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RESEARCH ARTICLE

Multiple layers of regulation influence cell integrity control by the PKC ortholog Pck2 in fission yeast

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ABSTRACT

The fission yeast protein kinase C (PKC) ortholog Pck2 controls cell wall synthesis and is a major upstream activator of the cell integrity pathway (CIP) and its core component, the MAP kinase Pmk1 (also known as Spm1), in response to environmental stimuli. We show that *in vivo* phosphorylation of Pck2 at the conserved T842 activation loop during growth and in response to different stresses is mediated by the phosphoinositide-dependent kinase (PDK) ortholog Ksg1 and an autophosphorylation mechanism. However, T842 phosphorylation is not essential for Pmk1 activation, and putative phosphorylation at T846 might play an additional role in Pck2 catalytic activation and downstream signaling. These events, together with turn motif autophosphorylation at T984 and binding to small GTPases Rho1 and/or Rho2, stabilize Pck2 and render it competent to exert its biological functions. Remarkably, the target of rapamycin complex 2 (TORC2) does not participate in the catalytic activation of Pck2, but instead contributes to *de novo* Pck2 synthesis, which is essential to activate the CIP in response to cell wall damage or glucose exhaustion. These results unveil a novel mechanism whereby TOR regulates PKC function at a translational level, and they add a new regulatory layer to MAPK signaling cascades.

KEY WORDS: MAP kinase, Pck2, Ksg1, Tor1, Pmk1, Cell integrity, *S. pombe*

INTRODUCTION

In eukaryotic organisms, the protein kinase C (PKC) family is a group of serine/threonine kinases belonging to the AGC class that play essential roles in signaling pathways controlling cell growth, morphogenesis, differentiation and cell death (Newton, 2010). PKC isoforms share a basic structure consisting of a variable N-terminal regulatory domain followed by a highly conserved C-terminal kinase domain. The core of the kinase domain contains three conserved phosphorylation sites that are crucial for catalytic activity, known as the activation loop, the turn motif and the hydrophobic motif (Freeley et al., 2011; Newton, 2010). The 3-phosphoinositide-dependent kinase-1 (PDK1), an AGC kinase family member itself, is the activation loop kinase of most PKCs and other AGC kinases such as PKC-related protein kinases (PRK1 and PRK2; also known as PKN1 and PKN2, respectively, in mammals) or protein kinase A (PKA) (Newton, 2010). Phosphorylation of the activation loop of PKCs is crucial for their activation, with the sole exception of PKCδ (Liu et al., 2006). Interestingly, mammalian PKC isoforms like PKCθ or PKCδ become phosphorylated at the activation loop through PDK1-independent mechanisms involving autophosphorylation or unknown kinase(s) (Freeley et al., 2011). The turn and hydrophobic motifs in mammalian PKCs are phosphorylated either by mammalian target of rapamycin (mTOR) or by an autophosphorylation mechanism. However, the catalytic activity requirement for the phosphorylation of these motifs varies widely among the family members (Freeley et al., 2011), suggesting that alternative mechanisms regulate their activation and downstream signaling functions.

The fission yeast *Schizosaccharomyces pombe* has two PKC orthologs named Pck1 and Pck2. The *pck1Δ* or *pck2Δ* single mutants are viable, but simultaneous deletion is lethal (Arellano et al., 1999). The N-terminus of both kinases contains two HR1 Rho-binding repeats resembling those present in mammalian PRK1 and PRK2, and these repeats bind to active GTPases Rho1 or Rho2 (Arellano et al., 1999; Villar-Tajadura et al., 2008). Pck1 and Pck2 are unstable, and interaction through their N-terminal PEST sequences with GTP–Rho1 or GTP–Rho2 increases their stability (Arellano et al., 1999; Villar-Tajadura et al., 2008). Rho1-mediated stabilization promotes increased phosphorylation of Pck2 at T842 within the activation loop (Sayers et al., 2000). GTP-bound Rho2 might also stabilize Pck2, because most Rho2 effects are mediated by Pck2 (Calonge et al., 2000; Ma et al., 2006), and Pck2 is stabilized in cells lacking the Rho2 GTPase-activating protein (GAP) Rga2 (Villar-Tajadura et al., 2008). Rho1 and Rho2, acting through Pck2, coordinately regulate the biosynthesis of (1,3) β-D-glucan (Rho1) and α-glucan (Rho2), the two main cell wall polymers in fission yeast (Arellano et al., 1999; Calonge et al., 2000). In addition, Rho1, Rho2 and Pck2 are key upstream activators of the cell integrity MAPK pathway (CIP), whose central element, Pmk1 (also known as Spm1), becomes activated under adverse conditions and regulates cell separation, morphogenesis, cell wall construction or ionic homeostasis (Madrid et al., 2006; Pérez and Cansado, 2010; Toda et al., 1996). Rho1 and Rho2 support Pmk1 activity during vegetative growth primarily through Pck2 (Barba et al., 2008; Sánchez-Mir et al., 2014b). However, whereas the Rho2–Pck2 branch is fully responsible for MAPK activation in response to hyper- and hypo-osmotic stress, both Rho1 and Rho2 target Pck2 for signaling cell wall damage to the CIP (Barba et al., 2008; Sánchez-Mir et al., 2014b). In addition, Pmk1 activation under

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osmotic stress is quick and transient, whereas MAPK activation induced by cell wall stress or glucose deprivation is delayed and progressive (Barba et al., 2008; Madrid et al., 2013), suggesting the existence of specific factors determining the kinetics and magnitude of the response.

The PDK1 ortholog Ksg1, which is essential for vegetative growth in fission yeast, phosphorylates the activation loop of AGC kinases such as Gad8, Pka1, and Psk1 (Matsuo et al., 2003; Nakashima et al., 2012; Niederberger and Schweingruber, 1999; Tang and McLeod, 2004). T842 within the activation loop of Pck2 is indeed phosphorylated *in vivo*, but the identity of Ksg1 as the upstream activating kinase remains unexplored. Strains expressing conditional *ksg1* thermosensitive alleles show marked morphology defects and sensitivity to staurosporine, a potent PKC inhibitor (Gräub et al., 2003), suggesting that, in fission yeast, Ksg1 might control the CIP through Pck2. In this work, we conducted a comprehensive study to investigate how Pck2 activity is regulated *in vivo* in fission yeast during growth and in response to stress, by using the CIP as a biological reporter. Our results show a complex scenario in which Ksg1 phosphorylation-dependent and -independent mechanisms coordinately regulate Pck2 activation and stabilization. Remarkably, the TORC2 complex regulates *de novo* Pck2 synthesis during stress, which is required for specific activation of the CIP in response to cell wall stress or glucose limitation.

RESULTS

The PDK1 ortholog Ksg1 positively regulates the activation of the cell integrity MAPK pathway

Cells expressing the thermosensitive *ksg1-208* allele show a mutant phenotype above 34.5℃ and normal growth at 25℃ (Gräub et al., 2003). To explore a functional crosstalk between Ksg1 and the Pmk1 MAPK cascade, control and *ksg1-208* strains expressing a genomic version of Pmk1 tagged with the HA6H epitope at its C-terminus were analyzed. Basal Pmk1 phosphorylation levels in growing *ksg1-208* cells were reproducibly lower than those of control cells at the permissive and restrictive temperatures (Fig. 1A). This decrease was not further enhanced by incubation at higher temperatures (36℃; not shown). Relative to control, treatment of *ksg1-208* cells with 0.6 M KCl prompted a progressive decrease in Pmk1 activation at either semipermissive (32℃) or restrictive (34.5℃) temperatures (Fig. 1B). Similarly, Pmk1 activation during glucose deprivation became strongly abrogated at 34.5℃ in *ksg1-208* cells (Fig. 1C). Also, in *ksg1-208* cells experiencing cell wall stress induced by caspofungin (a β-glucan synthase inhibitor) or Calcofluor, Pmk1 activation was lower than in control cells even at the permissive temperature (Fig. 1D). Importantly, expression of a wild-type version of Ksg1 in caspofungin-treated *ksg1-208* cells rescued Pmk1 activation, whereas a catalytically dead version of the kinase did not (supplementary material Fig. S1A). Moreover, activation of Sty1, the core member of the stress-activated protein kinase (SAPK) pathway (Pérez and Cansado, 2010), was very similar in control and *ksg1-208* cells in response to salt stress or absence of glucose (supplementary material Fig. S1B), indicating that Ksg1 function does not crosstalk with the SAPK pathway. These results point to Ksg1 as a specific positive regulator of the CIP during vegetative growth and in response to stress.

The increased basal Pmk1 phosphorylation that was induced upon *rho2+* overexpression in control cells was abrogated in *ksg1-208* cells incubated at permissive or restrictive temperatures (Fig. 1E). Rho2 targeting to the plasma membrane, which is essential for downstream signaling (Sánchez-Mir et al., 2014a), remained unaltered in *ksg1-208* cells incubated at 34.5℃ (not shown), suggesting that Rho2 acts upstream of Ksg1 in the regulation of the CIP. However, Pmk1 hyperphosphorylation in control cells overexpressing *pck2+* was not suppressed in *ksg1-208* cells at the restrictive temperature (Fig. 1F), supporting the idea that Pck2 is likely to act downstream of Ksg1 during activation of the CIP. As compared to controls, the incubation of *ksg1-208* cells at 34.5℃ resulted in a decreased amount of glucose being incorporated into the cell wall (~30%), and a marked drop in α- and β-glucan content (Fig. 1G), the synthesis of which depends upon the activities of Pck2 and both Pck1 and Pck2, respectively (Calonge et al., 2000). Indeed, whereas the decrease in α-glucan was similar in both *ksg1-208* and *pck2Δ* cells incubated at 34.5℃, this was not the case for β-glucan content (Fig. 1G). Thus, both Pck2 and Pck1 are likely targets for Ksg1 during the synthesis of cell wall α- and β-glucans.

Ksg1 associates with Pck2 and phosphorylates T842 within its activation loop *in vivo*

Although Pck1 interacts *in vivo* with Ksg1, a Ksg1–Pck2 interaction has not been demonstrated (Gräub et al., 2003). Cells expressing N-terminally HA-tagged versions of either Pck1 or Pck2 under the control of the medium-strength thiamine-repressible promoter were incubated with GST-tagged versions of either a wild-type or a catalytically dead version of Ksg1 (*K128E* mutant). Under these conditions, HA–Pck1 or HA–Pck2 were detected after purification of both GST-tagged wild-type and mutant Ksg1 with GSH-beads, but not after purification of GST alone (Fig. 2A). Hence, both Pck1 and Pck2 associate with Ksg1 *in vivo*. Amino acid sequence alignment of selected human and yeast AGC and PKC family kinases predicts that the conserved T842 within the activation loop of Pck2 might be phosphorylated by Ksg1 (Fig. 2B). We obtained a rabbit polyclonal phospho-specific antibody that recognizes Pck2 phosphorylated at T842 (supplementary material Fig. S1C,D). Next, we performed *in vitro* kinase assays using bacterially expressed GST-tagged versions of wild-type or catalytically dead Ksg1 as activating kinases, together with wild-type or T842A mutant GST-tagged versions of Pck2. Incubation with the anti-phospho-T842 antibody revealed a strong phosphorylation signal in assays performed with wild-type versions of Ksg1 and Pck2 (Fig. 2C, lane 5). T842 phosphorylation was absent when employing the kinase-dead version of Ksg1 (lane 6) or the T842A-mutated version of Pck2 (lane 7), but it was still detected when using catalytically dead Pck2 as the substrate (K712W, lane 10). These observations confirm that Ksg1 phosphorylates Pck2 at T842 *in vitro*, and that this event is not due to Ksg1-induced autophosphorylation at this site. Pck2 also harbors a conserved phosphorylatable threonine residue at position 984 within the turn motif domain, and a serine residue at position 1002 corresponding to the hydrophobic motif (Fig. 2B). As shown in Fig. 2D (lanes 3, 5 and 9), replacement of either T984 (turn motif), S1002 (hydrophobic motif) or both motifs with alanine did not prevent Ksg1 from phosphorylating Pck2 at T842 *in vitro*. Notably, mutation of two conserved aromatic phenylalanine residues located within the hydrophobic domain (F998A F1001A mutant; Fig. 2B), strongly reduced Ksg1-dependent *in vitro* phosphorylation of Pck2 at T842 (Fig. 2D, lane 7). This domain might behave as a docking site for Ksg1 to achieve further phosphorylation of the activation loop of Pck2 at T842.

To confirm that Ksg1 phosphorylates Pck2 at T842 *in vivo*, we obtained *pck2Δ* (control) and *pck2Δ ksg1-208* strains expressing a

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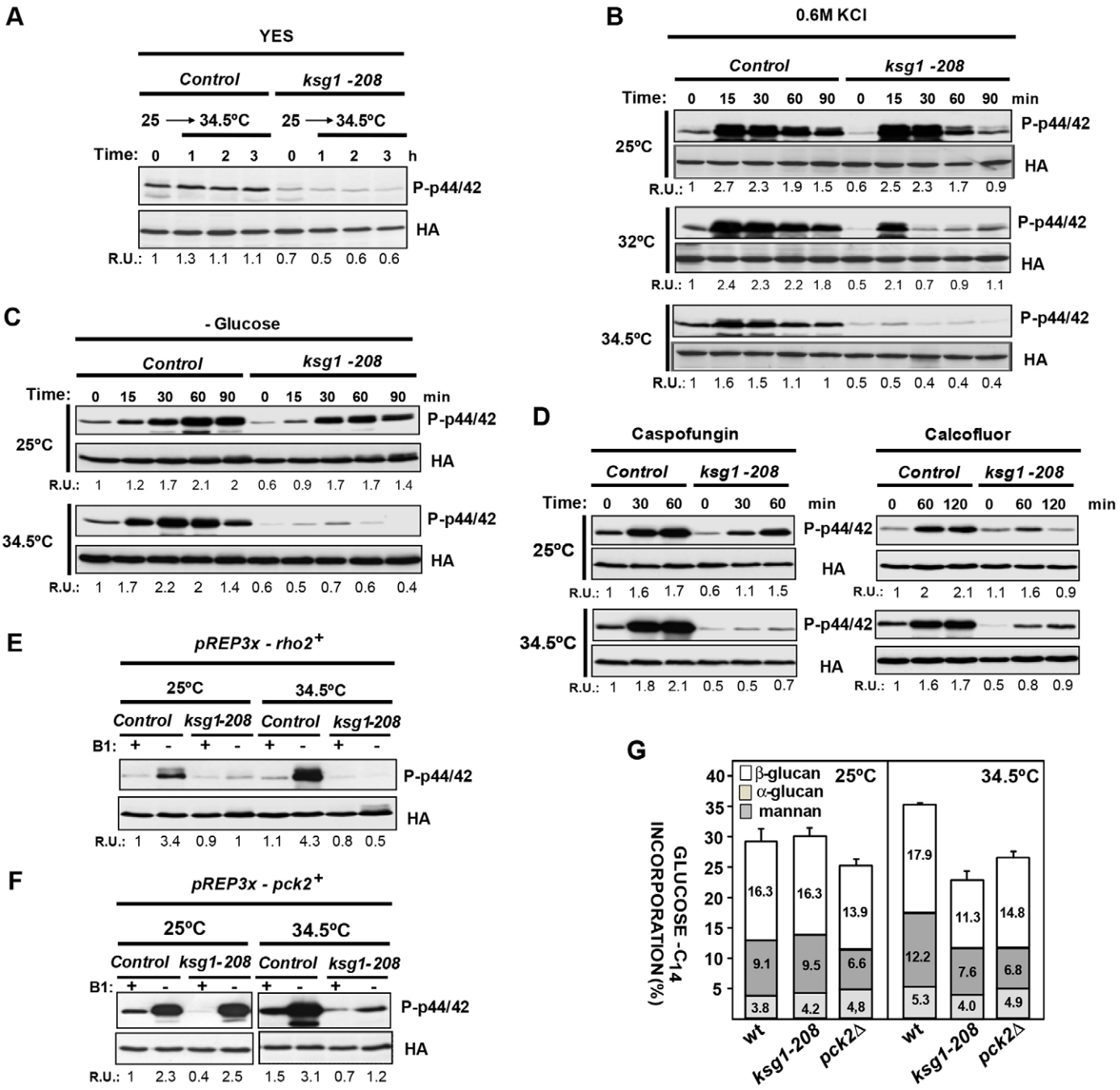

Fig. 1. Ksg1 is a positive regulator of the cell integrity MAPK pathway in fission yeast. (A) Strains MI200 (control) and MM667 (*ksg1-208*) were grown at 25˚C in YES medium to early log phase and then incubated at 34.5˚C for the times indicated. The Pmk1 fusion protein was purified by affinity chromatography, and activated and total Pmk1 were detected by immunoblotting with anti-phospho (P)-p44/42 and anti-HA antibodies, respectively. R.U., relative units. (B) Pmk1 activation in strains described in A growing at 25˚C in YES medium, and incubated at either 32 or 34.5˚C for 1 h with 0.6 M KCl. (C) Pmk1 activation in strains described in A growing in YES medium plus 7% glucose at 25˚C, incubated at 34.5˚C for 1 h, and then transferred to the same medium with 3% glycerol. (D) Pmk1 activation in strains described in A growing at 25˚C in YES medium, incubated at 34.5˚C for 1 h and treated with either 1 μg/ml caspofungin or 1 mg/ml Calcofluor. (E,F) Strains described in A were separately transformed with pREP3X-*rho2*$^{+}$ (E) or pREP3X-*pck2*$^{+}$ (F) and grown at either 25˚C or 34.5˚C for 18 h in the presence or absence of thiamine (B1). (G) Cell wall composition analysis. Control, *ksg1-208* and *pck2Δ* strains were grown in YES medium at either 25 or 34.5˚C and ^{14}C-glucose was added at 6 h before harvesting. wt, wild type. Error bars represent standard deviations for the total carbohydrate values.

genomic version of Pck2 fused to a single HA epitope at its C-terminus. Levels of total (anti-HA antibody) and T842-phosphorylated (anti phospho-T842 antibody) Pck2 were low in both mutants growing at the permissive temperature (25˚C; Fig. 2E). However, transfer of control cells from 25 to 36˚C prompted a clear increase in the amount of both phosphorylated and total Pck2, with a notable rise in the ratio of phospho-T842 to total Pck2 (Fig. 2E). Importantly, the amount of both total and T842-phosphorylated Pck2 species was reduced in *ksg1-208* cells at longer incubation times at the restrictive temperature (Fig. 2E).

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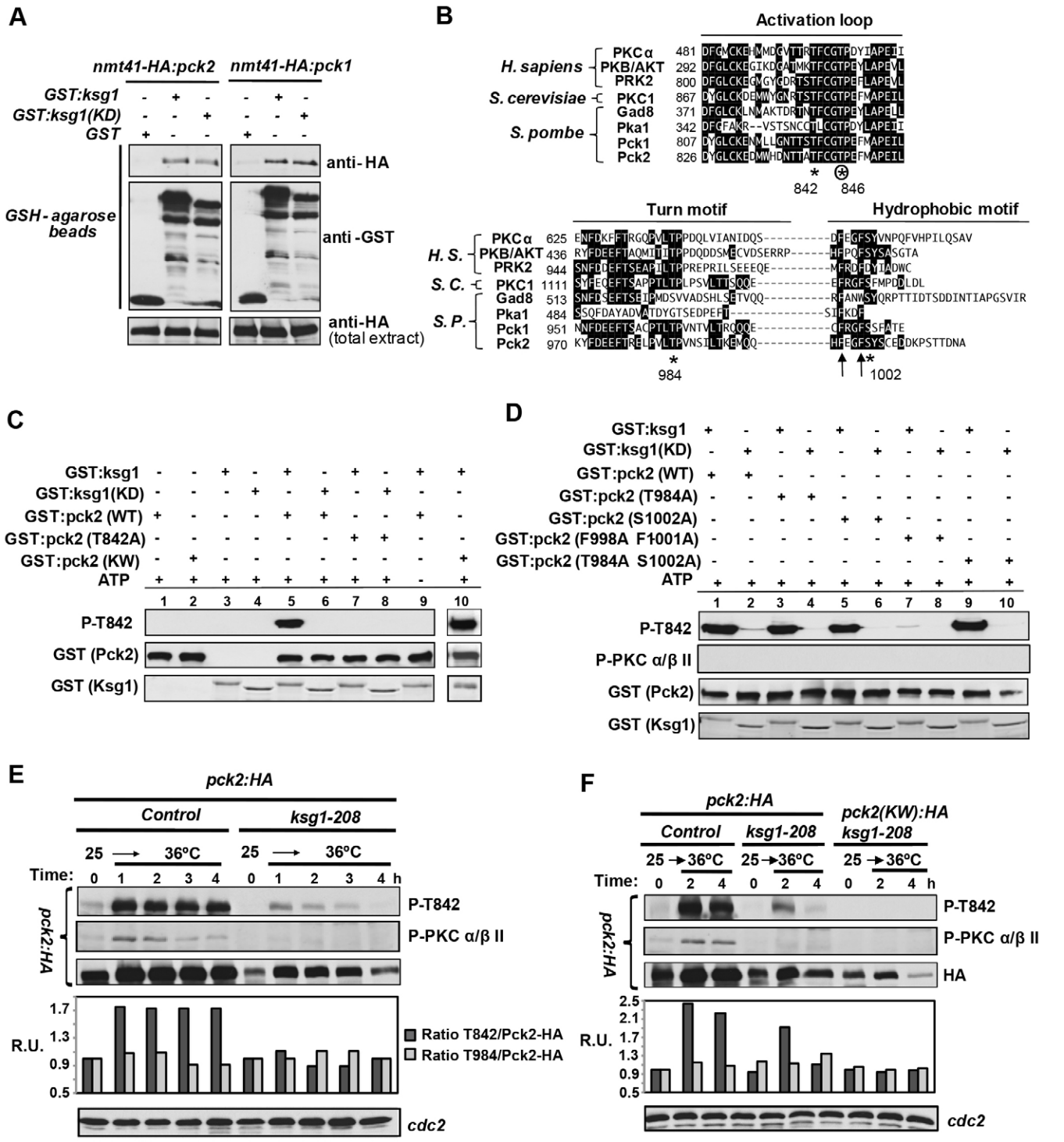

Fig. 2. See next page for legend.

Therefore, Ksg1 phosphorylates Pck2 at T842 *in vivo*, and both phosphorylated and total Pck2 levels increase under thermal stress. Ksg1 might be involved in Pck2 synthesis and/or stability, as indicated by the drop in the amount of Pck2 in *ksg1-208* cells at the restrictive temperature. Pck2 T842 phosphorylation was virtually undetectable in *ksg1-208* cells expressing a catalytically dead version of Pck2 (K712W mutant) at the restrictive temperature, which was accompanied by an additional reduction in the amount of total Pck2 (Fig. 2F). These results imply that both Ksg1 and an autocatalytic mechanism are responsible for the *in vivo* activation loop phosphorylation of Pck2 at T842 (see below).

By using a commercial anti-phospho-PKCα/βII antibody we detected specific *in vivo* turn motif phosphorylation of Pck2 at T984 in growing cells expressing the Pck2–HA fusion (supplementary material Fig. S1E,F). The amount of phosphorylated T984 increased in control cells incubated at the restrictive temperature, but not in

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Fig. 2. Ksg1 phosphorylates Pck2 *in vivo* at T842. (A) Strains PPG5.23 (*nmt41:HA-pck2*) and PPG5.16 (*nmt41:HA-pck1*) were grown in EMM2 medium without thiamine for 24 h, and cell extracts were incubated with bacterially expressed GST, GST:ksg1(KD) (kinase-dead mutant) or GST:ksg1. GST and GST fusions were purified with glutathione–agarose, resolved by SDS-PAGE and detected with anti-HA and anti-GST antibodies. (B) Amino acid sequence alignment of activation domains in selected AGC family kinases. Conserved canonical phosphorylated residues within the activation loop, turn motif and hydrophobic motif are shown with asterisks. The circled asterisk marks the alternative putative phosphorylation residue within the activation loop. Arrows indicate conserved phenylalanine residues relevant for docking to PDK. *H.S.*, *Homo sapiens*; *S.C.*, *Saccharomyces cerevisiae*; *S.P.*, *Schizosaccharomyces pombe*. (C) GST:pck2 (wild type, wt), GST:pck2-T842A and GST:pck2-K712W (kinase dead, KD) fusions were incubated at 30˚C for 1 h with either GST:ksg1(KD) or GST:ksg1 with or without ATP. Reaction mixtures were resolved and hybridized with anti-phospho (P)-T842 or anti-GST antibodies. (D) The assay described in C was performed by employing as substrates GST:pck2 (wild type), GST:pck2-T984A, GST:pck2-S1002A, GST:pck2-F998A F1001A and GST:pck2-T984A S1002A. The mixtures were resolved and detected with anti-phospho-T842, anti-phospho-PKCα/β II and anti-GST antibodies. (E) Strains MM913 (Pck2-HA; Control) and MM1100 (Pck2-HA; *Ksg1-208*) were grown at 25˚C in YES medium and then incubated at 36˚C. Cell extracts were resolved by SDS-PAGE and detected with anti-phospho-T842, anti-phospho-PKCα/β II and anti-HA antibodies. Anti-cdc2 was used as loading control. R.U., relative units. (F) Extracts from strains MM913, MM1100 and MM1130 (Pck2-K712W-HA, *Ksg1-208*) were hybridized with anti-phospho-T842, anti-phospho-PKCα/β II and anti-HA antibodies (total Pck2) following the procedure described in E.

ksg1-208 cells (Fig. 2E), suggesting that Ksg1 mediates the turn motif phosphorylation of Pck2. However, Ksg1 was unable to phosphorylate T984 *in vitro* (Fig. 2D). Notably, *in vivo* T984 phosphorylation was absent from cells expressing a catalytically dead version of Pck2 (supplementary material Fig. S1E), and was not significantly affected in Pck2-T842A cells as compared to control cells (supplementary material Fig. S1F). Hence, an autocatalytic mechanism might be responsible for *in vivo* Pck2 turn motif phosphorylation.

Functional significance of activation domain phosphorylation of Pck2

Evidence obtained with PKC isoforms from other organisms supports the idea that Ksg1-mediated phosphorylation of the activation loop of Pck2 at T842 should be essential to regulate its biological activity. Ectopic expression of the *pck2+* gene induces a marked increase in Pmk1 phosphorylation and morphological alterations leading to cell death (Fig. 3A–C) (Arellano et al., 1999; Ma et al., 2006; Sánchez-Mir et al., 2014b). Overexpression of the Pck2-T842A allele did not enhance Pmk1 phosphorylation (Fig. 3A), alter cell morphology (Fig. 3B) or inhibit cell growth (Fig. 3C), supporting the biological relevance of *in vivo* Ksg1-dependent phosphorylation of Pck2 at T842. By contrast, overexpression of the turn motif (T984A) or hydrophobic motif (S1002A) mutants caused Pmk1 hyperphosphorylation and was lethal (Fig. 3A–C). Overexpression of a Pck2-F998A F1001A mutant still increased Pmk1 phosphorylation, but cell morphology and viability remained unaffected (Fig. 3A–C), thus confirming the functional relevance of the hydrophobic motif during Pck2 function *in vivo*.

To gain a precise understanding of the impact of Pck2 phosphorylation on its biological activity, we evaluated the ability of strains harboring genomic versions of wild-type or distinct mutated alleles of Pck2 to suppress the defective signaling to the CIP in a *pck2Δ* background. Basal Pmk1 phosphorylation in cells expressing the Pck2 mutants T842A, T984A, T984A S1002A and F998A F1001A, was quite similar to that of those harboring the wild-type kinase (Fig. 3D). Conversely, cells expressing the constitutively active (T842D) or hydrophobic motif (S1002A) Pck2 mutants displayed increased Pmk1 phosphorylation (Fig. 3D), suggesting that whereas T842 phosphorylation positively influences basal Pmk1 activation, putative S1002 phosphorylation negatively regulates Pmk1 activity. Accordingly, strains expressing Pck2 alleles with phosphomimetic mutations in this residue (S1002E, S1002D) showed decreased Pmk1 activity in comparison with that of control cells (Fig. 3D). Basal MAPK activity in cells of a double T842A T984A mutant was low and quite similar to that of cells expressing catalytically dead versions of Pck2 (K712W and K712E; Fig. 3D), although still higher than in *pck2Δ* cells (Fig. 3D). The VIC phenotype (growth in the presence of $MgCl_2$ plus the specific calcineurin inhibitor FK506), provides a highly sensitive biological readout for Pmk1 activity *in vivo* (Sánchez-Mir et al., 2014b). As described previously (Ma et al., 2006; Sánchez-Mir et al., 2014b), *pck2Δ* cells showed a strong VIC phenotype that was absent from control cells and most Pck2 mutants assayed, even at low $MgCl_2$ concentrations (Fig. 3E). Interestingly, cells carrying either the T842A T984A mutant or the catalytically dead versions of Pck2 were VIC positive but unable to grow in the presence of 0.1 M $MgCl_2$ (Fig. 3E), showing higher basal Pmk1 phosphorylation than in *pck2Δ* cells and retention of the signaling capacity to the Pmk1 cascade. Taken together, these results show that, in fission yeast, the concerted *in vivo* phosphorylation of Pck2 at T842 and T984 is important to maintain basal activity of the CIP.

Fission yeast *pck2Δ* cells are sensitive to growth inhibition in the presence of caspofungin (>0.8 µg/ml), and this phenotype was suppressed by expressing wild-type Pck2 (control) or the Pck2 mutants T842D, T984A, S1002A and T984A S1002A (Fig. 3F). However, expression of the T842A T984A double mutant or catalytically dead versions of Pck2 (K712W; K712E) poorly suppressed the caspofungin sensitivity of *pck2Δ* cells (Fig. 3F). These results support the idea that *in vivo* phosphorylation at both the activation loop and the turn motif is important for Pck2 regulation of cell wall homeostasis. The observation that caspofungin sensitivity is higher in *pck2Δ* cells than in catalytically dead or T842A T984A Pck2 mutants also reveals that Pck2 function is exerted, at least in part, independently of its catalytic activity.

T842, T846 and T984 are required for Pck2 activation and downstream signaling to the cell integrity MAPK pathway in response to stress

Our results indicate that fission yeast basal Pck2 activity does not become substantially abolished by lack of activation loop phosphorylation at T842. To further analyze this issue, we performed a comparative study using Pmk1 phosphorylation as a biological reporter for Pck2 activity in strains expressing distinct Pck2 mutant alleles under different stresses (hypertonic stress, hypotonic shock or cell wall stress). These stimuli are mostly transduced to the CIP in a Pck2-dependent fashion (Barba et al., 2008). Thus, a fission yeast mutant carrying a Pck2 kinase-dead allele (K712W) showed very low Pmk1 activation in response to the above stimuli, and this signaling defect was further aggravated in *pck2Δ* cells (Fig. 4A). Notably, Pmk1 phosphorylation levels in Pck2-T842A cells under the above stresses were quite similar to those of control cells except during caspofungin treatment (Fig. 4A). Similar to T842A cells, strains expressing Pck2

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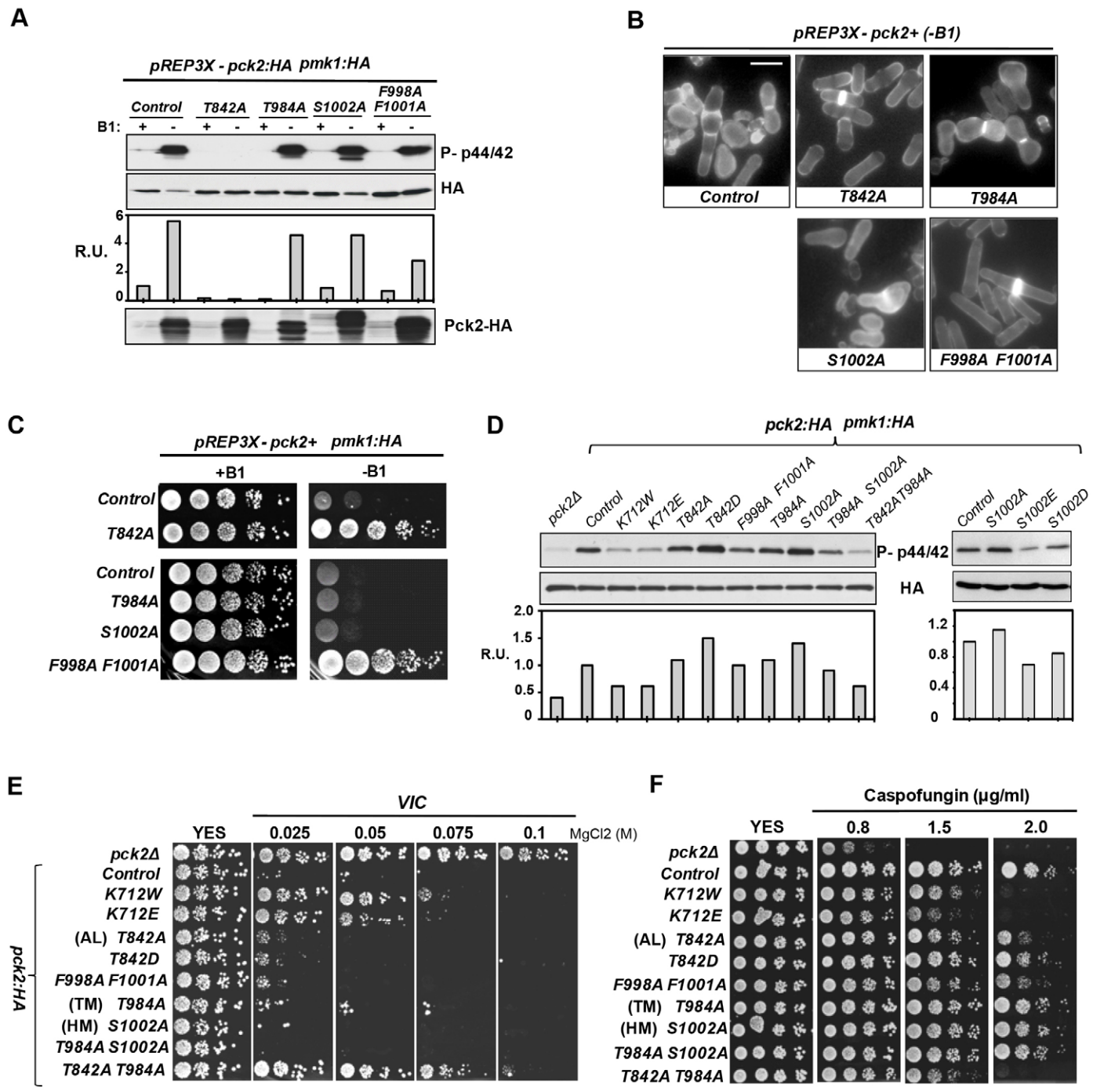

Fig. 3. Functional significance of activation domain phosphorylation of Pck2. (A) Strain MI200 was transformed with plasmids pREP3X-Pck2-HA (Control), pREP3X-Pck2.T842A-HA, pREP3X-Pck2.T984A-HA, pREP3X-Pck2.S1002A-HA or pREP3X-Pck2.F998A F1001A-HA, and grown for 18 h with or without thiamine (B1). Activated and total Pmk1 were detected by immunoblotting with anti-phospho (P)-p44/42 and anti-HA antibodies, respectively. R.U., relative units. (B) Cell morphology of transformants described in A growing in the absence of thiamine was analyzed by fluorescence microscopy after staining with Calcofluor White. Scale bar: 10 μm. (C) Serially diluted cells from transformants described in A were spotted on EMM2 plates with or without 5 mM thiamine, and incubated for 4 days at 28˚C. (D) Activated and total Pmk1 were detected in GB3 (*pck2Δ*), MM913 (control) and strains expressing Pck2–HA versions with indicated mutations in a Pmk1-HA6H background in YES medium. (E) Serially diluted cells of strains described in D were spotted on YES plates supplemented with either 0.025–0.1 M $MgCl_2$ plus 1 μg/ml FK506 (VIC) or (F) 0.8–2.0 μg/ml caspofungin, and incubated for 3 days at 28˚C. AL, activation loop; TM, turn motif; HM, hydrophobic motif.

mutants T984A (turn motif phosphorylation) or F998A F1001A (hydrophobic motif) were slightly defective for Pmk1 activation only in response to cell wall damage (Fig. 4B). Relative to control cells, Pmk1 activation was rather enhanced in Pck2-S1002A cells (Fig. 4B), indicating that putative phosphorylation at this residue might negatively regulate both basal and stress-induced Pck2 activation along with signaling to Pmk1. We noticed that, as compared with T842A cells, stress-induced Pmk1 activation

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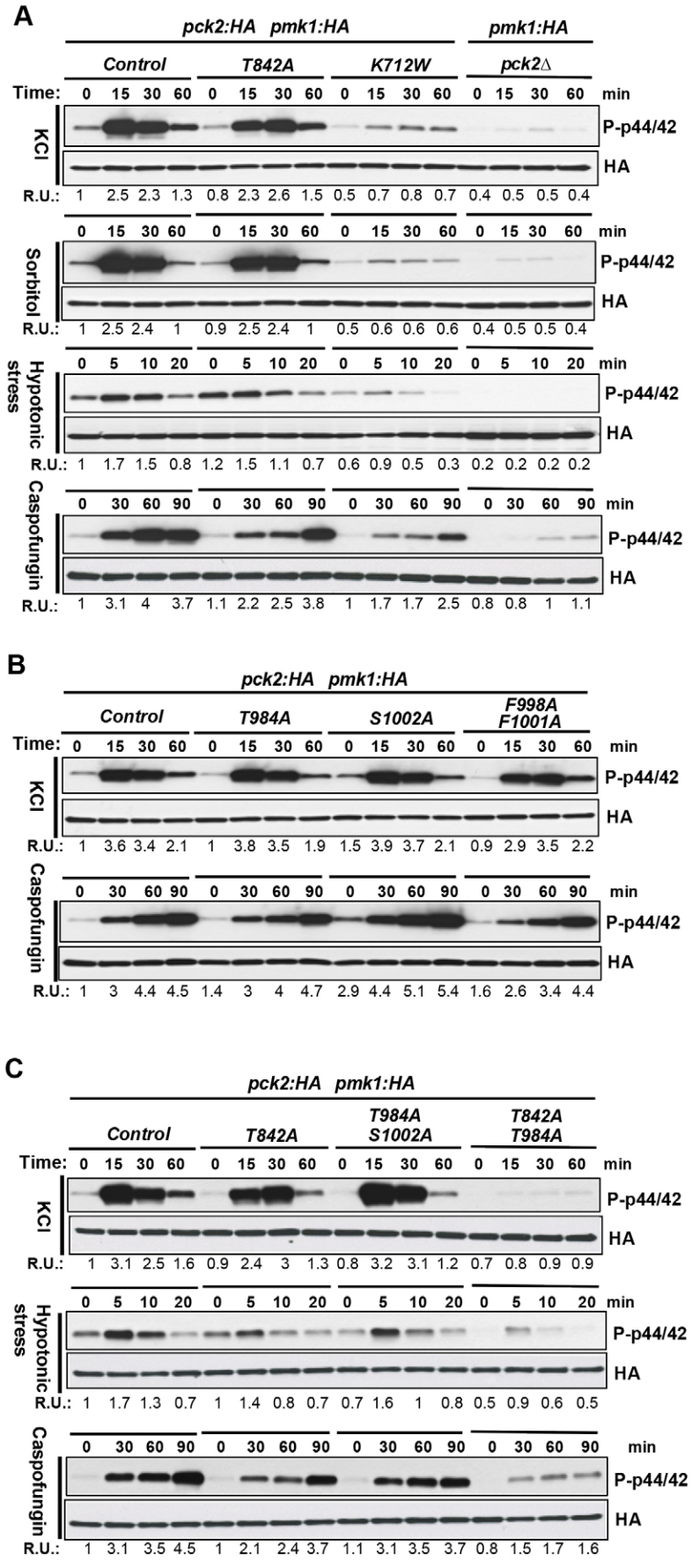

Fig. 4. T842 and T984 within the Pck2 activation domain are required for downstream signaling to Pmk1 during stress. (A) Strains MM913 (Pck2–HA; control), MM918 (Pck2.T842A–HA), MM1022 (Pck2.K712W–HA) and GB3 (*pck2Δ*), expressing a genomic Pmk1–HA6H fusion, were grown in YES medium with either 0.6 M KCl or 1 M sorbitol, subjected to a hypotonic stress or treated with 1 μg/ml caspofungin. Aliquots were harvested at the times indicated and activated and total Pmk1 were detected with anti-phospho (P)-p44/42 and anti-HA antibodies, respectively. R.U., relative units. (B) Pmk1 activation in cultures of strains MM913, MM1009 (Pck2.T984A–HA), MM1011 (Pck2.S1002A–HA) and MM998 (Pck2.F998A F1001A–HA), treated with either 0.6 M KCl or 1 μg/ml caspofungin. (C) Pmk1 activation in cultures of strains MM913, MM918, MM1042 (Pck2.T984A S1002A–HA) and MM1123 (Pck2.T842A T984A–HA) under hypotonic stress or treated with either 0.6 M KCl or 1μg/ml caspofungin.

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became strongly compromised in cells expressing the Pck2-T842A T984A allele (Fig. 4C), and that this decrease was very similar to that of cells expressing catalytically dead versions of Pck2 (K712E; K712W) or a T842A T984A S1002A mutant (not shown). Therefore, both T842 and T984 modulate Pck2 activity and signaling to the CIP during the growth of fission yeast and in response to stress.

Impaired activity of Ksg1, which phosphorylates Pck2 at T842 *in vivo*, affects Pmk1 phosphorylation (Fig. 1). The expendable nature of T842 phosphorylation during Pmk1 activation might be due to the existence of alternative Ksg1 phosphorylation sites within the Pck2 activation loop contributing to signaling. Mass spectrometry and phosphoproteomic analyses indicate that the invariant threonine residue located four amino acids downstream of the conserved PDK1 site in different human PKC isoforms, equivalent to T846 in the Pck2 sequence (Fig. 2B), is indeed phosphorylated *in vivo* (Oppermann et al., 2009; Phanstiel et al., 2011). In comparison with that of control cells, T842 and T984 phosphorylation remained unaffected in T846A cells (Fig. 5A,B), suggesting that this mutation does not alter the conformation of the activation loop. However, the levels of both total Pck2 and Pck2 phosphorylated at T984 in the turn motif were lower in T842A T846A cells than in the T842A or T846A single mutants (Fig. 5B). In this regard, T842A T846A cells displayed a moderate VIC phenotype, although weaker than that of T842A T984A or T842A T846A T984A mutants (Fig. 5C). Remarkably, Pmk1 activation decreased strongly in T846A cells under saline stress or caspofungin treatment (Fig. 5D). Moreover, simultaneous mutation of both T842 and T846 almost completely abrogated the osmotic-stress-induced MAPK activation, as observed also in the T842A T846A T984A triple mutant or in *pck2Δ* cells (Fig. 5E). Such results suggest that, in addition to T842, putative phosphorylation at T846 within the activation loop of Pck2 might play a relevant role for its catalytic activation and downstream signaling functions.

Rho1 and Rho2 GTPases and activation domain phosphorylation are essential to maintain increased Pck2 levels during stress

As observed for thermal stress (Fig. 2E,F), the levels of both total and T842-phosphorylated Pck2 increased progressively in control cells subjected to a hypertonic stress with KCl or after cell wall stress with caspofungin (Fig. 6A). These stress-induced increases were attenuated in cells expressing a catalytically dead version of the kinase (K721W; Fig. 6A), confirming the suggestion that autophosphorylation partially regulates both processes. However, Pck2 levels in cells lacking T842 phosphorylation (T842A mutant) increased as much as those of control cells and independently of the stressful stimulus (Fig. 6B). Also, phosphorylation at T842 was lower in cells expressing a version of Pck2 with a mutation within the hydrophobic domain (F998A F1001A; Fig. 6B). This mutant was not phosphorylated by Ksg1 *in vitro* (Fig. 2D), highlighting the functional relevance of the hydrophobic domain for activation loop phosphorylation of Pck2. As compared to control cells, simultaneous mutation of the conserved phosphorylation residues at the turn and hydrophobic motifs (T984A S1002A mutant) did not alter either total or T842-phosphorylated Pck2 levels in KCl- or caspofungin-treated cultures (Fig. 6C), but they were clearly reduced in stressed cells expressing either T842A T984A (Fig. 6C) or T842A T846A (Fig. 6D) double mutants.

Active Rho1 and Rho2 interact with Pck1 and Pck2, causing their stabilization and increased phosphorylation of Pck2 at T842 (Arellano et al., 1999; Sayers et al., 2000). Also, the increased total and T842-phosphorylated Pck2 levels in control cells under salt stress became strongly attenuated in *rho2Δ* cells expressing the hypomorphic Rho1 allele *rho1-596* (supplementary material Fig. S2A) (Viana et al., 2013). Taken together, these results indicate that, in *S. pombe*, the concomitant phosphorylation of the activation domain (at T842, T846 and T984) and Rho1/Rho2 stabilization increase Pck2 levels in response to stressful situations.

Increased Pck2 levels are required for the activation of the cell integrity pathway in response to cell wall stress

Compared with those of untreated cultures, both total and T842-phosphorylated Pck2 levels decreased quickly in growing cells treated with the protein synthesis inhibitor cycloheximide (Fig. 7A, upper panels), supporting the idea that Pck2 is an unstable protein with a short half-life (Villar-Tajadura et al., 2008). This decrease was parallel to a delayed reduction in basal Pmk1 phosphorylation but not in total Pmk1 (Fig. 7A, lower panels), which suggested a strong correlation between *de novo* Pck2 synthesis and activation of the Pmk1 pathway. If a rise in Pck2 levels was essential for downstream signaling to the cell integrity pathway during stress, it would be predicted that blocking Pck2 synthesis would result in defective Pmk1 activation. However, although cycloheximide treatment inhibited Pck2 synthesis and T842 phosphorylation during salt stress, Pmk1 activation remained unaffected (supplementary material Fig. S2B), suggesting that *de novo* Pck2 synthesis does not participate in MAPK activation by this stress. Nevertheless, cycloheximide abolished Pmk1 activation at earlier times in cells under cell wall stress with caspofungin (Fig. 7B, upper panels), and this was followed at longer incubation times by a slight increase in MAPK activity, which mimicked a very modest rise in both total and T842-phosphorylated Pck2 (Fig. 7B, upper panels). As compared with those of untreated cells, cycloheximide pretreatment did not alter Ksg1, Rho2, Mkh1 (MAPKKK) and Pek1 (MAPKK, also known as Skh1) protein levels in the presence of caspofungin (Fig. 7B, lower panels). These results support the notion that *de novo* synthesis of Pck2 is required for activation of the Pmk1 pathway during cell wall stress.

TORC2 positively regulates Pck2 synthesis and activation of the Pmk1 pathway during cell wall stress and in the absence of glucose

The above results prompted us to identify putative regulatory networks responsible for increased Pck2 synthesis in response to stress. Fission yeast TOR kinases Tor1 and Tor2 are essential to coordinate cell growth and cell size within nutritional and environmental contexts (Davie and Petersen, 2012). Using a mutant expressing the thermosensitive allele of Tor2 (*tor2-51*), the core kinase of the TORC1 complex (Alvarez and Moreno, 2006), we found that Pmk1 activity was rather similar in cells incubated at permissive or restrictive temperatures under salt or cell wall stress (supplementary material Fig. S3A). In response to a saline stress, Pmk1 activation was also identical in control and in mutant cells lacking the TORC2 complex kinase Tor1 (supplementary material Fig. S3B). Under these conditions, the levels of Pck2 and T842 phosphorylation were lower in *tor1Δ* cells at early time-points, although they reached levels similar to those of control cells at longer incubation times (supplementary material Fig. S3B). Notably, both Pck2 levels and T842 phosphorylation were strongly reduced in *tor1Δ* cells treated

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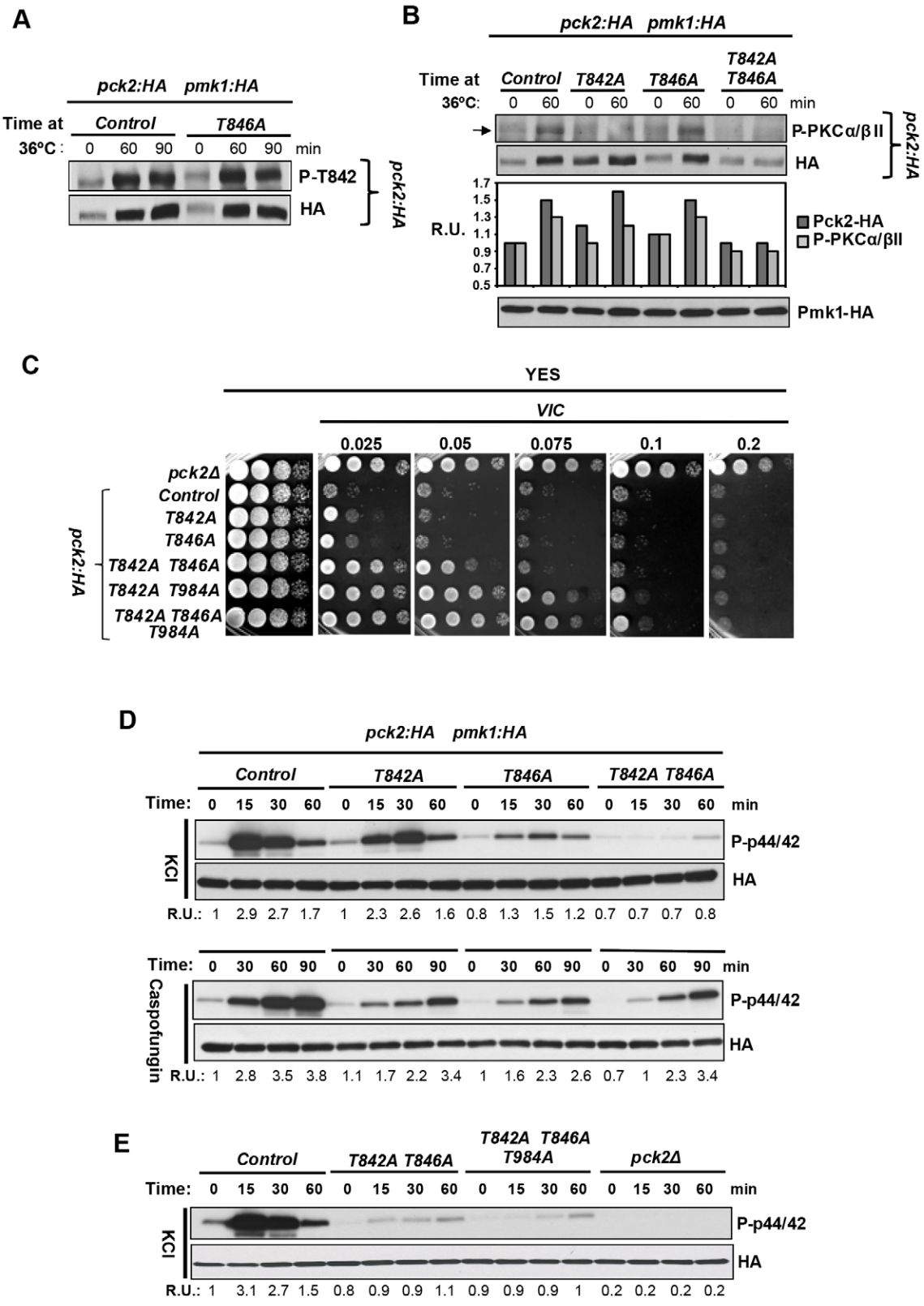

Fig. 5. Role of T846 during catalytic activation of Pck2 and signaling to Pmk1. (A) Strains MM913 (Pck2–HA; control) and MM916 (Pck2.T846A–HA), expressing a genomic Pmk1–HA6H fusion, were grown in YES medium at 28˚C (0) and incubated at 36˚C. Cell extracts were resolved by SDS-PAGE and detected with anti-phospho (P)-T842 and anti-HA antibodies. (B) Strains MM913 (Pck2–HA; control), MM918 (Pck2.T842A–HA), MM916 (Pck2.T846A–HA) and MM921 (Pck2.T842A T846A–HA), expressing a genomic Pmk1–HA6H fusion, were grown in YES medium at 28˚C (0), or incubated at 36˚C for 1 h. Cell extracts were resolved and detected with anti-phospho-PKCα/β II and anti-HA antibodies. R.U. relative units. (C) Serially diluted cells of strains described in B, plus strains MM1123 (Pck2.T842A T984A–HA) and MM931 (Pck2.T842A T846A T984A–HA), were spotted on YES plates supplemented with 0.025–0.2 M $MgCl_2$ plus 1 μg/ml FK506 (VIC), and incubated for 3 days at 28˚C. (D,E) Pmk1 activation in cultures of strains described in C, treated with 0.6 M KCl or 1 μg/ml caspofungin.

with caspofungin, resulting in decreased Pmk1 activation (Fig. 8A, upper panels). Rho2, Mkh1 and Pek1 protein levels remained unaltered in these cells (Fig. 8A, lower panels). Moreover, as compared to *ksg1-208* cells, both total and T842-phosphorylated Pck2 were almost undetectable in *ksg1-208 tor1Δ* cells at the restrictive temperature (Fig. 8B). The fission yeast TORC2 complex is composed of Tor1, Rictor homolog Ste20 (also known as Ste16), Lst8 homolog Pop3 (also known as Wat1) and Bit61 (Otsubo and Yamamato, 2008). Whereas *tor1Δ*, *ste20Δ* and *sin1Δ* mutants were moderately sensitive to growth inhibition

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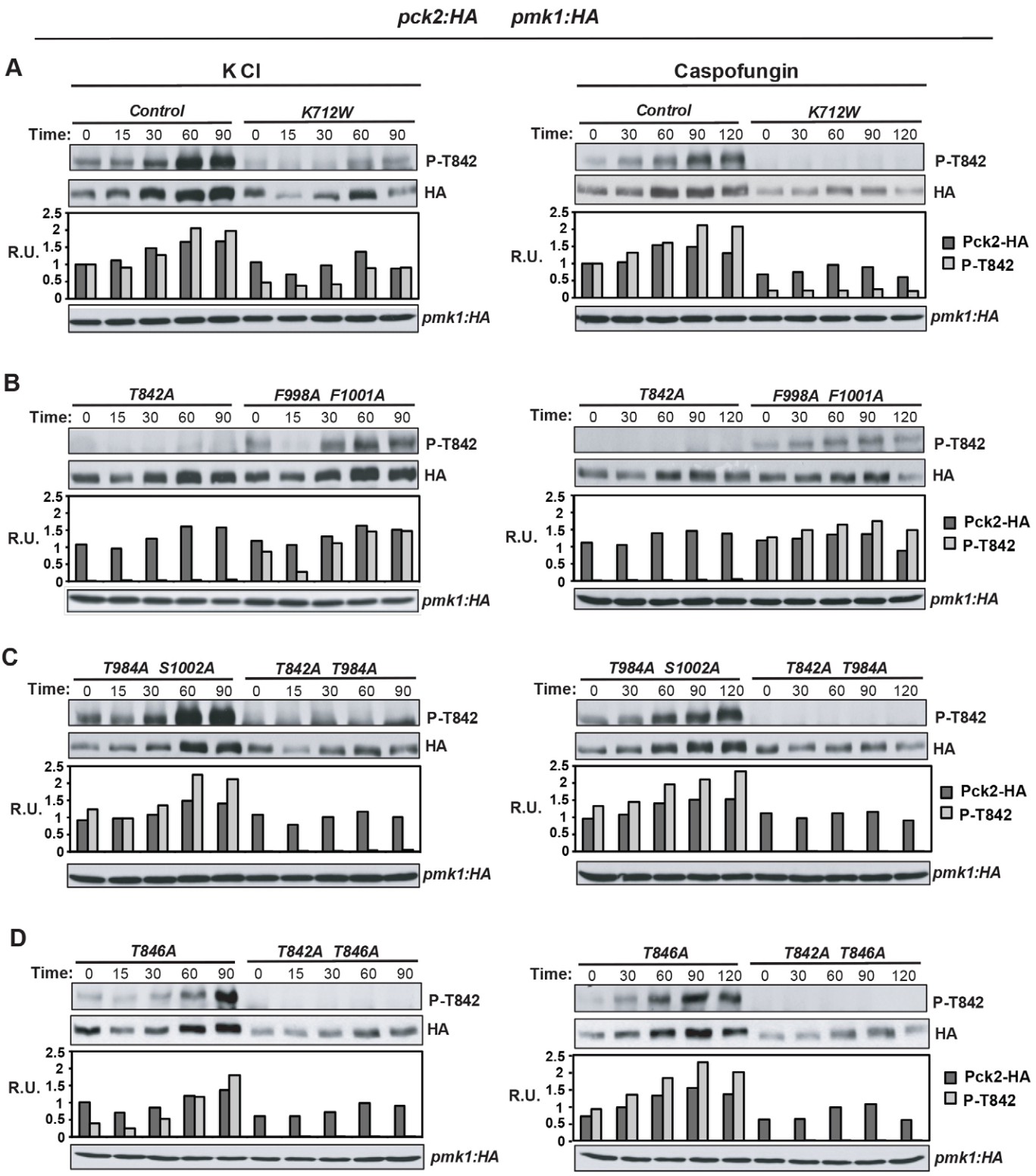

Fig. 6. Activation domain phosphorylation accounts for increased Pck2 levels during stress. (A) Strains MM913 (Pck2–HA; control) and MM1022 (Pck2.K712W–HA), (B) MM918 (Pck2.T842A–HA) and MM998 (Pck2.F998A F1001A–HA), (C) MM1042 (Pck2.T984A S1002A–HA) and MM1123 (Pck2.T842A T984A–HA), (D) MM916 (Pck2.T846A–HA) and MM921 (Pck2.T842A T846A–HA), expressing a genomic Pmk1–HA6H fusion, were grown in YES medium and treated with 0.6 M KCl or 1 μg/ml caspofungin. Cell extracts were resolved by SDS-PAGE and detected with anti-phospho (P)-T842 and anti-HA antibodies. R.U. relative units.

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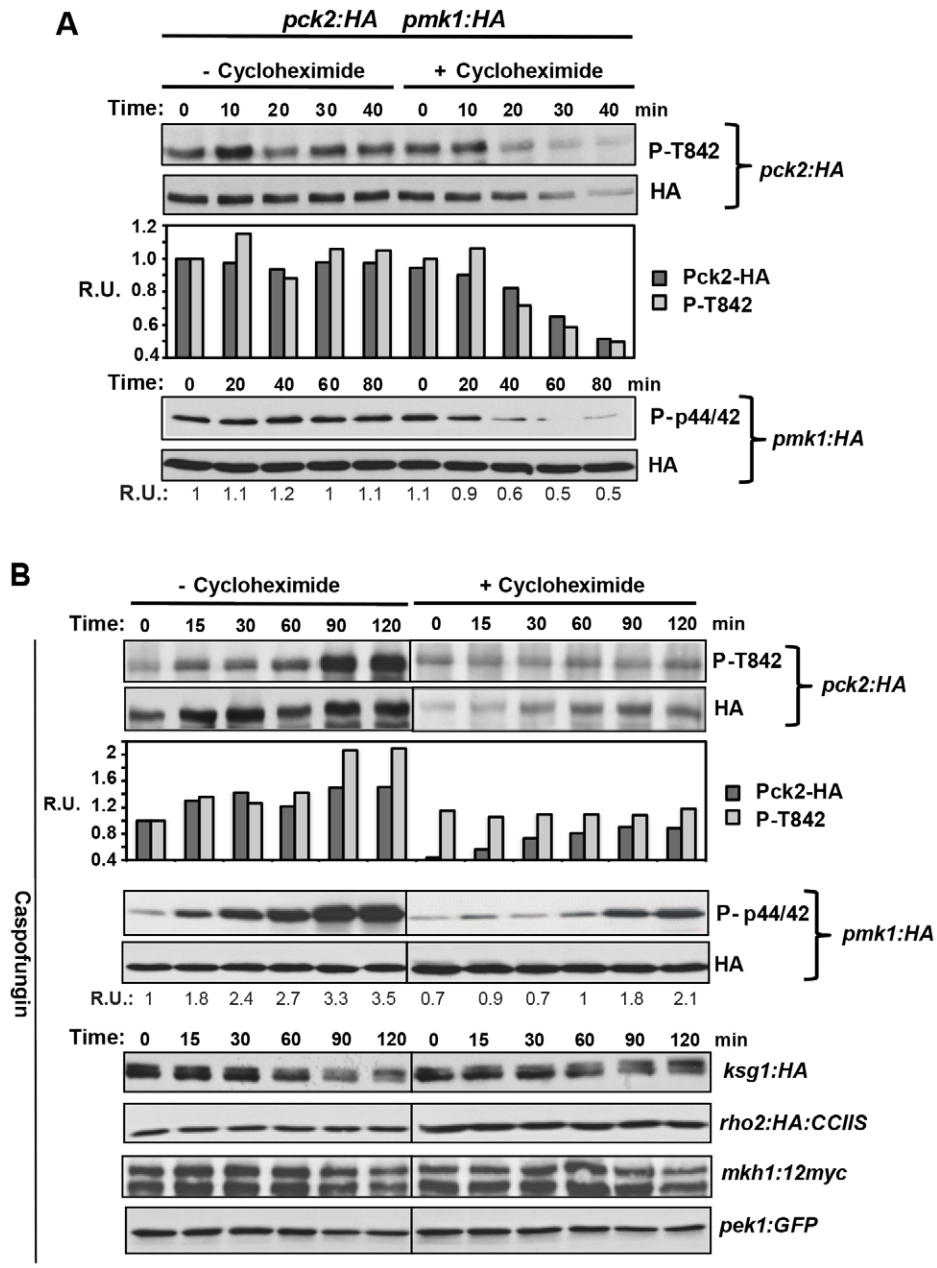

Fig. 7. Increased *de novo* Pck2 synthesis is required for activation of the cell integrity pathway in response to cell wall stress. (A) Control strain MM913 (Pck2–HA, Pmk1–HA6H) was grown to early log phase and incubated with or without 100 μg/ml cycloheximide for the indicated times. Cell extracts were resolved by SDS-PAGE and detected with anti-phospho (P)-T842 and anti-HA antibodies. Activated and total Pmk1 were detected as in Fig. 1. R.U., relative units. (B) Upper panels, a growing culture of control strain MM913 was incubated with or without 100 μg/ml cycloheximide for 1 h, and treated with 1 μg/ml caspofungin. Detection of T842-phosphorylated and total Pck2, as well as active and total Pmk1, was performed as described above. Lower panels, strains MM1372 (Ksg1–HA), LSM400 (Rho2–HA-CCIIS), LS210 (Mkh1–12myc) and MI401 (Pek1–GFP) were treated as above, and the correspondent fusions were detected with anti-HA, anti-myc or anti-GFP antibodies.

in the presence of caspofungin, *pop3Δ* and *bit61Δ* cells remained unaffected by the drug (Fig. 8C). Interestingly, *Δtor1* Pck2-K712W double mutants showed additive caspofungin sensitivity as compared with that of *Δtor1* or Pck2-K712W cells (supplementary material Fig. S3C), suggesting that TORC2 controls cell wall integrity through Pck2-dependent and independent mechanisms. Taken together, these findings suggest that Tor1, as part of the TORC2 complex, enhances Pck2 synthesis, which contributes to the timely activation of the Pmk1 pathway in response to cell wall damage. Alternatively, TORC2 might stabilize Pck2 under conditions of stress, allowing it to accumulate at a constant translational rate. However, cycloheximide pre-treatment did not further reduce the low Pck2 levels of *tor1Δ* cells in the presence of caspofungin (supplementary material Fig. S3D), thus supporting the notion that Tor1-dependent regulation of Pck2 levels is likely exerted at a translational level. Cycloheximide pre-treatment suppresses Pmk1 activation during glucose exhaustion (Madrid et al., 2013), implying that progressive MAPK activation under these conditions also requires *de novo* Pck2 synthesis. Remarkably, in the absence of glucose, the amounts of both total and T842-phosphorylated Pck2 were significantly reduced in *tor1Δ* cells, together with decreased Pmk1 activation (Fig. 8D). Hence, Tor1 likewise regulates Pck2 synthesis and CIP activation during glucose limitation.

DISCUSSION

In this work, we show that Ksg1, the essential PDK1 ortholog in fission yeast, is a positive regulator of the CIP, as Pmk1 activity is abolished in cells expressing the *ksg1-208* thermosensitive allele during growth at the restrictive temperature and under multiple stresses. We also demonstrate that, *in vivo*, Ksg1 phosphorylates the highly conserved T842 within the activation loop of Pck2, a major upstream activator of the CIP (Barba et al., 2008; Ma et al.,

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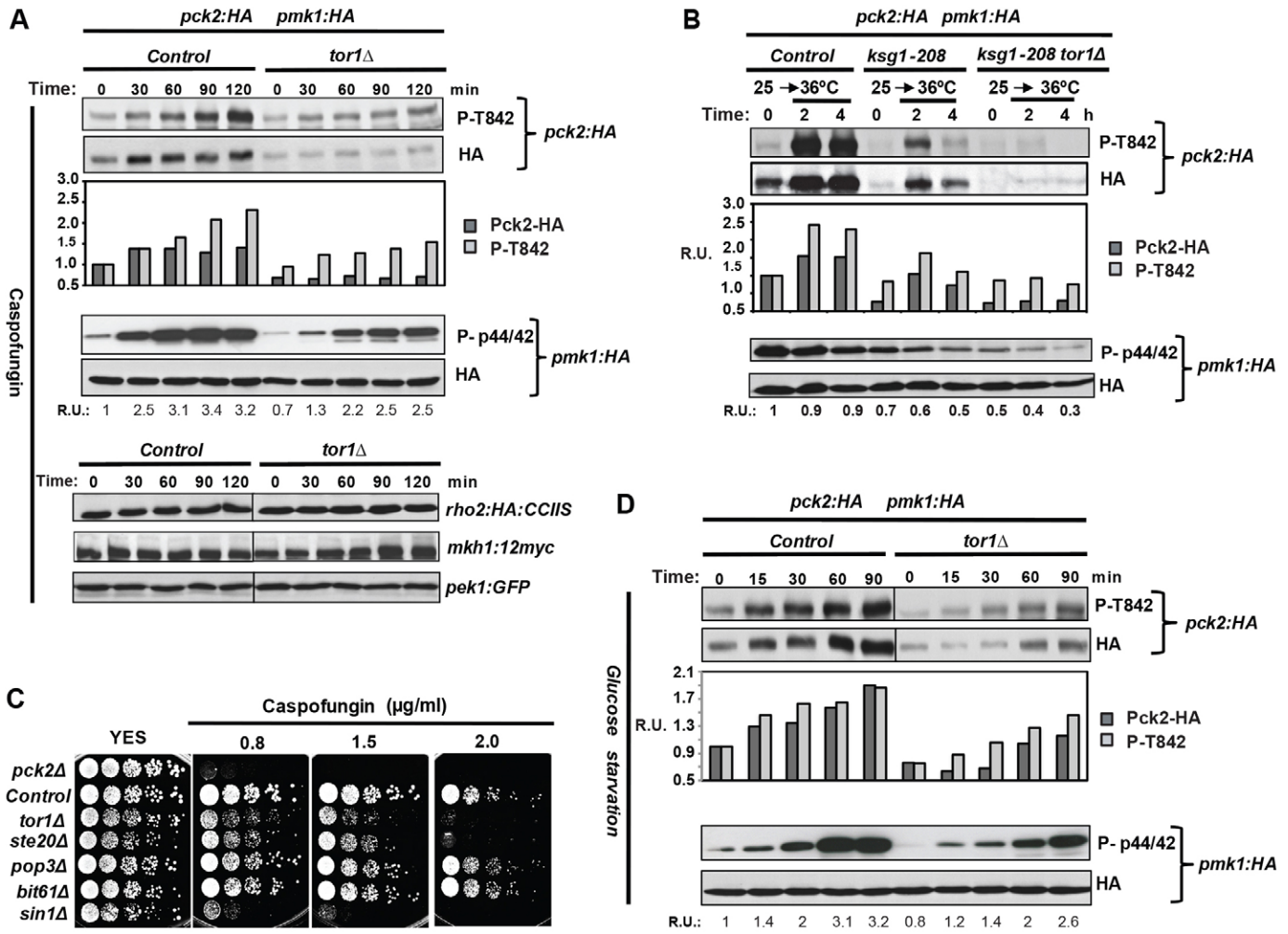

Fig. 8. The TORC2 complex positively regulates Pck2 synthesis and Pmk1 activation in response to specific stresses. (A) Upper panels, growing cultures of strains MM913 (Pck2–HA; control) and MM1205 (*tor1Δ* Pck2–HA) expressing genomic Pmk1–HA6H fusions were treated with 1 μg/ml caspofungin. Cell extracts were resolved by SDS-PAGE and detected separately with anti-phospho (P)-T842 and anti-HA antibodies. Activated and total Pmk1 were detected with anti-phospho (P)-p44/42 and anti-HA antibodies, respectively. Lower panels, strains LSM400 (Rho2–HA-CCIIS), LS210 (Mkh1–12myc) and MI401 (Pek1–GFP) were treated as above, and the correspondent fusions were detected with either anti-HA, anti-myc or anti-GFP antibodies. R.U., relative units. (B) Strains MM913 (control), MM1100 (Pck2–HA, *Ksg1-208*) and MM1257 (Pck2–HA, *Ksg1-208 tor1Δ*), expressing a genomic Pmk1–HA6H fusion, were grown at 25°C and then incubated at 36°C. Cell extracts were resolved and hybridized as indicated in A, and activated and total Pmk1 were detected as described above. (C) Serially diluted cells of strains GB3 (*pck2Δ*), MI200 (control), MM1194 (*tor1Δ*), MM1242 (*ste20Δ*), MM1241 (*pop3Δ*), MM1240 (*bit61Δ*) and MM1214 (*sin1Δ*) were spotted on YES plates supplemented with either 0.8–1.5 μg/ml caspofungin and incubated for 3 days at 28°C. (D) Strains MM913 (control) and MM1205 (*tor1Δ*), expressing a genomic Pmk1–HA6H fusion, were grown in YES medium with 7% glucose and shifted to the same medium without glucose and supplemented with 3% glycerol. Cell extracts were resolved by SDS-PAGE and detected with anti-phospho-T842 and anti-HA antibodies. Activated and total Pmk1 were detected as described above.

2006). Phosphorylation of the activation loop of PKC isoforms from higher eukaryotes is, in most cases, PDK1 dependent, with exceptions such as PKCθ or PKCδ (Freeley et al., 2011; Liu et al., 2006). This applies at least in part to Pck2, because T842 phosphorylation is absent from *ksg1-208* cells expressing a catalytically dead version of the kinase. Thus, both autophosphorylation and a Ksg1-dependent mechanism are involved in phosphorylation of the activation loop of Pck2.

Ksg1-dependent T-loop phosphorylation of Pka1, Gad8 or Psk1 is essential for catalytic activation and function *in vivo* (Matsuo et al., 2003; Nakashima et al., 2012; Tang and McLeod, 2004). In budding yeast, phosphorylation of the activation loop of the PKC ortholog Pkc1 at T983 by the redundant PDKs Pkh1 and Pkh2 is also indispensable for its catalytic and biological activity (Roelants et al., 2004). By contrast, phosphorylation of Pck2 at T842 is dispensable for the activation of Pmk1 during growth and stress. Moreover, turn motif phosphorylation at T984 is required for catalytic activation of Pck2, as the Pck2 signaling was suppressed in cells expressing a Pck2-T842A T984A double mutant. Activation loop phosphorylation is crucial for the activation of most PKC isoforms except PKCδ, because the presence of a pair of phenylalanine residues surrounding the T507 phosphorylation site stabilizes PKCδ activity in the absence of phosphorylation (Liu et al., 2006). Equivalent amino acids are, however, absent from Pck2, suggesting that other residues within the activation loop might become phosphorylated *in vivo* alternatively or in addition to T842. Indeed, T846 is also important for Pck2 activation and downstream signaling

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functions, given that T842A T846A cells displayed complete abrogation of Pmk1 activation upon salt stress, as compared with T842A or T846A single mutants. Residues equivalent to T846 are phosphorylated *in vivo* in several human PKC isoforms (Phanstiel et al., 2011), hinting that this post-translational modification might be evolutionarily conserved. Unfortunately, we have been unable to raise specific antibodies to detect specific T846 phosphorylation *in vivo*. Moreover, *in vitro* phosphorylation of Pck2 by Ksg1 was not further reduced in the T842A T846A mutant as compared with that of the T842A mutant (supplementary material Fig. S2C). This suggests that either T846 phosphorylation might take place only under certain physiological conditions, for instance in the presence of Rho1 or Rho2 GTPases, or that a kinase different from Ksg1 is responsible for this modification. The lack of increase in T984 phosphorylation in heat-shocked T842A T846A cells suggests a scenario where, in response to stress, *in vivo* Ksg1-mediated phosphorylation at T842, and likely T846, of the activation loop in Pck2 seems to be important for subsequent turn motif autophosphorylation at T984 in order to render the kinase fully functional. However, the observation that the VIC phenotype is stronger in the T842A T846A T984A triple mutant than in the T842A T846A double mutant (Fig. 5C) suggests that this functional interdependency might not exist during vegetative growth.

Conserved phenylalanine residues at the hydrophobic motif in human atypical PKC isoforms PKCζ and PRK2 define a docking site for PDK1 to mediate phosphorylation of the activation loop (Balendran et al., 2000). Both *in vitro* and *in vivo* Ksg1-dependent phosphorylation at T842 becomes partially compromised in cells expressing a Pck2-F998A F1001A mutant, strongly suggesting that a similar docking mechanism involves phosphorylation of the activation loop of Pck2 by Ksg1. By contrast, putative phosphorylation of S1002 at the hydrophobic motif appears to negatively regulate Pck2 catalytic activity, because although a S1002A mutant increased both T842 and basal Pmk1 phosphorylation, MAPK activity was reduced in S1002E or S1002D phosphomimetic mutants. Again, Pck2 resembles human PKCδ in that phosphorylation of the hydrophobic motif negatively regulates its catalytic activity (Karmacharya et al., 2010). In budding yeast, phosphorylation of Pkc1 at the Pdk1 site was dispensable for its ability to confer myriocin resistance (Roelants et al., 2004). However, the fact that *pkc1Δ* cells are not viable without osmotic support complicated the interpretation of this observation. We found that mutants expressing non-catalytic Pck2 show some measurable biological activity as compared to *pck2Δ* cells, implying that Pck2 functions are partially dependent on the protein itself rather than on its catalytic activity. This scaffold-like role of Pck2 might go unnoticed for PKC isoforms from higher organisms owing to their essential nature.

Rho1 and Rho2 promote the stabilization of Pck2 and concomitant increase in Ksg1-dependent phosphorylation at T842 (Sayers et al., 2000; Villar-Tajadura et al., 2008). Pck2 *de novo* synthesis and phosphorylation of the activation loop and turn motif *in vivo* increase progressively in response to high temperature, salt or cell wall stress, thus revealing that, in fission yeast, Pck2 behaves as stress-response-inducible protein. Therefore, maintenance of high Pck2 protein levels during stress relies on a complex mechanism involving *in vivo* phosphorylation plus Rho1- or Rho2-mediated stabilization. Importantly, while phosphorylation of the activation loop of Pck2 is a general requirement for downstream activation of the Pmk1 cascade, increased Pck2 synthesis is essential to trigger proper MAPK activation in response to cell wall damage or glucose starvation. This is based on the finding that Pmk1 phosphorylation kinetics under both conditions parallels the increase in total Pck2, and that, contrary to the remaining components of the cascade, Pck2 levels decreased significantly upon protein synthesis inhibition. Osmotic-stress-induced Pmk1 activation is a quick and transient event that does not require new protein synthesis (Madrid et al., 2006). However, MAPK activation under cell wall stress resembles a positive-feedback mechanism, where low Pmk1 activation at early time-points is followed by a progressive increase until reaching its maximum. The extent of the adaptive responses needed to cope with cell wall stress might explain the need for increased Pck2 synthesis to activate the CIP under this specific stimulus.

Which mechanisms are responsible for increased synthesis of Pck2 during stress? Tor1 kinase, as part of the TORC2 complex, appears to be involved in such translational control, as demonstrated by the defective Pck2 synthesis shown in *tor1Δ* cells under different stressful situations. This is particularly evident during cell wall damage or glucose deprivation, situations where a rise in Pck2 synthesis is required to allow efficient activation of the Pmk1 pathway. In mammalian cells, the mTORC2 complex regulates phosphorylation of the turn motif of some PKCs to enhance their stability and folding (Freeley et al., 2011). However, Pmk1 activation, which becomes severely attenuated during salt stress in Pck2-T842A T984A cells, remained unaffected in Pck2-T842A *tor1Δ* cells (supplementary material Fig. S3E). Therefore, Tor1 is not involved in phosphorylation of the turn motif of Pck2 and might regulate increased Pck2 synthesis during stress through a phosphorylation-independent mechanism.

The results summarized in supplementary material Fig. S4 reveal that, in fission yeast, both Ksg1 and an autophosphorylation mechanism promote *in vivo* phosphorylation of the activation loop of Pck2 at T842, and likely T846, during growth and in response to multiple environmental stresses. These events, in addition to turn motif autophosphorylation at T984 and binding to Rho1 and/or Rho2, stabilize and render Pck2 catalytically competent to modulate the activation of the Pmk1 MAPK cascade and to elicit cell adaptation to stress by independent means, such as the incorporation of structural glucans into the cell wall. In addition, the TORC2 complex favors *de novo* Pck2 synthesis during stress, which is necessary to allow Pmk1 activation in response to specific environmental insults, such as cell wall stress or glucose limitation. Although TORC2–Gad8 signaling is required for adequate cellular response to stress, it has been shown recently that the CIP negatively regulates the TORC2–Gad8 pathway (Cohen et al., 2014). Therefore, we envisage a scenario where TORC2 will positively influence CIP signaling but will subsequently undergo CIP-mediated inhibition, thus modulating the stress response in a highly dynamic and flexible fashion.

Gad8, which undergoes *in vivo* phosphorylation of the turn motif and hydrophobic motif in a TORC2-dependent manner, has been shown to co-purify with several ribosomal proteins and to regulate the phosphorylation of ribosomal protein S6 (Du et al., 2012). Furthermore, protein levels of several PKC isoforms are strongly reduced in murine embryonic fibroblasts (MEFs) lacking mTORC2 components (Facchinetti et al., 2008; Ikenoue et al., 2008). These data, along with the fact that mTORC2 co-purifies with ribosomes (Zinzalla et al., 2011), raise the possibility that, in fission yeast, the TORC2 complex might be also associated with

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ribosomes, providing a suitable platform to regulate Pck2 translation and MAPK activation in response to environmental changes.

MATERIALS AND METHODS

Strains, media, growth conditions and gene disruption

S. pombe strains used in this work (supplementary material Table S1) were derived from control strain MI200, which expresses a genomic Pmk1–HA fusion (Madrid et al., 2006). Strains JM1250 (Sty1-HA6H) and MM1224(*tor2-51*) were provided by Jonathan Millar (University of Warwick, UK) and Sergio Moreno (Universidad de Salamanca, Spain), respectively. They were grown in rich (YES) or minimal (EMM2) medium with 2% glucose plus supplements (Moreno et al., 1991). Transformants expressing pREP3X-based plasmids were grown in liquid EMM2 with thiamine (5 mg/l) and were either plated in solid medium with or without the vitamin or transferred to EMM2 lacking thiamine (Pmk1 activation assays). The *tor1*$^+$, *ste20*$^+$, *pop3*$^+$, *bit6*$^+$ and *sin1*$^+$ null mutants were obtained by open reading frame (ORF) deletion and replacement with the *hphMX6* (hphR) or the G418 (*kanR*) cassettes by a PCR-mediated strategy using plasmids pFA6a-*hphMX6* or pFA6a-*kanMX6* (Bähler et al., 1998).

Gene fusion, site-directed mutagenesis and expression plasmids

To construct the integrative plasmid pJK148-Pck2:HA, the *pck2*$^+$ ORF plus regulatory sequences was amplified by PCR using fission yeast genomic DNA as the template and the 5′ oligonucleotide Pck2*Xba*I-F2 (supplementary material Table S2), which hybridizes 724– 703 bp upstream of the *pck2*$^+$ ATG start codon and contains a *Xba*I site, and 3′ oligonucleotide Pck2HA*Sma*I-R, which hybridizes at the 3′ end of the *pck2*$^+$ ORF and incorporates a 64-nucleotide sequence (underlined in supplementary material Table S2) encoding one HA epitope (sequence GYPYDVPDYA) and a *Sma*I site. PCR fragments were digested with *Xba*I and *Sma*I and cloned into the integrative plasmid pJK148. The Pck2 ORF contained an *Nru*I site that was deleted using the mutagenic 5′ oligonucleotide Pck2NruIX-F and the 3′ oligonucleotide Pck2NruIX-R with plasmid pJK148-Pck2:HA as template. The mutagenized Pck2 sequence was digested with *Xba*I and *Sma*I and subcloned to generate pJK148-Pck2NruIΔ:HA. Integrative plasmids expressing HA-fused Pck2 mutants were obtained by site-directed mutagenesis PCR using the plasmid pJK148-Pck2NruIΔ:HA as template and the mutagenic oligonucleotide pairs described in supplementary material Table S2. Once confirmed, the mutagenized Pck2 sequences were subcloned into pJK148. The resulting integrative plasmids were digested at the unique *Nru*I site within *leu1*$^+$, and transformed into *pck2Δ* strain GB3. Transformants *leu1*$^+$ were obtained and the fusions were verified by both PCR and western blot analysis. Wild-type and mutant Pck2 overexpression constructs were obtained by PCR amplification of the corresponding ORF using the above plasmids as templates with the 5′ oligonucleotide Pck2OE-F (*Xho*I site) and the 3′ oligonucleotide Pck2OE-R (*Sma*I site). Purified PCR products were digested with *Xho*I and *Sma*I and cloned into pREP3X (Siam et al., 2004).

A GST-fused wild-type Pck2 construct was obtained by PCR employing yeast genomic DNA as template and the oligonucleotides Pck2pKG-F (*Sma*I site) and Pck2pKG-R (*Xba*I site). The construct was digested with *Sma*I and *Xba*I and cloned into the plasmid pGEX-KG (Guan and Dixon, 1991) to generate pGEX-KG-Pck2. Mutant Pck2 constructs were obtained by site-directed mutagenesis using pGEX-KG-Pck2 as a template and the corresponding mutagenic oligonucleotide pairs (supplementary material Table S2), digested with *Sma*I and *Xba*I, and cloned into pGEX-KG. The Ksg1–GST fusion protein was obtained by PCR employing the oligonucleotide pair Ksg1pKG-F (*Sma*I site) and Ksg1pKG-F (*Nco*I site). A kinase-dead version of Ksg1 (K128R mutant) was obtained by site-directed mutagenesis with the plasmid pGEX-KG-Ksg1 as a template and the mutagenic oligonucleotides Ksg1K128R-F and Ksg1K128R-R. Constructs were digested with *Sma*I and *Nco*I and cloned into pGEX-KG.

Stress treatments and detection of activated Pmk1 and Sty1

Log-phase cell cultures (OD_{600} 0.5) were supplemented with either KCl (Sigma Chemical), caspofungin (Sigma Chemical) or Calcofluor White (Sigma Chemical). Hypotonic treatment was achieved by transfer of growing cells in YES medium supplemented with 0.8 M sorbitol (Sigma Chemical) to the same medium without polyol. In glucose starvation experiments, cells grown in YES medium with 7% glucose were resuspended in the same medium lacking glucose and osmotically equilibrated with 3% glycerol. Preparation of cell extracts, purification of HA-tagged Pmk1 or Sty1 with Ni^{2+}-NTA–agarose beads (Qiagen), and SDS-PAGE was performed as described previously (Madrid et al., 2006; Madrid et al., 2007). Dual phosphorylation in either Pmk1 or Sty1 was detected with rabbit polyclonal anti-phospho-p44/42 (Cell Signaling Technology) or mouse monoclonal anti-phospho-p38 (Cell Signaling Technology), respectively. Total Pmk1 or Sty1 were detected with mouse monoclonal anti-HA antibody (12CA5, Roche Molecular Biochemicals). Anti-PSTAIR (anti-Cdc2, Sigma Chemical) was occasionally used for loading control. Immunoreactive bands were revealed with an anti-mouse-IgG horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma) and the ECL system (Amersham-Pharmacia).

Detection of total and phosphorylated Pck2

Cell extracts were prepared using Buffer IP (50 mM Tris-HCl pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM β-mercaptoethanol, 10% glycerol, 0.1 mM sodium orthovanadate, 1% Triton X-100 and protease inhibitors). Equal amounts of total protein were resolved in 6% SDS-PAGE gels and transferred to Hybond-ECL membranes. To detect phosphorylation of the activation loop of Pck2 at T842, an anti-phospho-polyclonal antibody was produced by immunization of rabbits with a synthetic phosphopeptide corresponding to residues surrounding Thr842 of Pck2 (GenScript). A rabbit polyclonal anti-phospho-PKCα/βII antibody (Cell Signaling Technology) was used to detect turn motif phosphorylation of Pck2 at T984. Total Pck2 was detected with mouse monoclonal anti-HA antibody.

Kinase assays

In vitro kinase reactions were performed as described previously (Takada et al., 2007), with purified bacterially expressed GST–Ksg1 or GST–Ksg1-K128R (kinase dead) as activating kinases, and either wild-type or mutant GST-fused Pck2 constructs as substrates. GST-tagged fusion proteins were detected with polyclonal goat anti-GST antibody conjugated to horseradish peroxidase (GE Healthcare) and the ECL system.

Cell wall analysis

Labeling and fractionation of cell wall polysaccharides was performed as described previously (Sánchez-Mir et al., 2012).

Plate assay of stress sensitivity for growth

Decimal dilutions of *S. pombe* control and mutant strains were spotted in duplicate on YES solid medium or in the same medium supplemented with different concentrations of $MgCl_2$ (Sigma Chemical), FK506 (Alexis Biochemicals) or caspofungin. Plates were incubated at 28℃ for 3 days and then photographed.

Fluorescence microscopy

Calcofluor White was employed for cell wall and septum staining as described previously (Alfa et al., 1993). Images were taken on a Leica DM 4000B fluorescence microscope with a 100× objective and captured with a cooled Leica DC 300F camera and IM50 software.

Reproducibility of results

Densitometric quantification of western blot signals was performed using ImageJ (Schneider et al., 2012). Experiments were repeated at least three times with similar results. The data show the mean±s.d. and/or representative results.

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Competing interests
The authors declare no competing interests.

Author contributions
M.M. and J.C. conceived of the experiments. M.M., R.J., L.S.-M., T.S., A.F., J.V.-S. and P.P. performed the experiments. M.M., M.G. and J.C. wrote the manuscript.

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