Hog1 activation delays mitotic exit via phosphorylation of Net1

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Adaptation to environmental changes is crucial for cell fitness. In *Saccharomyces cerevisiae*, variations in external osmolarity trigger the activation of the stress-activated protein kinase Hog1 (high-osmolarity glycerol 1), which regulates gene expression, metabolism, and cell-cycle progression. The activation of this kinase leads to the regulation of G1, S, and G2 phases of the cell cycle to prevent genome instability and promote cell survival. Here we show that Hog1 delays mitotic exit when cells are stressed during metaphase. Hog1 phosphorylates the nucleolar protein Net1, altering its affinity for the phosphatase Cdc14, whose activity is essential for mitotic exit and completion of the cell cycle. The untimely release of Cdc14 from the nucleolus upon activation of Hog1 is linked to a defect in ribosomal DNA (rDNA) and telomere segregation, and it ultimately delays cell division. A mutant of Net1 that cannot be phosphorylated by Hog1 displays reduced viability upon osmostress. Thus, Hog1 contributes to maximizing cell survival upon stress by regulating mitotic exit.

cell cycle | mitosis | osmostress | Net1 | MAPK

Upon sudden environmental changes, cells must induce a rapid and transient adaptive response to ensure survival. The response to variations in extracellular osmolarity has been evolutionarily conserved, and it involves the activation of mitogen-activated protein kinase (MAPK) signaling cascades. In *Saccharomyces cerevisiae*, the effector of the high-osmolarity glycerol (HOG) pathway is the Hog1 MAPK, a functional homolog of p38 in higher eukaryotes. Upon phosphorylation, Hog1 induces a cytoplasmatic response that acts on glycerol and ion transporters, metabolism, and translation. Additionally, Hog1 rapidly translocates into the nucleus, where it modulates transcription to control gene expression and alters cell-cycle progression (reviewed in refs. 1–3).

The effect of osmostress on cell-cycle progression has been addressed extensively in budding yeast (4–15). These studies have unraveled a series of Hog1-dependent events that are finely tuned to prevent genetic instability and ensure maximal survival. Activation of the HOG pathway during each phase of the cell cycle leads to an alteration in the speed of progression, and the mediators of this transient effect are phase-specific. Cells in G1 transiently arrest the cell cycle upon exposure to osmostress. This event is dependent on Hog1 in a dual manner: 1) stabilization of the cyclin-dependent kinase inhibitor Sic1 (10); and 2) down-regulation of G1 cyclins via phosphorylation of the transcription regulators Whi5 and Msa1 (10, 11, 16). The G1-to-S transition is also delayed via Hog1-induced transcriptional inhibition of Clb5 (4), whereas S phase is regulated not only by delaying the accumulation of Clb5 and Clb6 (15) but also by directly acting on components of the replicative machinery such as Mrc1 (8). Hog1 also impinges on the G2-to-M transition by down-regulating Clb2 expression and stabilizing Swe1, a negative regulator of Cdc28 whose degradation is required for entry into mitosis (5, 7, 17). Moreover, Hog1 controls the levels of a long noncoding RNA on *CDC28* to facilitate cell-cycle reentry upon stress (12). Finally, exit from mitosis appears to be promoted upon osmostress in late anaphase-arrested mutants via modulation of the release of the phosphatase Cdc14 (14). However, the effect of osmostress on early mitotic cells is still unclear.

In an unperturbed cell cycle, progression through mitosis depends on Cdc14 (reviewed in refs. 18 and 19). The activity of Cdc14 is blocked from G1 to metaphase as a result of its binding to the nucleolar protein Net1 (20, 21). Cdc14 release and activation occur in two steps. The first drives Cdc14 relocalization from the nucleolus to the nucleus and depends on the activation of Cdc5 (22–25) and FEAR (Cdc fourteen early anaphase release) pathway (reviewed in refs. 18, 19, and 26). This initial release is mediated by the activation of the Clb2–Cdc28 complex, which phosphorylates Net1 on at least six sites, thereby destabilizing the Net1–Cdc14 complex (22, 27, 28). This release was recently reported to additionally depend on nucleolar ribosomal DNA (rDNA) condensation (29). The functions of nuclear Cdc14 are essential for the establishment of a successful anaphase, and they include regulation of the anaphase spindle (30, 31), chromosome movements, and positioning of the anaphase nucleus (32) and segregation of rDNA (33–36) and telomeres (37, 38). The second step, activated during late mitosis and

Significance

Proper chromosome segregation is critical for the maintenance of genomic information in every cell division, which is required for cell survival. Cells have orchestrated a myriad of control mechanisms to guarantee proper chromosome segregation. Upon stress, cells induce a number of adaptive responses to maximize survival that range from regulation of gene expression to control of cell-cycle progression. We have found here that in response to osmostress, cells also regulate mitosis to ensure proper telomeric and rDNA segregation during adaptation. Osmostress induces a Hog1-dependent delay of cell-cycle progression in early mitosis by phosphorylating Net1, thereby impairing timely nucleolar release and activation of Cdc14, core elements of mitosis regulation. Thus, Hog1 activation prevents segregation defects to maximize survival.

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dependent on Cdc14 nuclear localization, promotes the full release of Cdc14 into the cytoplasm and relies on the MEN (mitotic exit network) (reviewed in refs. 39 and 40). As a result of MEN activation, Cdc14 is phosphorylated at sites adjacent to its nuclear localization signal and is consequently retained in the cytoplasm (41). Cytoplasmatic Cdc14 directly promotes mitotic exit via dephosphorylation of the APC activator Cdh1, the transcription factor Swi5, and the Cdc28 inhibitor Sic1. Additionally, cytoplasmatic Cdc14 is required for completion of mitosis as it dephosphorylates a number of Cdc28 substrates, erasing the phosphorylation marks accumulated during the cell cycle (42–45). Among its cytoplasmatic targets, Cdc14 is also responsible for activating the RAM (regulation of Ace2 and morphogenesis) pathway, which leads to the transcriptional activation of genes responsible for cell separation (46, 47), thereby ensuring timely septum disruption after cytokinesis (reviewed in refs. 48–50).

Here we show that the activation of Hog1 in metaphase leads to delayed mitosis. This defect was not found to be linked to mitotic spindle formation or elongation, or to nuclear division. In contrast, the timely release of Cdc14 was affected upon genetic activation of Hog1. Hog1 phosphorylated the nucleolar protein Net1 and thus negatively regulated Cdc14 release. Correspondingly, a Net1 unphosphorylatable mutant partially rescued the Cdc14 localization defect. Additionally, Hog1 activation resulted in defective segregation of the late segregating regions (rDNA

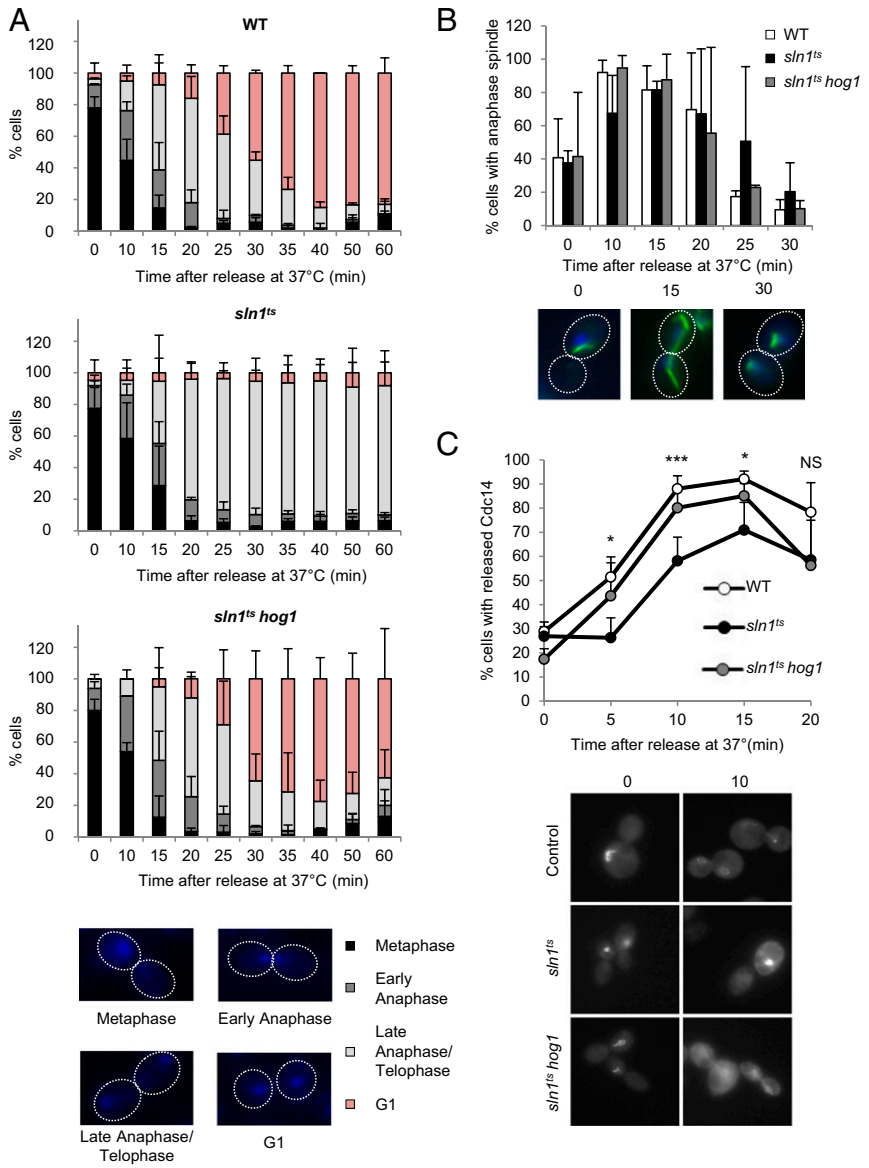

Fig. 1. Hog1 activation induces a defect in cell division and Cdc14 release in metaphase-arrested cells. (*A*) *GAL1p-CDC20* cells were synchronized in metaphase in YPRaff at 25 °C for 3 h and switched to 37 °C for 1 h before release upon galactose addition. Nuclear dynamics were monitored by DAPI staining. Data represent mean and SD. Representative images of the WT strain show the temporal progression of nuclear division by DAPI staining. (*B*) Cells were treated as in *A*. Mitotic spindle length was measured by immunofluorescence (α-tubulin). Data represent mean of the percentage of cells with anaphase spindles (>2 μm) and SD. Representative microscopy images of the WT strain show how the mitotic spindles (α-tubulin, green) elongate over time in relation to nuclear division (DAPI, blue). (*C*) Cells were treated as in *A*. Cdc14 release was analyzed by fluorescence microscopy on fixed cells using strains bearing GFP-tagged Cdc14. Data represent mean and SD. Asterisks indicate the Student's *t* test assuming unequal variance analysis comparing the WT with *sln1*^{ts} (NS, no significance, $P > 0.05$; *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.005$). Representative images correspond to Cdc14 signal at time 0 and 10 min after release at the restrictive temperature.

and telomeres), which was rescued by the Net1 unphosphorylatable mutant. Remarkably, this mutant is partially osmosensitive. Thus, Net1 is a target of Hog1 required to facilitate osmoadaptation during early stages of mitosis.

Results

Hog1 Activation Induces a Defect in Cell Division and Cdc14 Release in Metaphase-Arrested Cells. To study whether osmostress resulted in a delay after G2, we synchronized cells at early mitosis by means of expressing *CDC20* under the control of the inducible galactose promoter (*GAL1p-CDC20*), which arrested cells in metaphase. The *GAL1p-CDC20* expression system consists of the replacement of the *CDC20* promoter by the *GAL1* promoter, and thus cells arrest in metaphase in the absence of galactose and reenter into the cell cycle again in the presence of galactose. This is a well-established and accepted tool for the synchronization of cells when analyzing specific mitotic events (e.g., refs. 51 and 52). The ability of cells to progress into a new cell cycle was analyzed by flow cytometry after release in control conditions or upon osmostress (*SI Appendix*, Fig. S1*A*). Control cells progressed normally through mitosis and entered a new cell cycle 50 to 60 min after release. Remarkably, when released in the presence of 0.4 M NaCl, cells showed progression to a new G1 phase only after 90 min. Additionally, when mitosis progression was monitored by means of degradation of the mitotic cyclin Clb2, a delay in Clb2 degradation was observed in osmostressed compared with untreated cells (*SI Appendix*, Fig. S1*B*). These data indicate that osmostress induces a striking delay after release from early mitosis. However, after an initial delay, cells were able to restore cell-cycle progression (*SI Appendix*, Fig. S1*A*), suggesting that their viability is not compromised. *GAL1p-CDC20* cells promoted osmoadaptation upon stress similar to wild-type (W303) cells (*SI Appendix*, Figs. S1*C* and S2*C*). Similar results were obtained when cells were synchronized using α-factor and released in control conditions or stressed with 0.4 M NaCl when entering mitosis (*SI Appendix*, Fig. S2 *A* and *B*). This is consistent with the idea that cells are able to adapt to the environmental change and survive despite being exposed to stressful conditions. Of note, cells expressing *CDC20* from the *MET3* promoter [where Cdc20 expression was regulated by the presence of methionine in the culture media (53)] also showed delayed progression into a new cell cycle upon stress when compared with control conditions (*SI Appendix*, Fig. S2*D*), thereby indicating that the phenotype observed upon osmostress does not depend on the synchronization method.

To verify the involvement of the MAPK pathway in the observed phenomena upon osmostress after release from early mitosis synchronized cells, we compared a *GAL1p-CDC20* strain (from here on, WT) with a strain additionally bearing a temperature-sensitive *SLN1* allele (*sln1^{ts}*), which leads to constitutive genetic activation of Hog1 independent of external stimuli when exposed to the restrictive temperature (54, 55). It is worth mentioning that activation of the HOG pathway by genetic manipulation has served to unravel the direct role of Hog1 in the cell cycle (4, 7, 8, 10, 15). Cell-cycle progression and Clb2 protein dynamics were monitored under these conditions. Consistent with what was observed upon salt addition, genetic activation of Hog1 promoted a delay in the progression into a new G1 phase for cells released from metaphase arrest at 37 °C (*SI Appendix*, Fig. S1*D*). This delay was also reflected in a defect in full timely degradation of Clb2 in the *sln1^{ts}* strain (*SI Appendix*, Fig. S1*E*). Remarkably, for both cell-cycle progression and Clb2 degradation and reaccumulation, *HOG1* deletion in the *sln1^{ts}* background fully suppressed the delay caused by Hog1 activation (*SI Appendix*, Fig. S1 *D* and *E*). Taken together, these data indicate that Hog1 mediates a delay when cells are released from early mitosis.

Then, we attempted to determine which mitotic event(s) was impaired upon stress. Initially, we monitored the dynamics of nuclear division by DAPI staining of *GAL1p-CDC20* cells released from metaphase arrest at the restrictive temperature (Fig. 1*A*). In the WT strain, most of the cells at time 0 showed a clear metaphase phenotype, with one nuclear signal localized on the mother cell. Over time, the percentage of binucleated anaphase cells progressively increased, then augmenting the percentage of late anaphase/telophase cells (separated nuclei with mother and daughter cells still attached to one another), to finally almost fully reach the typical G1 phenotype of separated cells with a concentrated nuclear signal at 40 min (Fig. 1 *A*, *Top*). *sln1^{ts}* nuclear distribution over time differed greatly from the WT. Cells progressed normally through anaphase but, although showing seemingly separated nuclei, failed to reach physical separation of mother and daughter (Fig. 1 *A*, *Middle*). Once again, *sln1^{ts} hog1* cells mimicked the behavior of the control strain (Fig. 1 *A*, *Bottom*). These data indicate that the activation of Hog1 results in a delay in the late stages of mitosis.

A crucial step of successful mitosis is the establishment of a functional spindle for chromosome segregation. To test whether mitotic spindle stability or elongation was affected, cells carrying *GAL1p-CDC20* were synchronized and released at the restrictive temperature, and tubulin was immunostained to measure spindle length in WT, *sln1^{ts}*, and *sln1^{ts} hog1* cells (Fig. 1*B*). All strains were observed to show a timely enrichment in the number of cells with anaphase spindles (>2 μm), thereby indicating that Hog1 activation does not impair mitotic spindle elongation. On the other hand, the persistence of longer spindles observed in *sln1^{ts}* cells suggests a delay in spindle disassembly.

The phosphatase Cdc14 is a master regulator of progression through mitosis (reviewed in refs. 56 and 57), and its localization is modulated by interaction with the nucleolar protein Net1 (20, 21). We thus assessed whether Cdc14 localization is impaired upon Hog1 activation. *GAL1p-CDC20* strains additionally bearing a enhanced GFP (yeGFP)-tagged version of endogenous Cdc14 were analyzed by fluorescence microscopy upon release from metaphase arrest at the restrictive temperature in WT, *sln1^{ts}*, and *sln1^{ts} hog1* strains on fixed cells (Fig. 1*C*). The control strain displayed a rapid release of Cdc14, which reached its maximum 15 min after release from metaphase arrest. In contrast, the *sln1^{ts}* strain failed to initiate Cdc14 release as promptly. Additionally, successful relocalization of Cdc14 in this strain occurred in only ∼71% of the cells compared with ∼92% of control cells at the same time point (15 min after release). *sln1^{ts} hog1* cells mimicked the pattern of the control strain. These observations indicate that Hog1 activation impairs the timely release of Cdc14. Given the crucial function of this phosphatase in mitotic exit, this finding could explain the delay observed in cell-cycle progression.

Hog1 Phosphorylates Net1 In Vitro and In Vivo. Upon stress-induced activation, the MAPK cascade, through its effector Hog1, is responsible for a rapid alteration in the phosphoproteome of the cell to mediate an effective response to environmental changes (58). We found that Cdc14 release is impaired upon genetic activation of Hog1 and thus tested whether this phosphatase is a direct target of Hog1. Cdc14 phosphorylation was assessed in an in vitro kinase assay using glutathione S-transferase (GST)-tagged versions of Cdc14, Hog1, and its activator Pbs2 (in its constitutively active form, Pbs2^{EE}) purified from *Escherichia coli* (Fig. 2*A*). Under the experimental conditions tested, Hog1 did not phosphorylate Cdc14. However, Cdc14 release has been reported to depend on its binding to the nucleolar protein Net1 and the initial release is in fact promoted by Clb2–Cdc28–dependent phosphorylation on Net1 (22, 28). We then hypothesized that Hog1 acts directly on Net1 to negatively regulate the release of Cdc14. To test for Net1 phosphorylation, we performed an in vitro

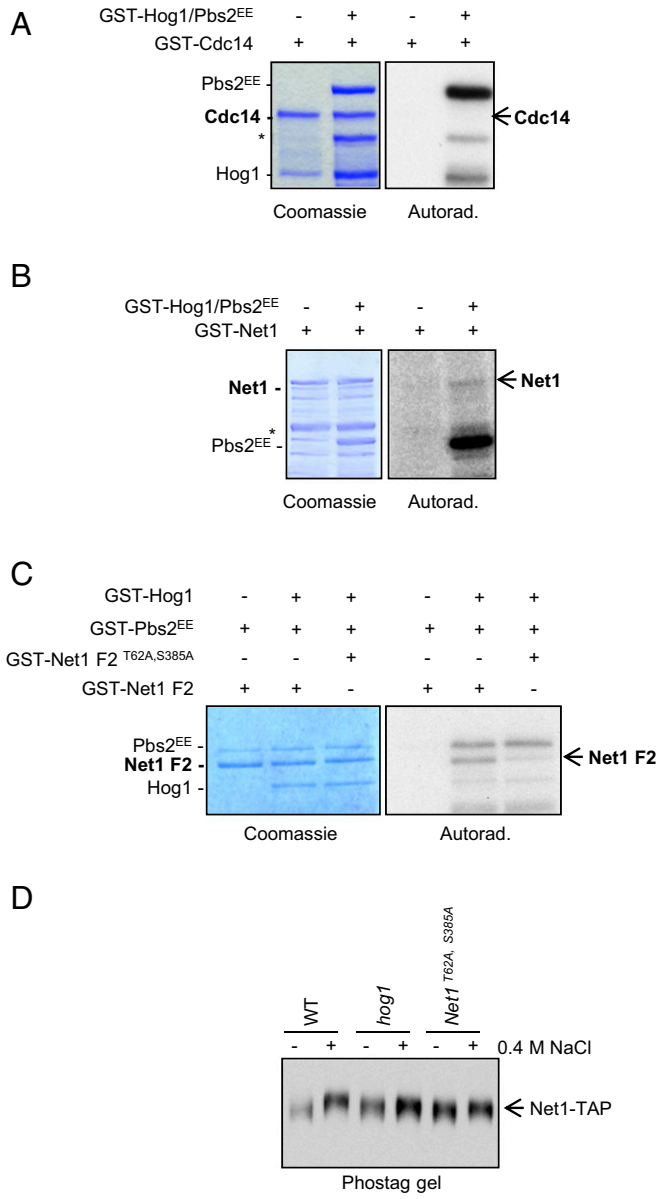

Fig. 2. Hog1 phosphorylates Net1 on T62 and S385 in vitro and in vivo. (*A*) Bacterially expressed GST-tagged Cdc14 was used as substrate for the in vitro kinase assay with GST–Hog1. Degradation/contaminating bands are indicated with an asterisk. (*B*) Bacterially expressed GST–Net1 was tested as substrate of GST–Hog1 in vitro. Degradation/contaminating bands are indicated with an asterisk. (*C*) Comparison of the phosphorylation signal of the wild-type fragment F2 of Net1 (amino acids 1 to 511) with the fragment mutated to alanine on T62 and S385 in an in vitro kinase assay with GST–Hog1. (*D*) *GAL1p-CDC20* cells bearing TAP-tagged endogenous Net1 were synchronized in metaphase in YPD and harvested in the control condition or after a 5-min treatment with 0.4 M NaCl. Protein extracts were analyzed in a 6% polyacrylamide gel containing 10 μM Phos-tag, and Net1 mobility was followed by Western blot (α-TAP).

kinase assay with GST-tagged Net1 (Fig. 2*B*). Net1 was phosphorylated by Hog1, thereby indicating that Net1 is a direct substrate of the kinase. To further explore Net1 as a substrate of Hog1, we screened the functional domains of Net1 (*SI Appendix*, Fig. S3*A*) and generated a series of GST-tagged truncations of the protein, which were tested for in vitro phosphorylation (*SI Appendix*, Fig. S3*B*). These analyses showed that the Hog1-dependent phosphorylation sites were present in the first 511 amino acids of the N terminus of the protein (a fragment we called F2). Strikingly, this region contains the Cdc14-binding domain (amino acids 1 to 341) (59) and Clb2–Cdc28 phosphorylation sites responsible for promoting Cdc14 release (22, 28). The Net1 F2 sequence contains several S/T-P sites that can be phosphorylated by Hog1. All these serine and threonine residues were mutated to alanine, alone or in combination, expressed as recombinant GST–Net1 F2, and tested for Hog1 phosphorylation in vitro as done previously (*SI Appendix*, Fig. S4). The combination of alanine mutations on T62 and S385 almost fully abolished the phosphorylation signal in comparison with the WT (Fig. 2*C*).

To validate Net1 as a target of the osmostress-induced response, we tested its phosphorylation status in vivo. *GAL1p-CDC20* strains bearing tandem affinity purification (TAP)-tagged endogenous Net1 were synchronized in metaphase, cultures were divided into two groups, and one was stressed with 0.4 M NaCl for 5 min (*SI Appendix*, Fig. S3*C*). Net1 mobility shift was analyzed on Phos-tag gels in WT cells and *hog1* and $Net1^{T62A,S385A}$ mutants (Fig. 2*D*). In response to osmotic shock, the control strain showed a reduced mobility shift of Net1 signal when compared with the untreated sample. Importantly, no clear differences between the treated and untreated *hog1* and $Net1^{T62A,S385A}$ mutant strains were observed. This result suggests that, upon osmotic stress, Net1 is phosphorylated in vivo by Hog1 on residues T62 and S385.

Hog1-Dependent Phosphorylation of Net1 Impairs Cdc14 Release. Since Hog1 phosphorylates Net1 and activation of this kinase alters Cdc14 release, we envisioned that these phosphorylations could affect the stability of the Net1–Cdc14 complex. To address this question, we tested whether Hog1-dependent phosphorylation of Net1 interferes with the capacity of Clb2–Cdc28 to modify Net1 itself, thus affecting Cdc14 release. To this end, we performed a two-step in vitro kinase assay in which GST–Net1 F2 was incubated sequentially with activated Hog1 and Clb2–Cdc28 (*SI Appendix*, Fig. S5*A*). The Clb2–Cdc28 complex phosphorylated Net1 in vitro, regardless of Hog1, indicating that Hog1 does not prevent Clb2–Cdc28 phosphorylation on Net1. To rule out that the residues required for phosphorylation of Hog1 and Clb2–Cdc28 could be the same, Net1 F2 wild type and T62A, S385A were tested in an in vitro kinase assay with Clb2–Cdc28 (*SI Appendix*, Fig. S5*B*). Clb2–Cdc28 could phosphorylate the alanine mutant, suggesting that Clb2–Cdc28 and Hog1 phosphorylations on Net1 occur on different residues.

Next, the interaction between Net1 and Cdc14 was reconstituted in vitro in a multistep binding assay in order to recapitulate the contribution of Clb2–Cdc28 and Hog1 to the stability of the complex (Fig. 3 *C* and *D* and schematic representation in Fig. 3 *A* and *B*). Briefly, purified Cdc14 and Net1 F2 proteins from *E. coli* were allowed to interact, and the complex was retained on an affinity resin. To promote the disassembly of the complex, samples were then incubated with Clb2–Cdc28 immunoprecipitated from metaphase-arrested yeast cells. To test whether Hog1 induced stabilization of the Net1–Cdc14 complex, purified Hog1 and $Pbs2^{EE}$ from *E. coli* were added to the reaction prior to Clb2–Cdc28. Samples were extensively washed to release all unspecifically bound proteins and analyzed by Western blot (Fig. 3*C*). Finally, the relative % of Net1 binding was quantified (Fig. 3*D*). The unspecific binding of Net1 to the resin was tested in the absence of Cdc14 (Fig. 3*C*, sample 1). The assay performed with unmodified Net1 F2 showed the interaction between Net1 and Cdc14 (Fig. 3 *A*, *C*, and *D*, sample 2). Additionally, as expected, the addition of Clb2–Cdc28 caused a marked destabilization of the complex (decreased GST–Net1 binding; Fig. 3 *A*, *C*, and *D*, sample 3), an observation consistent with the findings of a previous study (22). However, of note, when Hog1 was added to the reaction, the interaction between Net1 and Cdc14 appeared to be stabilized, even in the presence of Clb2–Cdc28 (Fig. 3 *A*, *C*, and *D*, sample 4). These results thus

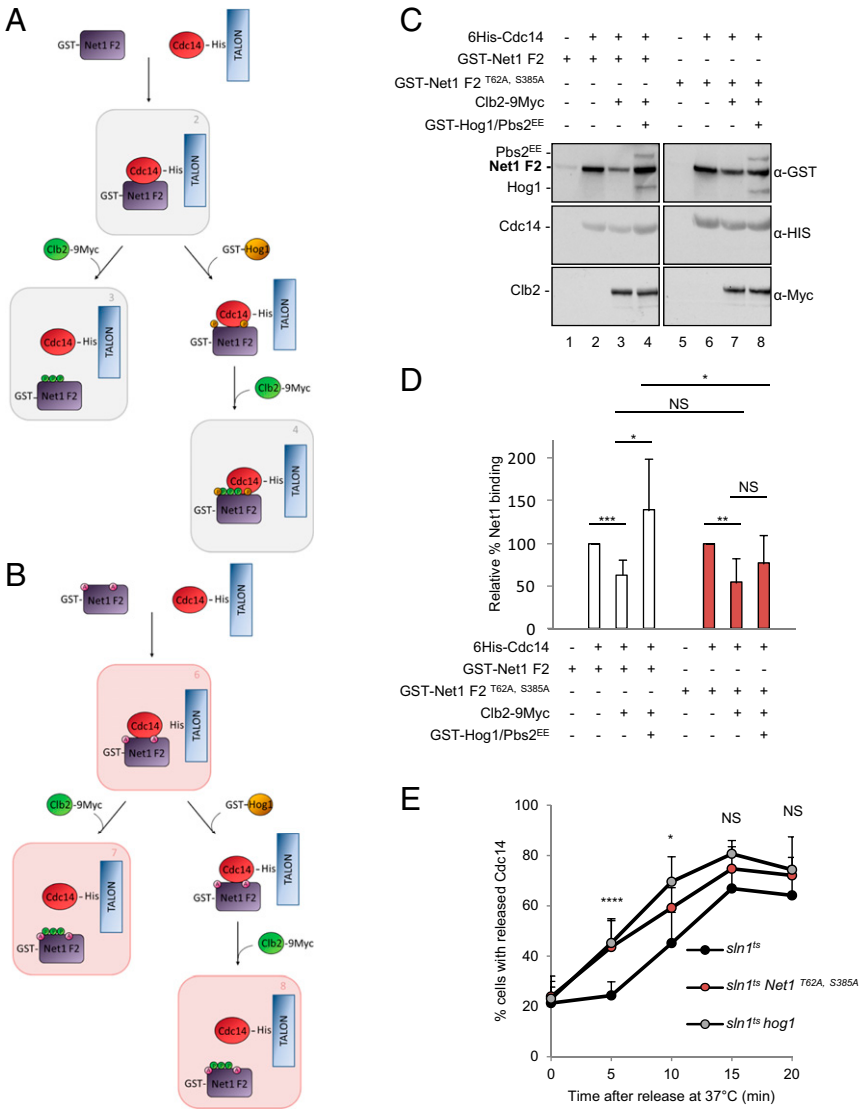

Fig. 3. Hog1-dependent phosphorylation of Net1 impairs Cdc14 release. (*A* and *B*) Schematic representation of the in vitro reconstitution experiment for Net1–Cdc14 binding. Briefly, purified Cdc14 and Net1 F2 proteins (unmodified or $Net1^{T62A,S385A}$) were allowed to interact, and the complex was retained on an affinity resin. To promote the disassembly of the complex, samples were then incubated with Clb2–Cdc28. Hog1/$Pbs2^{EE}$ were added to test whether Hog1 induced stabilization of the Net1–Cdc14 complex. (*C*) In vitro reconstituted Net1–Cdc14 interaction using purified proteins was monitored by Western blot, probing for Net1 (α-GST), Cdc14 (α-HIS), and Clb2 (α-Myc). (*D*) Quantification of the in vitro binding assays. Data represent mean and SD; asterisks indicate statistically significant differences by the Student's *t* test assuming unequal variance (NS, no significance, $P > 0.05$; *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$). (*E*) Cells were treated as in Fig. 1*A*. Cdc14 release was analyzed by fluorescence microscopy on fixed cells using strains bearing GFP-tagged Cdc14. Data represent mean and SD. Statistical analysis comparing $sln1^{ts}$ with $sln1^{ts}$ $Net1^{T62A,S385A}$. Asterisks indicate statistically significant differences by the Student's *t* test assuming unequal variance (NS, no significance, $P > 0.05$; *$P \leq 0.05$, ****$P \leq 0.001$).

indicate that Hog1 is indeed involved in modulating Cdc14 release. To further confirm the critical role of Hog1 phosphorylation on Net1 residues T62 and S385, the assay was then performed using the Net1 $F2^{T62A,S385A}$ mutant (Fig. 3 *C* and *D* and schematic representation in Fig. 3*B*). The reconstitution of the complex with Cdc14 was similar to that achieved with the wild-type Net1 (Fig. 3 *B–D*, sample 6 compared with sample 2), indicating that the two sites are not required to promote or sustain binding. Furthermore, the introduction of Clb2–Cdc28 resulted in the destabilization of the Net1–Cdc14 complex to a similar extent as that of wild-type Net1 (Fig. 3 *B–D*, sample 7 compared with sample 3). These data show that the modification of residues T62 and S385 per se does not interfere with the role of Clb2–Cdc28 in facilitating Cdc14 release from Net1. Importantly, prior addition of Hog1 to the reaction involving *Net1* $F2^{T62A,S385A}$ did not promote stabilization of the Net1–Cdc14 complex (Fig. 3 *B–D*, sample 8), thereby demonstrating that Hog1 does phosphorylate the two residues of Net1 to impair Cdc14 release. Of note, the presence of Hog1/Pbs2 alone did not alter Net1–Cdc14 complex stability (neither for the wild type nor for the alanine mutant) (*SI Appendix*, Fig. S5 *C* and *D*). To study whether Hog1-dependent phosphorylation of the residues T62 and S385 of Net1 is sufficient to cause the observed impairment of Cdc14 release (Fig. 1*C*), we monitored Cdc14 localization in $sln1^{ts}$, $sln1^{ts}$ $Net1^{T62,S385A}$, and $sln1^{ts}$ *hog1* strains upon release from metaphase arrest at the restrictive temperature, as previously described (Fig. 3*E*). The $sln1^{ts}$ strain showed a clear delay in Cdc14 release when compared with $sln1^{ts}$ *hog1*. The $sln1^{ts}$ $Net1^{T62A,S385A}$ strain showed overall higher levels of nucleolar release compared with $sln1^{ts}$ and promoted a prompt increase in

Cdc14 release already at 5 min after exit from metaphase arrest, as occurred in the *sln1^{ts} hog1* strain.

Altogether, these data indicate that Hog1-dependent phosphorylation of Net1 is required for the stabilization of Cdc14 binding and thus the prevention of Cdc14 release.

Hog1 Activation during Mitosis Impairs rDNA and Telomere Segregation. The early functions of Cdc14 include the promotion of rDNA and telomere segregation, so-called late segregating regions, as they divide after all other genomic regions have segregated (33–35, 37, 38). To test whether the segregation of these regions is affected upon activation of Hog1, we performed fluorescence time-lapse microscopy on *GAL1p-CDC20* strains bearing a TetO/TetR system, where a yellow fluorescent protein (YFP)-tagged reporter could be recruited to a specific genomic locus (60, 61). rDNA segregation was monitored over time after release from metaphase arrest at the restrictive temperature on WT, *sln1^{ts}*, *sln1^{ts} Net1^{T62A,S385A}*, and *sln1^{ts} hog1* strains bearing *TetR-YFP tetO:450Kb-ChrXII* (Fig. 4 *A* and *B*). The control strain started segregating the rDNA region at around 20 min after release, reaching ∼80% segregation at 30 min. As expected, the *sln1^{ts} Net1^{T62A,S385A}* and *sln1^{ts} hog1* strains showed similar behavior. Strikingly, the *sln1^{ts}* strain started to segregate the rDNA only at 30 min after release, and full release was not reached in ∼30% of the cells. These observations thus indicate that Hog1 activation has an impact on rDNA segregation.

Telomere segregation was monitored on a similar set of strains bearing *TetR-YFP tetO:1061Kb-ChrXII* (Fig. 4 *C* and *D*). In this case, the dynamics of segregation were slower and less homogeneous among cells of the same population, with the control strain reaching ∼80% segregation only at 60 min. Of note, *sln1^{ts} Net1^{T62A,S385A}* and *sln1^{ts} hog1* reached a similar extent of segregation. In contrast, the *sln1^{ts}* strain was unable to fully segregate the telomeric region of chromosome XII during the time course. The segregation of the telomeric region upon genetic activation of Hog1 was also assessed in cells reaching mitosis from α-factor block/release (*SI Appendix*, Fig. S6 *A* and *B*). Consistently, the *sln1^{ts}* strain displayed a clear defect in telomere segregation compared with *sln1^{ts} hog1* and this defect was almost fully reversed in the *sln1^{ts} Net1^{T62A,S385A}* mutant strain. This observation therefore indicates that Hog1 phosphorylation of Net1 affects the segregation of this genomic region. Additionally, the mutations T62A and S385A on Net1 did not have any effect on telomere segregation in a *SLN1* wild-type background (*SI Appendix*, Fig. S6*C*). Taken together, these data indicate that genetic Hog1 activation impairs the segregation of rDNA and

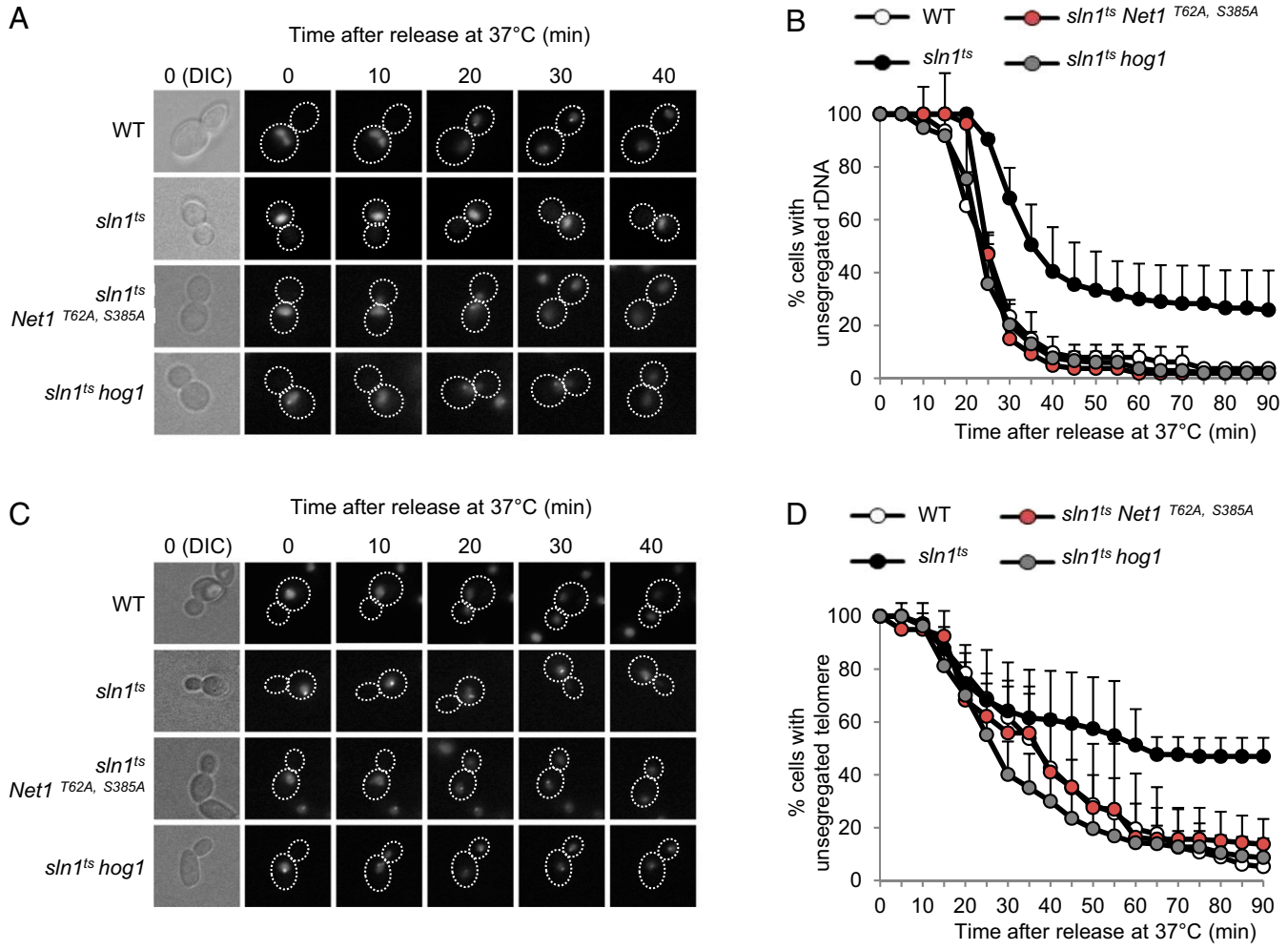

Fig. 4. Hog1 activation during mitosis impairs rDNA and telomere segregation. (*A*) *GAL1p-CDC20 TetR-YFP tetO:450Kb-ChrXII* cells were synchronized in metaphase in YPRaff at 25 °C for 3 h and switched to 37 °C for 1 h before release upon galactose addition. rDNA segregation was monitored by time-lapse microscopy. DIC, differential interference contrast. (*B*) Quantification of rDNA segregation (as in *A*). Data represent mean and SD. (*C*) *GAL1p-CDC20 TetR-YFP tetO:1061Kb-ChrXII* cells were synchronized in metaphase in YPRaff at 25 °C for 3 h and switched to 37 °C for 1 h before release upon galactose addition. Telomere segregation was monitored by time-lapse microscopy. (*D*) Quantification of telomere segregation (as in *C*). Data represent mean and SD.

telomeres. To measure whether these segregation defects compromise genomic integrity, we measured Rad52-YFP foci to assess the presence of recombination foci. *Net1*^{T62A,S385A} cells displayed significantly higher levels of Rad52 foci compared with Net1 wild-type cells (*SI Appendix*, Fig. S6*D*). Finally, to assess whether segregation defects could ultimately affect bud-neck structures, we monitored by time-lapse microscopy the localization of Myo1, a protein involved in bud-neck morphogenesis and cytokinesis (*SI Appendix*, Fig. S6 *E* and *F*). The *sln1*^{ts} strain released at the restrictive temperature displayed a clear defect in Myo1 localization compared with the WT. This delay in the actomyosin ring disassembly could be accountable for the pronounced defect, in contrast with the delay observed in Cdc14 release, in cell-cycle completion observed upon Hog1 activation.

Hog1-Dependent Phosphorylation of Net1 Is Required for Osmoadaptation. Hog1 activation in metaphase-arrested cells induces a delay in cell-cycle progression that appears to be linked to Net1 phosphorylation on residues T62 and S385. We thus tested whether the phosphorylation of Net1 is required to maximize cell survival upon osmostress. To this end, WT, *Net1*^{T62A,S385A}, and *hog1* strains were presynchronized in metaphase and released either in yeast extract peptone galactose (YPGal) or in YPGal supplemented with 1 M NaCl. Cell growth was monitored for 66 h after release in the two conditions (Fig. 5*A*). All strains were equally able to grow in YPGal (control), thereby indicating that neither *HOG1* deletion nor T62 and S385 mutations on Net1 affected cell fitness. When grown in media containing NaCl, the WT strain adapted over time and reached normal growth. As expected, the *hog1* strain did not proliferate under these conditions as it lacked the capacity to adapt to high osmolarity. Strikingly, the *Net1*^{T62A,S385A} strain showed osmosensitivity compared with the WT strain, thereby indicating that the T62 and S385 residues of Net1 are involved in osmoadaptation during mitosis. To corroborate this finding, we also tested the same set of strains for growth on solid YPGal media in the presence or absence of 0.8 M NaCl upon release from metaphase arrest (Fig. 5*B*). Once again, the WT strain grew in NaCl, whereas the condition was lethal for the *hog1* strain. Similar to what was observed in liquid media, the *Net1*$^{T62A,\ S385A}$ strain showed less growth than the WT cells on the NaCl plate. The reduced viability of the unphosphorylatable mutant of Net1 was also observed in asynchronous cultures (*SI Appendix*, Figs. S7 and S8 *A* and *B*), confirming its disadvantage for growth upon osmostress. Of note, *Net1*^{T62A,S385A} cells also displayed defective growth upon osmostress compared with a W303 wild-type background (*SI Appendix*, Fig. S8*C*). Altogether, these data show that Hog1 phosphorylation of Net1 on T62 and S385 is required for proper adaptation to high osmolarity.

Discussion

A key feature of living beings is their capacity to adapt to environmental changes and thus ensure maximal cell survival. Mitosis is a particularly vulnerable moment of the cell cycle involving the onset of a series of critical events, such as sister chromatid segregation, cytokinesis, and physical separation of mother and daughter cells. Defects in chromosome segregation and cell division can have dramatic effects on cell fate. For instance, in human cells, DNA missegregation can lead to genetic instability and tumorigenesis (62, 63). Therefore, upon perturbation of the extracellular environment, cell-cycle regulation must take place during all phases of the cycle, including mitosis, to ensure cell fitness.

Upon cellular stress, the G1, S, and G2 phases of the cell cycle are delayed (4, 5, 7, 8, 10–12, 15). In this study, we found that metaphase cells exposed to osmostress also showed a Hog1-dependent delay in cell-cycle progression. Additionally, the

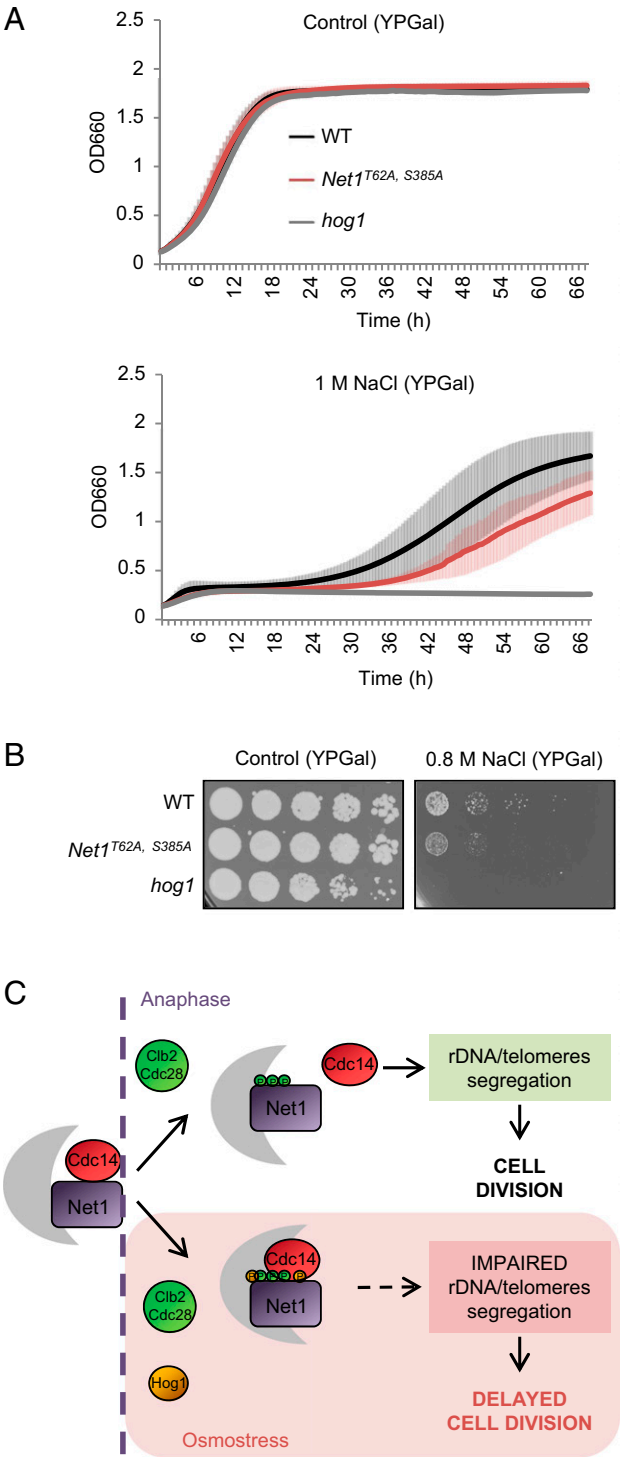

Fig. 5. Hog1-dependent phosphorylation of Net1 is required for osmoadaptation. (*A*) *GAL1p-CDC20* cells were synchronized in metaphase in YPRaff and released via galactose addition in YPGal (control) or in the presence of 1 M NaCl (osmostress). Growth was monitored at 25 °C every 30 min in a 96-well plate using a Synergy H1 MultiMode Reader. Data represent mean and SD. (*B*) *GAL1p-CDC20* cells were synchronized in metaphase in YPRaff and released via galactose addition before spotting fivefold dilutions on YPGal or YPGal-containing 0.8 M NaCl plates. (*C*) Schematic representation of Hog1-mediated osmoadaptation during mitosis. Hog1 phosphorylates Net1 residues T62 and S385, impairing Cdc14 release. As a result, rDNA and telomere segregation are affected, causing a delay in cell division.

dynamics of Cdc14 release from the nucleolus were delayed upon genetic activation of the kinase Hog1. Cdc14 is a master regulator of mitosis and its localization-dependent activation is crucial for cell-cycle completion (reviewed in refs. 39 and 64). Cdc14 nucleolar release during anaphase is promoted by Clb2–Cdc28–dependent phosphorylation on the N-terminal region of Net1 (22, 27). We therefore hypothesized that Hog1 alters the Net1–Cdc14 complex association via phosphorylation of one of the two proteins. We found that, at least in vitro, Hog1 phosphorylated Net1 on residues T62 and S385, which are in close proximity to the Cdc14-binding domain (59). Consistently, in vitro reconstitution of the Net1–Cdc14 interaction indicated that Hog1 interferes with the ability of Clb2–Cdc28 to promote disruption of the complex. In an unperturbed cell cycle, timely regulated accumulation of Clb2–Cdc28 facilitates Cdc14 release, which in turn promotes dephosphorylation of a number of targets and segregation of late genomic regions to finally allow for cell division. We speculate that, upon activation during mitosis, Hog1 phosphorylates Net1 on residues T62 and S385 and, by this means, transiently increases Net1 affinity for Cdc14 rendering the Net1–Cdc14 complex more resistant to dissolution upon Clb2–Cdc28 phosphorylation. This event affects Cdc14 timely release and in turn rDNA/telomere segregation, ultimately delaying cell division (see the schematic diagram in Fig. 5*C*). We therefore propose that cells execute a program to ensure osmoadaptation during early stages of mitosis via Hog1-dependent phosphorylation of Net1 on residues T62 and S385.

An important question regards the biological reason underlying a transient arrest of cell-cycle progression during the early stages of mitosis upon osmostress. Previously reported osmoadaptive responses that led to a delay in the transition through other cell-cycle phases could be explained by the need for the cell to prevent or regulate DNA replication (G1 and S phase, respectively) and postpone mitosis (G2) to avoid suboptimal execution of these crucial steps while adapting to environmental change. When cells are forced to adapt to osmostress while already committed to division, as occurs in metaphase, they might sustain the osmoadaptive program to maximize survival. Therefore, a delay in mitosis might be required in order to prevent defects in DNA segregation. In fact, the Net1 mutant that failed to delay Cdc14 release became osmosensitive. Although Cdc14 requirement and functions appear to differ slightly between organisms, all living beings most likely require the dephosphorylation of Cdk substrates to end a cell cycle and enter a new G1 phase. Of note, human Cdc14B rescues *cdc14* lethality in budding and fission yeast, thereby suggesting functional conservation (65, 66). Interestingly, disruption of the Cdc14B locus in human cells causes an increased incidence of rDNA anaphase bridges, thus pointing to a defect in segregation, despite no apparent impairment of cell-cycle progression (67). Given the increased complexity of human compared with budding yeast cells, a scenario in which additional factors complement or facilitate Cdc14 functions can be envisioned.

Budding yeast Cdc14 promotes the dephosphorylation of many proteins that are phosphorylated by cyclin-dependent kinases throughout the cell cycle (reviewed in refs. 42–44 and 56). It has been proposed that, in addition to Cdc14 activation, the Clb2–Cdc14 balance is important for execution of mitotic exit events (68, 69). Along this line, even nondramatic changes in the timing or in the number of Cdc14 molecules released can have noticeable effects on cell-cycle progression. Among the most extensively studied effects of Cdc14 release is the promotion of rDNA and telomere segregation via inhibition of transcription for completion of the cell cycle (33–35, 38). Consistently, here we report that Hog1 activation during metaphase not only causes an impairment in Cdc14 nucleolar release but is also linked to a defect in the timely segregation of the late segregating regions. This finding suggests that these cells do not have fully divided nuclei but might instead arrest with residual genomic material belonging to the late segregating regions stretched in-between mostly separated nuclei. Additionally, studies that analyzed the phenotype of thermosensitive mutants of Cdc14 (*cdc14-1* and *cdc14-3*) at the restrictive temperature reported cell-cycle arrest in late anaphase/telophase, with generally separated nuclear masses but unsegregated telomeres and rDNAs (34, 35, 60). The phenotype observed in the event of Hog1 genetic activation during metaphase, with initial impairment of the timely release of nucleolar Cdc14 and cells arrested in late mitosis, therefore resembles that reported in the literature for thermosensitive Cdc14 mutants. In contrast, the delay in metaphase observed in the present study differs from what was observed when mitotic cells bearing MEN mutants were driven to exit mitosis in response to hyperosmotic stress (14). We propose that this apparent discrepancy lies in the differential biochemical state in which cells encounter stress. MEN mutant cells should arrest in anaphase, presumably after Cdc14 has already been transiently released from the nucleolus into the nucleus as a function of FEAR activation (reviewed in refs. 18, 19, and 26). This condition is strikingly different from the metaphase arrest/release analyzed in our study, in which cells still have Cdc14 bound to Net1 and sequestered into the nucleolus. Under the conditions analyzed in their study (14), Hog1 might overrule the MEN requirement for Cdc14 release in order to maximize survival. Accordingly, Reiser and colleagues (14) found that the hypertonic environment per se does not accelerate exit from mitosis and that cells treated with sorbitol progressed through mitosis less efficiently than untreated cells. These data are fully consistent with our observations that mitotic exit is delayed in cells that face an osmotic shock before or during metaphase (with Cdc14 still sequestered in the nucleolus) and that Net1 is involved in the process of osmoadaptation. However, we find plausible that Hog1 may phosphorylate targets other than Net1 to modulate cell-cycle progression in mitosis.

At present, we also cannot rule out that the direct action of Cdc14 on promoting segregation of the late segregating regions is paired with additional functions of the phosphatase on other factors required for successful mitotic exit (i.e., Aurora B, as reported in ref. 35). On the other hand, upon hyperosmotic shock, Hog1-dependent phosphorylation of Net1 could not only impair Cdc14 release but also alter other functions of Cdc14 or Net1, functions that could be related to the stability of the nucleolus and rDNA silencing, or to additional activities of the phosphatase Cdc14. In the absence of additional evidence, we speculate that the Cdc14-related defect in full timely sister chromatid segregation could be reflected in a delay in organized septum formation by physical means, having unsegregated stretches of DNA lying in-between mother and daughter cells and ultimately obstructing proper septum formation. The exact mechanisms by which Cdc14 release, together with rDNA and telomere segregation defects, mediates the delay in cell-cycle progression deserves further attention.

In summary, here we report that activated Hog1 phosphorylates Net1, thus impairing Cdc14 nucleolar release and rDNA and telomere segregation, ultimately delaying cell-cycle progression. Here we identify a regulatory mechanism, through phosphorylation of Net1, by which cells maximize survival via the regulation of mitosis upon environmental changes.

Materials and Methods

Synchronization of *GAL1p-CDC20* cells in all experiments was achieved by incubating exponential cells in yeast extract peptone raffinose (YPRaff) for 2 to 4 h and released by inducing Cdc20 expression by galactose addition (1%). Activation of the HOG pathway was achieved by incubating cells at the indicated NaCl concentration or by genetic means using the *sln1^{ts}* allele at 37 °C. For immunofluorescence (IF) and DAPI staining experiments, cells were fixed overnight with 3.7% formaldehyde in IF buffer (0.1 M K_2HPO_4,

0.1 M KH_2PO_4, 0.5 mM $MgCl_2$, pH 6.4). Cells were then washed twice with IF buffer and once with sorbitol buffer (0.1 M K_2HPO_4, 0.1 M KH_2PO_4, 0.5 mM $MgCl_2$, 1.2 M sorbitol, pH 7.4). Each sample was digested in sorbitol buffer with 2 μL β-mercaptoethanol and 40 μg/μL zymolase T100 (AttendBio) for 30 min at 30 °C. For Cdc14 localization analysis, samples were collected every 5 min after release at 37 °C and fixed for 10 min on ice with 3.7% formaldehyde in phosphate-buffered saline (PBS). Cells were spun down, resuspended with a solution of 1% formaldehyde in PBS, and incubated for 10 min before extensive washes with 40 mM Tris (pH 8.8). For rDNA and telomere segregation analysis in metaphase-arrested cultures, cells were synchronized at the onset of metaphase, switched to 37 °C, and let attach to eight-well chambers pretreated with Con A, and unattached cells were washed away before release by galactose addition. For α-factor synchronization, cultures were arrested with 20 μg/mL α-factor for 3 to 4 h at 25 °C and released at 25 °C for 60 min before switching to 37 °C to promote genetic activation of Hog1 in mitosis. During this time, cells were let attach as before. For Myo1 localization analysis, cells were synchronized at the onset of metaphase, switched to 37 °C, and let attach to eight-well chambers pretreated with Con A, and unattached cells were washed away before analysis. In either case, images were acquired every 10 min for at least 90 min at 37 °C with a 100× oil objective lens in the YFP or Cherry spectrum. Analysis of electromobility shifts in Phos-tag gels was achieved in a 6% polyacrylamide gel supplemented with 10 μM Phos-tag (Wako) and 20 μM $MnCl_2$ for 3 h at 100 V on ice. Basic Hog1 kinase assays were performed using 1 μg of GST–Hog1 activated with 0.5 μg of GST–$Pbs2^{EE}$ in the presence of kinase buffer (*SI Appendix*) and 100 μM ATP. After 30 min at 30 °C, 2 μg of GST–Cdc14, GST–Net1, GST–Net1 F2, and GST–Net1 $F2^{T62A,S385A}$ was added to the Hog1/$Pbs2^{EE}$ mixture together with [^{32}P]ATP (0.1 mCi/mL; PerkinElmer) and incubated for 30 min at 30 °C. Full lists of reagents, strains, and plasmids and detailed protocols used in this article are provided in *SI Appendix, Methods*.

Data Availability. All data discussed in this study are included in the main text and *SI Appendix*.

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1. A. Duch, E. de Nadal, F. Posas, The p38 and Hog1 SAPKs control cell cycle progression in response to environmental stresses. *FEBS Lett.* **586**, 2925–2931 (2012).
2. E. de Nadal, G. Ammerer, F. Posas, Controlling gene expression in response to stress. *Nat. Rev. Genet.* **12**, 833–845 (2011).
3. H. Saito, F. Posas, Response to hyperosmotic stress. *Genetics* **192**, 289–318 (2012).
4. M. A. Adrover *et al*., Time-dependent quantitative multicomponent control of the G_1-S network by the stress-activated protein kinase Hog1 upon osmostress. *Sci. Signal.* **4**, ra63 (2011).
5. M. R. Alexander *et al*., Regulation of cell cycle progression by Swe1p and Hog1p following hypertonic stress. *Mol. Biol. Cell* **12**, 53–62 (2001).
6. Y. L. Chang *et al*., Yeast Cip1 is activated by environmental stress to inhibit Cdk1-G1 cyclins via Mcm1 and Msn2/4. *Nat. Commun.* **8**, 56 (2017).
7. J. Clotet *et al*., Phosphorylation of Hsl1 by Hog1 leads to a G2 arrest essential for cell survival at high osmolarity. *EMBO J.* **25**, 2338–2346 (2006).
8. A. Duch *et al*., Coordinated control of replication and transcription by a SAPK protects genomic integrity. *Nature* **493**, 116–119 (2013).
9. A. Duch *et al*., Multiple signaling kinases target Mrc1 to prevent genomic instability triggered by transcription-replication conflicts. *Nat. Commun.* **9**, 379 (2018).
10. X. Escoté, M. Zapater, J. Clotet, F. Posas, Hog1 mediates cell-cycle arrest in G1 phase by the dual targeting of Sic1. *Nat. Cell Biol.* **6**, 997–1002 (2004).
11. A. González-Novo *et al*., Hog1 targets Whi5 and Msa1 transcription factors to downregulate cyclin expression upon stress. *Mol. Cell. Biol.* **35**, 1606–1618 (2015).
12. M. Nadal-Ribelles *et al*., Control of Cdc28 CDK1 by a stress-induced lncRNA. *Mol. Cell* **53**, 549–561 (2014).
13. E. Radmaneshfar *et al*., From START to FINISH: The influence of osmotic stress on the cell cycle. *PLoS One* **8**, e68067 (2013).
14. V. Reiser, K. E. D'Aquino, L. S. Ee, A. Amon, The stress-activated mitogen-activated protein kinase signaling cascade promotes exit from mitosis. *Mol. Biol. Cell* **17**, 3136–3146 (2006).
15. G. Yaakov *et al*., The stress-activated protein kinase Hog1 mediates S phase delay in response to osmostress. *Mol. Biol. Cell* **20**, 3572–3582 (2009).
16. G. Bellí, E. Garí, M. Aldea, E. Herrero, Osmotic stress causes a G1 cell cycle delay and downregulation of Cln3/Cdc28 activity in *Saccharomyces cerevisiae*. *Mol. Microbiol.* **39**, 1022–1035 (2001).
17. K. King, H. Kang, M. Jin, D. J. Lew, Feedback control of Swe1p degradation in the yeast morphogenesis checkpoint. *Mol. Biol. Cell* **24**, 914–922 (2013).
18. A. Mocciaro, E. Schiebel, Cdc14: A highly conserved family of phosphatases with non-conserved functions? *J. Cell Sci.* **123**, 2867–2876 (2010).
19. E. Queralt, F. Uhlmann, Cdk-counteracting phosphatases unlock mitotic exit. *Curr. Opin. Cell Biol.* **20**, 661–668 (2008).
20. W. Shou *et al*., Exit from mitosis is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from nucleolar RENT complex. *Cell* **97**, 233–244 (1999).
21. R. Visintin, E. S. Hwang, A. Amon, Cfi1 prevents premature exit from mitosis by anchoring Cdc14 phosphatase in the nucleolus. *Nature* **398**, 818–823 (1999).
22. R. Azzam *et al*., Phosphorylation by cyclin B-Cdk underlies release of mitotic exit activator Cdc14 from the nucleolus. *Science* **305**, 516–519 (2004).
23. W. Shou *et al*., Cdc5 influences phosphorylation of Net1 and disassembly of the RENT complex. *BMC Mol. Biol.* **3**, 3 (2002).
24. S. Yoshida, A. Toh-e, Budding yeast Cdc5 phosphorylates Net1 and assists Cdc14 release from the nucleolus. *Biochem. Biophys. Res. Commun.* **294**, 687–691 (2002).
25. R. Visintin, F. Stegmeier, A. Amon, The role of the polo kinase Cdc5 in controlling Cdc14 localization. *Mol. Biol. Cell* **14**, 4486–4498 (2003).
26. J. M. Rock, A. Amon, The FEAR network. *Curr. Biol.* **19**, R1063–R1068 (2009).
27. E. Queralt, C. Lehane, B. Novak, F. Uhlmann, Downregulation of PP2A(Cdc55) phosphatase by separase initiates mitotic exit in budding yeast. *Cell* **125**, 719–732 (2006).
28. L. J. Holt *et al*., Global analysis of Cdk1 substrate phosphorylation sites provides insights into evolution. *Science* **325**, 1682–1686 (2009).
29. A. I. de Los Santos-Velázquez, I. G. de Oya, J. Manzano-López, F. Monje-Casas, Late rDNA condensation ensures timely Cdc14 release and coordination of mitotic exit signaling with nucleolar segregation. *Curr. Biol.* **27**, 3248–3263.e5 (2017).
30. T. Higuchi, F. Uhlmann, Stabilization of microtubule dynamics at anaphase onset promotes chromosome segregation. *Nature* **433**, 171–176 (2005).
31. M. Roccuzzo, C. Visintin, F. Tili, R. Visintin, FEAR-mediated activation of Cdc14 is the limiting step for spindle elongation and anaphase progression. *Nat. Cell Biol.* **17**, 251–261 (2015).
32. K. E. Ross, O. Cohen-Fix, A role for the FEAR pathway in nuclear positioning during anaphase. *Dev. Cell* **6**, 729–735 (2004).
33. A. Clemente-Blanco *et al*., Cdc14 inhibits transcription by RNA polymerase I during anaphase. *Nature* **458**, 219–222 (2009).
34. D. D'Amours, F. Stegmeier, A. Amon, Cdc14 and condensin control the dissolution of cohesin-independent chromosome linkages at repeated DNA. *Cell* **117**, 455–469 (2004).
35. M. Sullivan, T. Higuchi, V. L. Katis, F. Uhlmann, Cdc14 phosphatase induces rDNA condensation and resolves cohesin-independent cohesion during budding yeast anaphase. *Cell* **117**, 471–482 (2004).
36. J. Torres-Rosell, F. Machín, A. Jarmuz, L. Aragón, Nucleolar segregation lags behind the rest of the genome and requires Cdc14p activation by the FEAR network. *Cell Cycle* **3**, 496–502 (2004).
37. A. Clemente-Blanco *et al*., Cdc14 phosphatase promotes segregation of telomeres through repression of RNA polymerase II transcription. *Nat. Cell Biol.* **13**, 1450–1456 (2011).
38. J. Torres-Rosell, F. Machín, L. Aragón, Cdc14 and the temporal coordination between mitotic exit and chromosome segregation. *Cell Cycle* **4**, 109–112 (2005).
39. B. Baro, E. Queralt, F. Monje-Casas, Regulation of mitotic exit in *Saccharomyces cerevisiae*. *Methods Mol. Biol.* **1505**, 3–17 (2017).
40. M. Segal, Mitotic exit control: A space and time odyssey. *Curr. Biol.* **21**, R857–R859 (2011).
41. D. A. Mohl, M. J. Huddleston, T. S. Collingwood, R. S. Annan, R. J. Deshaies, Dbf2-Mob1 drives relocalization of protein phosphatase Cdc14 to the cytoplasm during exit from mitosis. *J. Cell Biol.* **184**, 527–539 (2009).
42. J. Bloom *et al*., Global analysis of Cdc14 phosphatase reveals diverse roles in mitotic processes. *J. Biol. Chem.* **286**, 5434–5445 (2011).
43. L. Kao *et al*., Global analysis of Cdc14 dephosphorylation sites reveals essential regulatory role in mitosis and cytokinesis. *Mol. Cell. Proteomics* **13**, 594–605 (2014).
44. T. Kuilman *et al*., Identification of Cdk targets that control cytokinesis. *EMBO J.* **34**, 81–96 (2015).
45. R. Visintin *et al*., The phosphatase Cdc14 triggers mitotic exit by reversal of Cdk-dependent phosphorylation. *Mol. Cell* **2**, 709–718 (1998).
46. J. Brace, J. Hsu, E. L. Weiss, Mitotic exit control of the *Saccharomyces cerevisiae* Ndr/LATS kinase Cbk1 regulates daughter cell separation after cytokinesis. *Mol. Cell. Biol.* **31**, 721–735 (2011).
47. P. R. Dohrmann *et al*., Parallel pathways of gene regulation: Homologous regulators SWI5 and ACE2 differentially control transcription of HO and chitinase. *Genes Dev.* **6**, 93–104 (1992).
48. M. A. Juanes, S. Piatti, The final cut: Cell polarity meets cytokinesis at the bud neck in *S. cerevisiae*. *Cell. Mol. Life Sci.* **73**, 3115–3136 (2016).

49. F. Meitinger, S. Palani, G. Pereira, The power of MEN in cytokinesis. *Cell Cycle* **11**, 219–228 (2012).
50. E. L. Weiss, Mitotic exit and separation of mother and daughter cells. *Genetics* **192**, 1165–1202 (2012).
51. R. Visintin, S. Prinz, A. Amon, CDC20 and CDH1: A family of substrate-specific activators of APC-dependent proteolysis. *Science* **278**, 460–463 (1997).
52. S. Prinz, E. S. Hwang, R. Visintin, A. Amon, The regulation of Cdc20 proteolysis reveals a role for APC components Cdc23 and Cdc27 during S phase and early mitosis. *Curr. Biol.* **8**, 750–760 (1998).
53. X. Mao, Y. Hu, C. Liang, C. Lu, MET3 promoter: A tightly regulated promoter and its application in construction of conditional lethal strain. *Curr. Microbiol.* **45**, 37–40 (2002).
54. T. Maeda, S. M. Wurgler-Murphy, H. Saito, A two-component system that regulates an osmosensing MAP kinase cascade in yeast. *Nature* **369**, 242–245 (1994).
55. F. Posas *et al*., Yeast HOG1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the SLN1-YPD1-SSK1 "two-component" osmosensor. *Cell* **86**, 865–875 (1996).
56. F. Stegmeier, A. Amon, Closing mitosis: The functions of the Cdc14 phosphatase and its regulation. *Annu. Rev. Genet.* **38**, 203–232 (2004).
57. M. Sullivan, D. O. Morgan, Finishing mitosis, one step at a time. *Nat. Rev. Mol. Cell Biol.* **8**, 894–903 (2007).
58. B. Soufi *et al*., Global analysis of the yeast osmotic stress response by quantitative proteomics. *Mol. Biosyst.* **5**, 1337–1346 (2009).
59. E. E. Traverso *et al*., Characterization of the Net1 cell cycle-dependent regulator of the Cdc14 phosphatase from budding yeast. *J. Biol. Chem.* **276**, 21924–21931 (2001).
60. F. Machín, J. Torres-Rosell, A. Jarmuz, L. Aragón, Spindle-independent condensation-mediated segregation of yeast ribosomal DNA in late anaphase. *J. Cell Biol.* **168**, 209–219 (2005).
61. C. Michaelis, R. Ciosk, K. Nasmyth, Cohesins: Chromosomal proteins that prevent premature separation of sister chromatids. *Cell* **91**, 35–45 (1997).
62. P. V. Jallepalli, C. Lengauer, Chromosome segregation and cancer: Cutting through the mystery. *Nat. Rev. Cancer* **1**, 109–117 (2001).
63. B. N. Zhang, A. Bueno Venegas, I. D. Hickson, W. K. Chu, DNA replication stress and its impact on chromosome segregation and tumorigenesis. *Semin. Cancer Biol.* **55**, 61–69 (2019).
64. F. Machín, O. Quevedo, C. Ramos-Pérez, J. García-Luis, Cdc14 phosphatase: Warning, no delay allowed for chromosome segregation! *Curr. Genet.* **62**, 7–13 (2016).
65. L. Li, B. R. Ernsting, M. J. Wishart, D. L. Lohse, J. E. Dixon, A family of putative tumor suppressors is structurally and functionally conserved in humans and yeast. *J. Biol. Chem.* **272**, 29403–29406 (1997).
66. M. D. Vázquez-Novelle, V. Esteban, A. Bueno, M. P. Sacristán, Functional homology among human and fission yeast Cdc14 phosphatases. *J. Biol. Chem.* **280**, 29144–29150 (2005).
67. E. Berdougo, M. V. Nachury, P. K. Jackson, P. V. Jallepalli, The nucleolar phosphatase Cdc14B is dispensable for chromosome segregation and mitotic exit in human cells. *Cell Cycle* **7**, 1184–1190 (2008).
68. C. Bouchoux, F. Uhlmann, A quantitative model for ordered Cdk substrate dephosphorylation during mitotic exit. *Cell* **147**, 803–814 (2011).
69. B. J. Drapkin, Y. Lu, A. L. Procko, B. L. Timney, F. R. Cross, Analysis of the mitotic exit control system using locked levels of stable mitotic cyclin. *Mol. Syst. Biol.* **5**, 328 (2009).