Hst3 Is Regulated by Mec1-dependent Proteolysis and Controls the S Phase Checkpoint and Sister Chromatid Cohesion by Deacetylating Histone H3 at Lysine 56*§

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Safia Thaminy†1, Benjamin Newcomb†1, Jessica Kim†, Tonibelle Gatbonton†, Eric Foss†, Julian Simon‡§, and Antonio Bedalov†1*

From the †Clinical Research Division and §Human Biology Division, Fred Hutchinson Cancer Research Center, Seattle, Washington 98109 and the ‡Department of Medicine, University of Washington, Seattle, Washington 98109

The SIR2 homologues HST3 and HST4 have been implicated in maintenance of genome integrity in the yeast Saccharomyces cerevisiae. We find that Hst3 has NAD-dependent histone deacetylase activity in vitro and that it functions during S phase to deacetylate the core domain of histone H3 at lysine 56 (H3K56). In response to genotoxic stress, Hst3 undergoes rapid Mec1-dependent phosphorylation and is targeted for ubiquitin-mediated proteolysis, thus providing a mechanism for the previously observed checkpoint-dependent accumulation of Ac-H3K56 at sites of DNA damage. Loss of Hst3-mediated regulation of H3K56 acetylation results in a defect in the S phase DNA damage checkpoint. The pathway that regulates H3K56 acetylation acts in parallel with the Rad9 pathway to transmit a DNA damage signal from Mec1 to Rad53. We also observe that loss of Hst3 function impairs sister chromatid cohesion (SCC). Both S phase checkpoint and SCC defects are phenocopied by H3K56 point mutants. Our findings demonstrate that Hst3-regulated H3K56 acetylation safeguards genome stability by controlling the S phase DNA damage response and promoting SCC.

Post-translational modification of histone proteins has been implicated in controlling many aspects of chromosome biology. Among the recently described histone modifications is acetylation of histone H3 on lysine 56 (Ac-H3K56) located at the lateral surface of the nucleosome core (1–4). Ac-H3K56 appears in S phase on newly synthesized histone H3 prior to its deposition onto chromatin, and Lys56 is deacetylated later in S phase following its deposition (5). Acetylation of H3K56 requires a histone chaperone, Asf1 (6, 7), and is carried out by a recently characterized acetyltransferase Rtt109 (8–11). Regulation of acetylation is critical, since cells with a mutant form of histone H3 that cannot be acetylated are DNA damage- and hydroxyurea (HU)-sensitive. In the presence of DNA damage, persistent checkpoint-dependent H3K56 acetylation suggested an interplay between classical DNA damage checkpoint proteins (e.g. Mec1) and regulators of H3K56 acetylation.

In response to genotoxic stress, Mec1, acting as a DNA damage sensor, activates the DNA damage response network by phosphorylating Rad53. Rad9 serves as an adaptor (12) that facilitates Mec1-mediated phosphorylation of Rad53 throughout the cell cycle. In addition, and specifically during S phase, Mec1 is capable of efficiently phosphorylating Rad53 in a RAD9-independent fashion (13, 14). The ability of Mec1 to transmit a DNA damage signal during S phase in the absence of RAD9 depends on several genes (e.g. TOF1, CSM3, and MRC1) that constitute an S phase-specific branch of the DNA damage response network (15). In addition to DNA damage signal transduction from Mec1 to Rad53, the members of the S phase-specific branch of the DNA damage response network play a separate role in ensuring the stability of the stalled replication forks (16, 17) and enabling the establishment of faithful sister chromatid cohesion SCC (18, 19). Their role in SCC appears to be distinct from DNA damage signaling, because mec1 mutants, although completely deficient in DNA damage-induced signal transduction, have intact SCC (19). S phase checkpoint proteins have been found to travel with the replication forks; in their absence, replication forks are prone to collapse and breakage (15), thus creating endogenous DNA damage during S phase and genome instability (20).

Two reports (21, 22) implicated the functionally redundant NAD-dependent deacetylases Hst3 and Hst4 and their DNA damage and Mec1-mediated transcriptional control (22) as regulators of H3K56 acetylation. However, the published studies have not determined how H3K56 regulation contributes to genome stability. In this paper, we report that Hst3 has NAD-dependent deacetylase activity in vitro, and we confirm Hst3 functions in vivo during S phase as an Ac-H3K56 deacetylase. Furthermore, we show that the Hst3 protein level is regulated by Mec1-dependent proteolysis during genotoxic stress. Lack of Hst3-mediated regulation of H3K56 generates a defect in the DNA damage signal transduction from Mec1 to Rad53, increases formation of foci containing endogenous DNA damage during S phase,

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† The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1 and Figs. 7–9.

§ This abbreviations used are: HU, hydroxyurea; MMS, methyl methanesulfonate; SCC, sister chromatid cohesion; GST, glutathione S-transferase; GFP, green fluorescent protein; aa, amino acid(s).
Mec1-dependent Proteolysis of Hst3

and impairs SCC. These observations suggest Hst3 and H3K56 acetylation safeguard genome stability by participating in the S phase-specific branch of the DNA damage response pathway.

EXPERIMENTAL PROCEDURES

Yeast Media and Strains—Yeast strains were grown in synthetic complete medium (SC) or selective synthetic drop-out medium containing 2% glucose or 2% galactose when mentioned. The yeast strains used in this study are listed in the supplemental materials (supplemental Table 1).

Plasmid Construction and Mutagenesis—The single Hst3 mutations F72A, N152A, D154A, and H187A were introduced into pRS414-HST3 by site-directed mutagenesis using the QuickChange kit (Stratagene). The glutathione S-transferase (GST)-Hst3 truncated (aa 46–377) was generated by PCR using a 5′-primer containing an EcoRI site (5′-GCGAGTTGAGCAGATCTAGG) and a 3′-primer containing a XhoI site (5′-CGCTCGAGTCGTTTATTTGCGCATCATTGTC). The PCR fragment was inserted between the EcoRI and Xhol sites of pGEX4-T1 (Amersham Biosciences). The PCR fragment was inserted between the EcoRI and Xhol sites of pGEX4-T1 (Amersham Biosciences). All constructs were confirmed by PCR-based sequencing.

Cell Cycle Synchronization—For G1 arrest experiments, cultures were grown at 30 °C to logarithmic phase and arrested with 2.5 μM aphidicolin. The yeast strains used in this study are listed in the supplemental materials.

DNA Damage Sensitivity Assay—5-Fold serial dilutions of yeast cells were plated on rich media containing the indicated concentration of either HU or methyl methanesulfonate (MMS). Plates were photographed after 3–4 days of growth at 30 °C. For MMS treatment in liquid culture, cells were grown to logarithmic phase, harvested, and resuspended in rich medium, and samples were taken at the indicated time points.

RESULTS

Hst3 Is an NAD-dependent Deacetylase That Controls H3K56 Acetylation in Vivo—In order to determine whether Hst3 has NAD-dependent deacetylase activity, we expressed and purified a truncated form of Hst3 (Fig. 1A, left) that retained the conserved NAD-dependent deacetylase domain but lacked 45 N- and 70 C-terminal amino acids (retaining aa 46–377) and used a chemically acetylated 20-aa peptide derived from the histone H3 globular domain or a tetraacetylated histone H4 peptide (20,000 cpm) with or without 1 mM NAD and with or without 5 mM nicotinamide in a 20-μl reaction containing 50 mM Tris·HCl (pH 8.0), 150 mM NaCl, and 1 mM dithiothreitol overnight at 30 °C. The reaction was stopped by the addition of 5 μl of 1 N HCl and 0.15 N acetic acid. Released [3H]acetate was extracted with 500 μl of ethyl acetate, and 250 μl of the extract was subjected to scintillation counting.

Western Blot Analysis—Yeast whole cell extracts were prepared using NaOH lysis as described previously (23). Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Blots were probed with antibodies against TAP (1:5000 dilution; Open Biosystems), Ac-H3K56 (1:4000 dilution; Upstate Biotechnology, Inc.), actin (1:400 dilution; Neo-markers), HA (1:1000 dilution; Covance), and ubiquitin (1:5000; Ubp4D1; Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Purification of Recombinant Protein and Deacetylation Assay—A histone deacetylase assay was performed using bacterially expressed and purified GST-Hst3 (aa 46–377) and GST-Sir2 proteins as previously described (24). The GST-Sir2 expression plasmid was a gift from J. Boeke (25). The truncated Hst3 (aa 46–377) and Sir2 proteins were expressed as GST fusion proteins from Amersham Biosciences pGEX plasmids in the Escherichia coli DH5-α strain. The cells were grown in LB with 100 mg/liter ampicillin at 37 °C. When the A600 reached 0.8–1.0, 1 mM isopropyl-1-thio-β-D-galactopyranoside was added. The cells were then induced at room temperature and harvested after 5 h of induction. The purification was performed according to the methods recommended by the manufacturer.

A histone deacetylase assay was performed using bacterially expressed and purified proteins as previously described (24). Histone H4 N-terminal peptide (SGRKGGKGLGKGGAK-RHR; aa 2–20) and histone H3 peptide (GTVVALERRFQKSTELLIR; aa 44–63) with the N-terminal amino group acetylated during peptide synthesis were acetylated chemically using the HDAC assay kit (Upstate Biotechnology). One microgram of purified truncated Hst3 or Sir2 proteins was incubated with [3H]acetylated histone H3 or H4 peptide (20,000 cpm) with or without 1 mM NAD and with or without 5 mM nicotinamide in a 20-μl reaction containing 50 mM Tris·HCl (pH 8.0), 150 mM NaCl, and 1 mM dithiothreitol overnight at 30 °C. The reaction was stopped by the addition of 5 μl of 1 N HCl and 0.15 N acetic acid. Released [3H]acetate was extracted with 500 μl of ethyl acetate, and 250 μl of the extract was subjected to scintillation counting.

Hst3 deacetylates acetyl-K56 in vitro (Fig. 1A, right). Hst3 also deacetylated a tetraacetylated histone H4 N-terminal peptide and this NAD-dependent deacetylase activity was inhibited by nicotinamide (Fig. 1A, bottom right). These results demonstrate that Hst3 is an NAD-dependent histone deacetylase.

To determine whether HST3 deacylates Ac-H3K56 in vivo, we used an antibody that recognizes Ac-H3K56 to monitor this protein modification in wild type and hst3 hst4 mutants in log-arithmically growing cells, in α factor-induced G1-arrested cells, and in synchronized cells following release from α factor. We found hyperacetylation of H3K56 in cells lacking HST3 and HST4 in both G1-arrested and logarithmically growing cells (Fig. 1B). After release from α factor, in wild type cells, H3K56 acetylation peaked at 50 min and then decreased, whereas the
Ac-H3K56 level in strains lacking HST3 and HST4 was markedly elevated throughout the cell cycle, and its fluctuation was largely abolished (supplemental Figs. 7A and 8A). These results suggest that Hst3 deacetylates H3K56 in vivo. Furthermore, consistent with a requirement for the enzymatic activity of Hst3 in genotoxic stress resistance and Ac-H3K56 deacetylation, we found that point mutants in Hst3 known to abolish enzymatic activity of NAD-dependent deacetylases (27) or treatment with nicotinamide phenocopied the DNA damage sensitivity and H3K56 hyperacetylation of HST3;4 deletion mutants (supplemental Fig. 7B and C). These findings are consistent with those previously published by other groups (21, 22).

Hst3 level, similarly to Ac-H3K56, fluctuates during the cell cycle, peaking at the end of S phase 60 min after release from α factor, declining in mitosis, and is low in the subsequent G1 phase (supplemental Fig. 7A). In order to determine whether the observed decline of Hst3 following S phase is mediated through proteasome degradation, cells were released into S phase from α factor-induced G1 arrest into medium containing the proteasome inhibitor MG132, and Hst3 level was monitored by Western blot. Proteasome inhibition led to an increase in Hst3 level and the appearance of multiple Hst3 forms with very low mobility, suggestive of polyubiquitylation (Fig. 1C, top). Probing of an Hst3 immunoprecipitate from a log phase culture in the presence of MG132 with a specific anti-ubiquitin antibody confirmed that low mobility forms of Hst3 correspond to polyubiquitylated forms of the protein (Fig. 1C, bottom). These results suggest that ubiquitin-mediated proteolysis contributes to cell cycle fluctuations in Hst3 levels.

Hst3 is down-regulated during DNA damage response in an MEC1-dependent fashion. The previously published observation that Ac-K56H3 persists in cells exposed to DNA-damaging agents in a DNA damage checkpoint-deficient mec1 mutant (2) led us to test the hypothesis that Hst3 level is regulated by genotoxic stress. Wild type and checkpoint-deficient mec1 cells were released from α factor-induced G1 arrest in media with or without 0.032% MMS. Hst3 levels were monitored using Western blots (Fig. 1D) and cell cycle progression by flow cytometry (supplemental Fig. 8A). We observed a marked difference in Hst3 kinetics between cells released in the presence or absence of MMS. Unlike cells released in medium lacking MMS, which showed an increase in Hst3 level that peaked at 60 min, cells released in MMS did not
show a significant increase in Hst3 level. Consistent with previous observations (2), MMS treatment was associated with increased levels of Ac-H3K56 (supplemental Fig. 8A). In contrast to wild type cells, MMS treatment did not alter Hst3 kinetics in checkpoint-deficient mec1 cells (Fig. 1D). This result links MEC1 with down-regulation of Hst3 protein in response to DNA damage. Importantly, down-regulation of Hst3 during genotoxic stress is critical for the ability of cells to survive genotoxic stress, since we found that overexpression of Hst3 resulted in MMS sensitivity comparable with that of cells lacking HST3 and HST4 (supplemental Fig. 8, B and C). This result is consistent with earlier reports by Maas et al. (22) and underscores the importance of tight control of Hst3 protein level during genotoxic stress.

**FIGURE 2.** Hst3 is subjected to Mec1-dependent phosphorylation and ubiquitylation and proteasome-mediated degradation during genotoxic stress. A, the strains HST3-TAP sml1 (14611), labeled WT, HST3-TAP mec1 sml1 (14652), labeled Δmec1, and HST3-TAP mec1 tel1 sml1 (14804), labeled Δmec1 Δtel1, were grown to midlog phase, arrested in G1 with α factor, and released in medium containing MG132 with or without 0.032% MMS. Samples were taken at the indicated times and processed for Western blot analysis (left) and flow cytometry (right). Anti-TAP antibodies were used to measure levels of Hst3. Proteasome inhibition prevents genotoxic stress-induced decline in Hst3 level. The arrow points to a lower mobility form of Hst3 that is shown in Fig. 2B to correspond to phosphorylated Hst3. Rapid phosphorylation of Hst3 is dependent on Mec1, whereas the residual phosphorylation is Mec1-independent and reflects cell cycle progression rather than DNA damage response. B, Hst3 is phosphorylated during genotoxic stress. A whole cell lysate from cells treated with MMS and MG132 (80 min time point from Fig. 2A) was prepared under denaturing conditions and was treated with λ-phosphatase with and without phosphatase inhibitor mixture for 30 min. Hst3 mobility was evaluated by Western blotting using anti-TAP antibodies. Phosphatase treatment increases Hst3 mobility. C, Mec1 promotes ubiquitylation of Hst3 during genotoxic stress. The HST3-TAP sml1 strain (14611), labeled WT, and HST3-TAP mec1 sml1 strain (14652), labeled Δmec1, were arrested in G1 with α factor and released into medium containing 0.032% MMS and MG132. Samples were taken at the indicated times and processed for Western blot analysis. The anti-TAP antibody was used to analyze Hst3 level and mobility, and actin was probed as a loading control. The arrow in the upper panel indicates the phosphorylated form of Hst3; the polyubiquitylated forms of Hst3 (polyub Hst3) are indicated in the middle panel. D, genotoxic stress promotes MEC1-dependent proteolysis of Hst3. Logarithmically growing wild type (HST3-TAP sml1, strain 14611) and mec1 cells (HST3-TAP mec1 sml1, strain 4652), were resuspended in medium with or without 0.032% MMS. The samples were collected immediately before (time 0) or at the indicated time points following the addition of cycloheximide (35 μg/ml) and analyzed for Hst3 and actin level. The intensity of the bands corresponding to Hst3 was quantified by densitometry and normalized to actin level, and the time Hst3/actin action curve was fitted using Prism software to calculate Hst3 half-life with and without MMS (wild type, 8.5 and 3.5 min; mec1, 9 and 8 min with and without MMS, respectively).
from MMS- and MG132-treated cells (Fig. 2B), demonstrating that the lower mobility forms of Hst3 under genotoxic stress correspond to phosphorylated protein. Thus, proteasome inhibition specifically stabilizes phosphorylated forms of Hst3. The phosphorylated form of Hst3 during genotoxic stress could not be detected without proteasome inhibition, presumably due to its short half-life. Phosphorylation of Hst3 appeared to be reduced in mec1 cells relative to wild type cells (Fig. 2A). Because mec1 cells exposed to MMS still exhibited residual phosphorylation of Hst3, we wanted to determine whether this residual phosphorylation is eliminated in cells lacking both MEC1 and TEL1 (Fig. 2A). Tel1 acts in parallel with Mec1 as a DNA damage sensor, and the two kinases often phosphorylate common substrates (15). Cells lacking both MEC1 and TEL1 exhibit the same degree of deficiency of Hst3 phosphorylation as mec1 cells (Fig. 2A), suggesting that the observed residual phosphorylation is not Mec1/Tel1-dependent. In order to evaluate the possibility that MEC1-independent phosphorylation of Hst3 does not occur in response to DNA damage but instead reflects cell cycle progression over the course of the experiment, we released mec1 cells from α factor arrest into medium containing MG312 only. In this condition, both wild type and mec1 cells exhibited Hst3 phosphorylation comparable with the residual phosphorylation observed in mec1 cells during genotoxic stress (Fig. 2A); thus, MEC1-independent phosphorylation is not a response to genotoxic stress. We conclude that the rapid and almost complete phosphorylation of Hst3 that occurs during genotoxic stress is dependent on MEC1, whereas the residual phosphorylation of Hst3 observed in the absence of MEC1 is independent of genotoxic stress and depends instead on cell cycle progression.

Rapid Mec1-dependent phosphorylation of Hst3 during genotoxic stress, coupled with the requirement for Mec1 protein kinase for down-regulation of Hst3 protein level, led us to evaluate the possibility that Hst3 protein is targeted for proteasome-mediated proteolysis during genotoxic stress through Mec1-dependent phosphorylation. We found that phosphorylation of Hst3 in wild type cells is accompanied by the appearance of ubiquitylated protein forms that are barely detectable in mec1 cells, consistent with phosphorylation-dependent ubiquitylation of Hst3 (Fig. 2C). To determine whether increased Mec1-mediated ubiquitylation of Hst3 during genotoxic stress promotes its instability, we next evaluated the effects of MMS on Hst3 protein stability in wild type and mec1 cells. We found that Hst3 protein has a half-life of 8.5 and 9 min in wild type and mec1 cells, respectively (Fig. 2D). Genotoxic stress decreases the half-life of Hst3 to 3.5 min in wild type cells, and this decrease appears to be dependent on Mec1, since we observe that Hst3 half-life does not significantly decrease in a mec1 mutant (9 min versus 8 min without and with MMS, respectively). These findings show that Hst3 level during genotoxic stress is regulated through Mec1-dependent proteolysis. Together, our results support the hypothesis that genotoxic stress leads to rapid Mec1-dependent phosphorylation of Hst3, its ubiquitylation, and subsequent proteasome-mediated degradation.

HST3 and HST4 Participate in the S Phase-specific Branch of the DNA Damage Response Network through Control of H3K56 Acetylation—The finding that Hst3 is expressed during S phase combined with the observation that hst3 hst4 mutants are sensitive to DNA-damaging agents led us to evaluate these mutants for sensitivity to HU. Because MMS sensitivity, created by the loss of HST3 and HST4, has been previously shown to be synthetic with the lack of RAD9 (28), we also tested hst3 hst4 rad9 triple mutants for sensitivity to HU. We found that these triple mutants have a growth defect and are sensitive to concentrations of HU that do not have an appreciable effect on growth of either hst3 hst4 double mutants or rad9 single mutants (Fig. 3A). This result demonstrates that loss of HST3 and HST4 makes cells vulnerable to replication stress in the absence of RAD9.

The synthetic phenotype of the lack of both HST3 and HST4 and of RAD9 under conditions of genotoxic stress is consistent with the properties of genes that control the S phase-specific branch of the DNA damage response pathway, such as MRC1, CS3M, or TOF1 (14, 29). In order to determine whether lack of HST3 and HST4, similar to the lack of other S phase-specific checkpoint genes, creates a defect in Rad53 phosphorylation, we first evaluated hst3 hst4 double mutants for DNA damage-induced up-regulation and phosphorylation of Rad53. Rad53 phosphorylation, following MMS-inflicted DNA damage, was monitored by Western blot through the change in the electrophoretic mobility of HA-tagged Rad53. We found that cells lacking HST3 and HST4 have a modest defect in both Rad53 induction and MMS-induced Rad53 phosphorylation (Fig. 3B, top and bottom). Importantly, this defect was further exacerbated in the triple hst3 hst4 rad9 mutants (Fig. 3B, bottom); the deficiency in DNA damage-induced phosphorylation of Rad53 in this triple mutant was comparable with that of mutants lacking MEC1. This result demonstrates that the combination of deletion of hst3, hst4, and rad9 causes a deficiency in the DNA damage response and supports the idea that Hst3 and Hst4 play an important role in the S phase-specific branch of the DNA damage response pathway. We also observed that the combined lack of HST3, HST4, and RAD9 creates a partial defect in delaying S phase progression in response to genotoxic stress (supplemental Fig. 9A). Deletion of the RTT109 gene, which encodes for an H3K56 acetyltransferase, also created synthetic sensitivity to HU (Fig. 3C) and synthetic Rad53 phosphorylation defect (Fig. 3D) with the loss of RAD9. Importantly, HST3 and HST4 loss of function phenotypes, including synthetic HU sensitivity with the loss of RAD9 (Fig. 3E), Rad53 phosphorylation defect (Fig. 3F), and S phase delay defects (supplemental Fig. 9B), were phenocopied by H3K56 point mutants, demonstrating that loss of H3K56 regulation is the critical event that leads to the observed checkpoint phenotypes in cells lacking HST3 and HST4. The observation that the nonacetylatable allele of histone H3 (K56R), the H3K56 mutant mimicking constitutive acetylation (K56Q), the deletion of HST3 and HST4, or the deletion of RTT109 all generated similar phenotypes suggests that reversible acetylation of H3K56 participates in the intra-S phase checkpoint.
HST3 and HST4 Loss of Function Mutants Accumulate Endogenous DNA Damage during S Phase—During the analysis of the role of HST3 and HST4 in Rad53 phosphorylation, we observed an Rad53 phosphoshift in hst3 hst4 mutants even in the absence of treatment with MMS (Fig. 3B, top). Furthermore, the Rad53 phosphoshift generated by the loss of HST3 and HST4 was abolished by deletion of RAD9, consistent with the activation of the DNA damage response by endogenous DNA damage. In order to determine whether hst3 hst4 mutants, like mutants in other intra-S phase DNA damage response genes (e.g. TOP1 and MRC1), exhibit endogenous DNA damage during DNA replication (12, 22), we evaluated the spontaneous formation of DNA damage-associated checkpoint protein foci in hst3 hst4 cells during S phase (as determined by budding). Ddc2, a checkpoint protein that physically associates with Mec1, is recruited to sites of DNA damage, and Ddc2-GFP foci are observed at sites of DNA damage during Ddc2-GFP foci are observed at sites of DNA damage during checkpoint activation (20). We compared the frequency of cells with Ddc2-GFP foci in logarithmically growing wild type cells and hst3 hst4 double mutants. As previously reported in wild type (20), only a small fraction of cells, 5% of total cells or 9% of budded cells, which are enriched for cells in S phase, exhibited spontaneous Ddc2 foci (Fig. 4, A and B). The frequency of total or budded cells containing Ddc2-GFP foci in cells lacking HST3
Figure 4. Ddc2-GFP foci formation at sites of spontaneous DNA damage in hst3 hst4 mutants. A, cells carrying an endogenous DDC2-GFP fusion gene were grown to log phase and visualized by deconvolution microscopy. Data were derived from three independent wild type or hst3 hst4 cultures, and at least 120 cells from each culture were scored for the presence of Ddc2-GFP foci. Foci were counted in all cells or in S phase cells as judged by budding. B, Ddc2-GFP foci in wild type (14944) cells and hst3 hst4 (15018) cells. Cells are shown on the left (Trans), GFP fluorescence is shown in the center (FITC), and the merged image is shown on the right (Merge). C, left, wild type (14141), hst3 hst4 (14299), csm3 (14460), hst3 hst4 csm3 (14814), mrc1 (14818), and hst3 hst4 mrc1 (14819) cells were assayed for HU sensitivity. 5-Fold serial dilutions were plated on YEPD with and without 40 mM HU. Plates were incubated for 3 days at 30 °C.

Since our data strongly suggested a role of HST3 HST4 in the intra-S phase checkpoint, we decided to determine the relationship between HST3 HST4 and the S phase-specific DNA damage response genes TOFI, MRC1, and CSM3 through epistasis analysis. We found that loss of function of HST3 and HST4 creates synthetic HU sensitivity with the loss of function of CSM3 and synthetic lethality with the loss of TOFI (Fig. 4C). The triple hst3 hst4 mrc1 mutant also exhibited slightly increased HU sensitivity relative to hst3 hst4 double mutant. Therefore, it appears that HST3 and HST4 belong to a separate epistasis group within the intra-S phase checkpoint pathway. This finding, together with the observation of synthetic phenotypes of MRC1 and TOFI or CSM3 (30), indicates that intra-S phase checkpoint genes do not form a linear pathway.

HST3- and HST4-mediated Regulation of H3K56 Is Required for Efficient SCC—Because several of the genes that control the S phase-specific branch of the DNA damage network also exhibit defects in SCC (15), we sought to determine whether HST3 and HST4 were also required for SCC. To monitor SCC, we used strains expressing bacterial DNA-binding proteins (Lac repressor and TET repressor) fused to green fluorescent protein (GFP) and containing their respective DNA binding repeats (lactose and tetracycline operators) integrated at several different chromosomal locations. We first introduced HST3 and HST4 deletions, both individually and in combination, in a strain that has Lac operator repeats integrated at a site near the centromere of chromosome III (SBY 885). Cells were grown to midlog phase and then arrested in G1, using α factor or G2/M using nocodazole. As expected, two separate GFP signals were observed in only 5% of wild type cells arrested in G2/M, indicating that 95% of the sisters remain paired prior to anaphase (Fig. 5A).

As in wild type cells, in mutants lacking either HST3 or HST4, two GFP signals were observed in less than 6% of cells. In contrast, strains lacking both HST3 and HST4, arrested in G2/M, had 18% of cells with two GFP signals. The two GFP signals in hst3 hst4 double mutants were observed only in G2/M arrest and not in G1-arrested cells, indicating a defect in SCC and not a chromosome duplication. In order to determine whether deacetylase activity of Hst3 and Hst4 is required for faithful SCC, we evaluated SCC in wild type cells treated with 50 mM nicotinamide. We have shown earlier that nicotinamide inhibits its NAD-dependent deacetylase activity of Hst3 in vitro (Fig. 1A) and leads to hyperacetylation of H3K56 in vivo (supplemental Fig. 7C). Nicotinamide treatment of wild type cells phenocopied the loss of SCC observed in hst3 hst4 double mutants (Fig. 5A), indicating that deacetylase activity of Hst3 and Hst4 is required for their function in SCC. Loss of SCC was also
observed at loci on the arm of chromosome V and near a telomere on chromosome IV (Fig. 5B). The SCC defect of HST3 HST4 deletion mutants was phenocopied by histone K56Q and K56R mutants (Fig. 5C), thus demonstrating that Hst3 affects SCC through control of H3K56 acetylation.

Using conditional inactivation of Hst3 function with 50 mM nicotinamide in synchronous cells following the release from α-factor arrest, we found that Hst3 function in SCC is no longer required after S phase has been completed and SCC had been established (cohesion maintenance) but is required during S phase (Fig. 5D), consistent with a role of HST3 during SCC establishment.

In order to further characterize the role of HST3 and HST4 in SCC, we evaluated whether loss of HST3 and HST4 causes a synthetic interaction with mutations in MCD1, which encodes a component of the cohesin complex. We used a temperature-
sensitive allele of *MCD1, mcd1-1*, that fails to grow at 33 °C due to loss of SCC. We found that *hst3 hst4 mcd1-1* triple mutants fail to grow at 30 °C, whereas *hst3 hst4* double mutants and *mcd1-1* single mutants are capable of growth at this temperature (Fig. 5E). This synthetic interaction further confirms the role of *HST3* and *HST4* in SCC.

**DISCUSSION**

During S phase, newly synthesized histone H3 is acetylated at Lys56 and deposited into nucleosomes during DNA replication and becomes largely deacetylated by the G2 phase of the cell cycle (2). We show that Hst3 deacetylates a Lys56-containing histone H3 peptide in an NAD-dependent fashion *in vitro* and provide strong support for the hypothesis that Hst3 and Hst4 are critical enzymes for deacetylating H3K56 *in vivo*. Consistent with previous reports (21, 22), we show, first, that deletion of *HST3* and *HST4* leads to an increase in global H3K56 acetylation. Second, cell cycle-dependent oscillations in Ac-K56H3 are abolished in cells lacking *HST3* and *HST4*. Third, point mutations that disrupt NAD-dependent deacetylase activities also lead to increased H3K56 acetylation and recreate the phenotypes of *hst3* deletion mutants.

When cells are exposed to DNA damage, the Ac-H3K56 modification persists and has been shown to be retained specifically at sites of DNA damage in a checkpoint-dependent fashion (2). The finding that Hst3 is down-regulated when cells are exposed to DNA-damaging agents and that this down-regulation requires the DNA damage sensor Mec1 provides a mechanism for the persistence of Ac-H3K56 during genotoxic stress at the sites of DNA damage. Previous reports show that Hst3 protein levels appear to be down-regulated during genotoxic stress through a transcriptional mechanism (22). Our results demonstrate that during genotoxic stress Hst3 levels are also controlled through Mec1-dependent phosphorylation and subsequent ubiquitylation and degradation by the proteasome. Further studies are required to determine whether phosphorylation of Hst3 during genotoxic stress is carried out by Mec1 directly, by its downstream kinases, or both. Control of Hst3 protein stability is a particularly appealing mechanism, because, unlike transcriptional regulation, which can control only the global Hst3 level, checkpoint-regulated Hst3 protein stability may direct Hst3 protein degradation specifically to sites of DNA damage. This provides an explanation for the observed accumulation of Ac-H3K56 specifically at sites of DNA damage.

Perturbation of the H3K56 acetylation/deacetylation cycle phenocopies the loss of function mutations in S phase-specific DNA damage response genes (*e.g. TOFI, CSM3, or MRC1*) (14, 19, 29), including 1) a DNA damage signal transduction defect that acts in parallel with Rad9 to transmit the signal from Mec1 to Rad53 and a consequent defect in slowing down S phase when encountering DNA damage; 2) accumulation of endogenous DNA damage during S phase; and 3) defects in sister chromatid cohesion. Based on these observations and synthetic interactions with *TOFI*, *MRC1*, and *CSM3*, we propose that *HST3*, *HST4*-mediated control of H3K56 acetylation shares the functions of intra-S phase checkpoint genes in DNA damage signal transduction (14, 26), stabilization of stalled replication forks (17), and establishment of sister chromatid cohesion (19). How does increased H3K56 acetylation at the sites of DNA damage contribute to DNA damage resistance? Our finding that Hst3-regulated acetylation of H3K56 plays a role in enabling Mec1 to phosphorylate Rad53 in the absence of Rad9 supports the model that reversible H3K56 acetylation and Rad9 act in parallel pathways for transmitting a DNA damage signal from Mec1 to Rad53 and a consequent defect in slowing down S phase when encountering DNA damage.

**FIGURE 6.** Left, Hst3 and H3K56 act in parallel with Rad9 during DNA replication checkpoint. Right, model for targeted proteolysis of Hst3 and local Ac-H3K56 control in the S phase-specific DNA damage response and SCC. Ac-H3K56, targeted to the sites of DNA damage through regulated Hst3 proteolysis, serves to recruit DNA repair and cohesion factors. Global loss of Hst3 activity (e.g. *HST3* deletion) leads to ubiquitous H3K56 acetylation, whereas excessive Hst3 activity (e.g. *HST3* overexpression) leads to global loss of Ac-H3K6. Both of these conditions disrupt specific targeting of Ac-H3K56 to the sites of DNA damage, and consequently, the efficient recruitment of DNA repair factors is abrogated.
DNA damage response proteins to sites of DNA damage. This model is consistent with the observation that unacytlatable histone H3 (H3K56R), complete loss of H3K56 acetylation (RTT109 deletion), and the histone H3 mutant that mimics constitutive acetylation (K56Q) all elicit phenotypes similar to that of loss of \textit{HST3} and \textit{HST4} and also with the observation that overexpression of \textit{HST3} results in a degree of DNA damage sensitivity similar to that of \textit{hst3 hst4} double mutants. Since multiple S phase DNA damage checkpoint proteins have been shown to move along with the replication fork (16, 29), it is possible that Hst3 also moves with the replication complex, where it controls the acetylation state of H3K56 and serves a role in S phase checkpoint signaling, stabilization of stalled replication forks, and establishment of SCC.

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