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Cip1 tunes cell cycle arrest duration upon calcineurin activation

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Cells exposed to environmental stress arrest the cell cycle until they have adapted to their new environment. Cells adjust the length of the arrest for each unique stressor, but how they do this is not known. Here, we investigate the role of the stress-activated phosphatase calcineurin (CN) in controlling cell cycle arrest in *Saccharomyces cerevisiae*. We find that CN controls arrest duration through activation of the G1 cyclin–dependent kinase inhibitor Cip1. Our results demonstrate that multiple stressors trigger a G1/S arrest through Hog1-dependent down-regulation of G1 cyclin transcription. When a stressor also activates CN, this arrest is lengthened as CN prolongs Hog1-dependent phosphorylation of Cip1. Cip1 plays no role in response to stressors that activate Hog1 but not CN. These findings illustrate how stress response pathways cooperate to tailor the stress response and suggest that Cip1 functions to prolong cell cycle arrest when a cell requires additional time for adaptation.

cell cycle | stress response | calcineurin | Cip1

Significance

To ensure their survival, cells arrest the cell division cycle when they are exposed to environmental stress. The duration of this arrest is dependent upon the time it takes a cell to adapt to a particular environment. How cells adjust the amount of time they remain arrested is not known. This study investigates the role of the phosphatase calcineurin in controlling cell cycle arrest duration in yeast. We show that calcineurin lengthens arrest by prolonging Hog1-dependent activation of the poorly characterized cyclin-dependent kinase inhibitor Cip1. Cip1 only impacts cell cycle arrest in response to stressors that robustly activate calcineurin, suggesting that Cip1 is a context-specific regulator that differentially adjusts the length of arrest depending on the particular stressor.

Environmental conditions, including nutrient availability, temperature, pH, and osmolarity, can fluctuate rapidly. Thus, cells must coordinate their growth and division with environmental changes to ensure that they divide only when conditions are favorable. When cells attempt to divide in stressful environments, they do so at the cost of both cell integrity and future viability (1–3). To adapt to their environment, cells induce a transient cell cycle arrest and simultaneously promote stress-specific changes in gene expression and posttranslational modifications (4, 5). As adaptation occurs, some posttranslational modifications and gene expression changes are reversed, and cells resume cycling.

Cells often arrest in G1 phase, prior to DNA replication, when environmental conditions are unsuitable for cell division (6). Progression from G1 into S phase is controlled by a stoichiometric balance of positive and negative cell cycle regulators (7, 8). During G1, cells increase the expression of G1 cyclins, which then bind to and activate cyclin-dependent kinases (CDKs). As the levels of active G1 cyclin/CDK complexes rise, inhibitory signals from cyclin-dependent kinase inhibitors (CKIs) are overwhelmed, and cells can pass through the G1/S transition. Simply shifting this stoichiometric balance in the opposite direction will prevent cell cycle progression. Cells commonly achieve this shift by promoting 1) the down-regulation and/or destruction of cyclins and 2) the accumulation and activation of CKIs (9, 10).

The stress-activated MAPK p38/Hog1 is a conserved regulator of cell cycle arrest and adaptation. In mammals, p38 is activated by diverse stressors, including osmotic shock, arsenite, heat shock, and ultraviolet (UV) radiation (11). p38 inhibits the G1/S transition, as well as other cell cycle stages, to promote adaptation in response to stress (12, 13). To arrest cells in G1, p38 down-regulates cyclin gene expression and modulates the levels and activity of CKIs (3, 14–18). In budding yeast, the p38 homolog Hog1 also responds to a wide array of environmental stressors. However, the role of Hog1 in cell cycle regulation is best characterized in response to osmotic stressors such as NaCl, KCl, and sorbitol (19, 20). Similar to p38, Hog1 promotes a G1/S cell cycle arrest by down-regulating the G1 cyclins, Cln1 and Cln2, and promoting the activation and accumulation of the G1 CKI Sic1 (Fig. 1*A*) (21–23). An additional Hog1-regulated CKI, Cip1, was recently identified and found to act redundantly with Sic1 in response to osmotic stress (24).

Several factors impact the length of time cells remain arrested after they are exposed to environmental stress. One factor that affects cell cycle arrest duration is the severity of the stress. For example, arrest duration in yeast scales with increasing concentrations of osmotic stressors such as NaCl (23). In some cases, cells tailor their response to particular stressors by simultaneously activating multiple stress-response pathways (25–30). One example of this type of regulation occurs when yeast cells are exposed to $CaCl_2$, which triggers a longer cell cycle arrest than NaCl or sorbitol even at concentrations of $CaCl_2$ that cause a smaller increase in osmolarity (26). Although all three of these stressors similarly activate the MAPK Hog1, $CaCl_2$ also activates the calcium/calmodulin-activated

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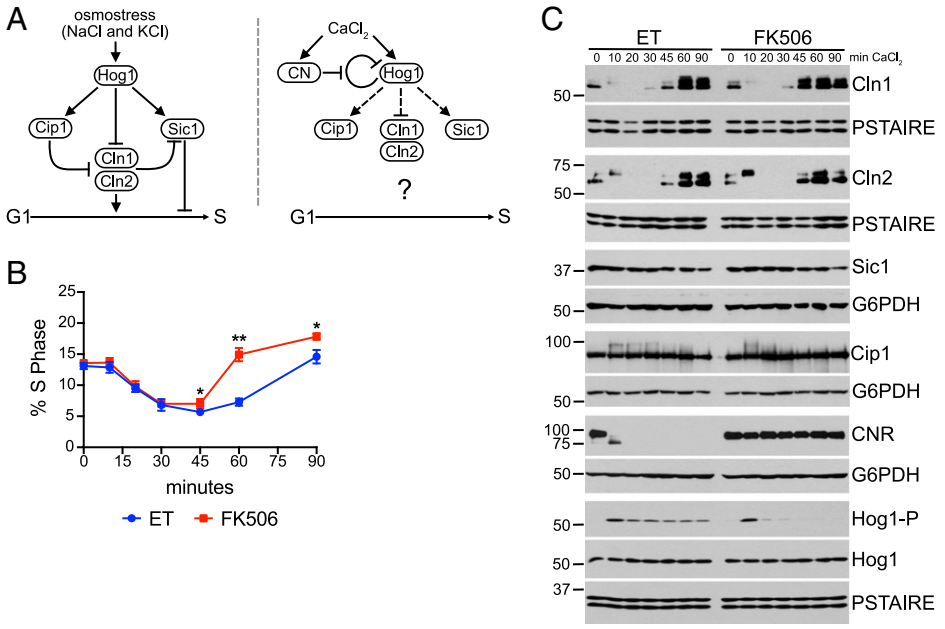

Fig. 1. CN controls the duration of $CaCl_2$-induced changes in G1/S cell cycle regulators. (*A*) Model of Hog1-dependent regulation of the G1/S transition in response to osmostress (*Left*) and potential regulation of G1/S proteins by CN-Hog1 cross-talk during $CaCl_2$ stress (*Right*). (*B*) Cells were pretreated with ET buffer or FK506 for 15 min before the addition of $CaCl_2$. DNA content was measured by flow cytometry and the percentage of cells in S phase from an average of $n = 3$ experiments is shown. Error bars represent the SD from the mean. A paired *t* test was used to calculate the statistical significance between ET- and FK506-treated samples at each time point. $^*P < 0.05$, $^{**}P < 0.01$. See also *SI Appendix*, Fig. S1A. (*C*) Cells were treated as in *B*, and samples were collected at the indicated times. Western blots were performed for Cln1-3V5, Cln2-3V5, Sic1-3V5, Cip1-13Myc, and GFP-Crz1$^{14-424}$ (CNR), phosphorylated/active Hog1 (Hog1-P), and total Hog1. PSTAIRE and G6PDH are shown as loading controls. Additional information about CNR can be found in *Materials and Methods*.

phosphatase calcineurin (CN). When Hog1 and CN are simultaneously activated, CN disrupts a negative feedback loop to prolong Hog1 activation, which then increases cell cycle arrest duration. Interestingly, $CaCl_2$-induced arrest can be initiated in the absence of CN, but activation of CN is required to maintain the arrest over time. The downstream effectors that enforce this prolonged CN-dependent arrest are not known (Fig. 1*A*).

Here, we investigated how CN prolongs the duration of Hog1-mediated cell cycle arrest to better understand how cells adjust arrest duration in response to different environmental stressors. We analyzed the temporal regulation of G1/S cell cycle regulators in response to CN activation and discovered that cell cycle arrest is controlled by two Hog1-dependent mechanisms that differentially require CN activity. First, Hog1 triggers a rapid CN-independent arrest in response to $CaCl_2$ by decreasing G1 cyclin expression. CN then extends this arrest by prolonging the Hog1-dependent activation of the poorly characterized G1 CKI Cip1. Notably, Cip1 does not contribute to normal cell cycle progression and has no effect on arrest dynamics in response to stressors that activate Hog1 but not CN. These findings demonstrate that a major function of Cip1 is to tune the length of cell cycle arrest in response to stressors, which enables cells to arrest for a duration appropriate for adaptation to different environments.

Results

CN Controls the Duration of $CaCl_2$-Induced Changes in G1/S Cell Cycle Regulators. To study how CN controls cell cycle arrest we investigated the cellular response to $CaCl_2$. In these conditions, cells transiently arrest for ~90 min, until they adapt and resume cycling (Fig. 1*B*) (26). The initial arrest that occurs is CN independent since it occurs with identical timing whether or not CN is inhibited with the drug FK506 (tacrolimus). In contrast, CN is required to maintain the arrest since FK506-treated cells resume cycling by ~60 min (Fig. 1*B*). Our aim was to identify the factors that mediate this extended CN-dependent cell cycle arrest.

We previously showed that CN increases the duration of $CaCl_2$-induced G1/S cell cycle arrest beyond the period observed for canonical osmostressors, in part by prolonging the activation of Hog1 (Fig. 1*C*) (26). Hog1 arrests cells at the G1/S transition in response to canonical osmostressors by modulating the expression and activity of the G1 cyclins *CLN1* and *CLN2* (22, 23, 31) and the G1 CKI Sic1 (21, 23). The recently identified CKI Cip1 may also contribute to arrest, but evidence suggests it is functionally redundant with Sic1 (24). We hypothesized that CN may extend $CaCl_2$-mediated cell cycle arrest by prolonging the Hog1-dependent repression of G1 cyclins, activation of CKIs, or both (Fig. 1*A*).

To investigate these possibilities, we examined the expression of G1/S regulators in response to $CaCl_2$. Cells were also treated with FK506 to identify CN-dependent changes. $CaCl_2$ stress robustly activates both CN and Hog1, which can be detected by the dephosphorylation and nuclear translocation of a previously described CN reporter (CNR) and the activating phosphorylation on Hog1 (Fig. 1*C* and *SI Appendix*, Fig. S1 *B* and *C*). There was no change in expression of the G1 cyclin *CLN3* (*SI Appendix*, Fig. S1*D*). In contrast, transcription of the downstream cyclins *CLN1* and *CLN2* rapidly decreases following exposure to $CaCl_2$ (26). Consistent with their mRNA levels, Cln1 and Cln2 protein levels were reduced within 10 min after the addition of $CaCl_2$, and this down-regulation was independent of CN activity (Fig. 1*C*). However, the duration of cyclin down-regulation was influenced by CN activity since both proteins accumulated earlier in FK506-treated cells (compare 45-min time points). Importantly, the onset of cyclin down-regulation preceded the initial CN-independent cell cycle arrest that occurs in response to $CaCl_2$ (Fig. 1 *B* and *C*). These data suggest that down-regulation of Cln1 and Cln2 could contribute to the initiation of $CaCl_2$-induced cell cycle arrest.

To determine whether activation of CKIs could also contribute to CN-regulated cell cycle arrest, we examined the expression of Sic1 and Cip1. Surprisingly, we did not observe any $CaCl_2$-induced changes in *SIC1* mRNA or protein (Fig. 1*C* and *SI Appendix*, Fig. S1*E*), even though Hog1-dependent stabilization of Sic1 was previously shown to regulate arrest in response to NaCl (21). Although there was a modest increase in *CIP1* mRNA, Cip1 protein levels did not change following the addition of $CaCl_2$ (*SI Appendix*, Fig. S1*F* and Fig. 1*C*). However, Cip1 was phosphorylated in response to $CaCl_2$, and the length of time that Cip1 remained phosphorylated was CN dependent (compare 20- to 60-min time points, Fig. 1*C*). Interestingly, Cip1 phosphorylation paralleled cell cycle arrest dynamics and cells did not resume cycling until phosphorylation decreased to prestress levels (Fig. 1 *B* and *C*). Since phosphorylation activates Cip1 (24), these data suggest that Cip1 could control the duration of G1 arrest by inhibiting G1 cyclin/CDK complexes and raises the possibility that Cip1 has a nonredundant role during adaptation to $CaCl_2$.

Hog1 Is Required for G1 Cyclin Down-Regulation and Cip1 Phosphorylation. Activation of CN affected the duration of both cyclin down-regulation and Cip1 phosphorylation, and changes in these G1/S regulators correlated with the timing of Hog1 activation (Fig. 1*C*). To directly test whether Hog1 was required for the $CaCl_2$-induced changes we observed, we monitored the expression of G1/S regulators in *hog1*Δ cells following treatment with $CaCl_2$. CN was activated normally in *hog1*Δ cells (*SI Appendix*, Fig. S1*G*); thus, any changes in the cyclins or Cip1 could be attributed to a specific loss of *HOG1*. As expected, Cln1 and Cln2 protein levels were maintained in the absence of *HOG1*, consistent with the expression of their mRNAs (*SI Appendix*, Fig. S1*H*) (26). Hog1 was also required for Cip1 phosphorylation (*SI Appendix*, Fig. S1*H*), similar to prior observations in cells exposed to KCl (24). Asynchronous *hog1*Δ cells had a lower fraction of cells in S phase (*SI Appendix*, Fig. S1*I*); however, there was no significant change in the fraction of S phase cells in response to $CaCl_2$, consistent with the expression and activation of G1/S regulators remaining unchanged (*SI Appendix*, Fig. S1*H*). Taken together, these data demonstrate that Hog1 is required for cyclin down-regulation, Cip1 phosphorylation, and cell cycle arrest in response to $CaCl_2$, similar to its role in the response to canonical osmostressors.

Down-Regulation of G1 Cyclin Transcription Initiates Cell Cycle Arrest. Since cyclin down-regulation preceded the onset of cell cycle arrest (Fig. 1 *B* and *C*), we hypothesized that Hog1-dependent down-regulation of cyclin transcription may trigger the arrest. However, cyclin levels could also decrease if $CaCl_2$ stress induced destabilization of cyclin proteins. To test this possibility, we examined the stability of Cln1 and Cln2 in the presence or absence of $CaCl_2$ (Fig. 2*A*). Both cyclins had very short half-lives (<5 min) and were not further destabilized by $CaCl_2$, consistent with the possibility that depletion of cyclin proteins results from transcriptional shutoff of the *CLN* genes. To determine whether transcriptional down-regulation was required to initiate arrest, we disrupted transcriptional regulation of *CLN1* by integrating the *GPD1* promoter upstream of *CLN1* in the genome, which resulted in consistent, elevated expression of Cln1 (Fig. 2*B*) (32). When compared with *CLN1* cells, *GPD1p-CLN1* blocked the stress-induced down-regulation of Cln1 and prevented the initial decrease in S phase that occurs after $CaCl_2$ addition (Fig. 2 *B* and *C*). This result suggested that

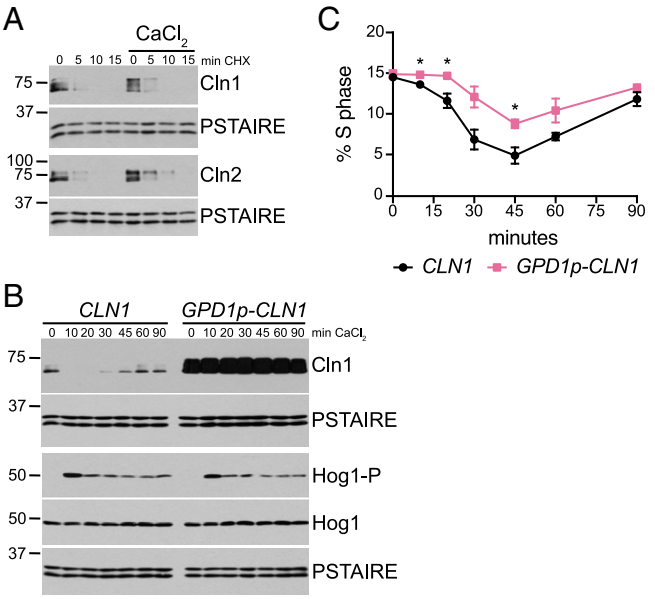

Fig. 2. Constitutive *CLN1* expression delays the onset of cell cycle arrest. (*A*) Where indicated, cells were pretreated with $CaCl_2$ for 2 min before the addition of cycloheximide (CHX). Samples were collected at the indicated times and Western blots were performed for Cln1-3V5 and Cln2-3V5. PSTAIRE is shown as a loading control. (*B*) *CLN1* and *GPD1p-CLN1* cells were treated with $CaCl_2$, and samples were collected at the indicated times. Levels of Cln1-3V5, total Hog1, and Hog1-P were monitored by Western blot. PSTAIRE is shown as a loading control. (*C*) DNA content from the cells in *B* was measured by flow cytometry and the percentage of cells in S phase from an average of $n = 3$ experiments is shown. Error bars represent the SD from the mean. Statistical significance between *CLN1* and *GPD1p-CLN1* samples at each time point was determined using a paired *t* test. $^*P < 0.05$. See also *SI Appendix*, Fig. S2*A*.

cyclin down-regulation is required to trigger cell cycle arrest in response to $CaCl_2$. Interestingly, cyclin expression from the *GPD1* promoter did not prevent cells from arresting at later time points since the percentage of S phase cells began to decrease 30 min after the addition of $CaCl_2$ in *GPD1p-CLN1* cells (Fig. 2*C*). As expected, Hog1 activity was not affected in *GPD1p-CLN1* cells, consistent with Hog1 acting upstream of *CLN* transcription (Fig. 2*B*). These data demonstrate that Hog1-dependent down-regulation of *CLN* transcription is required to initiate $CaCl_2$-induced cell cycle arrest but is not sufficient to maintain arrest at later time points.

The CKI Cip1 Controls Cell Cycle Arrest Duration. Our data suggested that Cip1 may also contribute to the prolonged CN-dependent cell cycle arrest in response to $CaCl_2$ (Fig. 1*C*). If true, this would be a unique Cip1-specific role, since Cip1 acts redundantly with Sic1 to regulate arrest in response to KCl (24). To test this possibility, we examined how cell cycle dynamics differed in *cip1*Δ cells. Following exposure to $CaCl_2$, *cip1*Δ cells initiated an arrest with timing identical to control cells but were unable to maintain the arrest at later time points (Fig. 3*A*). This result is nearly identical to what is observed when CN is inhibited with FK506 (Fig. 1*B*) or when cross-talk between CN and Hog1 is genetically disrupted (26), suggesting that Cip1 may mediate the prolonged CN-dependent cell cycle arrest. To confirm that Cip1 mediates arrest at the G1/S transition, we synchronized *CIP1* and *cip1*Δ cells and monitored their cell cycle progression following release from a G1 arrest into media with or without $CaCl_2$. Consistent with the asynchronous data, deletion of *CIP1* partially prevented the dramatic G1/S delay that occurs in response to $CaCl_2$ (Fig. 3*B*). A similar rescue is observed in

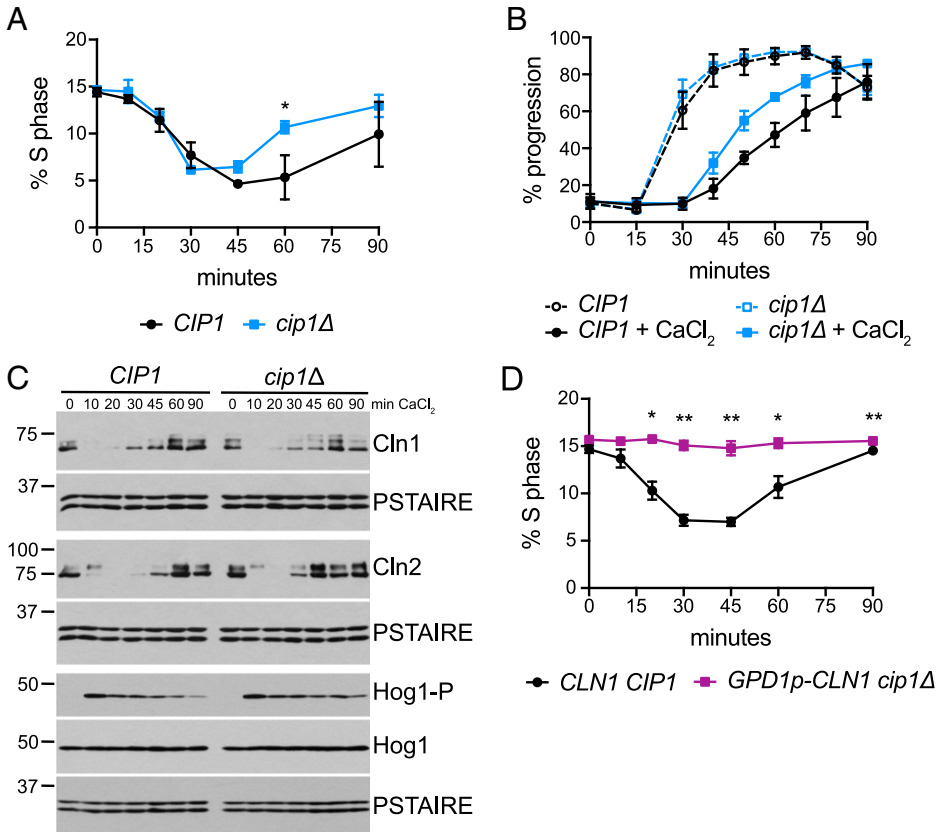

Fig. 3. Cip1 increases cell cycle arrest duration independent of cyclin down-regulation. (*A*) *CIP1* and *cip1*Δ cells were exposed to $CaCl_2$, and DNA content was measured by flow cytometry at the indicated times. The percentage of cells in S phase was calculated from an average of $n = 3$ experiments and error bars are shown representing the SD from the mean. For each time point, a paired *t* test was used to determine statistical significance between *CIP1* and *cip1*Δ samples. $*P < 0.05$. See also *SI Appendix*, Fig. S2*B*. (*B*) *CIP1* and *cip1*Δ cells were released from an alpha factor arrest into media with or without $CaCl_2$. The percentage of cell cycle progression was calculated as described in *Materials and Methods*. An average of $n = 3$ experiments is shown, and error bars represent the SD from the mean. See also *SI Appendix*, Fig. S2*C*. (*C*) *CIP1* and *cip1*Δ cells were treated as in *A*, and samples were collected at the indicated times. Levels of Cln1-3V5, Cln2-3V5, Hog1-P, and total Hog1 were monitored by Western blot. PSTAIRE is shown as a loading control. (*D*) *CLN1 CIP1* and *GPD1p-CLN1 cip1*Δ cells were treated with $CaCl_2$. DNA content was measured and the percentage of cells in S phase from an average of $n = 3$ experiments is shown. Error bars represent the SD from the mean. Statistical significance between *CLN1 CIP1* and *GPD1p-CLN1 cip1*Δ samples was calculated for each time point using a paired *t* test. $*P < 0.05$, $**P < 0.01$. See also *SI Appendix*, Fig. S2*D*.

synchronized cells upon deletion or inhibition of CN as well as genetic disruption of cross-talk between CN and Hog1 (26). Cip1 was not required for normal cell cycle progression since *cip1*Δ cells and control cells released with identical timing in the absence of $CaCl_2$ (Fig. 3*B*). Together, these data suggest that CN prolongs cell cycle arrest through Hog1-dependent activation of Cip1 and demonstrate a previously uncharacterized role for Cip1 in extending cell cycle arrest duration.

Since expression of *CLN1* from the *GPD1* promoter prevented the initial $CaCl_2$-induced arrest, and deletion of *CIP1* prevented the maintenance of the arrest, we hypothesized that cyclin down-regulation and Cip1 activation might act sequentially to control the duration of G1/S arrest. This model fits with the known functions of these regulators: Cip1 inhibits Cln1/Cdk1 and Cln2/Cdk1 complexes, as well as Cln3/Cdk1, which functions upstream of Cln1 and Cln2 (24, 33). Because of this, Cip1 is not expected to be necessary for arrest at early time points when Cln1/2 proteins are not expressed. However, at later time points when cyclin protein expression is restored, Cip1 may prevent premature activation of the Cln/Cdk1 complexes. Notably, as Cln1 and Cln2 levels accumulated ~30 to 45 min after $CaCl_2$ addition, phosphorylated forms of both proteins were evident earlier in *cip1*Δ cells compared with control cells (Fig. 3*C*, compare upper bands at 45 min). This observation is consistent with the possibility that Cip1 inhibits autophosphorylation of Cln1 and Cln2 (34). In addition, $CaCl_2$-induced cell cycle arrest was completely abolished in *GPD1p-CLN1 cip1*Δ cells that express elevated Cln1 and lack Cip1 (Fig. 3*D*). These data support the model that cyclin down-regulation initiates a cell cycle arrest in response to $CaCl_2$, which is then maintained by Cip1 through the extended inhibition of cyclin/CDK complexes.

Multisite Phosphorylation of Cip1 Is Required to Prolong Cell Cycle Arrest. Having identified a role for Cip1 in controlling the duration of cell cycle arrest, we wanted to better understand how Cip1 is regulated during adaptation to $CaCl_2$. Although *CIP1* transcription can be induced by the stress-activated transcription factors Msn2 and Msn4 (24), we observed only a modest transcriptional induction of *CIP1* in response to $CaCl_2$ (*SI Appendix*, Fig. S1*F*), and there was no change in Cip1 protein levels (Fig. 1*C*), which suggested that Cip1 does not control arrest through an increase in expression. However, Cip1 phosphorylation mirrored cell cycle arrest dynamics (Fig. 1 *B* and *C*), so we examined whether phosphorylation of Cip1 was required to prolong $CaCl_2$-induced arrest. Hog1 was previously shown to phosphorylate Cip1 at three N-terminal sites (T65, T69, and T73) to activate the protein in response to KCl (Fig. 4*A*) (24). We first tested whether a previously described mutant that cannot be phosphorylated at these sites (3TA) blocked the Cip1-mediated arrest in response to $CaCl_2$. Surprisingly, although the 3TA mutation prevented the observable Cip1 phosphoshift (Fig. 4 *B* and *C*), it had only a minor effect on

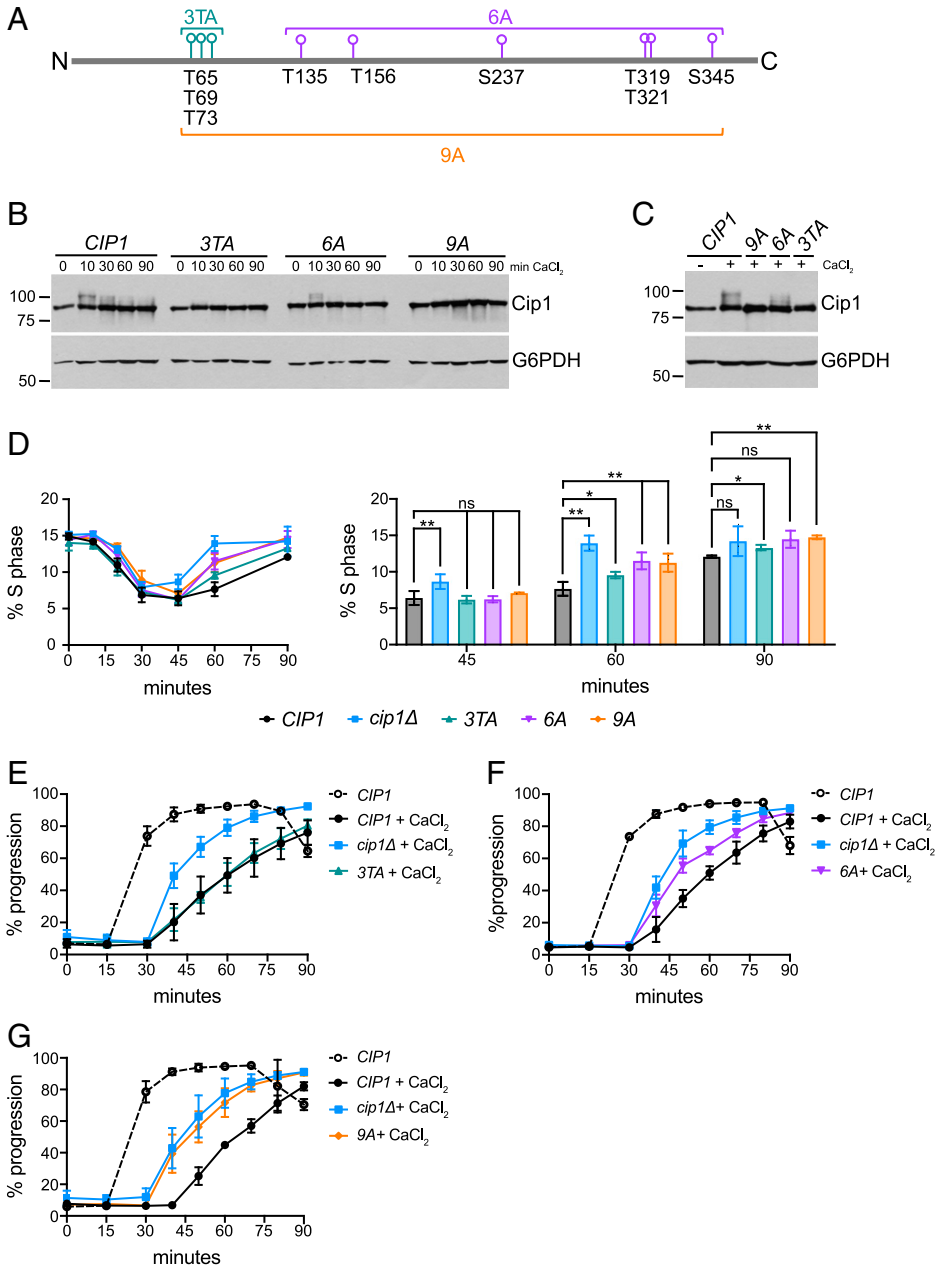

Fig. 4. Multisite phosphorylation of Cip1 is required to prolong cell cycle arrest. (*A*) Schematic of Cip1 protein and phosphorylation sites based on published work (3TA) or minimal S/T-P consensus sequence. (*B* and *C*) *CIP1*, *cip1-3TA* (*3TA*), *cip1-6A* (*6A*), and *cip1-9A* (*9A*) cells were treated with $CaCl_2$. Samples were collected at the indicated times and Western blots were performed against Cip1-13Myc (*B*). Direct comparison of Cip1 phosphorylation following 10 min of $CaCl_2$ treatment in *CIP1 3TA*, *6A*, and *9A* is shown in *C*. G6PDH is shown as a loading control. (*D*) *CIP1*, *cip1*Δ, *3TA*, *6A*, and *9A* cells were treated as in *B* and *C*. DNA content was measured by flow cytometry and the percentage of cells in S phase was calculated from an average of $n = 3$ experiments. Comparison of % S phase at the 45-, 60-, and 90-min time points is shown in the bar graph on the *Right*. Error bars represent the SD from the mean. For each time point, the statistical significance between *CIP1* and the indicated mutant was determined using a paired *t* test. $*P < 0.05$, $**P < 0.01$; ns, nonsignificant. See also *SI Appendix*, Fig. S2*E*. (*E–G*) *CIP1*, *cip1*Δ, *3TA* (*E*), *6A* (*F*), and *9A* (*G*) cells were released from an alpha factor arrest with and without $CaCl_2$. The percentage of cell cycle progression was calculated as described in *Materials and Methods*. Average values from at least $n = 3$ experiments are shown in each plot. Error bars represent the SD from the mean. See also *SI Appendix*, Fig. S2 *F–H*.

the duration of cell cycle arrest in asynchronous cells and had no detectable effect in synchronized cells (Fig. 4 *D* and *E*). Therefore, we considered whether Hog1 might phosphorylate additional sites on Cip1 following treatment with $CaCl_2$. Cip1 contains six additional putative phosphosites (T135, T156, S237, T319, T321, and S345) based on the minimal consensus sequence (S/T-P) recognized by Hog1 (Fig. 4*A*) (35) and Hog1-dependent phosphorylation of one of these sites (S345) has been reported during osmostress (36). Mutation of these six sites (6A) decreased both the extent and duration of Cip1 phosphorylation in response to $CaCl_2$ (Fig. 4 *B* and *C*) and partially prevented the Cip1-mediated arrest in both asynchronous and synchronized cells (Fig. 4 *D* and *F*). Finally, we examined a mutant in which all S/T-P sites were mutated to alanine (9A) and phosphorylation was completely prevented (Fig. 4 *A–C*). Similar to the 6A mutant, the 9A mutant partially prevented the Cip1-mediated arrest that occurs in asynchronous cells (Fig. 4*D*). However, the 9A mutant was indistinguishable from *cip1*Δ in synchronized cells (Fig. 4*G*), suggesting that phosphorylation of multiple sites throughout Cip1 is required to maintain cell cycle arrest in response to $CaCl_2$. These data are consistent with a model in which multisite phosphorylation

activates Cip1 and extends the length of cell cycle arrest as cells adapt to $CaCl_2$.

The minimal Hog1 consensus sequence is also the consensus sequence recognized by CDK (35, 37), which raised the possibility that the cell cycle effects we observed in the 6A and 9A phosphomutants resulted from interfering with CDK-dependent phosphorylation of Cip1. However, Cip1 did not undergo any detectable phosphorylation during the cell cycle in the absence of stress, suggesting that Cip1 is not a CDK substrate (*SI Appendix*, Fig. S3*A*). Furthermore, cells expressing the 9A mutant progressed through an unperturbed cell cycle with identical timing to control cells, demonstrating that Cip1 phosphorylation at S/T-P sites is not required for normal cell progression (*SI Appendix*, Fig. S3*B*). Taken together, our data suggest that Cip1 is not phosphorylated by CDK and is instead specifically phosphorylated during stress by the MAPK Hog1.

Strong CN Activation Is Required for Prolonged Cip1-Mediated Arrest. Our findings suggest that the primary function of Cip1 is to increase the duration of cell cycle arrest when cells are exposed to stressors that require additional time for adaptation. If this is true, we would not expect Cip1 to be required for arrest in response to stressors that trigger only a brief cell cycle delay, such as NaCl (26). Interestingly, CN is reported to be activated by NaCl and KCl (38), both of which are canonical Hog1-activating stressors (19). However, the relative strengths with which CN is activated by these different stressors has never been directly examined. We hypothesized that differences in the activation strength of CN could differentially affect Hog1-dependent activation of Cip1 and in turn alter cell cycle arrest dynamics.

To determine how strongly CN is activated in response to different stressors, we directly compared CN activation following treatment with 0.2 M $CaCl_2$, 0.4 M NaCl, or 0.4 M KCl by monitoring the dephosphorylation and nuclear localization of CNR. Although NaCl and KCl are reported to activate CN, there was no detectable dephosphorylation of CNR in response to either of these stressors (Fig. 5*A*). Moreover, although NaCl and KCl induced nuclear localization of CNR in a fraction of cells, it was a much smaller fraction than observed with $CaCl_2$ (Fig. 5 *B* and *C*). Therefore, although CN is strongly activated by 0.2 M $CaCl_2$, it is only weakly activated in response to 0.4 M NaCl or 0.4 M KCl, despite the greater increase in osmolarity with these latter stressors. As previously observed (26), Hog1 was only transiently activated after the addition of NaCl or KCl (Fig. 5*A*). Therefore, the strength of CN activation correlates with the duration of Hog1 activation.

Since Hog1 activation in response to NaCl and KCl was very brief, we examined whether these stressors also resulted in more transient cyclin down-regulation and Cip1 phosphorylation. In asynchronous cells, Cln1 and Cln2 levels decreased between 10 and 20 min after the addition of either NaCl or KCl (Fig. 5*A*). However, down-regulation of both cyclins occurred to a lesser extent than what was observed in response to $CaCl_2$. Similarly, while Cip1 was phosphorylated within 10 min after treatment with NaCl or KCl, phosphorylation was no longer evident by the 20-min time point, whereas it persisted after exposure to $CaCl_2$ (Fig. 5*A*). CN inhibition had no effect on the regulation of the cyclins or Cip1 in response to NaCl or KCl, consistent with the fact that CN was not strongly activated in these conditions (*SI Appendix*, Fig. S4 *A* and *B*). Notably, we did not observe any changes in the levels of Sic1 in response to NaCl or KCl (Fig. 5*A*), which is inconsistent with the proposed model that Sic1 stabilization triggers arrest in response to these stressors (21). Together, these results demonstrate that the timing of cyclin down-regulation and Cip1 phosphorylation mirror Hog1 activity in response to multiple stressors.

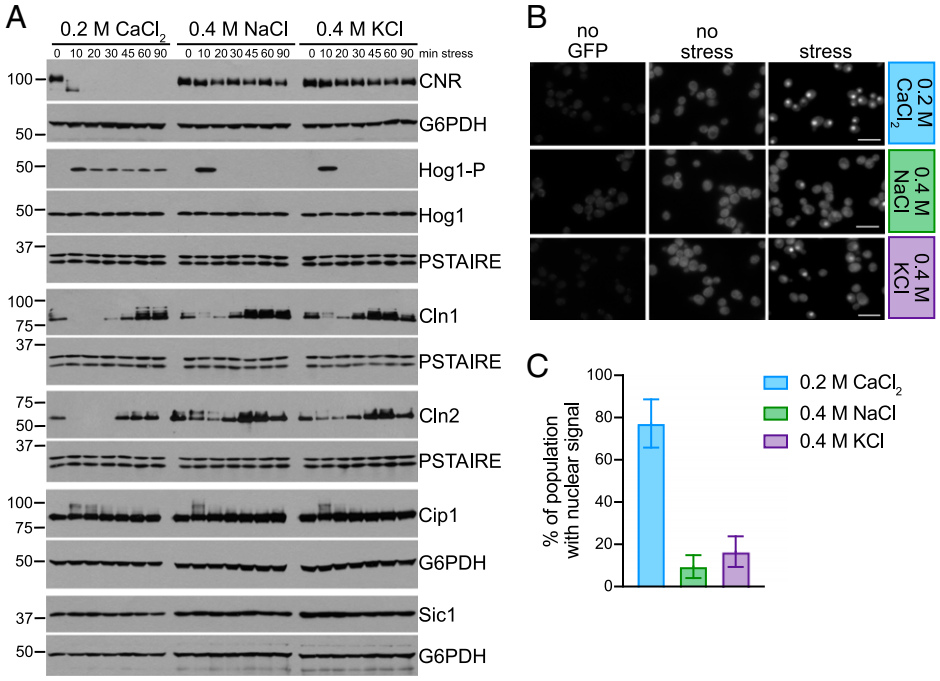

Fig. 5. Prolonged Cip1-mediated arrest requires strong CN activation. (*A*) Cells were treated with 0.2 M $CaCl_2$, 0.4 M NaCl, or 0.4 M KCl and samples were collected at the indicated times. Western blots were performed for CNR, Hog1-P, total Hog1, Cln1-3V5, Cln2-3V5, Cip1-13Myc, and Sic1-3V5. G6PDH and PSTAIRE are shown as loading controls. (*B*) Cells expressing CNR were treated with 0.2 M $CaCl_2$, 0.4 M NaCl, or 0.4 M KCl for 5 min and prepared for imaging as described in *Materials and Methods*. Representative images are shown. (Scale bar, 10 μm.) (*C*) The percentage of cells containing nuclear CNR signal was calculated from cells in *B*, and an average of $n = 3$ experiments is shown. Error bars represent the SD from the mean. Note that the same data from $CaCl_2$ treatment is also presented in *SI Appendix*, Fig. S1*C*.

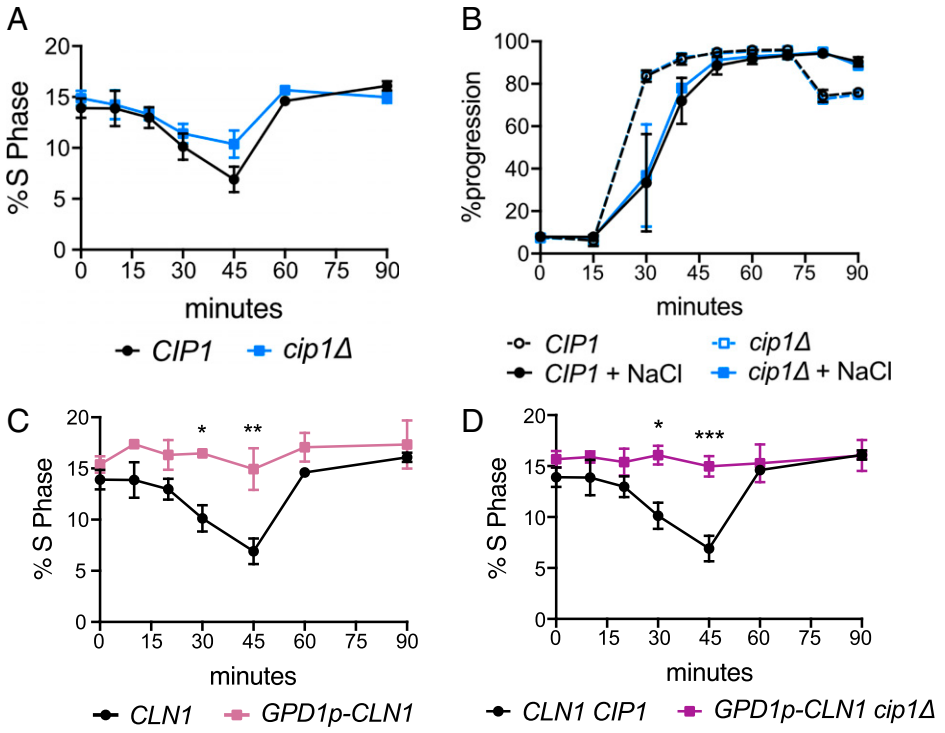

Fig. 6. Cip1 is not required for arrest in response to NaCl. (*A*) *CIP1* and *cip1*Δ cells were treated with 0.4 M NaCl, and samples were collected at the indicated times. DNA content was measured and the percentage of cells in S phase from an average of $n = 3$ experiments is shown. Error bars represent the SD from the mean. Statistical significance between *CIP1* and *cip1*Δ samples was determined for each time point using a paired *t* test. Differences at each time point were nonsignificant. See also *SI Appendix*, Fig. S5*A*. (*B*) *CIP1* and *cip1*Δ cells were released from an alpha factor arrest into media with or without 0.4 M NaCl. Values for the percentage of cell cycle progression were calculated as described in *Materials and Methods*. An average of $n = 3$ experiments is shown. Error bars represent the SD from the mean. See also *SI Appendix*, Fig. S5*B*. (*C*) *CLN1* and *GPD1p-CLN1* cells were treated with 0.4 M NaCl. DNA content was measured and the percentage of cell in S phase from an average of $n = 3$ experiments is shown. Error bars represent the SD from the mean. Statistical significance between *CLN1* and *GPD1p-CLN1* samples was determined for each time point using a paired *t* test. $^*P < 0.05$, $^{**}P < 0.01$. See also *SI Appendix*, Fig. S5*A*. (*D*) *CLN1 CIP1* and *GPD1p-CLN1 cip1*Δ cells were treated with 0.4 M NaCl. DNA content was measured and the percentage of cells in S phase from an average of $n = 3$ experiments is shown. Error bars represent the SD from the mean. Statistical significance between *CLN1 CIP1* and *GPD1p-CLN1 cip1*Δ samples was determined for each time point using a paired *t* test. $^*P < 0.05$, $^{***}P < 0.0005$. See also *SI Appendix*, Fig. S5*A*.

Since the brief Cip1 phosphorylation that occurred in response to NaCl and KCl coincided with the down-regulation of Cln1 and Cln2, we predicted that Cip1 would not be required for the transient cell cycle arrest that occurs in response to these stressors. Importantly, the overall duration of NaCl- and KCl-induced arrest was shorter than observed following $CaCl_2$ treatment (compare Fig. 6*A* and *SI Appendix*, Fig. S6*A* with Fig. 1*B*), consistent with the more transient activation of Hog1 in response to NaCl and KCl. Moreover, both asynchronous and synchronized *cip1*Δ cells arrested with nearly identical timing to control cells following exposure to NaCl and KCl (Fig. 6 *A* and *B* and *SI Appendix*, Fig. S6 *A* and *B*), in support of our model. In contrast, expression of *CLN1* from the *GPD1* promoter prevented cells from arresting in response to both NaCl and KCl (Fig. 6*C* and *SI Appendix*, Fig. S6*C*), and combining *GPD1p-CLN1* with *cip1*Δ had no additional effect on cell cycle arrest dynamics (Fig. 6*D* and *SI Appendix*, Fig. S6*D*). Taken together, these data demonstrate that Hog1 promotes cyclin down-regulation to trigger cell cycle arrest in response to multiple osmostressors. However, when a stressor also activates CN, CN increases arrest duration by prolonging Cip1 phosphorylation, which provides cells with additional time to adapt.

Discussion

Maintaining a cell cycle arrest for the appropriate amount of time in response to unique environmental stressors is crucial for adaptation and cell viability. Insufficient arrest may lead to damage and cell death, whereas an unnecessarily long arrest may cause cells to be outcompeted for available nutrients. In response to $CaCl_2$, the phosphatase CN promotes a prolonged G1 cell cycle arrest through a previously unknown mechanism (26). Here, we set out to examine the effect of CN activation on G1 cell cycle regulators and assess the relative contributions of these proteins to CN-dependent arrest. This analysis revealed that CN prolongs G1 arrest by maintaining Hog1-dependent activation of the poorly characterized cell cycle inhibitor Cip1. Although $CaCl_2$-induced arrest is initiated by a Cip1-independent mechanism, our findings demonstrate that Cip1 is required to prolong cell cycle arrest until cells have adapted to their environment. Cip1 does not appear to have any role in an unperturbed cell cycle; therefore its primary function may be to coordinate cell cycle arrest and adaptation to stress.

Our work also provides mechanistic insight into how CN and Hog1 coordinately regulate cell cycle arrest dynamics during adaptation to $CaCl_2$. CN and Hog1 are simultaneously activated in response to $CaCl_2$ stress and, together, modulate positive and negative G1 cell cycle regulators. Within 10 min of $CaCl_2$ exposure, Hog1 promotes the down-regulation of *CLN1* and *CLN2* transcription to initiate cell cycle arrest (*SI Appendix*, Fig. S1*H* and Fig. 2 *B* and *C*) (26). Hog1-dependent down-regulation of these transcripts likely occurs through a previously described mechanism in which Hog1 phosphorylates transcription factors that regulate the *CLN* genes (22). As the cyclins are down-regulated, Hog1 simultaneously phosphorylates and activates the G1 CKI Cip1 (*SI Appendix*, Fig. S1*H*). At these early time points Cip1 is not required for the arrest, which is expected, since Cip1 inhibits Cln/CDK complexes,

and the cyclins are not expressed at these time points. However, Cip1 remains phosphorylated until 60 to 90 min after the addition of $CaCl_2$ and is required to maintain cell cycle arrest later time points (Figs. 1*C* and 3*A*). Cip1 likely maintains cell cycle arrest by inhibiting Cln/CDK complexes as they reaccumulate in the cell (Fig. 3*D*). While Hog1 alone is responsible for the initial phosphorylation and activation of Cip1 (*SI Appendix*, Fig. S1*H*), activation of CN is required to prolong Cip1 phosphorylation and cell cycle arrest over time (Fig. 1*C*). Approximately 90 min after the addition of $CaCl_2$, cells adapt and resume cycling as cyclin levels and Cip1 phosphorylation return to prestress levels.

Our data strongly suggest that Hog1-dependent down-regulation of *CLN* transcription triggers cell cycle arrest in response to $CaCl_2$ (Fig. 2 *B* and *C*). This conclusion is supported by the fact that *CLN1/CLN2* down-regulation occurs within 10 min after $CaCl_2$ addition, before there is any detectable change in the cell cycle (Fig. 1 *B* and *C*). In contrast, the CKI Sic1 does not appear to be involved in initiating arrest, since its levels remain unchanged (Fig. 1*C* and *SI Appendix*, Fig. S1*E*). This result was unexpected since an increase in *SIC1* mRNA and Hog1-dependent stabilization of Sic1 protein have been reported in response to NaCl (21). However, in the previous study, the Hog1-activating stress was not applied until after cells were released from a G1 arrest, which may account for this discrepancy. We cannot completely rule out some involvement of Sic1 in $CaCl_2$-induced arrest since it is not possible to functionally separate down-regulation of Cln1 and Cln2 from activation of Sic1. Phosphorylation of Sic1 by G1 cyclin/CDKs promotes its ubiquitination and degradation (39); therefore *CLN* down-regulation in response to $CaCl_2$ prevents Sic1 degradation. In addition, when *CLN1* is overexpressed from the *GPD1* promoter (Fig. 2*B*), it inherently decreases Sic1 stability and could mask any involvement that Sic1 may have in the $CaCl_2$ arrest. However, since Cln1/2 act upstream of Sic1, and they are down-regulated prior to any detectable effect on the cell cycle, their down-regulation is most likely to initiate $CaCl_2$-induced arrest.

While CN was not required to trigger cell cycle arrest in response to $CaCl_2$ (Fig. 1*B*), we demonstrate that CN tunes arrest duration by maintaining Hog1-dependent phosphorylation of Cip1. Our findings demonstrate that prolonged multisite phosphorylation of Cip1 increases the duration of arrest in response to $CaCl_2$ (Fig. 4). Along with three previously described phosphosites (T65, T69, and T73) (24), we show that additional S/T-P sites within Cip1 are phosphorylated in response to $CaCl_2$, and phosphorylation of these sites is required to prolong arrest. Maximal Cip1 phosphorylation cannot be achieved when only the T65, T69, and T73 phosphosites are present (compare 6A with wild type [WT] in $CaCl_2$, Fig. 4*C*), indicating that additional S/T-P sites within the 6A group (T135, T156, S237, T319, T321, and S345) must also be phosphorylated during $CaCl_2$ stress. Although it is unclear whether all six sites are phosphorylated during stress, a phosphoproteomic study identified Hog1-dependent phosphorylation of S345 in Cip1 during NaCl stress (36), and S345 phosphorylation has also been observed in response to other Hog1-activating stressors (40). Together, this evidence strongly suggests that S/T-P sites outside of the N terminus are phosphorylated by Hog1 to promote Cip1-mediated arrest. Notably, *CIP1* transcription is also thought to be stimulated by the Hog1-regulated transcription factors Msn2/4 in response to multiple stressors, including KCl (24). However, in contrast to the nearly eightfold increase in *CIP1* expression that has been observed in response to KCl, *CIP1* expression only increased approximately twofold following treatment with $CaCl_2$ (*SI Appendix*, Fig. S1*F*), and there was no increase in total Cip1 protein levels (Fig. 1*C*). This suggests that phosphorylation is the primary mechanism regulating Cip1 during adaptation to $CaCl_2$.

Since Hog1 and CDK both phosphorylate S/T-P sites, this raised the possibility that Cip1 may be regulated by CDK in addition to Hog1. However, we did not detect any Cip1 phosphorylation during an unstressed cell cycle, suggesting that Cip1 is not a CDK substrate (*SI Appendix*, Fig. S3*A*). In addition, if Cip1 were a CDK substrate, we would expect that G1 cyclin/CDK complexes would regulate its activity since Cip1 functions at the G1/S transition (24, 33, 41). However, G1 cyclin/CDK complexes are unable to phosphorylate Cip1 in vitro (42). Finally, we found that neither deletion of *CIP1* nor mutation of all S/T-P sites in the protein affected progression through an unperturbed cell cycle (Fig. 3*B* and *SI Appendix*, Fig. S3*B*). These results argue that Cip1 does not have a cell cycle–regulatory role in the absence of stress. We note that a previous study suggested that Cip1 could be phosphorylated throughout the cell cycle by CDK (33). The reason for this discrepancy is not clear; however, that study also reported that *cip1*Δ cells progressed through the cell cycle following release from alpha factor with similar timing as wild-type cells, in agreement with our results.

CN is activated by many environmental stressors in addition to $CaCl_2$, including alkaline pH, cell wall stress, and toxic cations (38). However, the relative strength of CN activation across multiple stress conditions has never been directly compared. Here, we have simultaneously monitored CN activation in response to multiple osmotic stressors. We find that the strength of CN activation differs in response to unique stressors and this, in turn, affects the duration of Hog1 activity (Fig. 5). In the future it will be important to examine a broad range of stress conditions to determine whether CN and Hog1 regulate cell cycle dynamics through Cip1 during adaptation to these conditions in addition to $CaCl_2$.

Finally, our work suggests that specific CKIs may coordinate cell cycle arrest and adaptation in response to distinct environmental insults. Cip1 was previously shown to be functionally redundant with the related CKI Sic1 when Hog1 is activated by KCl (24). We have identified a nonredundant role for Cip1 in dramatically increasing G1/S cell cycle arrest duration when a stressor engages both Hog1 and CN (Fig. 3 *A* and *B*). Our findings suggest that individual CKIs may have specific roles in coordinating changes in the cell cycle with adaptation to environmental perturbations. Analogous mechanisms likely exist in mammals; for example, the mammalian homolog of Cip1, p21, controls arrest duration in response to DNA damage (6). Like yeast, mammalian cells are sensitive to changing external environments and must coordinate adaptation to these changes with an appropriate cell cycle arrest to protect cell viability (3). Future work aimed at identifying mammalian CKIs that impact arrest duration in response to different environmental conditions is needed to investigate this possibility.

Materials and Methods

Yeast Strains, Plasmids, and Growth Conditions. A complete list of strains is included in *SI Appendix*, Table S1. All experiments were carried out in strains carrying a deletion in the CN effector, *CRZ1*, to increase the amount of time it takes for cells to adapt to $CaCl_2$ stress. CN-Hog1 cross-talk occurs similarly in both WT and *crz1*Δ cells (26). All strains containing deletions and/or epitope tags were generated using standard methods as previously described (43, 44). Cip1 phosphomutants were generated using Hi-Q String gene fragments

(Thermo Fisher) containing the desired mutations that were then ligated into pGEM-T Easy (Promega) along with a 13MYC-His3Mx or 13MYC-KanMx PCR product using the NEBuilder HiFi Kit (New England Biolabs). The resulting insert was PCR amplified and integrated at the genomic locus using standard techniques. All mutations were confirmed by sequencing.

Strains were grown overnight at 30 °C in rich media (YM-1 complete media with 2% dextrose). Cells were shifted into synthetic complete media (C media with 1% ammonium chloride, 2% dextrose) for all experiments (45).

Monitoring CN Activation. CN activity was monitored using a previously described reporter in which GFP is fused to a fragment of the CN target Crz1 (CNR). Upon activation, CN dephosphorylates CNR and promotes its nuclear localization (26, 46, 47). A decrease in CNR levels can also be used as a marker of CN activity since CN activation decreases CNR stability (47).

Stress Experiments. Cells were grown to midlogarithmic phase in synthetic complete media. Where indicated, cells were pretreated for 15 min with ET buffer (90% ethanol, 10% Tween-20) or 1 μg/mL FK506 (LC Laboratories) before the addition of 200 mM $CaCl_2$, 400 mM NaCl, or 400 mM KCl.

Flow Cytometry. Equivalent optical densities (0.15 OD 600 nm) of cells were fixed overnight at 4 °C in 70% ethanol. After sonication, cells were treated with 0.25 mg/mL RNase A (Biomatik) for 1 h at 50 °C followed by digestion with 0.125 mg/mL Proteinase K (Biomatik) for 1 h at 50 °C and staining with 1 μM Sytox Green (Invitrogen). DNA content was measured using a Guava EasyCyte HT flow cytometer (Millipore) and analyzed with FlowJo software (FlowJo, LLC). The population of cells with a DNA content between the 1C unreplicated peak and 2C replicated peak was used to calculate the percent of S phase cells. For synchronized cells, percent cell cycle progression was calculated using the following equation: % progression = ((H – 1C)/(2C-1C)) × 100, where H = histogram mean fluorescence intensity (MFI), 1C = MFI of 1C DNA content peak, and 2C = MFI of 2C DNA content peak (48). Average values from a minimum of three experiments are shown with error bars representing SD from the mean.

Western Blots. Equivalent optical densities (1 OD 600 nm) of cells were lysed in cold TCA buffer (10 mM Tris·HCl pH 8.0, 10% trichloroacetic acid, 25 mM NH_4OAc, 1 mM Na_2 ethylenediaminetetraacetic acid) and incubated on ice. Following centrifugation to clarify lysates, pellets were resuspended in resuspension solution (0.1 M Tris·HCl pH 11.0, 3% sodium dodecyl sulfate [SDS]) and boiled at 95 °C for 5 min. Clarified samples were centrifuged and the supernatants were transferred to new tubes containing 4× sample buffer (250 mM Tris·HCl pH 6.8, 8% SDS, 40% glycerol, 20% β-mercaptoethanol) and boiled at 95 °C for an additional 5 min. Western blotting was performed using antibodies against Hog1 (sc-165978, Santa Cruz Biotechnology), phosphorylated Hog1 (anti-phospho-p38 MAPK, 9211L; Cell Signaling Technology), GFP (clone JL8,63268, Clontech), V5 (R96025, Invitrogen), MYC (clone 9E10, M5546; Sigma), PSTAIRE (P7962, Sigma), and G6PDH (A9521, Sigma).

CNR Imaging. Imaging experiments were carried out using an endogenously integrated, N-terminal GFP-tagged $Crz1^{14\text{-}424}$ truncation (CNR), which lacks its DNA binding domain. Activation of calcineurin was monitored by both dephosphorylation and nuclear localization of CNR. Cells were pretreated for 15 min with ET buffer or FK506 prior to addition of 200 mM $CaCl_2$, 400 mM NaCl, or 400 mM KCl. At the indicated times, equivalent optical densities (1 OD 600 nm) of cells were collected, centrifuged, and resuspended in a small volume of synthetic complete media. Cells were imaged on a Nikon Eclipse E400 microscope. Equivalent exposure times were used to capture all images and adjustments for brightness/contrast were performed equally on all panels. A strain lacking CNR is shown as a negative control.

Cycloheximide-Chase Assays. Cells were pretreated for 15 min with ET buffer or FK506 before the addition of 200 mM $CaCl_2$. A total of 50 μg/mL cycloheximide (Biomatik) was added to block protein synthesis after 2 min of $CaCl_2$ treatment. Samples were collected for Western blot analysis at the indicated time points.

G1 Cell Cycle Synchronization. To arrest cells in G1, cells were treated with 10 μg/mL alpha factor (Biomatik) for 3 h (an additional 10 μg/mL alpha factor was added at 2 h). Alpha factor was removed from cells by centrifugation and washing. Cells were then released into media lacking alpha factor in the presence or absence of 200 mM $CaCl_2$, 400 mM NaCl, or 400 mM KCl.

Data Availability. All study data are included in the article and/or *SI Appendix*.

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