RESEARCH ARTICLE

STRESS SIGNALING

Time-Dependent Quantitative Multicomponent Control of the G₁-S Network by the Stress-Activated Protein Kinase Hog1 upon Osmostress

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Control of cell cycle progression by stress-activated protein kinases (SAPKs) is essential for cell adaptation to extracellular stimuli. Exposure of yeast to hyperosmotic stress activates the SAPK Hog1, which delays cell cycle progression through G₁ by direct phosphorylation of the cyclin-dependent kinase (CDK) inhibitor Sic1 and by inhibition of the transcription of the genes encoding the G₁ cyclins Cln1 and 2. Additional targets of Hog1 may also play a role in this response. We used mathematical modeling and quantitative in vivo experiments to define the contributions of individual components of the G₁-S network downstream of Hog1 to this stress-induced delay in the cell cycle. The length of the arrest depended on the degree of stress and the temporal proximity of the onset of the stress to the commitment to cell division, called "Start." Hog1-induced inhibition of the transcription of the gene encoding cyclin Clb5, rather than that of the gene encoding Cln2, prevented entry into S phase upon osmostress. By controlling the accumulation of specific cyclins, Hog1 delayed bud morphogenesis (through Clns) and delayed DNA replication (through Clb5). Hog1-mediated phosphorylation and degradation of Sic1 at Start prevented residual activity of the cyclin/CDK complex Clb5/Cdc28 from initiating DNA replication before adaptation to the stress. Thus, our work defines distinct temporal roles for the actions of Hog1 on Sic1 and cyclins in mediating G₁ arrest upon hyperosmotic stress.

INTRODUCTION

Stress-activated protein kinases (SAPKs) are essential for proper cell adaptation to extracellular stimuli (1). In budding yeast, increased extracellular osmolarity results in the activation of the p38-related stress-activated kinase Hog1, which elicits an extensive program required for cell adaptation, involving changes in gene expression, protein translation, and cell cycle progression (2–4). Diverse stresses, such as heat stress, extracellular hyperosmolarity, and DNA damage, critically affect progression through the cell cycle (5–8). When yeast cells are exposed to osmostress, the activation of the Hog1 SAPK causes a transient cell cycle delay in G₁, S, and G₂ phases, depending on where the cells are in the cell cycle when they experience the stress (8–12).

In yeast, control of the G₁-to-S transition is exerted at a particular point in the cell cycle, called "Start," by an extensive transcriptional program that mediates transcription of G₁- and S-phase cyclins (13–15) (Fig. 1). When cells reach Start, the burst of cyclin-dependent kinase (CDK) activity leads to initiation of budding, as well as phosphorylation and degradation of the CDK inhibitor Sic1 (16). DNA replication is initiated when the cyclin B/CDK complexes Clb5/Cdc28 or Clb6/Cdc28 (alternatively re-

ferred to as Clb5,6/Cdk1) phosphorylate components of the preinitiation complex (17, 18). At the end of G₁, the net activity of newly formed Clb5,6/Cdc28 depends on both the abundance of the Clb5,6 and the abundance of its inhibitor Sic1 (19). Thus, adequate progression into S phase requires degradation of Sic1 or sufficient activity of cyclin B–associated CDK activity to overcome Sic1-mediated inhibition (20, 21).

Cell cycle delay at G₁ imposed by Hog1 involves the down-regulation of *CLN1* and *CLN2* expression and the direct phosphorylation by Hog1 of Sic1 at Thr¹⁷³, which interferes with its ubiquitination (11, 22).

Studies with yeast having mutant forms of various cell cycle regulatory proteins or genetic nulls have elucidated mechanistic properties of the regulatory machinery at the G₁-S transition, including in cells that otherwise exhibit normal cell cycle progression (16, 21). Quantitative modeling approaches have also elucidated regulatory principles of cell cycle progression (23–25) as well as of mitogen-activated protein kinase (MAPK) signaling, of which SAPKs represent one class (26–28). Here, we combined quantitative in vivo experiments and mathematical modeling, with parameters constrained by the quantitative experimental data, to analyze the impact of Hog1 on the regulation of the G₁-S transition upon stress. With this integrated approach, we related distinct experimental results, conceptualized our findings, and used the resulting model to perform systematic quantitative analysis and make generalized conclusions. We show that the control of Sic1 degradation and *CLN1,2* and *CLB5* expression have different physiological and temporal roles in the regulation of the G₁-S network by the Hog1 upon hyperosmotic stress. Whereas transcriptional regulation of Clb5 was relevant when the stress occurred in early and middle G₁ phase, the Sic1-mediated delay was important for stress that occurred late in G₁ (after Start). Moreover, we determined that Sic1 was important for temporal coordination of DNA replication and budding.

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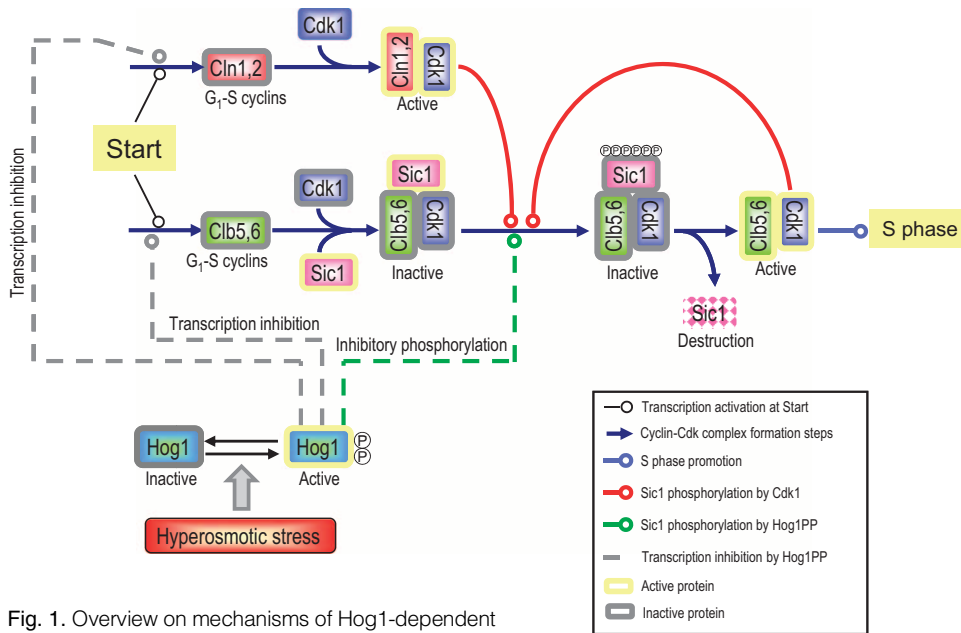‡Present address: Institute for Experimental Internal Medicine, Otto von Guericke University, Leipziger Strasse 44, 39120 Magdeburg, Germany.
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A Cell cycle progression
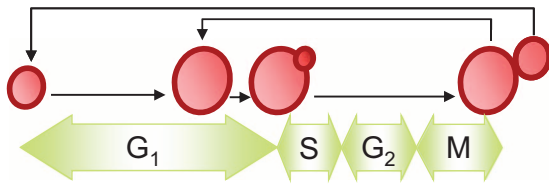
B Molecular network

Fig. 1. Overview on mechanisms of Hog1-dependent cell cycle regulation in G_1 and S phases. (**A**) Cell cycle of budding yeast. The red circles represent the yeast. Start occurs in G_1 and represents a commitment to begin cell division. The small daughter cell has a longer G_1 phase, whereas the mother cell reenters the cell cycle after mitosis closer to the G_1-to-S phase transition. (**B**) Regulation of the cell cycle by osmotic stress. The transition from Start to S phase is mediated by an increase in the abundance of cyclins Cln1 and Cln2 and Clb5 and Clb6 and their binding to the Cdk1 family kinase Cdc28. The Cln1/Cdc28 and Cln2/Cdc28 complexes are immediately active. Clb5/Cdc28 and Clb6/Cdc28 bind Sic1 and are inactive until multiple phosphorylation of Sic1 by Cln1/Cdc28 or Cln2/Cdc28 results in its degradation, releasing active Clb5/Cdc28 and Clb6/Cdc28. Cln1/Cdc28 and Cln2/Cdc28 coordinate bud formation, and Clb5/Cdc28 and Clb6/Cdc28 promote DNA replication. Hyperosmotic stress results in the phosphorylation of the stress-activated kinase Hog1. Active Hog1 interferes with normal G_1-S transition in two major ways: It inhibits transcription of the cyclin-encoding genes and phosphorylates Sic1 on residues that prevent its degradation.
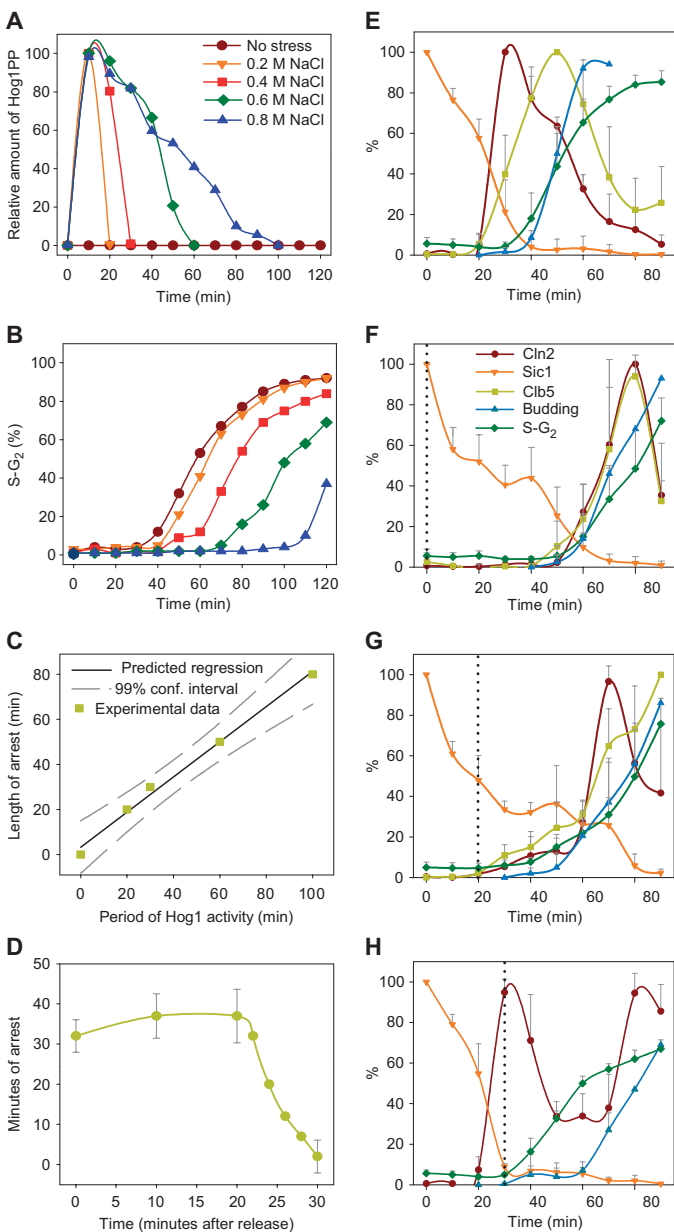
RESULTS

Cln1,2 and Sic1 play different roles in the Hog1-mediated cell cycle delay upon hyperosmotic stress

To understand and quantitatively assess the biological relevance of each regulatory element downstream of Hog1 in the G_1-S network, we experimentally quantified the impact of Hog1 on the cell cycle delay upon osmostress. We synchronized yeast cells with α factor, released the cells into fresh medium in the presence of different concentrations of NaCl (0 to 0.8 M NaCl), and then measured the time at which half of the cells

had replicated DNA, indicating that they were in S or G_2 (S-G_2) phase. Exposure of cells to increasing concentrations of NaCl resulted in longer duration of Hog1 phosphorylation, an indication of Hog1 activity (Fig. 2A and figs. S1 and S2), and longer delay of the cells in G_1, measured as DNA content analysis to detect cells in the S-G_2 phase (Fig. 2B and fig. S2). The duration of half-maximal Hog1 phosphorylation correlated with longer cell cycle delay in G_1 (Fig. 2C and fig. S2). Thus, these data suggested that the length of cell cycle delay at G_1 depends on the strength of extracellular osmolarity and correlates with the duration of Hog1 phosphorylation.

To determine whether the length of the cell cycle delay depended on the stage of G_1 in which cells were exposed to the hyperosmotic stress, we exposed the cells to medium containing 0.4 M NaCl at different times after release from synchronization. The length of delay was constant when cells were stressed within 20 min of removal of the pheromone, but the arrest shortened after this time (Fig. 2D). To avoid any indirect effect of α factor pheromone, we performed similar experiments with cells synchronized by elutriation (figs. S2 and S3), which selects newborn, small G_1 cells. Cells synchronized by elutriation are arrested early in G_1, whereas pheromone-synchronized cells are arrested late in G_1, near Start. Elutriated cells showed increased duration of Hog1 phosphorylation and cell cycle delay when subjected to different osmolarities at early G_1 and abrupt reduction in the delay when stressed at different time points after elutriation (fig. S2). Therefore, cells retain the ability to arrest for a specific period when stressed at different stages of G_1 until they reach a point when the ability to arrest becomes compromised.

We collected time course data sets by stressing cells at different times after release from pheromone and monitored the amounts of Sic1, Cln2, and Clb5, as well as the number of cells forming buds and of cells in S_1 or G_2. Wild-type cells bearing hemagglutinin (HA)–tagged Cln2 and Myc-tagged Sic1 or TAP (tandem affinity purification)–tagged Clb5 on their genomic loci were synchronized with α factor, released into fresh medium, and then subjected to osmostress (0.4 M NaCl) at different times (0, 20, or 30 min) after release (Fig. 2, E to H). Similar experiments were performed with cells arrested by elutriation (fig. S3). In the absence of stress, cells reached Start 20 min after release, as indicated by the onset of Cln2 production and Sic1 degradation (Fig. 2E). Passage through Start coincided with the time that pheromone-synchronized cells lost the ability to arrest at G_1 upon osmostress (Fig. 2D). Whereas in the absence of stress, there was a tight relationship between the lowest amounts of

Fig. 2. Hog1 activation correlates with cell cycle arrest in G_1 upon hyperosmotic stress and alters patterns of cyclin accumulation and Sic1 degradation. (**A**) W303 cells were synchronized and subjected to osmostress (0.2 to 0.8 M NaCl). Cultures were sampled every 10 min, and phosphorylated Hog1 (Hog1PP) was plotted as the percentage of its maximum level. Data are a representative experiment of three independent measurements. (**B**) DNA content was assessed by flow cytometry in cells treated as in (A) and plotted as the percentage of cells with duplicated DNA (representing cells in S or G_2). (**C**) Delay of DNA replication onset relative to control (measured as difference in time when half of the cells went into S or G_2 phases, in minutes) versus period of Hog1 activity (measured as period of at least half-maximal phosphorylation, in minutes) is shown for cells exposed to osmostress (0.2 to 0.8 M NaCl). (**D**) Relationship between the delay of replication onset and time of stress. Cells exposed to medium containing 0.4 M NaCl at the indicated times after release from synchronization were monitored by flow cytometry to determine DNA content. Data represent means and SD of three independent experiments. (**E**) Progression of YAN7 and YAN86 through the cell cycle in the absence of stress. (**F and G**) YAN7 and YAN86 cells were stressed with 0.4 M NaCl at 0 min (F) and 20 min (G) after release from pheromone. (**H**) YAN7 cells were stressed with 0.4 M NaCl 30 min after release from pheromone. In (E) to (H), YAN7 and YAN86 cells were analyzed in parallel. The cells were released from pheromone arrest and sampled every 10 min. Sic1 (orange), Cln2 (brown), and Clb5 (yellow) proteins (expressed as percentage of their maximal abundance), DNA content (green, representing cells in S or G_2), and budding index (blue) are depicted. Error bars represent means and SD of three independent experiments. Dotted lines in (F), (G), and (H) represent the time after release from pheromone arrest that osmotic stress was initiated.

after an osmostress at 30 min and a second peak appeared at 80 min (Fig. 2H), which was the time of the peak in Cln2 observed in cells stressed at earlier times (Fig. 2, F and G). These data suggest that although both processes are regulated simultaneously under normal conditions, they might be independently regulated by the SAPK in response to osmostress.

Clb5 is differentially down-regulated depending on the stage of G_1 in which cells are subjected to osmostress

Hyperactivation of Hog1 by the constitutive expression of a constitutively active form of Pbs2 (P_{GAL1}-Pbs2^{DD}) results in inhibition of *CLB5* expression, although it was not clear whether hyperosmotic stress also inhibits expression of this gene (*10*). To analyze the kinetics of Clb5 production upon stress, we analyzed the abundance of Clb5 after subjecting cells to hyperosmotic stress 0 or 20 min after release from pheromone arrest. Quantitative Western blots showed that, similar to Cln2, Clb5 accumulation was delayed upon osmostress (Fig. 2, E to G, and fig. S5). Expression of *CLB5* depends on MBF, which is a transcription factor complex containing Mbp1 and Swi4. Therefore, we asked whether *CLB5* alone or other genes regulated by MBF also exhibited altered expression patterns in cells subjected to hyperosmotic stress, and we found that expression of the MBF-regulated genes *RNR1*, *CDC21*, and *SWE1* was also delayed in response to stress (fig. S5A). Whereas Clb5 was undetectable when stress was applied just after release (Fig. 2F), we observed leaky production of Clb5 that reached ~20% of the maximum amount when cells were stressed 20 min after release, which is closer to Start (Fig. 2G and fig. S5B). However, this amount of Clb5 failed to induce DNA replication (Fig. 2G), suggesting that Sic1 might still control the activity of Clb5/Cdc28 activity under these conditions. We obtained similar results when cells were

Sic1, the peak of Cln2 protein, DNA replication, and budding (Fig. 2E), when cells were stressed, this relationship was partially lost (Fig. 2, F to H). When cells were stressed at Start (20 min after release from arrest), DNA replication and budding occurred before total degradation of Sic1 and the Cln2 peak (Fig. 2G). We also observed alterations in the timing of Cln2 production, Sic1 degradation, budding, and DNA replication in response when the cells were subjected to stronger hyperosmotic stress conditions (0.6 and 0.8 M NaCl) (figs. S1 and S2).

In the absence of stress, DNA replication and bud formation were induced almost simultaneously (Fig. 2E and fig. S1A). When the cells were stressed right after release from arrest (0 min), the concomitance of both processes was maintained (Fig. 2F). When the cells were stressed 30 min after release, there was no delay in DNA replication, but bud formation was delayed (Fig. 2H). Correspondingly, the amount of Cln2 decreased

Fig. 3. Scheme of the quantitative model and predictions of cell cycle progression for in silico mutants on Hog1 targets upon hyperosmotic stress. (**A**) Parsimonious model of G_1-S transition regulation by hyperosmotic stress, showing regulation of Cln2 and Clb5 activity and complex formation with Cdc28 (labeled as Cdk1) with focus on interaction with Sic1. Sic1 is produced from amino acids (AA) and constitutively degraded (five teal blue dots). It binds Clb5/Cdc28 at different stages and gets degraded after phosphorylation by Cln2/Cdc28. Phosphorylation by Hog1PP stabilizes Sic1 (blue arrows compared to red arrows for reactions without Hog1PP interaction). The interactions among Sic1, Cln2, Clb5, Cdc28, and Hog1PP are the core of the model. Peripheral model parts are simplified as black boxes. Details are in the Model Description of the Supplementary Materials. (**B** to **G**) Predictions for time courses of the percentage of cells in S-G_2 obtained with the unperturbed model and the model with the indicated perturbations in the absence of stress or in the presence of 0.4 M NaCl at 0 or 20 min after α factor release.

subjected to stress before or after Start in a second cell cycle after release from pheromone (fig. S4).

A mathematical model enables assessment of the effect of Hog1 on the G₁ regulatory network

We used the iterative approach of mathematical modeling, qualitative experimental testing, quantitative data retrieval, and model refinement to create a model with ordinary differential equations that described the temporal dynamics of the relevant components and complexes involved in Hog1-mediated regulation of the cell cycle in response to hyperosmotic stress (Fig. 3A; described in the Supplementary Materials, Model Description). The model comprises a detailed core module for the regulatory network of cell cycle from Start to S transition and its interactions with active Hog1, as well as three black box modules for (i) Hog1 activation by osmotic stress (Model Description, section 2.1), (ii) the inhibition of *CLN1,2* and *CLB5* expression by phosphorylated Hog1 (Model Description, section 2.2), and (iii) transition of cells from G₁ to S-G₂ phase (Model Description, section 2.3). The core module (Model Description, section 2.4) covers the interaction of Sic1 with the cyclin/CDK complexes as follows: Sic1 can bind to the Clb5/Cdk1 complex, thereby inactivating it. Bound and free Sic1 can be phosphorylated by Cln2/Cdk1, triggering Sic1 degradation and release of active Clb5/Cdk1. Active Hog1 can phosphorylate Sic1 at a different residue, stabilizing it. Black Box 1 simplifies the network responsible for osmotic stress response (*26*) to one input-output relationship governing measured Hog1PP (phosphorylated Hog1) dynamics. Black Box 2 functionally covers expression and protein production of Clb5 and Cln2 regulated by the transcription factor complexes MBF and SBF, respectively, and the modulating effect of Hog1PP (fig. S6). Black Box 3 stands for the effect of active Clb5/Cdk1 on the transition from G₁ to S-G₂ measured as DNA replication. We used quantitative data to parameterize the model for studying the regulatory properties of osmostress on G₁-to-S transition. One part of the data was used for parameter estimation (in-sample fit; see Model Description, section 3), and model simulations quantitatively reproduced experimentally determined dynamics (fig. S7). The other part of the data was used for model validation (out-sample fit; fig. S8). We used the parameterized model to simulate different experimental scenarios in which the coupling of Hog1 to various downstream components was isolated (Fig. 3, B to G).

The simulations yielded two immediate results with respect to the effect of the timing and strength of osmotic stress on cell cycle progression (Fig. 4). First, an increase in Hog1 activation due to stronger osmotic stress led to a longer cell cycle delay (Fig. 4, A to D), which was mediated by a slower accumulation of Cln2/Cdk1 caused by a Hog1PP-induced inhibition (Black Box 2) and Hog1PP-dependent Sic1 stabilization. Second, up to a critical point, cells with a particular amount of Hog1 activation exhibited a similar length of arrest, independently of the stage of G₁ in which Hog1 was activated (Fig. 4, E to G). However, when cells passed this critical point, they failed to arrest in G₁ (Fig. 4H). These predictions fit with our initial observations (Fig. 2), even though those initial data were not used for parameter estimation.

A quantitative mathematical model predicts and quantifies the differential roles of Hog1 targets on the cell cycle machinery at different stages of G₁

The model reproduced quantitatively (i) different lengths of arrest at G₁, (ii) the delay of Cln1,2 and Clb5 appearance, (iii) Sic1 degradation depending on the amount of stress and Hog1 phosphorylation, and (iv) the effect of hyperosmotic stress at different times in G₁. We used the model to perform a quantitative study of the relevance of the regulation of each element downstream of Hog1 by analyzing different in silico

"mutants" deficient in Hog1-mediated *CLN2* or *CLB5* down-regulation or for Hog1-mediated Sic1 stabilization (comparable with the Sic1[T173A] mutant). These analyses allowed us to characterize the cell cycle delay upon osmostress in virtual cells in which Hog1 can regulate all the elements in the G₁ network under its control except one (for example, in the *CLN2* in silico mutant condition, Hog1 can still stabilize Sic1 and down-regulate *CLB5* expression but cannot down-regulate *CLN2* expression). We simulated the three in silico mutants in response to osmostress before (0 min; Fig. 3, B to D) or after Start (Fig. 3, E to G). The simulation results indicated that a mutant deficient in stabilization of Sic1 mediated by Hog1 phosphorylation displayed only a minor defect on the delay in
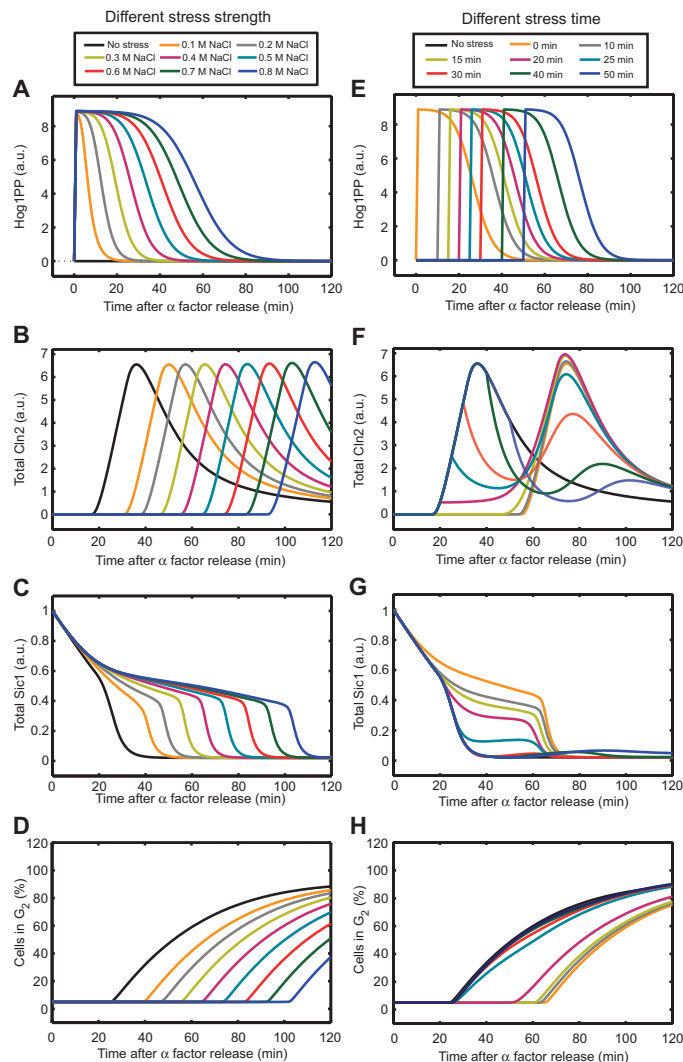

Fig. 4. Predictions of the model for different stresses. Using the model with estimated and tested parameter values, we systematically simulate different hyperosmotic stress conditions to investigate the temporal and quantitative effect of timing and strength of stress. (A to D) Different concentrations of NaCl were used to create different strengths of stress. (E to H) 0.4 M NaCl was applied at different times after release from pheromone-induced arrest to determine the effect of the timing of hyperosmotic stress on the cell cycle. For comparison, the scenario in the absence of stress (no stress) is shown in both cases as black lines. a.u., arbitrary units.

cell cycle progression caused by osmostress when cells were stressed shortly after release from arrest (Fig. 3B). However, when stressed after Start, the ability of cells to arrest upon stress was compromised (Fig. 4H and fig. S9). Cells deficient in the down-regulation of *CLN2* displayed a similar minor defect in delay of cell cycle progression when stressed after release from arrest (Fig. 3C) and only partially maintained their ability to arrest when stressed after Start (Fig. 3F and fig. S10). Thus, inhibition of expression of *CLN2* or Sic1 stabilization displayed a defect on osmotic stress–induced cell cycle arrest only when cells were stressed close to Start.

When simulations were performed with a mutant deficient in down-regulation of *CLB5* upon stress, cells showed almost a total inability to arrest both when stressed at the beginning of G_1 (Fig. 3D) and closer to Start (Fig. 3G and fig. S11). Thus, the model predicted that inhibition of *CLB5* expression by Hog1 is critical for the arrest at any stage of G_1. Thus, the model enabled quantification of the different contributions of elements downstream of Hog1 in G_1 arrest and suggested that, whereas inhibition of *CLN2* expression and Sic1 stabilization are important to prevent S-phase entry in response to stress occurring close to Start, inhibition of *CLB5* expression is critical in the response to osmotic stress occurring at any stage of G_1.

Sic1 restricts the activity of Clb5 when cells are stressed close to Start

The prediction that Sic1 stabilization would be important only in cells experiencing hyperosmotic stress close to Start but not before, together with the observation that the abundance of Clb5 was not properly reduced in cells stressed close to Start (Fig. 2G), suggested that the balance between Sic1 and Clb5 could be a key factor in maintaining the cell cycle delay in response to hyperosmotic stress. To assess the amounts of both proteins simultaneously, we genomically tagged Sic1 and Clb5 with the same tag in the same cell and then detected the two proteins by Western blotting. Cells were released from pheromone arrest in the absence of stress or after subjecting the cells to NaCl at time 0 (t_0) or close to Start (20 min after release, t_{20}). Either stress condition resulted in a similar stabilization of Sic1. However, the abundance of Clb5 was strongly reduced when the stress was applied at t_0, but its abundance was only partially reduced when the cells were stressed at t_{20} (Fig. 5A), which is consistent with our previous experiments (Fig. 2). In unstressed cells or cells stressed at t_0, there was minimal overlap between Sic1 and Clb5 (Fig. 5A); however, when cells were stressed at t_{20}, the Sic1 and Clb5 were simultaneously present (Fig. 5A). Therefore, we interpret this to mean that not stabilizing Sic1 when cells are subjected to stress before Start is not critical because Clb5 is not present as a result of the Hog1-mediated inhibition of its expression. However, once cells have passed Start and Clb5 has begun to accumulate, Sic1 stabilization is critical to prevent residual Clb5 from initiating DNA replication.

To assess whether leaky production of Clb5 at Start upon osmotic stress could result in onset of replication in the absence of Hog1-stabilized Sic1, we monitored Clb5-associated activity by following in vivo phosphorylation of Sld2 (a component of the replicative complex) (*17*, *18*). In the absence of stress, phosphorylation of Sld2 was concomitant to Clb5 induction, becoming detectable between 20 and 30 min after release of the cells from pheromone arrest (Fig. 5, A and B). When the cells were subjected to osmostress just after release from pheromone (0 min), Clb5 production (Fig. 5A) and Sld2 phosphorylation (Fig. 5C) were delayed in *sic1Δ* cells or cells containing either wild-type or the mutant nonphosphorylatable Sic1^[T173A] (Fig. 5C). Thus, these data suggested that the presence of Sic1 is not critical to delay Sld2 phosphorylation when cells are subjected to hyperosmotic stress before Start.
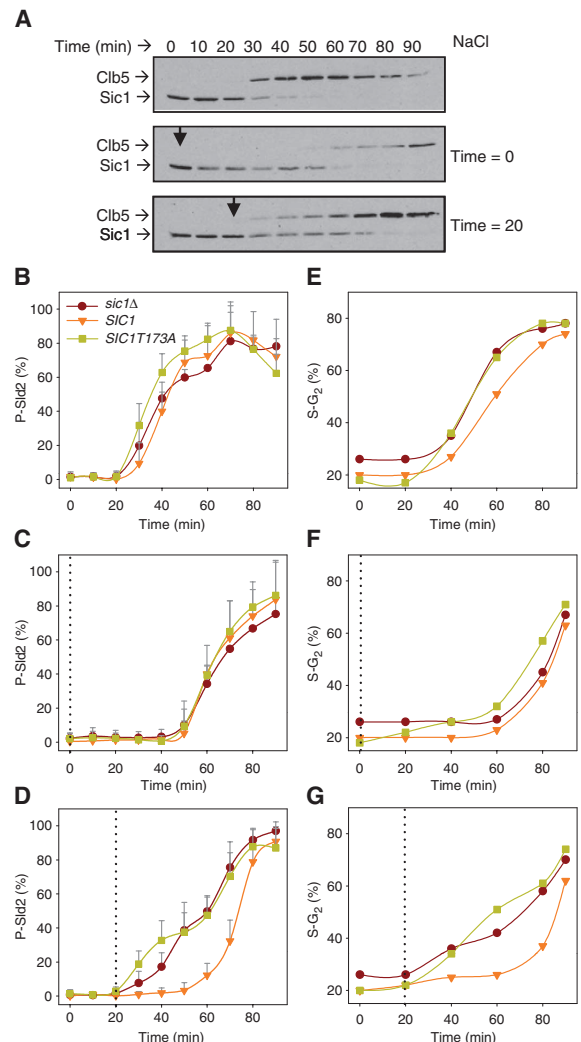
Fig. 5. Hog1-mediated Sic1 stabilization at Start is necessary to properly postpone Sld2 phosphorylation and progression into S phase upon osmostress. (**A**) YAN86 cells (expressing Clb5-TAP and Sic1-TAP) were collected at the indicated times under normal conditions (upper panel) and stressed immediately (middle panel) or 20 min (lower panel) after release from pheromone arrest. Total Sic1 and Clb5 protein amounts are plotted. Data represent three independent experiments. (**B**) *sic1Δ* cells bearing genomically tagged Clb5-TAP and Sld2-TAP (YAN37) and containing centromeric plasmids expressing Myc-tagged Sic1 (Sic1-9Myc) from the Sic1 promoter (pMZ55), or Sic1T173A (pMZ57), were released from α factor arrest and sampled every 10 min. Sld2 phosphorylation was assessed by quantitative Western blot (expressed as a percentage of the total amount of Sld2). Data are plotted as the means and SD of three independent experiments. (**C** and **D**) Cells like those described in (B) were released from arrest and stressed with 0.4 M NaCl at time 0 (C) or 20 min later (D), as indicated by vertical dotted lines, and Sld2 phosphorylation was assayed. (**E**) Cells like those in (B) were sampled at the indicated times to determine DNA content by flow cytometry. (**F** and **G**) Cells like those described in (B) were released from arrest and stressed with 0.4 M NaCl at time 0 (F) or 20 min later (G), as indicated by vertical dotted lines, and DNA content was assessed by flow cytometry. Data in (B) to (G) are plotted as the means of 10,000 cells.

In contrast, when these cells were subjected to osmostress closer to Start (20 min after release from pheromone), the results were different. Whereas wild-type cells arrested efficiently and displayed a strong synchrony in S-phase entry (sharp increase in Sld2 phosphorylation), $sic1\Delta$ cells or cells containing the mutant $SIC1^{T173A}$ allele showed partial and progressive phosphorylation of Sld2 earlier than wild-type cells (Fig. 5D). Correspondingly, DNA content analyses showed that despite progressing as wild-type cells under normal conditions (Fig. 5E) and arresting similarly to wild-type cells when stressed before Start (Fig. 5F), $sic1\Delta$ cells or cells containing the $SIC1^{T173A}$ mutation failed to properly arrest when stressed closer to Start (Fig. 5G). Therefore, as predicted by the model simulation for the $Sic1^{T173A}$ mutant, the role of Sic1 upon osmostress is to restrict the activity of Clb5 at Start, at a time in the cell cycle when the regulation by the Hog1 over cyclin production is insufficient to prevent DNA replication.

Inactivation of Sln1 ($sln1^{ts4}$) is a model for studying cell cycle arrest mediated by Hog1 without the complication of other responses to exposing the cells to hyperosmotic stress. Sln1 is an osmosensor that acts negatively on the HOG (high-osmolarity glycerol) pathway. Inactivation of the thermosensitive mutant of Sln1 ($sln1^{ts4}$) leads to sustained activation of Hog1 and delay of cell cycle progression (*11*), and the deletion of $SIC1$ or the $Sic1^{T173A}$ mutation is sufficient to overcome this cell cycle delay in G$_1$ (*11, 22*). To study the role of Sic1 and Clb5 under Sln1 inactivation, we genomically HA-tagged Sic1 and Clb5 in a $sln1^{ts4}$ strain and monitored the abundance of each protein after release from pheromone arrest at a permissive or restrictive temperature (fig. S12). The cell cycle arrest caused by $sln1^{ts4}$ inactivation was prevented by the deletion of $HOG1$, indicating that the observed effect is mediated by Hog1 (fig. S12). Consistent with a longer Hog1 activation, when $sln1^{ts4}$ was shifted to a nonpermissive temperature, Sic1 was stabilized permanently (fig. S12), much longer than we observed in response to osmotic stress (Fig. 5). However, at the nonpermissive temperature, Clb5 production was not efficiently repressed (fig. S12). These data suggest that, just as with osmotic stress that occurs close to Start, when Sln1 is inactive, stabilization of Sic1 is critical to prevent residual Clb5 activity from initiating DNA replication.

Regulation of Clb5, not Cln2, is critical for G$_1$ in response to hyperosmotic stress

The model predicted that down-regulation of $CLB5$ was critical to mediate G$_1$-S delay; therefore, preventing $CLB5$ down-regulation upon stress should prevent the delay in cell cycle progression (Fig. 3, D and G). The simulations obtained by challenging the model with in silico overexpression of $CLN2$ or $CLB5$ indicated complete abolishment of arrest in G$_1$ only when simulating overexpression of $CLB5$ and not $CLN2$ (Model Description, section 6.3, and fig. S13). We tested whether $CLB5$ overexpression in vivo could suppress cell cycle arrest in G$_1$ imposed by osmostress (Fig. 6). Whereas cells expressing $CLN2$ [under the $GAL1$ promoter ($GAL1::CLN2$)] exhibited a similar arrest as control cells in response to hyperosmotic stress (Fig. 6, A and B), cells expressing $CLB5$ [under the $GAL1$ promoter ($GAL1::CLB5$)] failed to arrest in response to osmostress (Fig. 6, D and E, and fig. S14), although these cells showed a delay in bud formation (fig. S14). $CLB5$ expression was induced only 20 min before release from pheromone; thus, the amount of Clb5 present in the cell was similar to that observed in wild-type cells without stress (Fig. 6F). Hence, these results agree with the in silico simulations (Fig. 3) and show that low amounts of Clb5 are sufficient to trigger S-phase entry, indicating that down-regulation of Clb5, rather than Cln2, is a critical event in the osmostress-induced G$_1$-S arrest.

Cells overexpressing $CLN2$ exhibited delayed onset of DNA replication when subjected to hyperosmotic stress (Fig. 6, A and B). To support these data, we expressed Cln2-HA under the control of the more precisely regulatable TetOn promoter to achieve amounts of the tagged protein similar to the amounts of endogenous Cln2 present in the absence of stress. The delay in Clb5 production was similar in cells that expressed $CLN2$ or control cells that did not express $CLN2$ (fig. S15). However, cells with constant $CLN2$ expression failed to exhibit an osmotic stress–induced delay in budding (fig. S15), suggesting that the function of Cln2 upon stress is mainly to coordinate budding with replication.
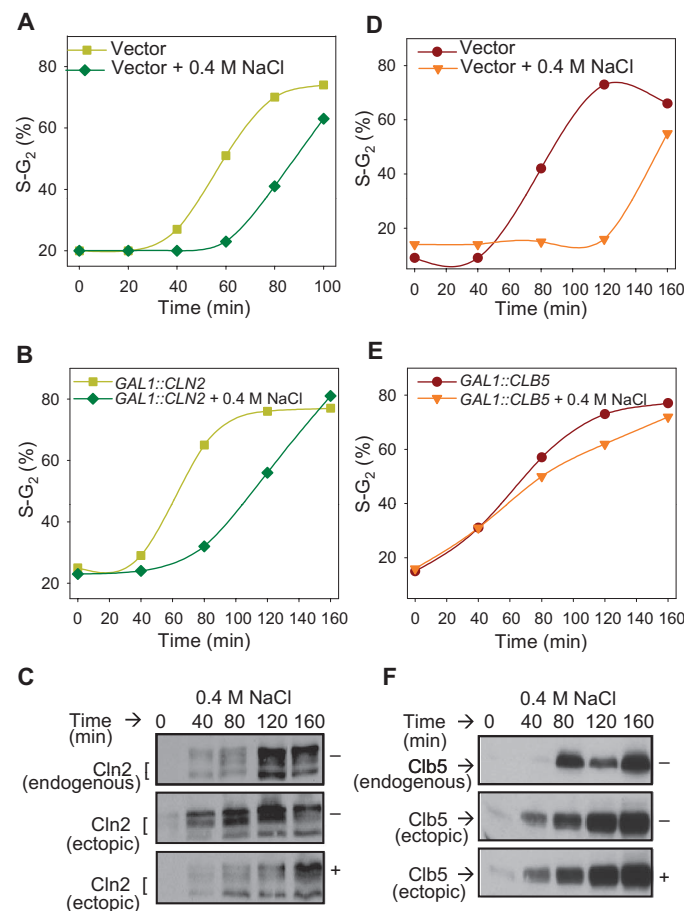

Fig. 6. Ectopic expression of $CLN2$ and $CLB5$ from the $GAL1$ promoter results in distinct effects on the osmostress-imposed delay in cell cycle progression. (**A** and **B**) W303 cells bearing empty plasmid (vector) (A) or pYES2-CLN2-HA (pCM249; $GAL1::CLN2$) (B) were synchronized with α factor in SD medium plus raffinose, and galactose was added 20 min before release in the absence or presence of 0.4 M NaCl. DNA content was determined by FACS. Data are representative of three independent experiments. (**C**) YAN32 cells and W303 $GAL1::CLN2$-expressing cells were sampled and analyzed at the indicated times by Western blotting to determine Cln2 abundance in the presence or absence of 0.4 M NaCl. (**D** and **E**) W303 cells bearing empty plasmid (vector) or pYES2-CLB5-6HA (pMAD25; $GAL1::CLB5$) were synchronized, treated, and analyzed as described for (A) and (B). (**F**) YAN79 and W303 $GAL1::CLB5$-expressing cells were sampled and analyzed at the indicated times by Western blotting to determine Clb5 abundance in the presence or absence of 0.4 M NaCl. Data in (A), (B), (D), and (E) represent three independent experiments. Data in (C) and (F) are representative of three independent experiments.

Fig. 7. Regulation of *CLB5* expression upon osmostress is independent of Cln2. (**A** and **B**) YAN32 cells were transformed with empty plasmid (A) or pYES2-Cln2-HA (B) and synchronized with pheromone in SD medium plus raffinose. Galactose was added 20 min before release from pheromone in the absence or presence of 0.4 M NaCl. Clb5 abundance was assessed by quantitative Western blot. (**C** and **D**) YAN32 (C) and YAN69 (*stb1 whi5*) (D) cells were synchronized with pheromone and released in the presence or absence of 0.4 M NaCl. Clb5 abundance was assessed by quantitative Western blot. (**E**) Total protein extracts were prepared from exponentially growing cells. Cln2/Cdc28 complexes were immunoprecipitated and visualized by immunoblotting (IP), and kinase activity was assessed by in vitro kinase assay with histone H1 as a substrate (P-H1). Wild-type cells without plasmid are shown as negative control (no tag). (**F**) Cells ectopically expressing *CLN2* from the *GAL1* promoter were grown to exponential phase in the presence of galactose and collected at the indicated times in the absence of stress (no stress) or after treatment with 0.4 M NaCl. Total RNA was extracted, and mRNAs were detected with specific probes. *ACT1* was detected as loading control. (**G**) Exponentially growing YGM62 cells (*BY4741 HOG1-6HA:HIS3*) were treated with 0.4 M NaCl and sampled at the indicated times for ChIP. *CLB5* promoter and *TEL1* region were amplified by PCR with specific oligonucleotide primers (upper panel). Quantification of Hog1 binding is shown in the lower plot. Filled bars represent Hog1 association in vivo by ChIP to *CLB5* promoter as fold induction relative to *TEL1*. Data are the means and SD of three independent experiments.

To examine whether Clb5 or Cln2 regulation was more important for Hog1-mediated cell cycle arrest in the absence of stress, we analyzed whether overexpression of *CLB5* or *CLN2* prevented cell cycle arrest mediated by hyperactivation of Hog1 at the nonpermissive temperature in *sln1^ts4* cells (figs. S12 and S14). Overexpression of *CLB5* abolished the Hog1-imposed arrest in G$_1$, whereas *CLN2* overexpression only had a minor effect, based on fluorescence-activated cell sorting (FACS) analysis. Together, our data indicate that under conditions that activate Hog1, such as hyperosmotic stress, Hog1-mediated inhibition of *CLB5* expression is a key determinant mediating cell cycle delay, whereas inhibition of *CLN2* expression seems to be important to coordinate bud formation with exit from G$_1$ in response to osmostress.

Hog1 mediates inhibition of *CLB5* expression independently from its effect on *CLN2* expression

It has been proposed that *CLB5* expression is controlled by a positive feedback loop exerted by G$_1$ cyclins (*29*). To test whether the effect of Hog1 was direct on *CLB5* rather than an indirect effect of Hog1 on Clns, we tested whether Cln2 was controlling *CLB5* expression upon stress. Although cells overexpressing *CLN2* entered S phase faster than control cells as suggested by the earlier production of Clb5, in response to stress, they delayed Clb5 production similarly to cells bearing empty plasmid (Fig. 7, A and B), suggesting that the effect of Hog1 on the regulation of Clb5 upon osmotic stress is independent of *CLN2* expression. We also measured the dynamics of Clb5 in the absence of *WHI5* and *STB1* (*whi5 stb1* strain), two connectors of the loop between Cln2 and the regulation of *CLB5* expression (*30*). In the *whi5 stb1* strain, regulation of the abundance of Clb5 upon stress was similar to that in wild-type cells (Fig. 7, C and D).

Because it is also possible that despite the presence of abundant Cln2 transcripts and protein, inhibition of Cln2/Cdc28 activity could inhibit *CLB5* expression. We followed *CLB5* expression under these experimental conditions and found that, whereas *CLN2* was constant (expressed from
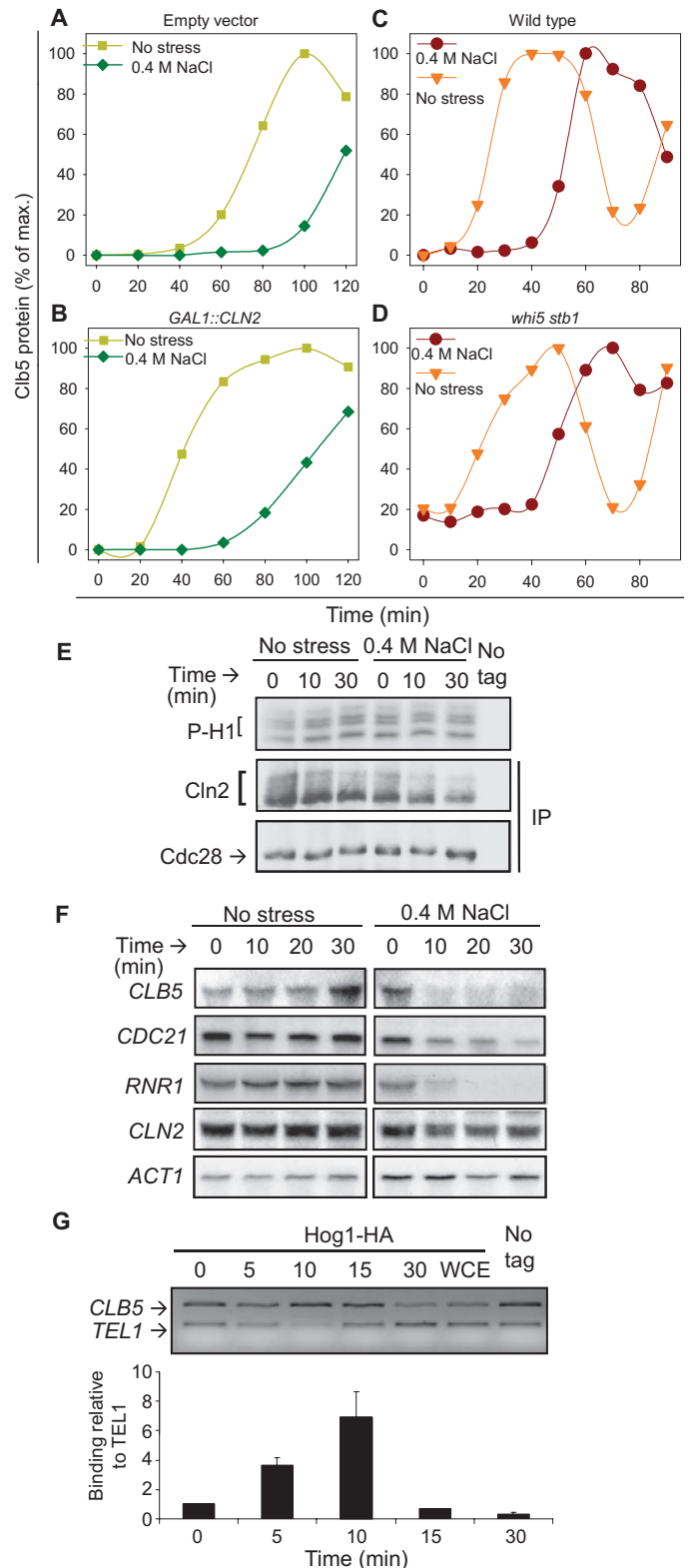
a heterologous promoter) (Fig. 7E), the abundance of *CLB5* transcripts was reduced in response to hyperosmotic stress (Fig. 7F). We also followed the expression of *RNR1* and *CDC21*, which are controlled by MBF, and these

were not affected by reduction in *CLN2* expression or a decrease in Cln2/Cdc28 activity (Fig. 7F). Furthermore, we prepared yeast extracts from asynchronous cells expressing tagged Cln2 from control cells or cells subjected to hyperosmotic stress and assayed the activity of Cln2/Cdc28. The kinase activity of Cln2/Cdc28 was similar to that in control cells (Fig. 7E), which is consistent with previous reports (*31*). These data suggest that the regulation of *CLB5* expression by Hog1 is independent of the regulation of *CLN2* expression and activity of Cln2/Cdc28.

Hog1 is recruited to osmostress-responsive promoters to regulate expression (*4*). Because Hog1 inhibited *CLB5* expression independently of Cln2 regulation, we performed chromatin immunoprecipitation (ChIP) experiments to assess whether Hog1 acted directly at the *CLB5* promoter. ChIP experiments with HA-tagged Hog1 showed that, in response to osmostress, Hog1 was recruited at the *CLB5* promoter (Fig. 7G) and recruitment occurred with similar kinetics to that reported for other osmostress-responsive genes (*32*), which suggests that Hog1 is acting directly at the *CLB5* promoter to regulate its expression in response to hyperosmotic stress.

DISCUSSION

Regulation of cell cycle progression by external stimuli requires complex regulatory mechanisms. Here, we have performed in vivo quantitative experiments complemented with a mathematical model, created by combining preexisting information about the molecular interactions, network refinement, and extensive parameter estimation from experimental data. We generated a model that defines the impact of a MAPK signaling pathway on the cell cycle machinery that extends beyond the published qualitative and semiquantitative models (*33*). Our analyses showed that in response to increasing osmolarities, Hog1 was phosphorylated for longer periods and that this correlated with the duration of cell cycle arrest. When cells were subjected to stress at different times between release from arrest with pheromone and Start, the duration of the osmotic stress–induced delay in the DNA replication was identical. Thus, to a given hyperosmotic stress, the arrest time required for adaptation was always similar, regardless of when in G_1 the cells experienced the stress.

Our experimental data and model analyses showed that neither inhibition of *CLN2* expression nor stabilization of Sic1 accounted for the cell cycle arrest mediated by Hog1. Indeed, cells overexpressing *CLN2* in a *sic1* background arrested in response to osmostress, which suggested that besides Sic1 stabilization and inhibition of *CLN2* expression, an additional mechanism contributed to the delay in cell cycle progression upon Hog1 activation. We showed that regulation of Clb5, the major S phase–promoting cyclin, was required for G_1 arrest upon hyperosmotic stress. Indeed, Clb5 production was delayed in response to osmotic stress, and Hog1 associated with the *CLB5* promoter, suggesting a direct role of the SAPK on transcriptional repression. Correspondingly, previous results showed that *CLB5* transcription was reduced upon Hog1 hyperactivation by constitutively active alleles of the pathway or heat shock (*6*, *11*). Thus, our data indicate that previously described components play restricted roles in delay of cell cycle progression and that they cooperate with Clb5, a newly defined regulatory element of the G_1 arrest, in response to osmotic stress. In a previous study (*11*), we showed that Sic1 stabilization was important for cell cycle delay. However, in those studies, cells were exposed to hyperosmotic stress after Start because they were allowed to adapt to the release medium before stress, which occurred 20 min after release. Thus, the data from that study is consistent with the data presented here, suggesting that Sic1 plays a critical role when hyperosmotic stress occurs close to or after cells pass Start.

Using mathematical modeling, we predicted the different roles of Cln2, Sic1, and Clb5 in regulating the G_1-S transition, and then we confirmed these predictions with in vivo experiments. The model also reproduced experimental results, such as the effect of the Sic1^{T173A} mutant. In addition, mathematical modeling combined with in vivo data permitted quantitative assessment of the relevance of each component in G_1 arrest through the use of in silico knockout of each specific component involved in G_1 regulation (regulation of Cln2 or Clb5 by Hog1 or Hog1-specific Sic1 phosphorylation). Phosphorylation of Sic1 by Hog1 has been shown to increase Sic1 stability (*11*). The model predicted that the role of Sic1 stabilization was to modulate cell cycle only at Start, for it also predicts that before Start, Clb5 production is inhibited and, therefore, the presence of its inhibitor, Sic1, is irrelevant. Correspondingly, we found that when cells were stressed close to Start, they showed a deficient arrest if they expressed a nonphosphorylatable Sic1 or genetically lacked Sic1, whereas wild-type cells were competent to arrest in this scenario. In cells in which both Sic1 and Clb5 were assessed simultaneously, we determined that the presence of Sic1 (stabilization) was critical at periods in the cell cycle when Clb5 production was not completely inhibited, such as after cells pass Start. Thus, our modeling and in vivo data showed that Hog1-mediated Sic1 phosphorylation played a key role in preventing a slow increase of Clb5 activity when this cyclin was not tightly down-regulated and that in the absence of Sic1 stabilization, the cells progressively initiate replication and fail to properly adapt to the osmotic stress. At Start, we found that deficiency in the inhibition of *CLN2* expression resulted in G_1-deficient arrest, similar to that observed in the absence of Sic1 stabilization. Therefore, from both in vivo and in silico data, we defined specific temporal roles for the regulation of Sic1 and *CLN2* expression in the hyperosmotic stress–induced arrest at G_1. In contrast, the in silico mutants showed that lack of inhibition of *CLB5* expression at any time in G_1 attenuated the ability of cells to delay cell cycle progression. Correspondingly, *CLB5* overexpression prevented cell cycle arrest upon osmostress at any stage of G_1.

Additionally, our results showed that the main role of Hog1-mediated inhibition of *CLN2* expression was to regulate budding. In normal cell cycle, budding occurred simultaneously with replication, but upon stress, budding correlates to *CLN2* expression independently of replication. Furthermore, we found that *CLB5* overexpression promoted replication, but not budding, upon osmotic stress. In contrast, in the presence of osmotic stress, *CLN2* overexpression promoted budding but not replication. Thus, we conclude that the main role of the down-regulation of *CLN2* by Hog1 is the coordination of the arrest in replication with cell morphogenesis.

Expression of *CLB5* may be controlled by a positive feedback loop exerted by G_1 cyclins (*29*). Here, our results showed that induction of *CLB5* expression was sufficient to abolish the delay in the onset of DNA replication caused by osmostress. In contrast, overexpression of *CLN2* did not abolish the delay in DNA replication upon osmostress. We also found that hyperosmotic stress did not affect Cln2 activity, consistent with previous reports (*31*). Furthermore, cells genetically impaired in the Cln2 feedback loop (*stb1 whi5* cells) showed the same delay in *CLB5* expression as wild-type cells, suggesting that in response to hyperosmotic stress the inhibition of *CLB5* expression is not mediated by a Cln2-dependent feedback loop. Hog1 associates with stress-responsive genes to modulate transcription. ChIP analyses showed that Hog1 was also recruited to the *CLB5* promoter in response to osmostress. Thus, we concluded that in response to osmostress, Hog1 directly affects *CLB5* rather than acts indirectly through *CLN*s.

Together, modeling and quantitative analyses have allowed us to define Clb5 as a previously unknown key regulator for the arrest at G_1 upon stress, acting independently of Cln2, and revealed quantitatively specific and distinct temporal roles of Sic1 and *CLN*s for cell adaptation in response to stress.

MATERIALS AND METHODS

Yeast strains and plasmids

The strains used were W303 (*MATa his3 leu2 trp1 ura3 ade2 can1*) and its derivatives YAN7 (*CLN2-3HA::KanMx SIC1-9Myc::TRPKl*), YAN32 (*CLN2-3HA::KanMx::Nat SIC1-9Myc::TRPKl CLB5-TAP::KanMx*), YAN72 (*CMVp(tetR-Ssn6-tet)::LEU2 CLB5-TAP::KanMX*), and YAN86 (*CMVp(tetR-Ssn6-tet)::LEU2 CLB5-TAP::URA3 SIC1-TAP::KanMX*); BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and its derivatives YAN37 (*CLB5-TAP::HIS3 sic1::KanMx::Nat SLD2-TAP::KanMx*), YAN69 (*CLN2-3HA::KanMx::Nat SIC1-9Myc::TRPKl CLB5-TAP::KanMx whi5::LEU2 stb1::HPH*), and YGM62 (*HOG1-6HA::HIS3*); and TM141 (*MATa his3 leu2 trp1 ura3*) and its derivatives YPC38 (*sln1-ts4*), YPC29 (*sln1-ts4 hog1::LEU2*), YAN80 (*Sln1-ts4 hog1::LEU2 Clb5-6HA::KanMx Sic16HA::TRPKl*), and YAN84 (*Sln1-ts4 hog1::LEU2 Clb5-6HA::KanMx Sic16HA::TRPKl*). Plasmids used: Sic1-Myc (pMZ55) and the mutant in the T173A (pMZ57) were cloned into pRS416 or pRS414 (pMZ65 and pMZ62, respectively). Full-length HA-*CLB5* was cloned into YCpIF16 (pMAD23) and into pYES2 (pMAD25) under the *GAL1* promoter. Full-length HA-*CLN2* was cloned into pYES2 controlled by the *GAL1* promoter (pCM249) and controlled by the tetO7 promoter (pCM254). These plasmids were provided by E. Garí (Universitat de Lleida).

Growth conditions, cell synchronization, and cytometry analyses

Cells were grown in yeast extract, peptone, and dextrose (YPD) at 25°C. Cell synchrony was accomplished by treatment of cells with α factor (40 μg/ml) for 3 hours at 25°C. In the case of *GAL1::CLB5* and *GAL1::CLN2* overexpression experiments, cells were grown overnight in SD medium with 2% raffinose and synchronized for 2 hours in the same medium at 30°C with pheromone. *GAL1* promoter was induced with 2% galactose 20 min before release from pheromone. For flow cytometry analyses, cells were fixed in ethanol, treated overnight with ribonuclease A (RNase A) at 37°C in 50 mM sodium citrate, stained with propidium iodide, and analyzed in a FACScan flow cytometer (Becton Dickinson). A total of 10,000 cells were analyzed, and the population of G_1 was quantified for each time point with WinMDI 2.9.

Western blotting and quantification analyses

Trichloracetic acid protein extracts were resolved in SDS–polyacrylamide gel electrophoresis (SDS-PAGE). Total amounts of the indicated proteins were detected by immunoblotting and chemiluminescence or infrared exposure for better quantification. Exposed films or filters were scanned in 16 bits per channel and quantified with the Odyssey application software 2.1.

Hog1 was detected with the anti-Hog1 antibody (Santa Cruz), phosphorylated Hog1 with an antibody that recognizes phosphorylated p38 (Cell Signaling), HA with the 12CA5 hybridoma, Myc with the 9E10 hybridoma, and PAP (Sigma) was used to detect TAP. Quantification analysis was performed by fluorescent detection with IRDye 800CW donkey anti-goat and IRDye 680 donkey anti-rabbit antibodies (LI-COR Biosciences).

Mathematical modeling

Details of the mathematical model, including the set of ordinary differential equations and the parameter values, are presented in the Supplementary Materials (Model Description). Parameter estimation was done with SBML-PET (*34*). The ordinary differential equations were solved with SBML-SAT (*35*) in MATLAB. SBML files for the model in the absence of stress (SBML model S1) and in the presence of stress (SBML model S2) are supplied as Supplementary Materials.

Chromatin immunoprecipitation

ChIP was performed as described previously (*32, 36*). Yeast cultures were grown to early log phase (optical density at 660 nm, 0.6 to 1.0) before aliquots of the culture were exposed to osmotic stress (0.4 M NaCl) for 5, 10, 15, or 30 min. For cross-linking, yeast cells were treated with 1% formaldehyde for 30 min at room temperature. Monoclonal anti-HA antibody was used to immunoprecipitate Hog1-HA. Primer mixes were adjusted for balanced signals with conventional polymerase chain reaction (PCR) analysis of the promoter region of *CLB5* between −400 and the ATG initiation codon. *TEL1* was amplified as a housekeeping gene (region 178 base pairs on the right arm of chromosome VI).

Cln2/Cdc28 kinase assay

Cln2-HA was immunoprecipitated from 4 to 5 mg of protein extracts [50 mM tris (pH 8), 150 mM NaCl, 15 mM EDTA, 15 mM EGTA, and 0.1% Triton X-100] with a polyclonal antibody recognizing HA and protein G–Sepharose. The in vitro kinase assay was performed as described in (*37*) with histone H1 as a substrate.

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