Sequential Counteracting Kinases Restrict an Asymmetric Gene Expression Program to early G1

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Gene expression is restricted to specific times in cell division and differentiation through close control of both activation and inactivation of transcription. In budding yeast, strict spatiotemporal regulation of the transcription factor Ace2 ensures that it acts only once in a cell's lifetime: at the M-to-G1 transition in newborn daughter cells. The Ndr/LATS family kinase Cbk1, functioning in a system similar to metazoan *hippo* signaling pathways, activates Ace2 and drives its accumulation in daughter cell nuclei, but the mechanism of this transcription factor's inactivation is unknown. We found that Ace2's nuclear localization is maintained by continuous Cbk1 activity and that inhibition of the kinase leads to immediate loss of phosphorylation and export to the cytoplasm. Once exported, Ace2 cannot re-enter nuclei for the remainder of the cell cycle. Two separate mechanisms enforce Ace2's cytoplasmic sequestration: 1) phosphorylation of CDK consensus sites in Ace2 by the G1 CDKs Pho85 and Cdc28/CDK1 and 2) an unknown mechanism mediated by Pho85 that is independent of its kinase activity. Direct phosphorylation of CDK consensus sites is not necessary for Ace2's cytoplasmic retention, indicating that these mechanisms function redundantly. Overall, these findings show how sequential opposing kinases limit a daughter cell specific transcriptional program to a brief period during the cell cycle and suggest that CDKs may function as cytoplasmic sequestration factors.

INTRODUCTION

Dynamic control of gene expression is critical for cellular differentiation. In cell fate determination, asymmetry of internal or external cues gives rise to divergent and transient gene expression patterns that specify cell type (Artavanis-Tsakonas *et al.*, 1995; Spana and Doe, 1995; Schuldt *et al.*, 1998). These asymmetric programs act in one daughter cell to define its identity and are then shut off as embryonic cells differentiate to form organs and tissues (Pesce and Scholer, 2001). The activation of transcription is clearly a critical point of regulation, but its controlled inactivation is also crucial for identity preservation in differentiating cells. Prolonged expression of key regulatory genes during development, for example, can lead to improper fate specification and abnormal organ or tissue development (Pesce and Scholer, 2000; Kos *et al.*, 2001). Determining how gene transcriptional programs are switched on and off is thus crucial to understanding how cells generate temporally restricted patterns of gene expression.

In budding yeast, a form of cell fate specification happens every division: a mother cell divides asymmetrically to produce a daughter that initiates a distinctive pattern of transcription. This gene expression program is turned on once in

the lifetime of a daughter cell, early in the first cell cycle, and is then shut off permanently. It is driven by asymmetric activation and segregation of the transcription factor Ace2, which induces expression of genes encoding enzymes that destroy the septum connecting mother and daughter cells at the end of cell division (Dohrmann *et al.*, 1992; Bidlingmaier *et al.*, 2001; Colman-Lerner *et al.*, 2001; Bourens *et al.*, 2008). This system limits expression of hydrolytic enzymes to a narrow time interval in newly born daughters, likely protecting cells from deleterious effects caused by their repeated expression. Ace2 also inhibits expression of the G1 cyclin *CLN3*, lengthening that phase of the cell cycle in daughter cells (Laabs *et al.*, 2003; Di Talia *et al.*, 2009).

Ace2's activation is closely coupled with the transition from mitosis to G1. The transcription factor is produced in early mitosis (Koch and Nasmyth, 1994) and is initially cytoplasmically localized, its nuclear import blocked by phosphorylation of three key sites on its nuclear localization sequence (NLS) by Cdc28/CDK1, a cyclin-dependent kinase (CDK; O'Conallain *et al.*, 1999; Sbia *et al.*, 2008). As mitosis progresses, this inhibition is presumably reversed by the activation of the phosphatase Cdc14, which specifically removes phosphorylations from proline-directed kinase sites. Ace2 then localizes to the daughter cell nucleus just before cytokinesis, while mother and daughter cells are still cytoplasmically contiguous (Mazanka *et al.*, 2008). Accumulation of Ace2 in the daughter cell nucleus is controlled by the NDR/Lats family protein kinase Cbk1, which is functionally restricted to the daughter cell (Colman-Lerner *et al.*, 2001; Weiss *et al.*, 2002; Nelson *et al.*, 2003; Jansen *et al.*, 2006; Mazanka *et al.*, 2008). Cbk1 phosphorylates Ace2's nuclear export sequence (NES), directly blocking its interaction with nuclear export machinery, and also modifies a site that likely increases the transcription factor's activity (Mazanka *et al.*, 2008).

Ace2's inactivation is also closely coordinated with G1 progression. The protein's nuclear abundance diminishes after cell separation, with a concomitant drop in transcription of its target genes (Sbia *et al.*, 2008). The mechanism by which the transcription factor is turned off is unknown. Intriguingly, Ace2 is stable throughout the cell cycle (Sbia *et al.*, 2008), including periods in which Cbk1 is active to promote normal cell morphogenesis (Weiss *et al.*, 2002; Jansen *et al.*, 2006). Thus, the transcription factor is permanently inactivated without degradation after briefly turning on expression of its target genes in early G1 daughter cells.

We sought to determine how Ace2 is turned off in early G1 and kept inactive through subsequent stages of the daughter cell's division. Our analysis focused on two poorly defined aspects of the transcription factor's activity cycle: maintenance of its activity and nuclear localization and the mechanism of its persistent inactivation. We found that Ace2's residence in the nucleus requires continuous rephosphorylation by Cbk1, indicating that persistence of Ace2-driven gene expression is controlled by a balance between activities of this positively acting kinase and a countervailing phosphatase. Once exported, Ace2 cannot re-enter the nucleus. This cytoplasmic sequestration can be established by the kinase activities of either Pho85 or Cdc28. These G1 CDKs act through two distinct and functionally redundant mechanisms: phosphorylation of consensus sites on Ace2, and a non-kinase function of Pho85 itself. Overall, these findings illustrate how sequentially acting kinases generate a brief pulse of transcription that occurs only once in a cell's life.

MATERIALS AND METHODS

GAL-CDC20 Cell Synchrony

Cells grown to log phase in YP + 2% galactose were filtered and resuspended into an equal volume of YP + 2% glucose and then grown at 30°C for 2 h until M phase arrest (assessed by accumulation of large-budded cells). Cells were filtered again, resuspended in YP + 2% galactose media, and grown at 30°C, with samples removed at indicated time points. Harvested cells were centrifuged and frozen in liquid nitrogen or directly analyzed for microscopy.

Kinase Inhibition

For *cbk1-as2* kinase inhibition, 25 μM 1NA-PP1 (gift of C. Zhang, University of California, San Francisco) in DMSO was added to cells immediately before analysis. *Cdc28-as1* cells were treated for 2 h with 5 μM 1NM-PP1 (Toronto Research Chemicals, Toronto, ON, Canada) in DMSO. *Pho85-as1* cells were treated for 2 h with 10 μM 1NA-PP1.

Microscopy

Time-lapse microscopy was performed using *GAL-CDC20* cells grown to log phase in synthetic media supplemented with 2% galactose and synchronized as described above. After M phase arrest, cells were released into synthetic media containing 2% galactose and mounted on agar pads, composed of synthetic media and 2% agarose. Cells were imaged using fluorescence/differential interference contrast microscopy with an Axiovert 200M (Carl Zeiss MicroImaging, Thornwood, NY). Images were taken every 3 min for 60 min. Photographs were taken with a Cascade II-512 camera (Photometrics, Tucson, AZ), and contrast enhancement was done using Openlab software (Improvision, Lexington, MA). Fluorescence quantification was done using Openlab software.

For *cbk1-as2* inhibition time-course experiments, *GAL-CDC20 cbk1-as2* cells were arrested and released as described above. Cells were mounted on concanavalin A (ConA) coated coverslips (Sigma, St. Louis, MO) in Petri dishes and incubated 5 min to ensure adhesion. Unadhered cells were rinsed off with synthetic media, and then cells were imaged as described above. Once appropriate fields were chosen, cells were immersed in synthetic media + 25 μM 1NA-PP1 and imaged for an additional 10 min.

For leptomycin B (LMB) microscopy experiments, asynchronous cultures were grown to log phase in synthetic media then incubated with rhodamine-conjugated ConA dye (Vector Laboratories, Burlingame, CA) for 5 min. Cells were washed with 2 volumes fresh media and grown for an additional 2–3 h at 30°C. Next, 10 ng/μl of LMB in ethanol (a gift of M. Yoshida, RIKEN Institute) was added, and cells were grown for 30 min at 30°C and then imaged in media. For experiments in which mother and daughter cells were not distinguished, cells were not pulse-labeled with rhodamine-conjugated ConA before LMB treatment.

For alpha arrest, BAR1 cells were grown to early log phase in synthetic media and then arrested using 10 μM α factor (GenScript, Piscataway, NJ).

Cells were incubated at 30°C for 2 h with additional 10 μM α factor added after 1 h. Cells were imaged as described above.

Cell separation experiments were performed using cells grown to log phase in rich media, sonicated twice for 15 s, and then imaged.

Immunoprecipitation and Western Blotting

Frozen cells were lysed and immunoprecipitated with anti-hemagglutinin (HA) mAb (12CA5, a gift of R. Lamb, Northwestern University) as described previously (Jansen *et al.*, 2006). Total lysate protein concentration was measured by Bradford assay (Bio-Rad, Richmond, CA), using BSA to generate a standard curve. Lysate concentration for each sample was normalized before immunoprecipitation. After immunoprecipitation and binding to rec-protein G 4B Sepharose beads (Invitrogen, Carlsbad, CA), samples were washed three times with yeast lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol) and immediately resuspended in 2× SDS-PAGE sample buffer and boiled. Immunoprecipitated samples were loaded onto 8% SDS-PAGE gels and transferred to PVDF membranes (Pall, Port Washington, NY). Blots were blocked for 30 min at room temperature in 10% BSA (Sigma) in Tris-buffered saline containing 20% Tween (TBST) and blotted with phospho-specific anti-pS122 (Open Biosystems, Huntsville, AL) diluted 1:200 in 1% BSA/TBST for 1.5 h, washed three times for 3 min each in TBST and incubated with Alexa 680–conjugated goat anti-rabbit secondary (Invitrogen) at 1:5000 in TBST. Blots were then washed five times for 3 min each in TBST and imaged using the LiCor Odyssey system (Lincoln, NE).

Blots were subsequently probed with 1:1000 anti-HA mAb for 1 h and washed three times for 3 min with TBST and then incubated with IRDye-800 goat anti-mouse secondary (Rockland, Gilbertsville, PA) 1:5000 for 45 min. Blots were washed five times for 3 min with TBST and imaged using the Odyssey.

For phosphatase treatment, immunoprecipitated Ace2-HA was washed three times in yeast lysis buffer and then two times in phosphatase buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 10 mM MnCl$_2$, 0.1 mg/ml BSA; Holt *et al.*, 2007). Reactions were carried out in 40 μl phosphatase buffer with 1 μl bacterially expressed recombinant CDC14 protein (a gift of C. Yoo, Northwestern University) or 1 μl λ phosphatase (New England Biolabs, Beverly, MA) or no phosphatase for mock treatment. Reactions were incubated for 35 min at 30°C with gentle shaking and washed twice with yeast lysis buffer. Samples were resuspended in 2× SDS-PAGE sample buffer run on 8% SDS-PAGE gels, transferred, and blotted as described above.

Frozen Ace2-HA and ace2-AP-HA pellets were resuspended in yeast lysis buffer and lysed using a homogenizer. Immunoprecipitations were performed as described above. After washes, Sepharose-bound substrates were stored at 4°C in 1:1 mix of yeast lysis buffer and 50% glycerol. Before kinase reactions, substrates were washed three times with kinase buffer (10% glycerol, 50 mM Tris-HCl, 150 mM NaCl, 10 mM MgCl$_2$, 1 mM dithiothreitol).

Protein Purification and Kinase Assays

BL21 (DE3) cells coexpressing pQE60 His-Pho85as and untagged Pcl1 were grown and treated as described in Jeffery *et al.* (2001). Briefly, cells were grown in LB/CARB/KAN at 37°C to log phase and then induced with 0.5 mM IPTG at 24° for 6.5 h. Cells were collected, pelleted, and resuspended in lysis buffer (10% glycerol, 50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 60 mM imidazole, 1 mM β-mercaptoethanol) and then frozen. Pellets were lysed by homogenization and then clarified at 15,000 rpm for 15 min at 4°C in a SA600 (Beckman, Fullerton, CA) rotor. Ni-NTA agarose beads (Qiagen, Chatsworth, CA) were added and incubated with lysate for 2 h at 4° rotating and then lysates were washed three times with 20 ml lysis buffer and eluted with high-imidazole lysis buffer (same as lysis buffer but with 300 mM imidazole) by gravity drip. Proteins were dialyzed into storage buffer (10% glycerol, 50 mM Tris-HCl, pH 7.5) and stored at −80°C. Protein concentration was measured by A$_{280}$ using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA).

Kinase assays were performed using immunoprecipitated Ace2-HA and ace2-AP-HA substrates that were resuspended in kinase buffer with 20 μM and 10 μCi/μl γ-^{32}P-ATP, and then 0.5 μM kinase was added to start the reaction. Reaction were incubated for 1 h at 30°C in kinase buffer then quenched by addition of 5× SDS-PAGE sample buffer, and incubated for 15 min at 85°C. Proteins were run on a 12% SDS-PAGE gel and transferred to PVDF membranes. Phosphorylation was detected using a Storm 860 Imager (Molecular Dynamics, Sunnyvale, CA). Blots were incubated simultaneously with anti-HA 1:1000 and anti-HIS 1:1000 (Santa Cruz) for 1.5 h, washed three times with TBST and incubated with Alexa 680 conjugated goat anti-rabbit secondary 1:5000 and IRDye-800 goat anti-mouse secondary 1:5000 for 45 min. Blots were washed four times with TBST and imaged on LiCor Odyssey. Quantifications were performed using ImageQuant (Molecular Dynamics) and Odyssey software.

Quantitative RT-PCR

Cells arrested for 2 h by glucose incubation using *GAL-CDC20* (as described above) were released into YP + 2% galactose and 5 OD$_{600}$s of cells were harvested every 10 min by centrifugation and immediately frozen in liquid nitrogen. RNA extraction and quantitative PCR were performed as described (Mazanka *et al.*, 2008) using *CTS1* and *ACT1* primers.

Table 1. Strains used in this study

Yeast strain	Genotype
ELY1144	*MATα cdc20Δ::LEU2 ade2::ADE2-GAL-CDC20 ACE2-GFP::KANMX cbk1Δ::TRP1 [cbk1-as2::URA3]*
ELY1310	*MATa crm1Δ::LEU2 [ACE2-GFP::URA3, crm1-T539C::HIS3]*
ELY1311	*MATa crm1Δ::LEU2 [NLS/NES-GFP::URA3, crm1-T539C::HIS3]*
ELY1340	*MATa cdc20Δ::LEU2 ade2::ADE2-GAL-CDC20 ACE2-HA::KANMX*
ELY1338	*MATa cdc20Δ::LEU2 ade2::ADE2-GAL-CDC20 ACE2-HA::KANMX cbk1Δ::TRP1*
ELY1353	*MATa crm1Δ::LEU2 pho85Δ::NAT [ACE2-GFP::URA3, crm1-T539C::HIS3]*
ELY1355	*MATa cdc20Δ::LEU2 ade2::ADE2-GAL-CDC20 ACE2-HA::KANMX cbk1Δ::TRP1 [cbk1-as2::URA3]*
ELY1376	*MATa crm1Δ::LEU2 pho85Δ::NAT [ace2-AAA-GFP::URA3, crm1-T539C::HIS3]*
ELY1444	*MATa crm1Δ::LEU2 [ace2-AP-GFP::URA3, crm1-T539C::HIS3]*
ELY1445	*MATa crm1Δ::LEU2 pho85Δ::NAT [ace2-AP-GFP::URA3, crm1-T539C::HIS3]*
ELY1450	*MATα cdc28-as1 crm1Δ::LEU2 [ACE2-GFP::URA3, crm1-T539C::HIS3]*
ELY1451	*MATα cdc28-as1 crm1Δ::LEU2 [ace2-AP-GFP::URA3, crm1-T539C::HIS3]*
ELY1493	*MATα cdc20Δ::LEU2 ade2::ADE2-GAL-CDC20 ACE2-HA::KANMX pho85Δ::HIS3 trp1::TRP1-pho85-as-MYC cdc28-as1*
ELY1498	*MATa cdc20Δ::LEU2 ade2::ADE2-GAL-CDC20 ACE2-GFP::KANMX MYO1-CHERRY::HIS3*
ELY1549	*MATa cdc20Δ::LEU2 ade2::ADE2 GAL-CDC20 ace2-AP-HA::KANMX*
ELY1551	*MATα cdc20Δ::LEU2 ade2::ADE2-GAL-CDC20 ACE2-HA::KANMX pho85Δ::HIS3 trp1::TRP1-pho85-as-MYC cdc28-as1 [ACE2-GFP::URA3]*
ELY1555	*MATa crm1Δ::LEU2 pho85Δ::NAT trp1::TRP1-pho85-as-MYC [ACE2-GFP::URA3, crm1-T539C::HIS3]*

RESULTS

Ace2's G1 Inactivation Coincides with Loss of Cbk1 Phosphorylation and Export from Nuclei

The fact that Ace2 remains stable as its target genes are turned off suggests that its inactivation involves export from nuclei. Thus, reduction of the transcription factor's nuclear concentration would coincide with inactivation of its target genes. We performed time-lapse microscopy to follow the localization of a C-terminally green fluorescent protein (GFP)-tagged Ace2 from late M phase through G1. To synchronize cells, we used a strain in which *CDC20*, a gene encoding a critical component of the anaphase promoting complex (APC), is under the control of a galactose inducible promoter (*GAL-CDC20*; Archambault *et al.*, 2004; see Table 1 for strains used in this study). In this strain, *CDC20* transcription is turned off upon incubation in glucose, leading to loss of APC activity and cell cycle arrest in metaphase with high mitotic Cdc28 activity. ***We found that Ace2 began to localize to nuclei 20 min after shift to galactose medium; by ~36 min it fully accumulated in daughter nuclei (Figure 1B). These localization kinetics are consistent with prior observations (Sbia *et al.*, 2008). Maximal accumulation of Ace2-GFP in daughter cell nuclei coincided with cytokinesis, as measured by disappearance of Myo1-Cherry from the bud neck, an established marker of cytokinesis (Figure 1B). Ace2-GFP remained strongly localized for ~20 additional minutes, after which the nuclear signal decreased. This was accompanied by an increase in cytoplasmic GFP fluorescence, consistent with shuttling of Ace2 out of nuclei (Figure 1B). Ace2-GFP became fully cytoplasmic by ~84 min after release from mitotic arrest, as shown by the representative cell in Figure 1B and previously observed by Sbia *et al.* (2008). An illustration summarizing Ace2 localization after the metaphase arrest and release is shown in Figure 1A. Ace2 G1 export was quantified by measuring the ratio of nuclear-to-cytoplasmic fluorescence for 10 cells (Figure 1C).

We performed quantitative real-time PCR (qRT-PCR) to determine if loss of Ace2 from nuclei coincided with loss of transcriptional activation of its target genes. Abundance of transcript from the Ace2-regulated *CTS1* gene increased after 40 min in galactose medium and peaked at 50 min, consistent with strong nuclear localization of Ace2 at this time (Figure 1D). Concurrent with loss of Ace2 from the nucleus, *CTS1* expression rapidly decreased by 60 min, reaching basal levels by 70 min. Therefore, loss of activation of Ace2 target genes coincides with Ace2 nuclear export during G1 phase.

Ace2's accumulation in daughter nuclei requires Cbk1 phosphorylation of two sites within its nuclear export sequence: serine 122 and serine 137. We previously demonstrated that these modifications directly block interaction with the exportin Crm1, thereby trapping Ace2 in daughter cell nuclei (Mazanka *et al.*, 2008). To directly examine the status of Ace2's phosphorylation by Cbk1 in vivo, we developed an antibody that specifically recognizes the phosphorylated form of serine 122 (anti-pS122). This antibody only recognized a form of Ace2 that was phosphorylated by Cbk1 and did not recognize λ phosphatase–treated Ace2 or Ace2 immunoprecipitated from *cbk1Δ* cells (Supplemental Figures 1 and 3). Serine 122 phosphorylation was undetectable in mitotically arrested *GAL-CDC20* cells, consistent with previously observed anaphase activation of Cbk1 (Jansen *et al.*, 2006; J. Brace, unpublished data). Phosphorylation of serine 122 became evident 30 min after release from mitotic arrest (Figure 1E). This modification was transient: it was undetectable by 50 min after release from arrest, coincident with Ace2's export from nuclei. Thus, Cbk1-mediated phosphorylation of Ace2 on serine 122 correlated with Ace2 nuclear accumulation.

Ace2's G1 Nuclear Localization Requires Continuous Rephosphorylation by Cbk1

Phosphorylation by Cbk1 is required for establishment of Ace2's nuclear localization, but it is not clear if the kinase is required for its maintenance. Cbk1 phosphorylation of Ace2 might occur once in late mitosis and be reversed upon activation of a G1 phosphatase, or it might be dynamic, with the kinase acting continuously in opposition to a phosphatase. To distinguish between these possibilities, we examined the effect of Cbk1 inhibition on the persistence of Ace2-GFP nuclear localization. To block Cbk1 activity, we used cells carrying the analog-sensitive *cbk1-as2* allele, which is inhibited by the cell-permeable compound 1NA-PP1. This inhibitor works quickly and specifically on analog-sensitive kinase alleles (Sekiya-Kawasaki *et al.*, 2003); in *cbk1-as2* cells it blocks only Cbk1 kinase activity, leaving all other kinases unperturbed (Bishop *et al.*, 1998, 2001; Weiss *et al.*, 2002).
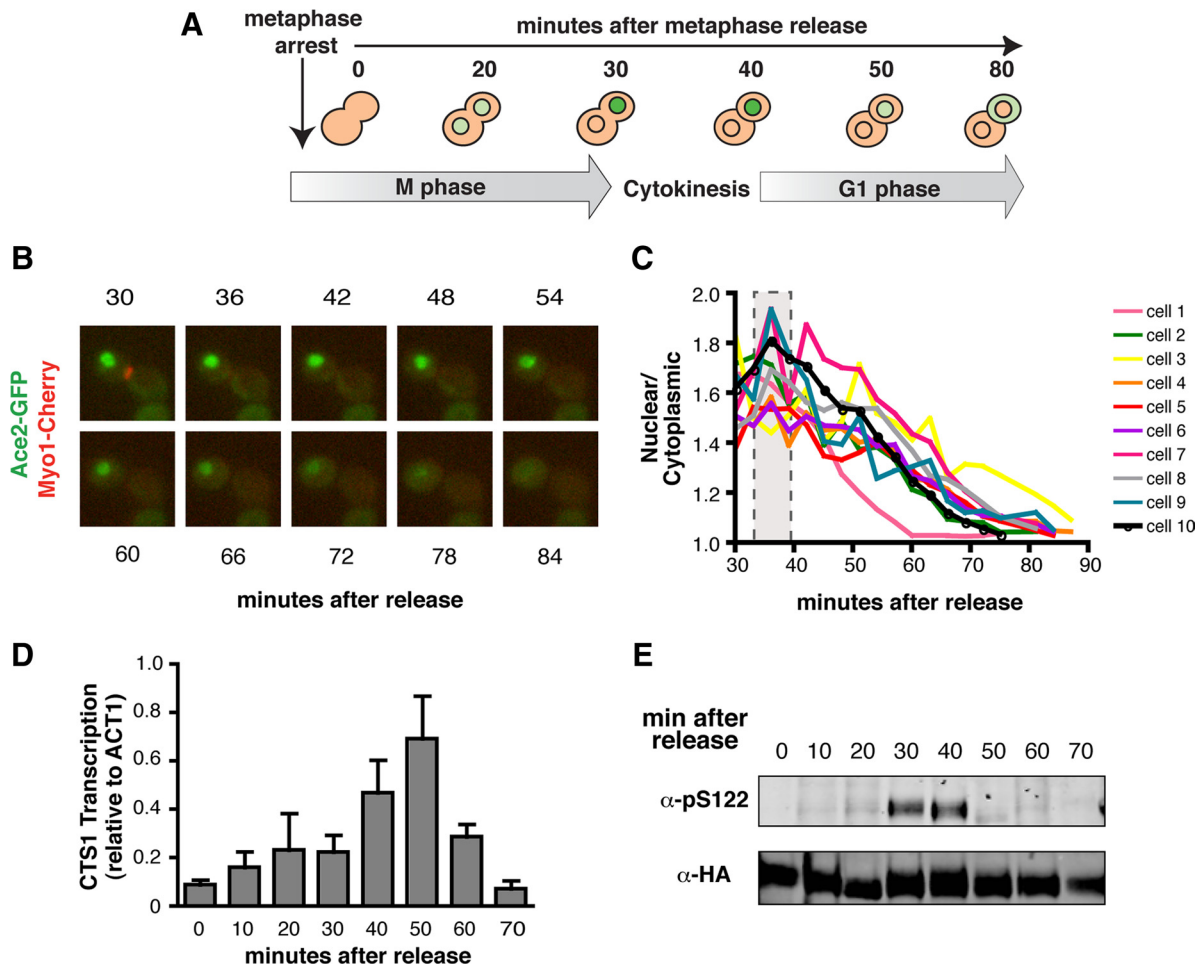
Figure 1. Ace2 is exported from nuclei in early G1, concurrent with loss of Cbk1 phosphorylation and inactivation of transcriptional activity. (A) Diagram of *GAL-CDC20* metaphase arrest and release. Illustrations summarize Ace2-GFP localization throughout the course of the release. (B) *GAL-CDC20 Ace2-GFP Myo1-CHERRY* cells were arrested in M phase by glucose incubation for 2 h and then released into galactose medium. Images were collected every 3 min. At 30 min after release, Ace2-GFP accumulated in daughter nuclei, and actomyosin ring contraction occurred at 36 min (signifying completion of cytokinesis). Loss of nuclear fluorescence was evident around 60 min. Ace2-GFP nuclear fluorescence was completely lost by 84 min, whereas cytoplasmic fluorescence of Ace2-GFP increased. (C) Quantification of 10 *GAL-CDC20 Ace2-GFP Myo1-CHERRY* time courses. The ratio of the average nuclear-to-cytoplasmic fluorescence is displayed for each time point. Time points of one are highlighted in black. Gray box represents distribution of timing of actomyosin ring contraction for all cells quantified. (D) Quantitative RT-PCR of Ace2 transcriptional target *CTS1* after *GAL-CDC20* arrest and release. Ace2 transcriptional activity peaked at 50 min after release and fell at 60–70 min. Averages of two independent trials are shown. Error bars, SEM. (E) *GAL-CDC20 Ace2-HA* cells were arrested and released as in A, and Ace2-HA was immunoprecipitated every 10 min after release. Blots were probed for phosphorylated S122 with a phospho-specific antibody (α-pS122) and α-HA. S122 phosphorylation was evident at 30 min and disappeared by 50 min.

We arrested cells in M phase using *GAL-CDC20* shutoff by glucose incubation, released into galactose medium, and waited 35 min for maximal accumulation of Ace2-GFP in daughter nuclei. We then flowed in medium containing 25 μM 1NA-PP1 and imaged the cells for 10 min. Within 2 min of inhibitor addition the amount of Ace2-GFP in nuclei began to diminish, and by 5 min Ace2-GFP was mainly cytoplasmic (Figure 2A). Measurement of the ratio of nuclear-to-cytoplasmic GFP fluorescence intensity (Figure 2B) demonstrated that Ace2-GFP rapidly exported from nuclei upon Cbk1 inhibition, compared with uninhibited control cells that remained in the nucleus throughout the time course. Therefore, continuous Cbk1 kinase activity is necessary to maintain Ace2's localization in daughter cell nuclei. We used anti-pS122 to assess the persistence of this phosphorylation after loss of Cbk1 activity: as above, we performed these experiments in M phase–synchronized cells,

adding 25 μM 1NA-PP1 after 30 min after release. Consistent with the rapid effect of Cbk1 inhibition on Ace2's localization, we found that serine 122 phosphorylation became undetectable after inhibitor addition (Figure 2C). This rapid loss of phosphorylation and immediate export of Ace2 upon Cbk1 inhibition indicates that the kinase normally phosphorylates Ace2 continuously during its nuclear residence and that this is reversed by one or more constitutively acting phosphatases.

Once Exported, Ace2 Is Sequestered in the Cytoplasm during G1

During early M phase, Cdc28 phosphorylates CDK consensus sites within Ace2's NLS, preventing the transcription factor from entering the nucleus (O'Conallain *et al.*, 1999; Sbia *et al.*, 2008). After the metaphase–anaphase transition this cytoplasmic retention is reversed, presumably by Cdc14 dephosphorylation of NLS sites, and Ace2 freely enters both
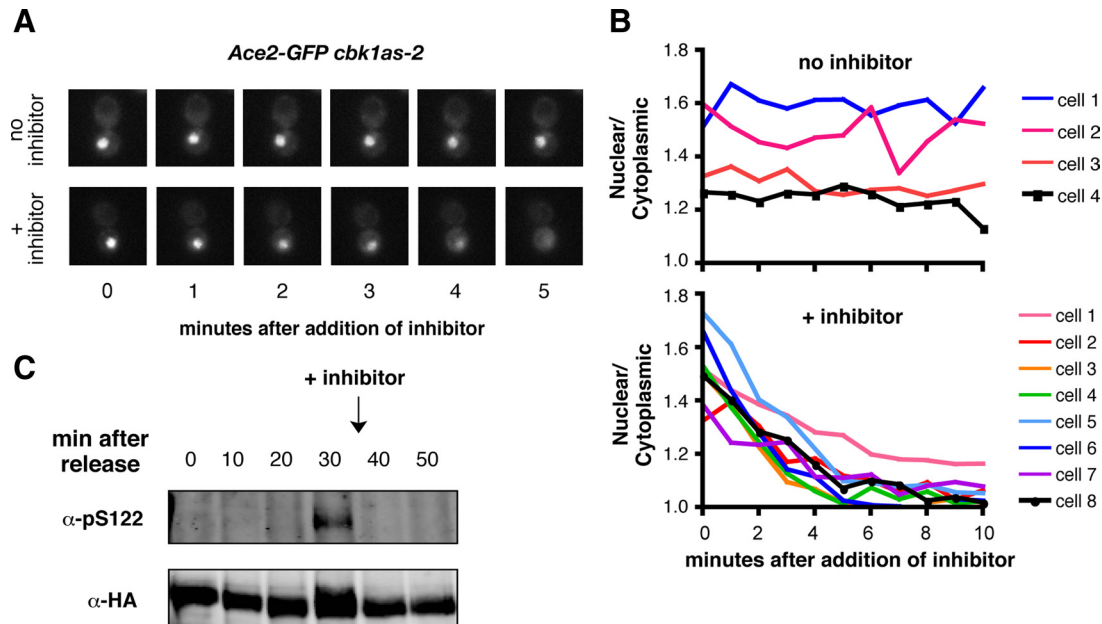
Figure 2. Ace2 requires continuous Cbk1 activity to remain in the nucleus. (A) *GAL-CDC20 Ace2-GFP cbk1-as2* cells were arrested in M phase and released into galactose medium. 25 μM 1NA-PP1 was added 35 min after release, and images were collected every minute after inhibition. Nontreated control cells are shown for comparison (above). Nuclear Ace2-GFP fluorescence decreased 2 min after Cbk1 inhibition and was absent after 5 min. (B) Quantification of time courses performed in A. For each time point, the ratio of nuclear-to-cytoplasmic fluorescence is shown. Top graph shows quantification of untreated control cells, bottom graph shows 1NA-PP1–treated cells. (C) *GAL-CDC20 Ace2-HA cbk1-as2* cells arrested and released as in A. 1NA-PP1 was added after sample collection at 30 min after release. Ace2-HA immunoprecipitates probed with α-p122 and α-HA show that S122 phosphorylation was undetectable after addition of 1NA-PP1.

mother and daughter nuclei. Subsequently, Cbk1 phosphorylation of Ace2 activates the transcription factor and promotes its nuclear accumulation in daughter cells by preventing nuclear export (Colman-Lerner *et al.,* 2001; Weiss *et al.,* 2002; Mazanka *et al.,* 2008). After cell separation the daughter cell is left with all of the Ace2 protein and must somehow keep it inactive until the subsequent mitosis. We hypothesized that Ace2's cytoplasmic retention might be re-established after its export from the nucleus, thereby excluding the transcription factor's access to target genes and maintaining its inactive state.

To determine if Ace2 can reenter nuclei after its export in G1, we examined Ace2-GFP localization in unbudded daughter cells after block of Crm1-mediated nuclear export. To inactivate Crm1, we used cells carrying the *crm1-T539C* allele, which renders this key nuclear export factor sensitive to the drug LMB (Neville and Rosbash, 1999). As a positive control for nuclear export block, we quantified localization of an NLS/NES-GFP construct with and without LMB. On treatment with inhibitor, NLS/NES-GFP strongly accumulated in nuclei of nearly all cells (Supplemental Figure 2). To restrict our analysis to G1 daughter cells, we labeled asynchronous cells briefly with rhodamine-ConA, which binds tightly to the cell wall and then washed out the dye and grew cells in fresh media for ~150 min. This treatment produces rhodamine-labeled mother cells and unlabeled daughter cells, allowing us to limit our analysis to G1 daughters in an asynchronous population.

In the absence of LMB, Ace2–GFP localized to nuclei of ~18% of G1 daughter cells (Figure 3A). This is consistent with the results shown in Figure 1A: Ace2 was present in nuclei for a portion of early G1 before its nuclear export. Blocking nuclear export with LMB did not significantly increase the fraction of G1 daughter cells with detectable nuclear Ace2-GFP. Similarly, inhibition of nuclear export did not cause

marked accumulation of Ace2-GFP in nuclei of small budded daughter cells, which correspond to S and G2 phase. As previously observed (Weiss *et al.,* 2002; Mazanka *et al.,* 2008), the number of large-budded cells with nuclear Ace2-GFP increased significantly upon LMB addition. These were cells in which anaphase had occurred, consistent with loss of Ace2 nuclear import block during mitotic exit (Sbia *et al.,* 2008;
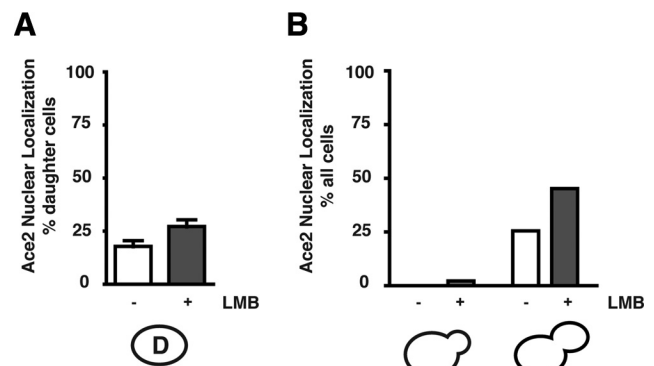

Figure 3. Ace2 nuclear import is blocked during most of the cell cycle. (A) Asynchronous *crm1-T539C Ace2-GFP* cells were pulse-labeled with Rhodamine-ConA for 5 min and grown in fresh media without ConA for approximately one doubling. Unlabeled G1 daughter cells were scored for Ace2 nuclear fluorescence with or without 30 min LMB treatment. The increase in Ace2 nuclear localization upon LMB addition is not statistically significant (p = 0.3, Student's two-tailed *t* test). Graph shows percentage of daughter cells with Ace2 nuclear localization. Each bar denotes the average of four independent trials; error bars, SEM. (B) Small and large-budded *crm1-T539C Ace2-GFP* cells from an asynchronous culture were scored for the presence or absence of Ace2 in nuclei with and without LMB treatment.

Figure 3, A and B; data not shown). Thus, once Ace2 is exported to the cytoplasm in early G1, it is retained there, and it cannot re-enter the nucleus in subsequent G1, S, and G2 phases.

G1 Cyclin-dependent Kinase Activity Is Required for Ace2 Cytoplasmic Sequestration

Because Ace2's phosphorylation by Cdc28 in M phase is important for its cytoplasmic sequestration in mitotic cells (O'Conallain *et al.,* 1999), we hypothesized that a CDK might also act in G1 to block the transcription factor's nuclear import. *S. cerevisiae* has six CDKs: Pho85 and Cdc28 are involved in cell cycle control, whereas Ctk1, Kin28, Sgv1, and Ssn3 are associated with the machinery of transcription and are not generally thought to function in cell cycle regulation (Morgan, 1997). Because both Pho85 and Cdc28 are strongly activated in G1 and function both in the cytoplasm and nucleus (Mendenhall and Hodge, 1998; Huang *et al.,* 2007), we considered these two kinases likely candidate regulators of Ace2's cytoplasmic retention.

We first sought to determine if Cdc28 activity is critical for Ace2's cytoplasmic retention in G1 cells, as it is in M phase. Because deletion of this CDK is lethal, we used an analog-sensitive allele, *cdc28-as1*, that can be specifically inhibited by the cell-permeable compound 1NM-PP1. We assessed nuclear import using *cdc28-as1 crm1-T539C* cells expressing Ace2-GFP. After pulse-labeling cells with rhodamine-ConA to allow identification of G1 daughters as in Figure 3A, we treated with 1NM-PP1 for 2 h to inhibit Cdc28 kinase activity. Importantly, this treatment causes late G1 cell cycle arrest just before bud emergence (Bishop *et al.,* 2000). Thus, all of the unbudded daughter cells scored in this experiment were arrested in late G1 (indicated on Figure 4A.) After *cdc28-as1* inhibition, we blocked *crm-T539C* function by LMB addition. For comparison, the fraction of unbudded daughter cells exhibiting nuclear Ace2-GFP after LMB treatment (Figure 3A) is shown as a black dashed line in Figure 4. We found that loss of Cdc28 activity did not reduce cytoplasmic sequestration of Ace2-GFP, with only ~3% of cells exhibiting nuclear localization of the protein upon LMB treatment (Figure 4A). This was notably lower than observed in asynchronous G1 daughter cells, consistent with all cells being arrested in late G1. These results indicate that Cdc28 is not solely responsible for blocking Ace2 nuclear import in G1.

The Pho85 CDK also functions in early G1, in association with multiple cyclin subunits (Espinoza *et al.,* 1994; Measday *et al.,* 1994, 1997). Pho85 is involved in transcriptional control of START and likely promotes degradation of Swi5, a transcription factor that is highly similar to Ace2 (Measday *et al.,* 2000; McBride *et al.,* 2001; Kung *et al.,* 2005; Huang *et al.,* 2007). We therefore evaluated the role of Pho85 activity in Ace2 cytoplasmic retention using the *pho85-as1* allele, which is sensitive to 1NA-PP1. To evaluate effectiveness of Pho85 inhibition, we examined localization of Pho4-GFP in *pho85-as1* cells with and without kinase inhibition. Absence of Pho85 function causes nuclear localization of Pho4 (O'Neill *et al.,* 1996; Kung *et al.,* 2005). We found that within 10 min of inhibitor addition Pho4-GFP localized strongly to nuclei of all cells, indicative of full kinase inhibition (Supplemental Figure 3). To assess Ace2 localization after Pho85 inhibition, we restricted our analysis to unbudded G1 daughter cells; unlike Cdc28 inhibition, loss of Pho85 function does not cause cell cycle arrest, and therefore the daughter cells we scored were distributed throughout G1. We found that the fraction of cells with Ace2-GFP nuclear localization was not significantly changed by inhibition of Pho85, indicating that

this CDK's kinase activity is not solely responsible for blocking Ace2's G1 nuclear import.

Pho85 and Cdc28 share overlapping substrates, and can redundantly phosphorylate common target proteins in vivo (Kung *et al.,* 2005). Cdc28 and Pho85 might therefore redundantly phosphorylate sites on Ace2 that block its nuclear entry, explaining why we never saw a full relief of nuclear import restriction when one or the other is inhibited. We reasoned that if this is the case, inhibiting the kinase activity of Pho85 and Cdc28 simultaneously should result in loss of cytoplasmic sequestration.

We used an Ace2-GFP expressing strain containing analog-sensitive alleles of both Cdc28 and Pho85 (*cdc28-as1* and *pho85-as1*), which can be specifically inhibited by the cell-permeable compounds 1NM-PP1 and 1NA-PP1, respectively. Combination of the *cdc28-as1 pho85-as1 crm1-T539C* proved synthetically lethal. Therefore, we assessed Ace2-GFP localization in *cdc28-as1 pho85-as1* cells without inhibition of Crm1-mediated nuclear export. We found that in the absence of kinase inhibitors nuclear Ace2-GFP was detectable in ~22% of G1 daughter cells, similar to the distribution seen in a wild-type strain. We treated cells with both 1NA-PP1 and 1NM-PP1 for 2 h, resulting in a late G1 arrest as described in Figure 4A and found that the number of cells with Ace2-GFP evident in nuclei increased dramatically, to ~78% (Figure 4B). Because Ace2's nuclear export was unrestricted in this experiment, this likely underestimates the disruption of Ace2 cytoplasmic retention caused by simultaneous inhibition of Cdc28 and Pho85. Taken together, these results indicate that kinase activity of either Pho85 or Cdc28 is critical for preventing Ace2's nuclear reentry in G1 (Figure 4C).

CDK phosphorylation consensus motifs are minimally serine or threonine followed by proline: Ace2 contains 21 such Ser/Thr-Pro motifs, and mass spectrometric analyses indicate that at least 15 of these are likely phosphorylated in vivo (Archambault *et al.,* 2004; Bodenmiller *et al.,* 2008; Holt *et al.,* 2009). We assessed phosphorylation of Ace2's at putative CDK sites by examining its electrophoretic mobility shifting before and after treatment with Cdc14, which specifically removes phosphorylations from serine or threonine residues that are followed by proline (Holt *et al.,* 2007). Ace2-HA immunoprecipitated from mitotically arrested cells showed a pronounced Cdc14-reversible shift that disappeared after release from arrest and then returned at ~30 min, whereas serine 122 was still phosphorylated (Supplemental Figure 4A). However, simultaneous inhibition of both *pho85-as1* and *cdc28-as1* did not eliminate G1 reappearance of Cdc14-reversible shifting (Supplemental Figure 4B). Thus, although these results are consistent with phosphorylation, they cannot resolve these modifications from phosphorylation by other proline-directed kinases.

Ace2 Cytoplasmic Retention Involves a Mechanism Independent of Direct CDK Phosphorylation

Because simultaneous inhibition of both Pho85 and Cdc28 dramatically reduced Ace2's G1 cytoplasmic retention, we next sought to determine if phosphorylation at CDK consensus sites is similarly critical for inhibition of Ace2's G1 nuclear import. We changed Ace2's phosphoacceptor residues to alanine in all of its 21 potential CDK consensus motifs, an allele denoted *ace2-AP* (Figure 5A). HA-tagged ace2-AP showed no significant Cdc14-reversible shifting in mitotically synchronized cells, consistent with absence of phosphorylation that is normally removed by this phosphatase (Supplemental Figure 4C). Cells carrying the *ace2-AP* allele had no cell separation defects (Supplemental Figure 5), and the ace2-AP-GFP protein was normally segregated to
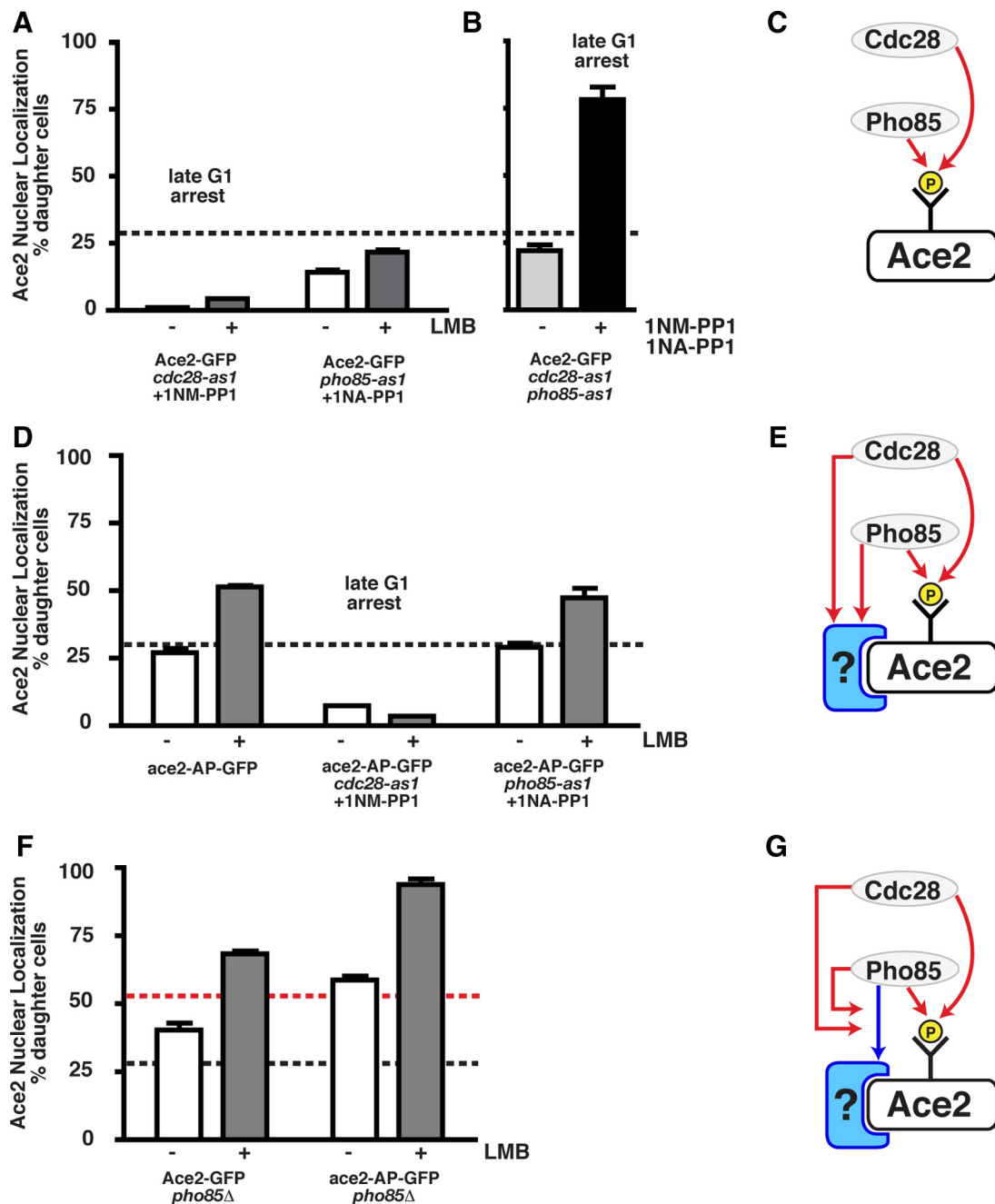
Figure 4. Ace2's nuclear import is unrestricted when *PHO85* is deleted and Ace2's CDK sites are eliminated. (A) G1 *crm1-T539C* daughter cells were scored for nuclear localization of Ace2-GFP with or without addition of LMB. *Cdc28-as1* cells were treated for 2 h with 5 μM 1NM-PP1 to inhibit kinase activity. Note that inhibition of *cdc28-as1* results in cell cycle arrest at late G1. Mother and daughter cells were distinguished as in Figure 3A. *Pho85-as1* cells were treated for 2 h with 10 μM 1NA-PP1, and importantly, this does not cause cell cycle arrest. Dotted line denotes percentage of wild-type G1 cells with Ace2 localized upon LMB addition as shown in Figure 3A. Error bars, SEM. (B) G1 daughter cells, differentiated from mothers as in A, were scored for Ace2 nuclear localization with and without simultaneous inhibition of *cdc28-as1* and *pho85-as1* with 5 μM 1NM-PP1 and 10 μM 1NA-PP1. Note that uninhibited cells are asynchronous, whereas doubly inhibited cells are all arrested in late G1. Dotted line denotes percentage of wild-type G1 cells with Ace2 localized upon LMB addition. Each bar denotes average of two independent trials; error bars, SEM. Significant increase in the number of cells with nuclear Ace2-GFP was evident upon inhibition of both kinases. (C) Cdc28 and Pho85 kinase activity is required for Ace2 cytoplasmic retention. (D) G1 *crm1-T539C* daughter cells of indicated genotypes from asynchronous cultures were scored for ace2-AP-GFP nuclear fluorescence with and without 30-min LMB treatment. Mother and daughter cells were distinguished, and *cdc28-as1* and *pho85-as1* cells were inhibited as in A. Graph shows percentage of daughter cells with ace2-AP-GFP nuclear localization for each strain. Each bar denotes the average of 3–5 independent trials; error bars, SEM. Dotted line denotes percentage of wild-type G1 cells with Ace2 localized upon LMB addition. (E) CDK phosphorylation on Ace2 does not fully block nuclear import, suggesting an additional mechanism independent of direct phosphorylation. (F) G1 *crm1-T539C* daughter cells were scored for nuclear Ace2 localization with and without LMB treatment as in A. Graph shows percentage of daughter cells with Ace2 localized to nuclei. Each bar denotes the average of 3–4 independent trials; error bars, SEM. Black dotted line indicates percentage of wild-type G1 cells with Ace2 localized after LMB treatment. Red dotted lines denotes percentage of G1 cells with ace2-AP localized to nuclei upon LMB addition as in D. (G) Ace2 cytoplasmic sequestration requires both direct phosphorylation at CDK sites (red lines) and an
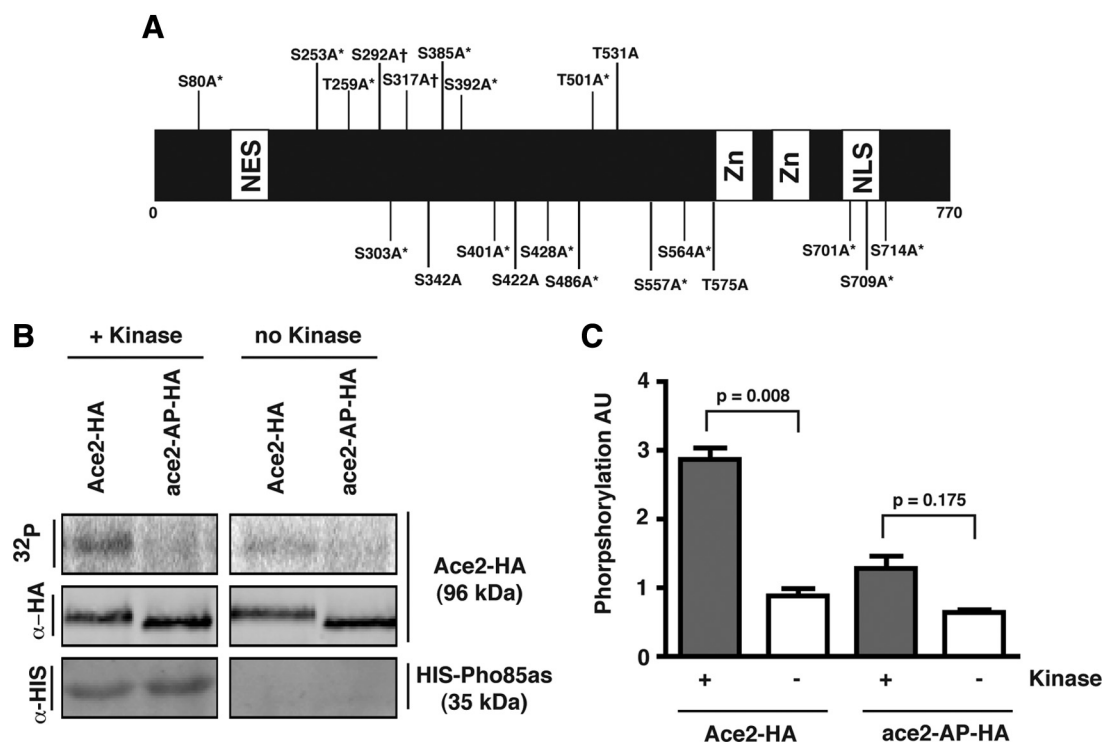
Figure 5. Pho85 does not phosphorylate non-consensus motifs within Ace2. (A) Diagram of the *ace2-AP* allele, denoting positions of all 21 putative CDK consensus motifs ([S/T]-P), in which serines and threonines were changed to alanine. Sites with evidence for in vivo phosphorylation by mass spectrometry are noted, with an asterisk (∗), indicating sites specifically identified, and dagger (†), indicating ambiguity of phosphorylation among sites within an examined peptide. (B) Ace2-HA and ace2-AP-HA substrates were immunoprecipitated from yeast cells and in vitro phosphorylated by bacterially expressed His-Pho85as/Pcl1 complex and γ-^{32}P-ATP for 1 h. Protein levels are shown. (C) Quantification of assays shown in B. Phosphorylation was normalized to total protein, and background levels were subtracted. Graph shows average of three independent trials; error bars, SEM. Ace2-HA is efficiently phosphorylated, whereas ace2-AP-HA cannot be statistically significantly phosphorylated over background.

the daughter cell nucleus (Supplemental Figure 5). Thus, CDK phosphorylation is not required for Ace2 transcriptional activity and asymmetry in normally cycling cells.

As above, we determined the fraction of G1 daughter cells with nuclear localization of ace2-AP-GFP, with and without inhibition of *crm1-T539C* by LMB. We found that ace2-AP-GFP accumulated in a significantly larger fraction of G1 daughter cells upon nuclear export block: to ~51% versus ~27% as seen in cells carrying Ace2-GFP (Figure 4D; black dashed line indicates wild type). However, ace2-AP-GFP was still cytoplasmically retained in about half of G1 daughter cells, indicating that eliminating phosphorylation of Ace2's CDK consensus sites only partially relieves nuclear import restriction of Ace2 during G1. Therefore, a mechanism independent of CDK consensus site phosphorylation on Ace2 must help block the transcription factor's nuclear import in G1.

How might Pho85 and Cdc28 block Ace2's nuclear import if not through direct phosphorylation of consensus sites within the transcription factor? To determine if either of these CDK activities are individually required for the remaining cytoplasmic retention of the ace2-AP protein, we examined nuclear accumulation of ace2-AP-GFP in *cdc28-as1* cells treated with 1NM-PP1 and *pho85-as1* cells treated with

1NA-PP1, with and without inhibition of nuclear export. Similar to results with Ace2-GFP shown in Figure 4A, Cdc28 inhibition dramatically decreased the number of cells with nuclear localization of ace2-AP-GFP, even when nuclear export is blocked with LMB (Figure 4D). Importantly, as shown above, these cells were all arrested in late G1 and were not distributed throughout G1 as were wild-type cells. We also found that inhibition of Pho85 did not alter the nucleo-cytoplasmic distribution of ace2-AP compared with cells with full Pho85 function (Figure 4D).

Ace2-AP's significant remaining cytoplasmic retention is surprising, given that inhibition of both Pho85 and Cdc28 nearly eliminate this sequestration, and suggests that the kinases may phosphorylate sites on Ace2 in addition to the consensus motifs. CDK–cyclin complexes have been shown to phosphorylate substrates at positions that do not match the minimal CDK consensus motif (Harvey *et al.,* 2005; Egelhofer *et al.,* 2008). It is therefore possible that Pho85 phosphorylates Ace2 at sites other than the 21 CDK consensus sites that are eliminated in the *ace2-AP* allele and could explain why ace2-AP remains partially cytoplasmic. To address this, we performed in vitro kinase assays using bacterially expressed His-Pho85as-Pcl1 complex and immunoprecipitated Ace2-HA or ace2-AP-HA as substrates. Pho85as-Pcl1 complex phosphorylated Ace2-HA (p = 0.008, two-tailed Student's *t* test), but did not significantly phosphorylate ace2-AP-HA (p = 0.175; Figure 5, B and C). These results strongly suggest that an additional mechanism contributes to blocking the transcription factor's nuclear import in G1 that is indepen-

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Figure 4 (cont). unknown phosphorylation-independent mechanism mediated by Pho85 (blue line), which is activated by Cdc28 or Pho85 kinase activity.

dent of direct CDK phosphorylation of Ace2 (Figure 4E). Intriguingly, this additional mechanism also requires Cdc28 and Pho85 kinase activity to function (Figure 4B). These results show that direct phosphorylation of Ace2's CDK sites is not strictly necessary for the transcription factor's cytoplasmic retention in G1.

The Pho85 Protein Is Required for Ace2's Phosphorylation-independent Cytoplasmic Retention

The above experiments show that the kinase activities of Cdc28 and Pho85 redundantly promote Ace2's cytoplasmic sequestration after it is exported from the nuclei of G1 daughter cells. To determine if the Pho85 protein has a kinase-independent role in blocking the transcription factor's nuclear import, we compared the effect of *pho85Δ* on Ace2-GFP G1 cytoplasmic retention with that of *pho85-as1* inhibition. An analogous comparison is not possible with Cdc28, which is essential for viability. As above, nuclear localization of Ace2-GFP in LMB-treated cells is indicated with a black dashed line; for further comparison, nuclear localization of ace2-AP-GFP in LMB-treated cells (Figure 4D) is indicated in Figure 4F with a dashed red line.

Remarkably, *pho85Δ* cells showed a dramatic reduction of Ace2 G1 cytoplasmic retention. In marked contrast to wild-type and 1NA-PP1–treated *pho85-as1* cells, the fraction of *pho85Δ* G1 daughter cells with Ace2-GFP localized to nuclei increased dramatically upon addition of LMB, to ~68% (Figure 4F). This effect was substantially greater than that of the ace2-AP allele, which accumulated in nuclei of ~51% of G1 daughter cells upon treatment with LMB. Additionally, an increased number of *pho85Δ* G1 daughter cells had detectable Ace2 in nuclei without LMB addition (Figure 4F, ~40 vs. ~18% for wild-type cells), suggesting that reducing the transcription factor's cytoplasmic retention results in increased nuclear concentration in many cells. Overall, these findings indicate that the Pho85 protein itself directly promotes a critical step in cytoplasmic sequestration of Ace2.

Neither the *ace2-AP* allele nor absence of Pho85 protein disrupted Ace2 cytoplasmic sequestration in all G1 daughter cells. Thus, it is possible that Ace2 is kept in the cytoplasm of by two separate mechanisms: direct phosphorylation of Ace2's CDK sites and a Pho85 function that is independent of its kinase activity. If so, the *ace2-AP* and *pho85Δ* effects should be additive. We therefore examined localization of ace2-AP-GFP in *pho85Δ* G1 daughter cells with and without LMB inhibition of *crm1-T539C*. We observed a dramatic additive effect, with ace2-AP-GFP localized to ~94% of G1 daughter cell nuclei of LMB-treated cells (Figure 4F). In fact, after excluding labeled mother cells (which did not contain Ace2) all *pho85Δ* cells had ace2-AP-GFP localized to nuclei (Supplemental Figure 6). Furthermore, ace2-AP-GFP localized to nuclei of 58% of *pho85Δ* G1 daughter cells in the absence of LMB, suggesting that Ace2's cytoplasmic retention is important for normal nucleo-cytoplasmic balance of the transcription factor once it is inactivated. Overall, these results further support Pho85's importance for cytoplasmic trapping of Ace2 in G1 through a mechanism that is independent of direct phosphorylation of the transcription factor's CDK sites (Figure 4G).

Previous analysis has shown that three CDK sites (threonine 575, serine 701, and serine 714) promote cytoplasmic retention of Ace2 in mitosis (O'Conallain *et al.,* 1999; Sbia *et al.,* 2008); in the previously characterized *ace2-AAA* allele all three sites are changed to alanine. The dramatic additive phenotype of ace2-AP and *pho85Δ* allowed us to compare the *ace2-AP* allele with *ace2-AAA*. As with ace2-AP-GFP, we found that ace2-AAA-GFP in a accumulated in ~60.5% of

pho85Δ cells without LMB added. However, upon addition of LMB, ace2-AAA-GFP only localized to nuclei in 74% of *pho85Δ* cells, whereas ace2-AP-GFP was found in 94% of nuclei (Supplemental Figure 7). Thus, additional CDK sites mutated in the *ace2-AP* allele appear to be involved in blocking Ace2 nuclear import in G1.

DISCUSSION

The Ace2-driven transcriptional program is a compelling example of temporally restricted gene expression. The transcription factor is active for a brief time in early G1 of newly born daughter cells, and once turned off it will never act again in that cell. Intriguingly, Ace2 is stable throughout cell division (Figure 1C and Sbia *et al.,* 2008), and its regulation is therefore a product of posttranslational control. How can a stable transcription factor's activity be restricted to a brief and precisely timed pulse, followed by persistent inactivation?

Our findings define two important phases of Ace2's regulatory cycle: how its interval of activation is maintained and how it is kept inactive once turned off in G1 daughter cells. Taking our findings here together with previous observations, we propose a series of regulatory events that control Ace2's nucleo-cytoplasmic distribution to generate a pulse of regulated transcription (Figure 6). Overall, this transcription factor's control exhibits a sequential logic: it is restricted to the cytoplasm in M phase, dynamically trapped in the nucleus during early G1, and then re-sequestered in the cytoplasm later in G1.

Early Phase: Establishment and Maintenance of Ace2 Activation

The initiation of Ace2's activation in mitosis is well defined (Figure 6A). Mitotic CDK phosphorylation of sites in Ace2's NLS prevents the transcription factor's nuclear import during mitosis, thereby blocking expression of its target genes (O'Conallain *et al.,* 1999; Sbia *et al.,* 2008). This inhibition forestalls production of enzymes that destroy the septum until the structure is actually built (J. Brace, unpublished data). Mitotic exit has been presumed to reverse these CDK phosphorylations through the action of Cdc14, and our results confirm this expectation (Supplemental Figure 4). Once dephosphorylated, Ace2's distribution reflects unhindered nucleo-cytoplasmic shuttling (Weiss *et al.,* 2002). Shortly after Ace2 is released from mitotic cytoplasmic retention, Cbk1 phosphorylates its NES, blocking its binding to nuclear export machinery and trapping Ace2 in the daughter cell nucleus (Mazanka *et al.,* 2008; Figure 6B).

The length of time that Ace2 resides in the daughter cell nucleus is then determined by the persistence of its NES-inactivating phosphorylation. We have shown that the persistence of phosphorylation within Ace2's NES requires constant Cbk1 activity: this modification was quickly lost when the kinase was inhibited, and Ace2 was rapidly exported to the cytoplasm. We conclude that Cbk1 continuously phosphorylates the transcription factor in the nucleus, and this is counteracted by a nuclear phosphatase. This phosphatase's identity is as yet unknown. Thus, a dynamic balance between NES phosphorylation and dephosphorylation likely defines the interval of Ace2 activation in G1 daughter cells.

It is unclear how the balance between these activities tips toward dephosphorylation of Ace2's NES sites. The unknown phosphatase may be dramatically up-regulated as G1 progresses, favoring dephosphorylation, or Cbk1 might be inactivated in G1 by an unknown mechanism. We favor the latter possibility: phosphorylation of Cbk1's C-terminal
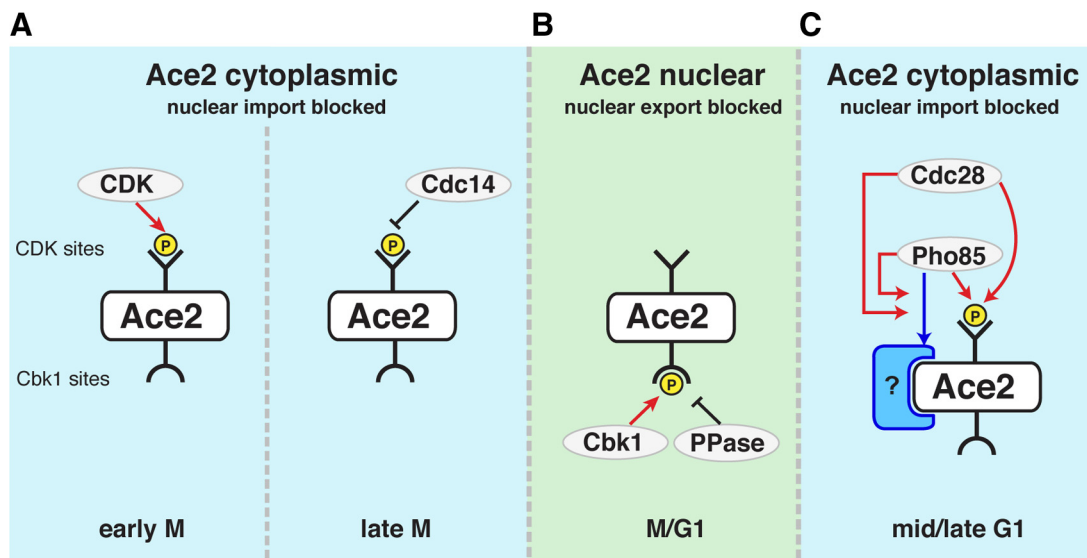
Figure 6. Temporal restriction of Ace2 activity through sequential opposing regulation of its nucleo-cytoplasmic trafficking. (A) In early mitosis, Ace2 nuclear import is restricted by phosphorylation of its CDK sites. After mitotic exit, release of Cdc14 results in dephosphorylation of these sites, allowing nuclear entry in mother and daughter cells. (B) Cbk1 is activated at the M/G1 transition and phosphorylates Ace2 in the daughter cell, promoting its activation and accumulation in its nucleus. Ace2's localization requires continuous Cbk1 phosphorylation that is constitutively counteracted by an opposing phosphatase. Ace2 export occurs upon Cbk1 inactivation or phosphatase up-regulation. (C) Once exported, Ace2's nuclear import is restricted by phosphorylation of its CDK sites by Pho85 and/or Cdc28 (red lines). In addition to direct regulation by phosphorylation of Ace2 CDK sites, Pho85 likely activates a mechanism that sequesters Ace2 in the cytoplasm independent of direct phosphorylation (blue lines). This additional mechanism requires Pho85 or Cdc28 kinase activity to function (red lines).

hydrophobic motif, shown to be essential for the kinase's regulation of Ace2, dramatically decreases following cytokinesis (Jansen *et al.*, 2006; J. Brace, unpublished data).

Late Phase: Inactivation and Cytoplasmic Sequestration of Ace2

Upon dephosphorylation, Ace2 is exported to the cytoplasm and retained, unable to re-enter the nucleus. Blocking nuclear export does not appreciably increase the fraction of G1 daughter cells with Ace2 nuclear localization, indicating that cytoplasmic sequestration takes hold very quickly. Such robust inhibition of Ace2's nuclear import in G1 may be important to prevent ectopic function of transcription factor, because Cbk1 is activated again in late G1 cells to promote normal polarized growth and cell expansion (Jansen *et al.*, 2006, 2009).

How is Ace2 trapped in the cytoplasm of G1 cells? We found that G1 CDK kinase activity is required for cytoplasmic retention of Ace2 once the transcription factor is exported from daughter cell nuclei. This is a redundant function of Pho85 and Cdc28: simultaneous inhibition of both of these CDKs nearly eliminates cytoplasmic retention of Ace2, whereas inhibition of either one alone has little effect. Consistent with this functional redundancy, these kinases can phosphorylate overlapping substrates (Kung *et al.*, 2005; Huang *et al.*, 2007). In our experiments, we found that loss of Cdc28 kinase activity actually led to a substantial reduction in the fraction of unbudded cells with Ace2 localized to nuclei. This is consistent with Cdc28 inhibition causing late G1 arrest; at this stage all cells presumably have exported Ace2 from nuclei and the protein is cytoplasmically trapped.

The simplest model is that direct CDK phosphorylation blocks Ace2's nuclear import in G1, similar to the transcription factor's established regulation in early M phase. However, we find that this is not the case. Direct phosphorylation

of its CDK sites, although important, cannot be the only way by which Ace2 is kept in the cytoplasm after its export from daughter cell nuclei. Ace2 lacking phosphoacceptor residues at all 21 minimal CDK consensus sites (the *ace2-AP* allele) exhibits only a partial loss of cytoplasmic sequestration. More dramatically, ace2-AP and wild-type Ace2 behave nearly identically in cells arrested at START by inhibition of Cdc28. Thus, phosphorylation of Ace2's CDK consensus sites may contribute to early cytoplasmic retention of the transcription factor after export, but is not necessary for sequestration in late G1 cells.

How is Ace2 kept in the cytoplasm without direct phosphorylation of its CDK sites? Our findings indicate that the Pho85 protein plays an important role in Ace2's G1 cytoplasmic sequestration, independent of its kinase function. Although loss of Pho85 kinase activity alone had no appreciable effect on Ace2 distribution, deletion of the *PHO85* gene reduced the transcription factor's retention in the cytoplasm of G1 daughter cells even more than elimination of all 21 of Ace2's CDK consensus phosphorylation sites. More dramatically, combining *pho85Δ* with the ace2-AP allele virtually eliminated the transcription factor's cytoplasmic retention in G1 daughter cells and even allowed Ace2 accumulation in nuclei of cells in S and G2 phases (Supplemental Figure 6).

We therefore propose that the Pho85 protein promotes a second mechanism that retains Ace2 in the cytoplasm, functioning independently of direct phosphorylation of the transcription factor's CDK sites (Figure 6C). We show that Pho85 does not significantly phosphorylate Ace2 at non-consensus sites in vitro, further supporting its role in promoting phosphorylation independent sequestration. This mechanism likely becomes quite robust by late G1, because the cytoplasmic retention of ace2-AP is significantly enhanced in cells arrested at START by inhibition of Cdc28. Intriguingly, the

significant disruption of Ace2's cytoplasmic retention caused by simultaneous inhibition of Pho85 and Cdc28 suggests that this second system requires CDK activity.

The secondary mechanism of sequestration could be mediated through physical association of Ace2 with Pho85 or an unknown factor (Figure 6C). Pho85 in complex with its cognate cyclins (Pcls) may bind stably to Ace2 in G1 daughter cells, blocking Ace2's nuclear import. Such associations, of Pho85 with its cyclins or the Pho85–Pcl complex with Ace2, might themselves be promoted by CDK phosphorylation. Cdc28 or Pho85 itself could accomplish such regulation. Intriguingly, recent proteome-scale identification of Cdc28 phosphorylation targets identified Pcl6, Pcl7, and Pcl8 as substrates, consistent with cross-talk between the systems (Holt *et al.*, 2009). It is also possible that CDK phosphorylation of an unknown factor regulates its binding to Ace2, promoting the transcription factor's sequestration in the cytoplasm.

Both mechanisms act in concert to exclude the transcription factor from the nucleus throughout the cell cycle until the subsequent mitosis. We speculate that direct phosphorylation provides an early block to Ace2's nuclear re-entry. It is possible that Pho85 and/or Cdc28 phosphorylate the transcription factor while it is still in the nucleus, because both are kinases are present there during G1 (Huh *et al.*, 2003). As G1 progresses, Pho85 and/or Cdc28 activate a secondary mechanism to enforce Ace2's cytoplasmic trapping. Pho85's kinase independent function may be the predominant method for sequestering Ace2 later in G1, because phosphorylation at Ace2's CDK sites is no longer sufficient for trapping during late G1 arrest. Such a multi-stage system might allow both rapid inactivation and long-term retention that is resistant to subsequent phosphatases that could reverse modification of the CDK sites.

Our results do not preclude the possibility that cytoplasmic sequestration of Ace2 in mitosis is considerably more complex than presently believed. The current model for Ace2's M phase control by CDK is based largely on the protein's constitutive nuclear localization when NLS-inhibiting CDK sites are mutated and its NES is disabled (Sbia *et al.*, 2008). Ace2 inactivation during G1 and mitosis serve different purposes and may be qualitatively different. During mitotic exit Ace2 is rapidly activated through the convergent action of Cdc14 and Cbk1 (J. Brace, unpublished data). This may require easily reversible nuclear import block. In contrast, during G1 once Ace2 is exported from the daughter cell nucleus it is held inactive, at least until that daughter undergoes its first cytokinesis as a new mother cell. Long-term inactivation of Ace2 in daughter cells may require redundant mechanisms to ensure premature activation does not take place.

Broadly speaking, these findings illustrate how cells use CDK oscillations to create a transient burst of transcription factor activity. In this case, a positive regulatory kinase is coordinated between waves of inhibitory mitotic and G1 CDK activity, and an opposing phosphatase helps define the interval of transcription factor activation. Functionally restricting the activating kinase to one daughter cell can make this a singular event in the cell's lifespan.

ACKNOWLEDGMENTS

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