternatively, ATP may increase TORC1 activity or act as a competitive inhibitor towards both compounds.

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

Ssp1 appears to regulate cell cycle re-entry, following Sty1-mediated Cdc25 inhibition, by suppressing Srk1 expression [35,36]. 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. *ssp1*Δ mutants exposed to 0.6 M KCl for 4 h became greatly elongated. Caffeine exacerbated this phenotype of *ssp1*Δ mutants exposed to osmotic stress (Figure 3B). At 35° C *ssp1*Δ mutants are unable to progress through mitosis [37]. This effect was clearly exacerbated by co-exposure to 10 mM caffeine (Figure 3C). In contrast, both rapamycin (200 ng/ mL) and torin1 (5 µM) were sufficient to overcome the *ssp1*Δ phenotype under these conditions (Figures 3C). 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 4A). Deletion of *ssp2* partially suppressed the effect of both caffeine and torin1 on cell cycle progression (Figure 4B). 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* [38,39], 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 4c). Co-deletion of *gsk3* and *ssp2* strongly suppressed

the effects of both caffeine and torin1 on cell cycle progression (Figure 4D). Additionally, caffeine failed to advance cell cycle progression in *gsk3*Δ *ssp1*Δ and *gsk3*Δ *amk2*Δ double mutants (Figure 4E). 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 [15,39]. 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 5A). In contrast, deletion of *gsk3*Δ strongly suppressed the ability of both caffeine and torin1 to drive cells into mitosis (Figure 5B). This differential effect on cell cycle progression was reflected in the ability of both compounds to enhance DNA damage sensitivity. Accordingly, the effect of caffeine and torin1 on DNA damage sensitivity was attenuated in *gsk3*Δ mutants (Figure 5C and 5D). It is unclear, why Gsk3 mediates the effects of caffeine in cells previously arrested with phleomycin but not under normal conditions (Figure 4A, 4C, 5A and 5B). Increased TORC1 activity in *gsk3*Δ mutants [38] 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 AMPK activity or 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 6A). Caffeine weakly induced eIF2α phosphorylation in contrast to direct TORC1 inhibition by torin1 (Figure 6B). Similarly, exposure to caffeine only moderately suppressed Maf1 phosphorylation (an indicator of TORC1 activity [15] in contrast to torin1 (Figure 6C). Accordingly, caffeine-induced Ssp2 phosphorylation was abolished in the *gsk3*Δ *ssp1*Δ [39] double mutant (Figure 6D). Furthermore, the co-deletion of *gsk3* and *ssp1* abolished caffeine but not torin1-induced eIF2α phosphorylation (Figure 6E). 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 [40].

Discussion

We have identified a novel role for the *S. pombe* 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 [5,23,30,41–43]. 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 [15,44]. Additionally, Gsk3 acts downstream of TORC2 to regulate cell division and genetically interacts with Ssp2 [38,39]. 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 [16,45,46]. 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 [9,47–51]. 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 [15,33,35,45].

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 7). 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 [5,30,39]. 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) [35–37,49,52,53]. 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* [9,48]. 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 [5,30]. 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. 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 [54,55]. 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 [46]. 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 [56,57]. Given that its effects on cell cycle progression

mimic environmental stress signalling, it is probable that caffeine activates TORC2 signalling [58]. This would in theory, result in suppressed Pub1 E3 ligase activity and increased Cdc25 expression [46,59]. 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 [48]. 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 [27,34,60]. 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 modulates cell cycle progression by, indirectly, inhibiting TORC1 activity in *S. pombe*. Exposure to caffeine activates the environmental stress response, leading to the Ssp2 dependent inhibition of TORC1. The inhibition of TORC1 activity, results in the downstream inhibition of $PP2A^{Pab1}$ activity and accelerated entry into mitosis [32]. 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 [7,61].

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. All 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-E. 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. *wt* cells were the wee1-6xHA strain (Table 1). 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 torin., adjusted for cell number, 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 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.** The indicated strains were treated as in a., adjusted for cell number and plated on YES agar plates for 3- 5 days. **c.** *ssp1*Δ mutants were grown at 35° C for 4 h and then exposed to 10 mM caffeine, 200 ng/ mL rapamycin or 5 μM torin1 for a further 2 and 24h. Scale bar= 10 μM.

Figure 4. Caffeine modulates cell cycle progression independently of Gsk3. A-E. Log phase *wt*, *gsk3*Δ and *gsk3*Δ *ssp2*Δ 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 5. 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 6. 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. Actin 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. **C.** Caffeine modestly inhibits TORC1 activity. Cells were treated as in A and probed with a mouse V-5 antibody to detect Maf1 phosphorylation. **D.** *wt* and *gsk3*Δ *ssp1*Δ mutants were incubated with 10 mM caffeine or 5 µM torin1 for 1 h. Cell lysates were resolved by SDS-PAGE and probed with antibodies specific for phospho-Ssp2 and total Ssp2. Actin was used to monitor gel loading. **E**. *wt* and *gsk3*Δ *ssp1*Δ mutants were incubated with 10 mM caffeine or 5 µM torin1 for 1 h and processed as in B.

Figure 7. 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/[35,53]
ssp2Δ	*h - ssp2::kanMX6, h + ssp2::kanMX6*	Moseley laboratory and Laboratory stocks/[35,53]
amk2Δ	*h - amk2::kanMX6*	Moseley laboratory/[35,53]
gsk3Δ	*h– gsk3::kanMX6*	Laboratory stocks/[39]
gsk3Δ ssp1Δ	*h– ssp1::kanMX6 gsk3::hphMX6*	Laboratory stocks/[39]
gsk3Δ ssp2Δ	*h– ssp2::kanMX6 gsk3::hphMX6*	Laboratory stocks/[39]
gsk3Δ amk2Δ	*h– amk2::natMX6 gsk3::hphMX6*	Laboratory stocks/[39]
ppk18Δ	*ppk18Δ::KanMX6 h+*	Moseley laboratory
igo1Δ	*h- igo1Δ::KanMX6*	Moseley laboratory/[35,53]
wee1-6xHA	*h- 3HA:6His:wee1 leu1-32 ura4-D18*	YGRC*
Maf1.pk	*maf1.pk::KanMX6*	Petersen laboratory/[15]

* Yeast Genetic Resource Center (YGRC) Japan

Figure 1

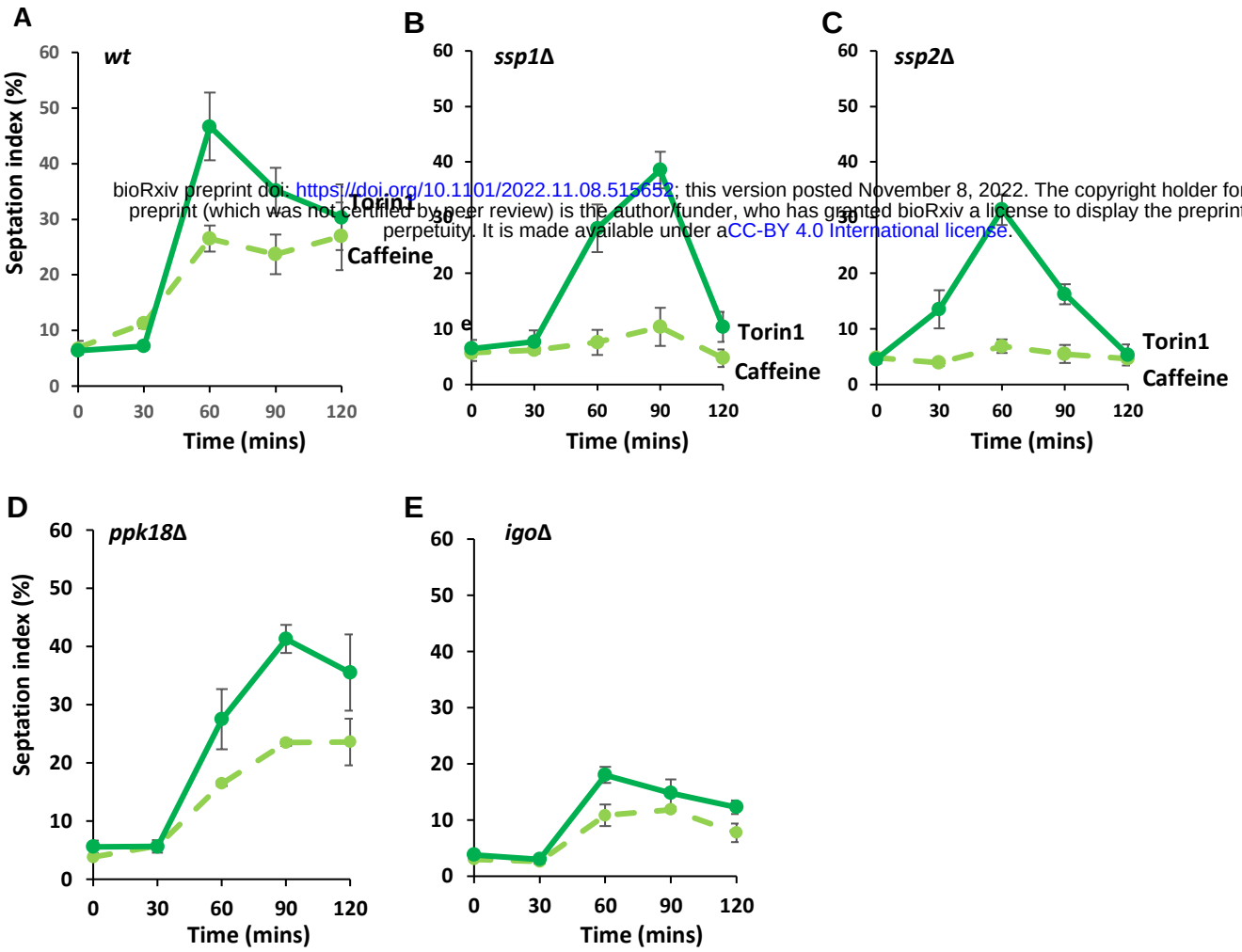

Figure 2

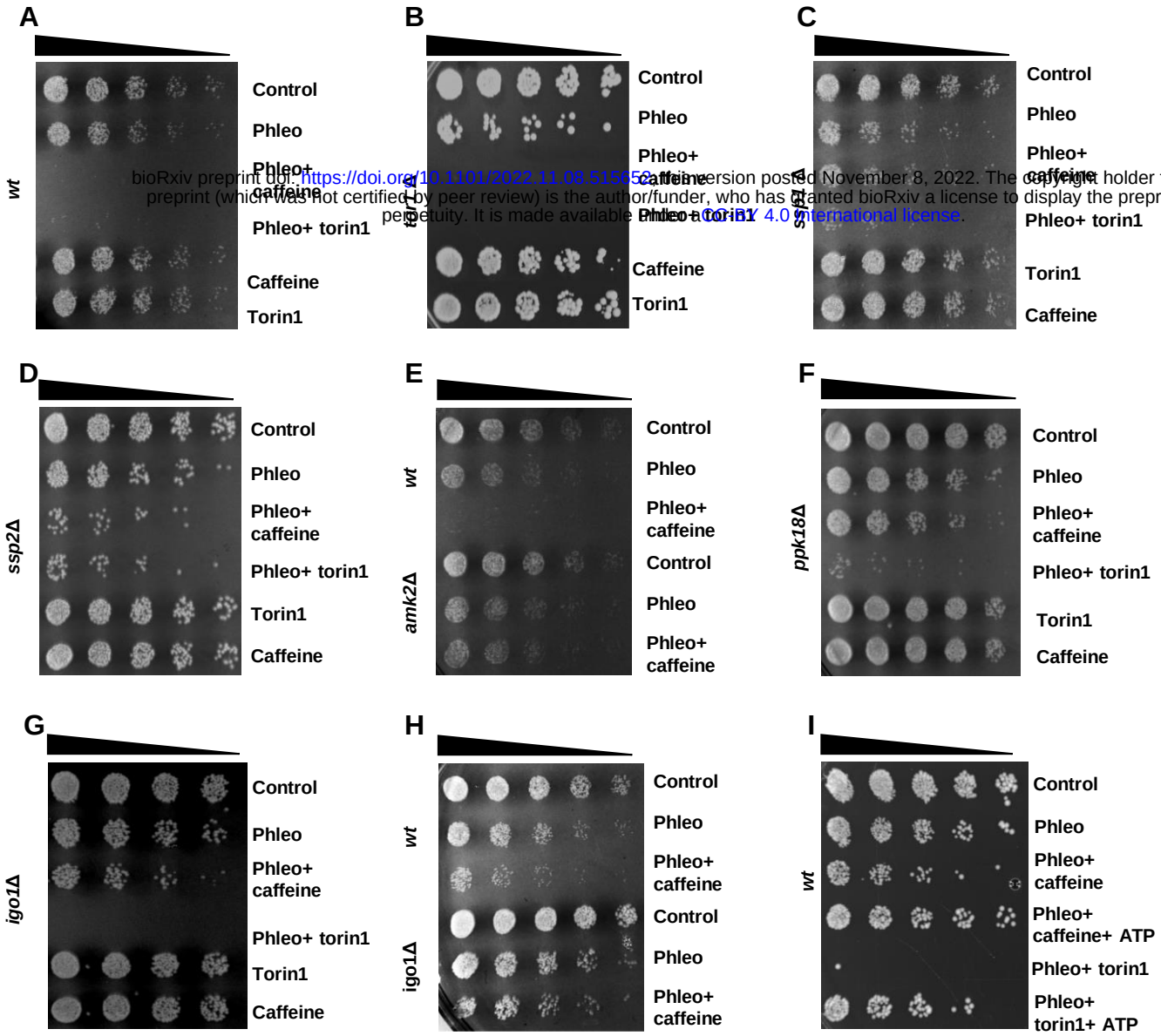

Figure 3

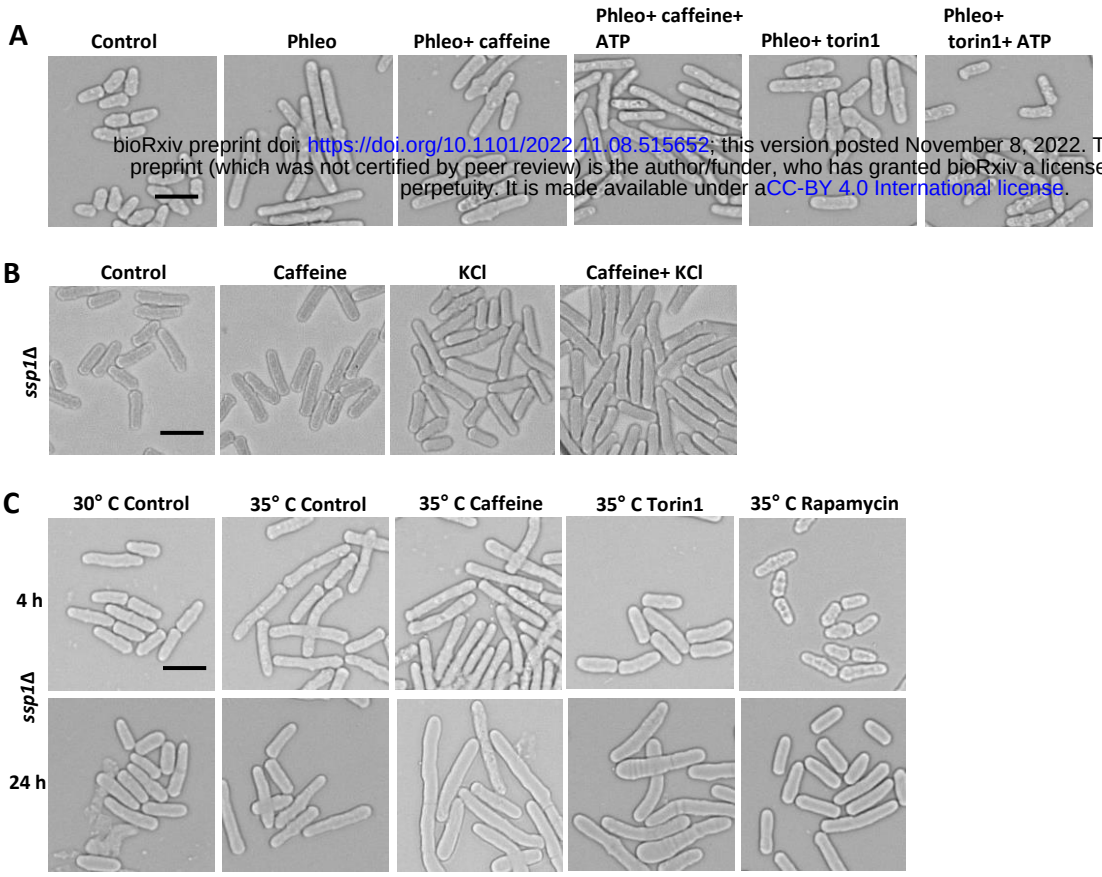

Figure 4

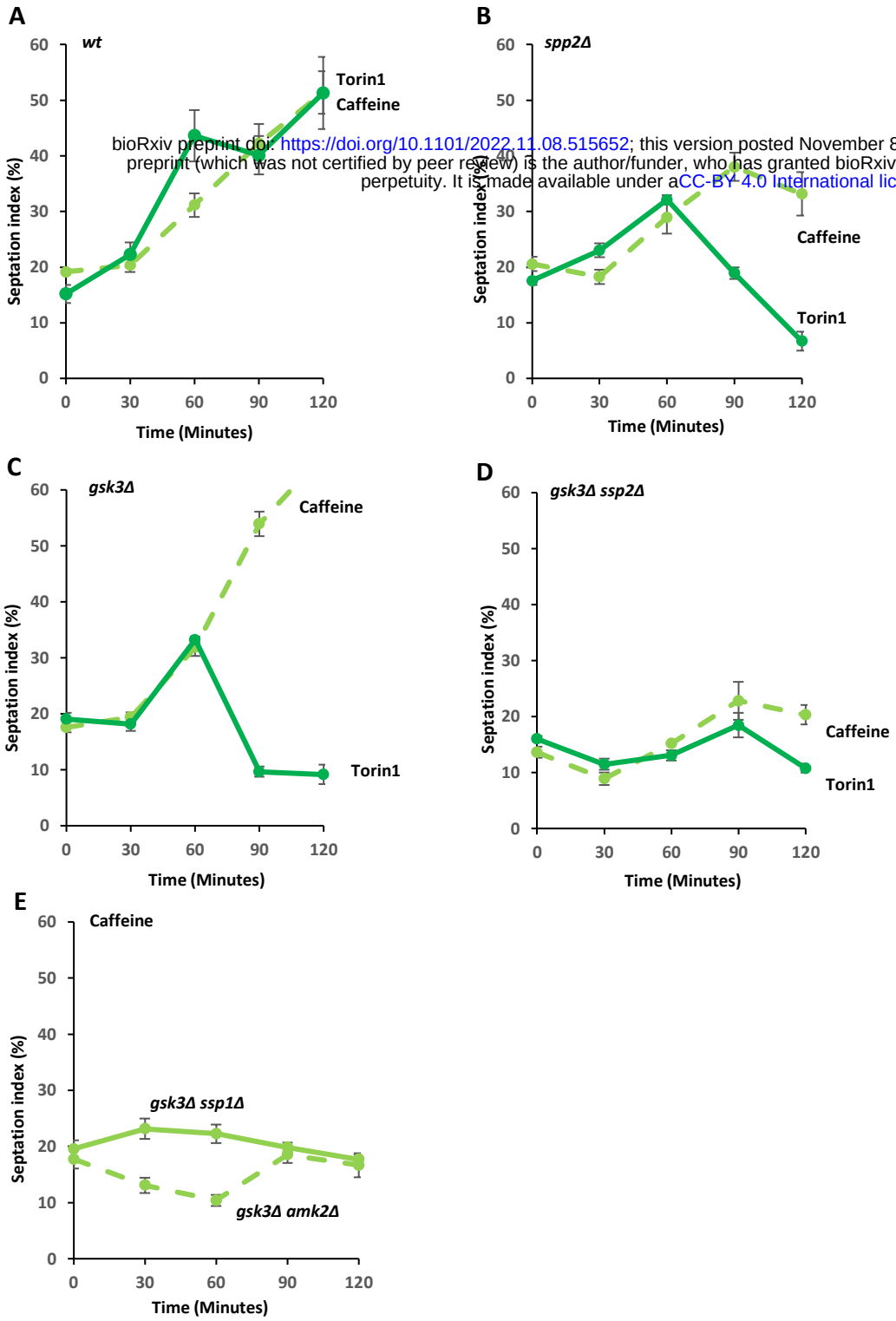

Figure 5

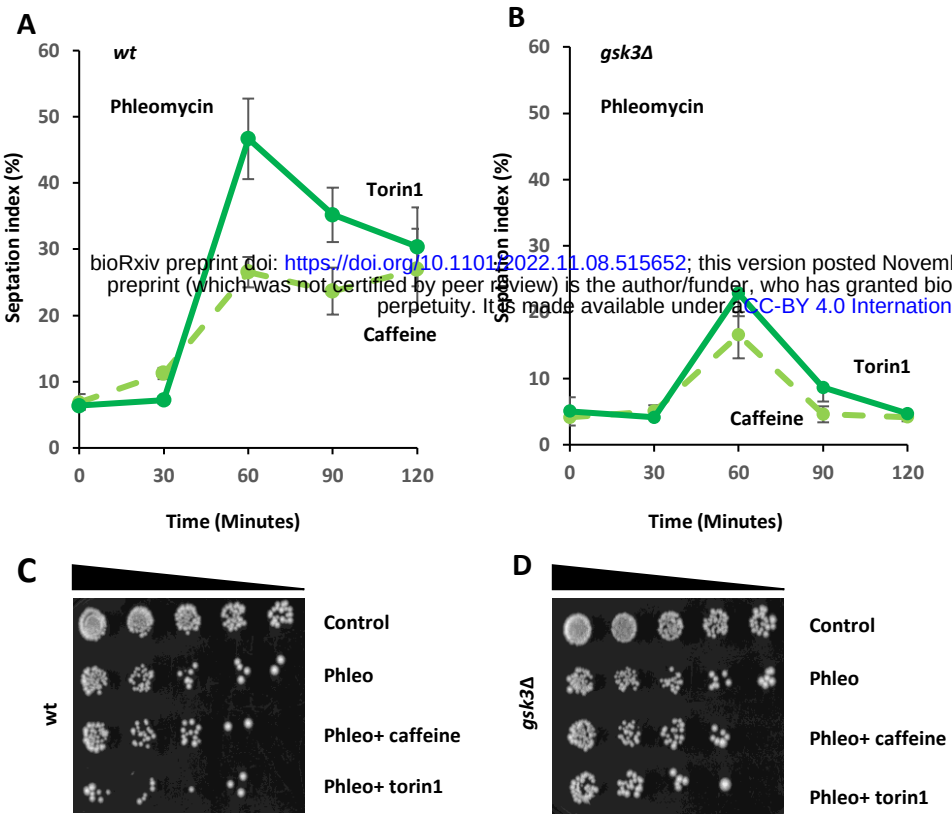

Figure 6

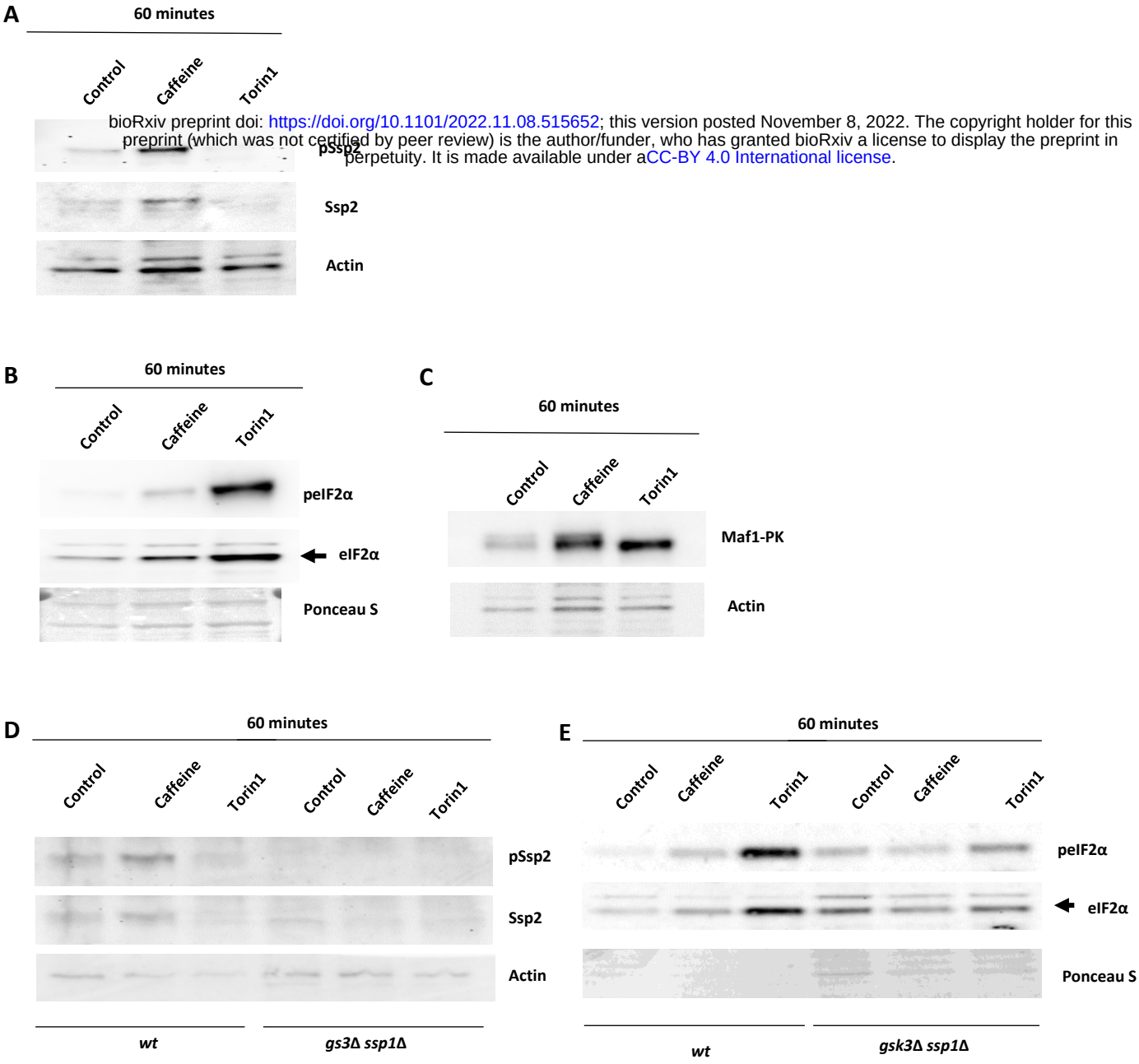

Figure 7