Regulation of the Histone Deacetylase Hst3 by Cyclin-dependent Kinases and the Ubiquitin Ligase SCF<sub>Cdc4</sub>*

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**Background:** A genome-wide wave of histone H3 acetylation and deacetylation occurs during the fungal cell cycle.

**Results:** Cyclin-dependent kinases and the ubiquitin ligase SCF<sub>Cdc4</sub> promote timely degradation of Hst3, a sirtuin involved in genome-wide histone H3 deacetylation.

**Conclusion:** Mutations that interfere with cell cycle-regulated degradation of the Hst3 histone deacetylase reduce cell fitness.

**Significance:** Similar regulatory mechanisms may exist in pathogenic fungi.

In <i>Saccharomyces cerevisiae</i>, histone H3 lysine 56 acetylation (H3K56ac) is a modification of new H3 molecules deposited throughout the genome during S-phase. H3K56ac is removed from the genome during G2 and/or M-phase (5) by the Hst3 histone deacetylase. This modification is mediated by the histone deacetylase Rtt109 in concert with the histone chaperone Asf1 (11–14). Hst56ac promotes cell survival in response to DNA damage. Cells that lack Hst3K56ac (<i>rut109Δ, asf1Δ</i>, or <i>Hst3K56R</i> mutants) are acutely sensitive to genotoxic agents that cause DNA damage during S-phase (4–7, 11, 12, 14, 15). In the absence of DNA lesions that interfere with completion of DNA replication, Hst3K56ac is removed from the genome during G<sub>2</sub> and/or M-phase (5) by the cell cycle-regulated histone deacetylases Hst3 and Hst4 (10, 16, 17).

Hst3 and Hst4 belong to the sirtuin family of nicotinamide adenine dinucleotide (NAD<sup>+</sup>)-dependent protein deacetylases (18, 19). The phenotypes of each single mutant are far less severe than those of the <i>hst3Δ hst4Δ</i> double mutant (10, 18). Consistent with this, Hst3K56ac is more abundant in cells lacking both Hst3 and Hst4 than in either single mutant strain, such that virtually all H3 molecules are Lys-56-acetylated throughout the cell cycle in <i>hst3Δ hst4Δ</i> cells (10). HST3 is expressed from G<sub>2</sub> until mitosis as part of the <i>CLB2</i> cluster, which includes many genes involved in the G<sub>2</sub>/M transition and mitosis (20–22). Hst3 is a protein with a relatively short half-life (17) that is synthesized in the G<sub>2</sub> phase and subsequently degraded in mitosis and/or the following G<sub>1</sub> phase (16). Hst4 levels peak later than Hst3 during the cell cycle but, like Hst3, Hst4 degradation is completed either in late stages of the cell cycle or before the beginning of S-phase in the two daughter cells (16). To perform its role in the DNA damage response, H3K56ac
must accumulate throughout the genome during S-phase. The molecular machinery that prevents premature deacetylation of H3K56 during S-phase by promoting destruction of Hst3 is not known.

The Skp1-Cullin-F-box (SCF) family of E3 ubiquitin ligases mediates the degradation of many cell cycle regulatory proteins and is conserved from yeast to humans. Phosphorylation of SCF substrates by cyclin-dependent kinases (CDKs) is often a prerequisite for substrate recognition, ubiquitylation, and subsequent degradation (23, 24). In S. cerevisiae, the SCF core complex is composed of the linker protein Skp1, the cullin scaffold protein Cdc53, and the RING domain protein Rbx1 (also known as Hrt1 or Roc1). SCF substrates are recruited by a variety of adaptor subunits known as F-box proteins, each of which recognizes specific sets of substrates. The SCF enzymes associate with the E2 ubiquitin-conjugating enzyme Cdc34, which directly builds lysine 48-linked polyubiquitin chains onto substrates that are bound by F-box protein subunits. All known substrates of the F-box protein Cdc4 require phosphorylation, often mediated by CDKs, as a prerequisite for recognition by Cdc4 (24, 25). These conditional recognition sites are referred to as Cdc4 phospho-degrons (CPD) and contain a minimal CDK consensus phosphorylation site (S/T)P. High affinity CPD consensus sites contain hydrophobic residues N-terminal to the phosphorylated residue and lack basic residues C-terminal to the phosphorylation site (26). However, many CPDs are suboptimal because they contain mismatches from the consensus, and this can create a requirement for multisite phosphorylation in Cdc4 recognition (27). In some cases, a non-CPD phosphorylation site at the +3 or +4 position relative to the CDK site contributes to high affinity recognition (28, 29), but this requirement is not universal (30). In the archetypal example of a Cdc4 substrate, multisite phosphorylation of the Cdk1 inhibitor Sic1 by Cln-Cdk1 activity in late G1-phase allows Sic1 recognition and polyubiquitylation by SCFCdc4 (24, 31). SCFCdc4 also controls the stability of other cell cycle regulators, such as the pheromone-activated inhibitor Far1, the DNA replication initiation factor Cdc6, and numerous other proteins. Genetic evidence suggests an additional function for Cdc4 at the G2/M transition (32), but the relevant substrates of SCFCdc4 at that stage of the cell cycle remain unknown. Like many aspects of the ubiquitin system, Cdc4 function is conserved in humans, in which the ortholog of Cdc4, known as FBW7, mediates timely degradation of many critical cell cycle and growth regulators (33).

Here, we investigate the mechanisms that control Hst3 degradation during the cell cycle. We find that Hst3 degradation can be completed prior to anaphase and demonstrate that Cdk1 directly phosphorylates Hst3 on two closely spaced threonine residues, which creates a diphospho-degron needed for SCFCdc4-mediated polyubiquitylation of Hst3. We also provide evidence that this cell cycle-regulated degradation of Hst3 is important for optimal proliferation. Our results define one of the post-translational mechanisms that restrict the presence of Hst3 to post-replicative phases of the cell cycle, a regulatory system that is important to ensure that H3K56ac can perform its role in response to DNA lesions that arise during replication.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains**—All strains used in this study are listed in Table 1. Yeast strains were generated by standard methods and grown under standard conditions unless otherwise stated.

**Construction of pCEN HST3-TAP Plasmids**—The original plasmid used to generate all pCEN-HST3-TAP constructs was the pCEN-URA3-HST3 plasmid that was described previously (10). We employed homologous recombination in yeast to replace the URA3 marker on the original plasmid with LEU2 and generate the pCEN-LEU2-HST3 plasmid. We used the same method to introduce a tandem affinity purification (TAP) tag at the C-terminal domain of Hst3 in pCEN-LEU2-HST3. The resulting construct was pCEN-LEU2-HST3-TAP that is referred to as pCEN-HST3-TAP throughout. Either or both Cdk1 sites of Hst3, Thr-380 and Thr-384, were mutated into alanine in pCEN-HST3-TAP by site-directed mutagenesis using the PfuTurbo DNA polymerase (Agilent) to generate pCEN-HST3 Thr-380A-TAP, pCEN-HST3 Thr-384A-TAP, and pCEN-HST3 2A-TAP, respectively. These plasmids were individually transformed into the hst3Δ hst4Δ [pCEN-URA3-HST3] strain (HWYG12) to generate the yeast strains NDY241, NDY244, NDY247, and NDY250 (Table 1). Because hst3Δ hst4Δ mutants accumulate spontaneous suppressors (18), the aforementioned strains were covered by the pCEN-URA3-HST3 plasmid encoding wild-type Hst3, in the event that Cdk1 site mutants of HST3 behaved as null mutations. Prior to each

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**Table 1**

| Yeast strains |
|----------------|
| NDY1 | W303 MATa leu2-3,112 ura3-1-trp1-1 his3-11,15 ade2-1 can1-100 HST3-TAP-HIS3MX6 |
| NDY7 | W303 MATa leu2-3,112 ura3-1 TRP1 his3-11,15 ade2-1 can1-100 [PST1] rad5-535 cdc23-1 HST3-TAP-HIS3MX6 |
| NDY224 | W303 MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 [PST1] rad5-535 PDSI-HA-URA3 HST3-TAP-HIS3MX6 |
| HWYG12 | FY MATa his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63 ura3-52 his3Δ3-KANMX hst4Δ TRP1 [pCEN-URA3-HST3] |
| NDY241 | FY MATa his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63 ura3-52 his3Δ3-KANMX hst4Δ TRP1 [pCEN-URA3-HST3] |
| NDY244 | FY MATa his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63 ura3-52 his3Δ3-KANMX hst4Δ TRP1 [pCEN-URA3-HST3] |
| NDY247 | FY MATa his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63 ura3-52 [pCEN-LEU2-HST3 TAP] |
| NDY250 | FY MATa his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63 ura3-52 his3Δ3-KANMX hst4Δ TRP1 [pCEN-URA3-HST3] |
| NDY343 | W303 MATa leu2-3,112 ura3-1-trp1-1 his3-11,15 ade2-1 can1-100 cdc34-2 HST3-TAP-HIS3MX6 |
| NDY344 | W303 MATa leu2-3,112 ura3-1-trp1-1 his3-11,15 ade2-1 can1-100 cdc34-2 HST3-TAP-HIS3MX6 |
| NDY196 | W303 MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 [PST1] rad5-535 cdc4-1 HST3-TAP-HIS3MX6 |
| NDY310 | W303 MATa cdc4Δ::TRP1 ade2-10 leu2-3,112 his3-11 trp1-1 ura3-1 HST3-TAP-HIS3MX6 |
| NDY318 | W303 MATa cdc4Δ::URA3 leu2-1,112 his3-11 trp1-1 [pCEN-cdc4-12-TRP1-LEU2] HST3-TAP-HIS3MX6 |
 Regulation of the Histone Deacetylase Hst3

experiment, strains were streaked on plates containing 5-fluoro-orotic acid to select against the pCEN-URA3-HST3 plasmid.

Time Course Experiments and Cell Synchronization— Cultures were grown to early exponential phase at 30 °C (or at 23 °C for thermosensitive mutants) overnight. Cells were arrested in G1 with 5 μg/ml α-factor for 2–3 h. To release cells from the G1 arrest, cell pellets were washed with water and resuspended in fresh medium containing 100 μg/ml Pronase. For nocodazole arrest, cells were released from G1 into fresh medium containing 15 μg/ml nocodazole added from a 15 mg/ml stock solution in dimethyl sulfoxide. To inactivate thermosensitive mutants, cultures were transferred from 23 °C to the restrictive temperature of 37 °C. During the time course, aliquots were taken for immunoblotting and determination of DNA content by flow cytometry. Samples for immunoblotting were immediately flash-frozen on dry ice. Flow cytometry samples were fixed in 70% ethanol and processed as described below.

Measurement of DNA Content by Flow Cytometry—DNA content was measured by fluorescence-activated cell sorting (FACS) using Sytox Green (Invitrogen) as the nucleic acid stain (34). About 2 × 10^6 cells were fixed in 70% ethanol for a minimum of 30 min at 4 °C. Cells were pelleted, resuspended in 500 μl of 50 mM Tris·HCl, pH 7.5, containing 400 μg/ml of ribonuclease A (Sigma), and incubated at 37 °C overnight. Cell pellets were resuspended in 200 μl of 50 mM Tris·HCl, pH 7.5, buffer containing 400 μg/ml protease K (Sigma) and incubated for 30 min at 50 °C. Cells were then resuspended in 500 μl of 50 mM Tris·HCl, pH 7.5. Samples were prepared by adding 100 μl of processed cell suspension to 900 μl of 50 mM Tris·HCl, pH 7.5, containing 1 μM Sytox Green (Invitrogen). FACS analysis was performed on BD Biosciences LSR II or FACSCanto II cytometers using the FACS Diva software. Histograms were generated using the FACS Express Version 3 software.

Immunoblots—Whole-cell yeast extracts were prepared from flash-frozen samples using a previously described alkaline method (35). For Hst3 immunoblots, whole-cell extracts were separated through SDS-12% polyacrylamide gels by electrophoresis. Proteins were transferred to PVDF membranes using standard Towbin buffer (25 mM Tris and 192 mM glycine) containing 5% methanol and 0.02% SDS on a Bio-Rad S.D. semi-dry transfer apparatus. Transfer settings were 1 mA/cm² of transfer area at 20 V for 2 h. TAP-tagged proteins were detected with a rabbit polyclonal antibody purchased from Open Biosystems. HA epitope-tagged proteins were detected with the 12CA5 mouse monoclonal antibody.

Mass Spectrometric Identification of Hst3 Phosphorylation Sites—Hst3 phosphorylation sites were identified during the course of a phosphoproteome study performed with wild-type cells. The Hst3 phosphorylation sites were identified from yeast whole-cell lysates without purification of the nontagged Hst3 protein, but the phosphoproteome study included a phosphopeptide enrichment step using titanium dioxide (36). The in vivo phosphorylation data were obtained from a SILAC experiment that used [13C]_α-[15N]_4-labeled heavy arginine, rather than the [12C]_α-[14N]_4-labeled natural form of arginine. Because of this, the mass of the doubly charged precursor peptide obtained from Hst3 in whole-cell lysates grown in SILAC medium is 5 Da higher than the theoretical mass of the natural peptide (Fig. 2C).

The Hst3 peptides phosphorylated in vitro by Clb2·CdK1 or Clb5·CdK1 were derived from recombinant GST-Hst3 containing only naturally occurring amino acids (Fig. 6B). The kinase assay reaction products were resolved through an SDS-12% polyacrylamide; the bands corresponding to GST-Hst3 were excised and processed as described previously for in-gel reduction/alkylation of cysteines, trypsin digestion, and peptide extraction (37). Phosphopeptide enrichment was not needed to identify Hst3 peptides phosphorylated in vitro by CdKs.

NanoLC-MS/MS—A nanoflow HPLC system (Eksigent, Thermo Fisher Scientific) was used for on-line reversed phase chromatographic separation. Peptides were loaded onto a 5-mm trapping column (300 μm inner diameter) in buffer A (0.2% formic acid) and separated through an 18-cm fused silica capillary analytical column (150 μm inner diameter). The trapping and separation columns were both packed with 300 Å Jupiter C18 reversed-phase material (Phenomenex). Peptides were eluted during a 60-min gradient from 5 to 40% buffer B (0.2% formic acid in acetonitrile). Peptides were eluted into the mass spectrometer at a flow rate of 600 nl/min. The total run time was ~70 min, including sample loading and column conditioning. Peptides were analyzed using an automated data-dependent acquisition on a Q-Exactive mass spectrometer. Each MS scan was acquired at a resolution of 70,000 full width at half-maximum (at m/z 200) for the mass range 300–2,000 with the lock mass option enabled (m/z 445.120025). This was followed by acquisition of up to 10 MS/MS data-dependent scans on the most intense ions using high energy collision-activated dissociation. Automatic gain control target values for MS and MS/MS scans were set to 1 × 10^6 (maximum fill time of 500 ms) and 1 × 10^5 (maximum fill time of 120 ms), respectively. The precursor isolation window was set to 2 Thomson with a normalized collision energy of 25 eV. The dynamic exclusion window was set to 30 s.

MS Data Processing and Analysis—MS data were analyzed using the MaxQuant software (version 1.3.0.3) (38, 39) and searched against the S. cerevisiae SwissProt subset of the UniProt database containing 6,630 entries. A list of 248 common laboratory contaminants included in MaxQuant was also added to the database as well as reversed versions of all sequences. For the search, the enzyme specificity was set to trypsin with a maximum allowed mass tolerance of 20 ppm for the first search (used for non-linear mass re-calibration) (40) and then to 6 ppm for the main search.

Phosphorylation of serine, threonine, and tyrosine residues was included as variable modification in the search, and carbamidomethylation of cysteines was included as fixed modification. The false discovery rate for peptide, protein, and site identification was set to 1%, and the minimal peptide length was set to 6.

Expression and Purification of Recombinant GST-Hst3— Residues Thr-380 and Thr-384 of S. cerevisiae Hst3 were mutated to alanines from a previously described plasmid (PHM187) (19). The ORFs encoding wild-type Hst3 and Hst3 T380A,T384A (HST3 2A) were amplified by PCR and inserted into the BamHI and EcoRI sites of the pGEX-2TK plasmid for expression of GST fusion proteins in Escherichia coli. The resulting expression constructs encode GST followed by a
RESULTS

Hst3 Can Be Degraded Prior to Anaphase—A previous study provided evidence that Hst3 was degraded at late stages of the cell cycle (16), but it was not clear exactly when degradation occurred. We first set out to establish when Hst3 is degraded during the cell cycle. Wild-type cells expressing TAP-tagged Hst3 were synchronized in G1 and released into the cell cycle in the presence of microtubule-depolymerizing agent nocodazole. Through activation of the spindle checkpoint, nocodazole prevents cells from entering anaphase. Immunoblots revealed that Hst3 was degraded in cells arrested in metaphase in the presence of nocodazole (Fig. 1A). The anaphase-promoting complex (APC) is an E3 ubiquitin ligase that promotes entry into anaphase by triggering the degradation of securin (S. cerevisiae Pds1) and unleashing separase (S. cerevisiae Esp1)-mediated cleavage of sister chromatid cohesion proteins (43, 44). To confirm that Hst3 degradation can occur prior to anaphase, we employed an APC-thermosensitive mutant (cdc23-1) expressing TAP-tagged Hst3. The cdc23-1 cells were released from G1 at the permissive temperature of 23 °C and later switched to the restrictive temperature to inactivate APC and block cells in metaphase. Consistent with our nocodazole result, Hst3 was degraded at the restrictive temperature for cdc23-1 cells (Fig. 1B). We also monitored the timing of Hst3 degradation in parallel with the APC substrate Pds1. For this experiment, cells co-expressing Hst3-TAP and Pds1-HA were released from G1 in the presence of nocodazole. Under these conditions, we found that Hst3-TAP was degraded, whereas Pds1-HA remained stable throughout our time course (Fig. 1C). Taken together, these results demonstrate that Hst3 degradation can occur prior to entry into anaphase and therefore does not require APC activity.

In Vivo Phosphorylation of Two Putative Cdk1 Sites in Hst3—To gain insight into molecular mechanisms that control the degradation of Hst3 before anaphase, we searched for the presence of putative regulatory motifs in the Hst3 sequence. Cdk1 is an integral component of the cell cycle control machinery that phosphorylates a myriad of proteins during the cell cycle. The consensus sequence for phosphorylation by Cdk1 is (S/T)PX(R/K), where the phosphorylation site is followed by a basic residue at the +3 position. However, many bona fide Cdk1 substrates are also phosphorylated at minimal (S/T)P dipeptides (45, 46). We found that the C-terminal domain of Hst3 contains a consensus Cdk1 site at Thr-384 that is preceded by a minimal Cdk1 site at Thr-380 (Fig. 2, A and B). These threonine residues are located outside the predicted catalytic core of Hst3 (Fig. 2A). Sequence alignments of Hst3 family members from fungal species revealed that these two putative...
phosphorylation sites of Hst3 and their spacing are conserved in the pathogenic fungi *Candida albicans*, *Candida tropicalis*, and *Candida glabrata* (Fig. 2B), suggesting that phosphorylation of Hst3 at these sites might have an important regulatory function. In support of this, mass spectrometry analysis of whole-cell lysates prepared from SILAC-labeled asynchronous cultures of *S. cerevisiae* revealed that Hst3 is indeed phosphorylated at both Thr-380 and Thr-384 in vivo (Fig. 2C).

**Phosphorylation of Threonines 380 and 384 Promotes Hst3 Degradation**—To uncover the physiological function of Hst3 phosphorylation at Thr-380 and Thr-384, we mutated either one or both Cdk1 sites of Hst3 into nonphosphorylatable alanines. The mutant proteins were named Hst3 T380A, Hst3 T384A, and Hst3 2A, respectively. Wild-type or Cdk1 site mutants of Hst3 were expressed from the natural HST3 promoter on a centromeric plasmid in *hst3/H9004 hst4/H9004* cells. When cells were released from G1 in the presence of nocodazole, our three Cdk1 site mutants of Hst3 were significantly more abundant than Hst3 (Fig. 3). Mutation of Thr-380 and/or Thr-384 resulted in elevated levels of Hst3 that, unlike wild-type Hst3, persisted for a prolonged period of time in nocodazole (Fig. 3). Moreover, the kinetics of Hst3 degradation after addition of cycloheximide was considerably shorter for wild-type Hst3 than for any of our three phosphorylation site mutants (Fig. 4). Collectively, these results suggest that phosphorylation of both Thr-380 and Thr-384 is necessary to create a phospho-degron that triggers Hst3 degradation.

**In Vivo Degradation of Hst3 Requires Cdc34 and the SCFCdc4 Ubiquitin Ligase**—Our results indicated that phosphorylation of Hst3 at Thr-380 and Thr-384 is a prerequisite for its degradation. This requirement is characteristic of SCF substrates (25). Hence, we tested whether the degradation of Hst3 was dependent on SCF activity by assessing Hst3 abundance in a temperature-sensitive *cdc53-1* mutant. *cdc53-1* cells were released from G1 in the presence of nocodazole at permissive temperature and later switched to the restrictive temperature. Late inactivation of *cdc53-1* was therefore performed to ensure that cells reached the time when Hst3 is normally degraded. Under these conditions, *cdc53-1* mutant cells clearly completed DNA replication and arrested with a 2C DNA content for up to 4 h (Fig. 5A, FACS profiles). Hst3 was not degraded in *cdc53-1* cells, even after 4 h at 37 °C in the presence of nocodazole (Fig. 5A). This is...
much longer than the time required for degradation of Hst3 in wild-type cells (Fig. 1). We employed a cdc34-2-thermosensitive mutant to assess whether the SCF-associated E2 enzyme Cdc34 was required for degradation of Hst3. cdc34-2 cells were released from G1 in medium containing nocodazole and later switched to the restrictive temperature. The cdc34-2 mutant failed to degrade Hst3 at restrictive temperature, even after 4 h in the presence of nocodazole (Fig. 5). These results demonstrate that SCF activity is required for Hst3 degradation prior to anaphase.

Hst3 degradation is mediated by an SCF-dependent mechanism (Fig. 5A). Moreover, mutation of the putative Cdk1 sites, Thr-380 and Thr-384, interferes with Hst3 degradation in vivo (Fig. 4). Some substrates of the F-box protein Cdc4 contain diphosphorylated degrons in which phosphorylation sites are 2–3 residues apart (28, 30). Therefore, we tested whether Cdc4 was required for degradation of Hst3. We monitored Hst3 levels in cells released from G1 in medium containing nocodazole in three thermosensitive mutants of CDC4, namely cdc4-1, cdc4-10, and cdc4-12. Similar to other SCF mutants, cdc4 mutants were initially released from G1 at permissive temperature and later switched to the restrictive temperature to allow cell cycle progression until the nocodazole arrest point. We found that all three cdc4 mutants failed to degrade Hst3 at restrictive temperature, even after 150–240 min in the presence of nocodazole (Fig. 5C–E). Taken together, our findings strongly suggest that Cdk1, Cdc34, and SCF Cdc4 are all necessary for degradation of Hst3 before entry into anaphase.

Hst3 Is Phosphorylated by Cdk5s and Ubiquitylated by SCF Cdc4 in Vitro—To establish whether Hst3 is a direct substrate of Cdk1 and SCF Cdc4, we conducted in vitro experiments with purified proteins. We first performed kinase assays with recombinant GST-Hst3 purified from E. coli as a substrate. Two kinases were used: S. cerevisiae Clb5-Cdk1 and Clb2-Cdk1. Based on incorporation of radiolabeled phosphate, both kinases were able to phosphorylate Hst3 (Fig. 6A). Moreover, phosphorylation of Hst3 was considerably reduced in the GST-Hst3 2A mutant, suggesting that Thr-380 and Thr-384 are prominent sites of phosphorylation for both Clb5- and Clb2-Cdk1 (Fig. 6A). However, both CDKs did phosphorylate the GST-Hst3 2A mutant to some extent (Fig. 6A and data not shown), arguing for the existence of phosphorylation sites other than Thr-380 and Thr-384. Mass spectrometry performed on the products of in vitro kinase assays showed that the tryptic peptide containing Thr-380 and Thr-384 was phosphorylated on both residues by Clb5- and Clb2-Cdk1 (Fig. 6B, and data not shown). We also detected peptides that were singly phosphorylated at either Thr-380 or Thr-384 (data not shown).

Next, we tested whether phosphorylation of Hst3 by CDKs was necessary for subsequent ubiquitylation by SCF Cdc4 in vitro. GST-Hst3 (wild-type or 2A mutant) purified from E. coli was first phosphorylated with either Clb5- or Clb2-Cdk1 or left untreated (Fig. 7, lower panel). The reaction products were then incubated with a ubiquitylation reaction mixture containing ubiquitin and the S. cerevisiae enzymes Uba1 (E1), Cdc34 (E2), and SCF Cdc4 (E3). Control reactions lacking CDKs resulted in the addition of only few ubiquitin moieties to GST-Hst3 wild-type or 2A mutant (Fig. 7, lanes 2 and 5). In contrast, reactions where wild-type GST-Hst3 was phosphorylated by either of the two CDKs generated polyubiquitylated forms of GST-Hst3 (Fig. 7, lanes 3 and 4, species labeled GST-Hst3 (Ub)n). Mutation of Hst3 Thr-380 and Thr-384 abolished the formation of

FIGURE 3. Mutation of either Thr-380 or Thr-384 increases the abundance of Hst3 at late stages of the cell cycle. A–D, cells expressing TAP-tagged wild-type or mutant forms of Hst3 were synchronized in G1, and released into the cell cycle in the presence of nocodazole at 30 °C. Aliquots of cells were processed to monitor DNA contents by FACS and Hst3-TAP proteins by immunoblotting. Ponceau S is shown as a loading control.

FIGURE 4. Mutation of either Thr-380 or Thr-384 reduces the rate of Hst3 degradation. A–D, asynchronous populations of cells expressing wild-type or mutant forms of Hst3-TAP were exposed to 35 μg/ml cycloheximide, and aliquots of cells were harvested at 5-min intervals to monitor the abundance of Hst3-TAP proteins by immunoblotting. Ponceau S is shown as a loading control.
polyubiquitylated forms of GST-Hst3 (Fig. 7, lanes 6 and 7). Our in vitro results therefore demonstrate that Hst3 phosphorylated by Cdk1 is a substrate for polyubiquitylation by Cdc34 and SCFCdc4.

Hst3 Phospho-degron Mutants Are Moderately Sensitive to MMS—We previously showed that both H3K56ac and DNA lesions sustained during S-phase persist for a long time after the bulk of the genome has been replicated (5, 15). After a prolonged period of time, cells lacking H3K56ac segregate chromosomes containing persistent DNA damage (15). This suggests that, prior to entry into anaphase, H3K56ac promotes cell survival by facilitating the repair of lesions that are refractory to repair. Consistent with this notion, H3K56ac is only removed following completion of DNA repair (5), and overexpression of Hst3 from a galactose-inducible promoter causes sensitivity to MMS (16). Based on this, we tested whether the increased

FIGURE 5. Cdc34 and SCFCdc4 are required for Hst3 degradation in vivo. A, inactivation of the SCF subunit Cdc53 stabilizes Hst3 in G2/M. cdc53-1 HST3-TAP cells were released from G1 in the presence of nocodazole at 23 °C and were switched to 37 °C after 120 min to inactivate SCF. B, Hst3 cannot be degraded in the absence of the E2 enzyme Cdc34. cdc34-2 HST3-TAP cells were released from G1 at 23 °C in the presence of nocodazole and were switched to 37 °C after 120 min to inactivate Cdc34. C–E, Hst3 degradation requires the F-box protein Cdc4. cdc4-1, cdc4-10, and cdc4-12 mutants expressing Hst3-TAP were released from G1 in the presence of nocodazole at 23 °C and were switched to 37 °C. A–E, aliquots of cells were analyzed by FACS to follow cell cycle progression, and Hst3 levels were monitored by immunoblotting with an antibody that detects the TAP epitope tag. Ponceau S staining is shown as loading control.

FIGURE 6. Clb5-Cdk1 phosphorylates Hst3 on Thr-380 and Thr-384 in vitro. A, kinase assays were performed with [γ-32P]ATP and either wild-type GST-Hst3 or the GST-Hst3-Thr-380A, Thr-384A (Hst3 2A) mutant as substrates. The reaction products were resolved through an SDS-12% polyacrylamide gel, and incorporation of radiolabeled phosphate was detected by autoradiography. Time 0 corresponds to samples collected before the addition of CDKs. B, GST-Hst3 was phosphorylated with cold ATP and Clb5-Cdk1, and the reaction products were analyzed by mass spectrometry. The doubly phosphorylated peptide that eluted at 34.8 min had a mass of 1413.592 Da that was within 2 ppm of its theoretical mass. The MS/MS spectrum shows abundant y- and b-fragment ions confirming the peptide sequence DSIGT(ph)PPT(ph)PLR, where the residues followed by (ph) are phosphorylated. Fragment ions at m/z 944.4 (y8*) and m/z 932.4 (b9*) are caused by losses of H3PO4 (98 Da) from the corresponding y8 and b9 fragment ions and provide additional evidence for phosphorylation of Thr-380 and Thr-384.
abundance of Hst3 caused by mutations of the Hst3 phospho-degron (Fig. 3) might result in genotoxic agent sensitivity. In agar plates, we observed that the single and double Cdk1 site mutants of Hst3 were moderately sensitive to MMS (Fig. 8A). To assess the phenotypes of our Hst3 mutants more quantitatively, we used an automated shaker-reader platform (47) that continuously monitors the cell density of cultures grown in the absence or presence of MMS. Quantitative growth curves (Fig. 8B) demonstrated that our mutants had longer doubling times than wild-type Hst3, and this phenotype was exacerbated when our Hst3 mutants is mild. This may reflect the fact that, even when the degron is doubly phosphorylated (Tables 2 and 3), we observed that the lag time before cells started proliferating exponentially was markedly longer (between 3 and 4 h) for the phospho-degron mutants (Table 2 and Fig. 8C). The double Cdk1 site mutant of Hst3 (Hst3 2A) did not give rise to more pronounced phenotypes than the two single site mutants (Tables 2 and 3). We note that, compared with Thr-380/Thr-384 can be phosphorylated by CDKs. It is also possible that phosphorylation of non-CDK sites may contribute to Hst3 degradation. Importantly, the requirement for dual phosphorylation of the Hst3 degron may help ensure that Hst3 is not degraded prematurely. HST3 is part of the CLB2 gene cluster, the expression of which rises dramatically during G2/M-phase due to a Clb2-dependent positive feedback loop (20–22). The two-phosphorylation site threshold may delay Hst3 degradation until a sufficient level of CDK activity has been reached to phosphorylate both residues (50). The time needed to reach this threshold in CDK activity would serve to transiently protect Hst3 from degradation and thus provide a window of time for completion of genome-wide H3K56 deacetylation.

For most Cdc4 substrates, it has not been determined whether a diphospho-degron alone is sufficient for in vivo recognition and degradation because a role for other potential weak CPD sites can only be ruled out by mutational elimination of all sites in the substrate. In the case of Sic1, the three high affinity diphospho-degrons present in the N-terminal region of the protein are not sufficient for recognition, and additional weak degrons are necessary for high affinity interaction between Sic1 and Cdc4 (30). Our nondirected phosphoproteome analyses were conducted without enrichment for Hst3 or proteasome inhibitor, and despite this, we readily identified an Hst3 peptide di-phosphorylated on Thr-380 and Thr-384 (Fig. 2C). This suggests that even when the degron is doubly phosphorylated, in vivo degradation of Hst3 is not extremely fast. Although phosphorylation of both Thr-380 and Thr-384 is needed for Hst3 degradation, it is possible that phosphorylation of other residues may be rate-limiting for rapid degradation of Hst3 in vivo. Consistent with this, our in vitro kinase assays (Fig. 6A) strongly suggest that residues other than Hst3 Thr-380/Thr-384 can be phosphorylated by CDKs. It is also possible that phosphorylation of non-CDK sites may contribute to Hst3 deg-
radation, as has been observed for some substrates of SCFCdc4 and SCFFbw7 (29, 49, 51).

Hst4 is redundant with Hst3 for H3K56 deacetylation, and interestingly, Hst4 was previously identified through a screen for proteins that directly bind to Cdc4 (25). Unlike the multiple CDK sites in Hst3, Hst4 contains only one minimal CDK phosphorylation site located at threonine 10. A singly phosphorylated peptide that contains this site binds to Cdc4 with moderate affinity (25). Moreover, Hst4 Thr-10 is followed at the Ser-11001 and Thr-11004 positions by a serine and a threonine residue. These residues may be phosphorylated by other kinases to create a higher affinity phospho-degron, as observed for Hst3, cyclin E, and other substrates (29). This versatility in phosphorylation requirements by the SCFCdc4/Fbw7 ubiquitin ligases enables coupling of multiple signaling events to substrate degradation. Elucidation of the exact structural determinants needed for recognition of phosphorylated Hst3/Hst4 by SCFCdc4 will be important to fully understand how the genome-wide deacetylation of H3K56 by these enzymes is regulated.

The observation that our Hst3 diphospho-degron mutant is undetectable in G1 (Fig. 3) implies that there must be at least one other unknown degron in Hst3. A complex cell cycle-dependent degradation system has been described for the replication initiation factor Cdc6, which is targeted by SCFCdc4 in

FIGURE 8. Stabilization of Hst3 reduces cell fitness. A, colony formation assays. The 5-fold serial dilutions of each strain were plated on SC plates with or without MMS, but containing 5-fluoroorotic acid (5-FOA) to select against the URA3 plasmid encoding wild-type HST3, thereby leaving LEU2 plasmids as the only source of Hst3. The SC-uracil plate containing MMS was incubated at 25 °C for 5 days, whereas the SC-uracil plates were incubated at 25 °C for 3 days. B, continuous monitoring of cell density. Cultures were grown to saturation at 25 °C in SC medium lacking leucine. Each strain was inoculated at A595 = 0.1 in 96-well plates containing YPD medium with or without MMS. Plates were incubated in a Sunrise (Tecan) plate reader, and cell density was monitored at 10-min intervals for up to 48 h. The average A595 of three replicates was plotted as a function of time. The growth curves corresponding to the three Hst3 mutants are superimposable. C, lag times are defined as the amount of time necessary for saturated cell populations to start proliferating following dilution to A595 = 0.1. The corresponding numerical data are shown in Table 2. D, doubling times of cells in exponential phase. The corresponding numerical data are shown in Table 3.
Regulation of the Histone Deacetylase Hst3

post-Start cells but by some other ubiquitin ligase in pre-Start cells (51, 52). Nonetheless, the SCFCdc4 diphospho-degron uncovered here enhances cell fitness by preventing excessive production of Hst3. Finally, we note that in addition to the cell cycle-regulated degradation of Hst3, another mechanism causes Hst3 to be phosphorylated, ubiquitylated, and degraded in response to genotoxic agents such as MMS (17). This regulation is dependent upon the key DNA damage response kinase Mec1 (17), but the Hst3 degron(s) or the ubiquitylation machinery involved in DNA damage-induced Hst3 degradation is not known. Thus, several lines of evidence suggest that Hst3 can be degraded through at least three distinct mechanisms. Further biochemical and molecular genetic studies will be essential to assess the respective importance of each Hst3 degradation mechanism in the regulation of H3K56ac and its role in DNA damage repair and the maintenance of genome stability.

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