Schizosaccharomyces pombe Hst4 Functions in DNA Damage Response by Regulating Histone H3 K56 Acetylation\*†

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The packaging of eukaryotic DNA into chromatin is likely to be crucial for the maintenance of genomic integrity. Histone acetylation and deacetylation, which alter chromatin accessibility, have been implicated in DNA damage tolerance. Here we show that Schizosaccharomyces pombe Hst4, a homolog of histone deacetylase Sir2, participates in S-phase-specific DNA damage tolerance. Hst4 was essential for the survival of cells exposed to the genotoxic agent methyl methanesulfonate (MMS) as well as for cells lacking components of the DNA damage checkpoint pathway. It was required for the deacetylation of histone H3 core domain residue lysine 56, since a strain with a point mutation of its catalytic domain was unable to deacetylate this residue in vivo. Hst4 regulated the acetylation of H3 K56 and was itself cell cycle regulated. We also show that MMS treatment resulted in increased acetylation of histone H3 lysine 56 in wild-type cells and hst4Δ mutants had constitutively elevated levels of histone H3 K56 acetylation. Interestingly, the level of expression of Hst4 decreased upon MMS treatment, suggesting that the cell regulates access to the site of DNA damage by changing the level of this protein. Furthermore, we find that the phenotypes of both K56Q and K56R mutants of histone H3 were similar to those of hst4Δ mutants, suggesting that proper regulation of histone acetylation is important for DNA integrity. We propose that Hst4 is a deacetylase involved in the restoration of chromatin structure following the S phase of cell cycle and DNA damage response.

DNA in eukaryotes is packaged into chromatin, and this packaging affects processes such as transcription, replication, repair, and recombination (14, 29). Compaction of DNA into chromatin reduces accessibility of DNA to various factors involved in these processes; therefore, cells have evolved different ways to counteract this inhibitory effect of chromatin (2, 45). Chromatin accessibility can be altered by posttranslational modifications of the histones, such as acetylation, ubiquitination, and phosphorylation (32, 55). These modifications are thought to alter chromatin structure, thereby regulating DNA metabolic processes. It has also been proposed that these modifications create a “histone code” that is utilized by nonhistone proteins targeting these proteins to sites of modification (49).

Acetylation of histones has been linked to transcriptionally active chromatin, but recent studies have demonstrated that this modification is also important in the DNA damage response and repair pathways (26, 36, 45, 55). In the budding yeast Saccharomyces cerevisiae, the histone acetyltransferase Esal acetylates the N-terminal tails of histone H4. This enzyme is also required for double-strand break repair via the nonhomologous end-joining pathway. Strikingly, the mammalian Esal homolog Tip60 is also required for DNA double-strand break repair (5, 27), suggesting conservation of the mechanism. Similarly, the SAGA acetyltransferase acetylates the N-terminal tails of histone H3, and this enzyme complex has also been shown to facilitate repair, leading to maintenance of genomic integrity (7, 11, 33, 53).

While these studies highlight the importance of N-terminal tail acetylation of the histones toward genomic integrity, recent studies showed that regulating the acetylation of the histone H3 core domain residue K56 was also important for S. cerevisiae cells to counter genotoxic stress (34). Data demonstrated that acetylation of histone H3 K56 occurred at the double-strand break, and these results led to the suggestion that acetylation of histone H3 K56 created a favorable chromatin environment for DNA repair. This acetylation is maintained in a checkpoint-dependent manner.

At the sites of DNA damage, acetylation is a transient effect. Histones are acetylated and then deacetylated, suggesting that histone deacetylases are likely to be involved in the process. Consistent with this view is the observation that Sin3, a component of Rpd3 histone deacetylase complex, renders cells defective in the nonhomologous end-joining repair pathway (23). The Esal- and Sin3-dependent acetylation and deacetylation of histone H4 imply that the turnover of acetyl groups is important for proper repair, just as acetylation and deacetylation cooperate to promote proper transcriptional regulation (26).

Sir2p and its family members are NAD-dependent histone deacetylases (12, 19, 22, 42). In Saccharomyces cerevisiae, Sir2 homologs Hst3 and Hst4 have been shown to be necessary for telomeric silencing and genomic stability (6, 9, 31, 38). Similarly, in mammalian cells, homologs of Sir2 are required for cell survival following DNA damage (30, 41). Interestingly, in the fission yeast Schizosaccharomyces pombe, Hst4 alone is the functional homolog of both Hst3 and Hst4 of S. cerevisiae. The
hst4Δ cells grow slower than wild-type cells do and have elongated cell morphology and fragmented DNA. Mutants show elevated chromosome loss rates in addition to having silencing defects at both telomeres and centromeres (18). These intriguing phenotypes led us to further investigate the role of Hst4 in the maintenance of genomic integrity.

This article presents the critical importance of Hst4 in regulating histone H3 K56 acetylation and thereby maintaining genomic integrity in S. pombe. While this work was in preparation and under review, three independent studies corroborating some of the findings reported here were published (9, 31, 56). In this study, we showed that S. pombe Hst4 was required for the deacetylation of histone H3 K56 and that in hst4Δ mutants and in a catalytic domain point mutant, H3 K56 acetylation increased dramatically. Here, we have provided evidence for the involvement of Hst4 in the cell cycle regulation of histone H3 K56 acetylation and also showed that the level of Hst4p itself is cell cycle regulated. The loss of Hst4 affected genomic integrity and resulted in DNA fragmentation, activation of the DNA damage checkpoint and loss of viability on exposure to DNA-damaging agents, such as methyl methanesulfonate (MMS) and camptothecin (CPT), which are genotoxic during the S phase of the cell cycle. We have also shown that in wild-type cells, MMS treatment resulted in an increase in acetylation of histone H3 K56 and a decrease in the level of Hst4. Furthermore, we found that some components of the DNA damage checkpoint were essential for the survival of cells lacking Hst4. Some of the phenotypes associated with cells lacking Hst4 were also observed in K56Q and K56R mutants of histone H3, suggesting that both H3 K56 acetylation and deacetylation are important in maintaining genomic integrity.

MATERIALS AND METHODS

Yeast strains, media, and culture conditions. The genotypes of the S. pombe strains used in this study are listed in Table 1. Standard culture conditions and genetic methods were used (40). S. pombe strains were grown in yeast extract plus supplements (YES) or Edinburgh minimal medium (EMM). Plates containing malt extract were used for sporulation. S. pombe cells were grown at 32°C on plates and in liquid culture unless otherwise mentioned. Crosses were carried out by mixing freshly grown cells on plates containing malt extract and sporulating for 3 days at room temperature before dissecting tetrads. Transformations were performed using the lithium acetate protocol (40), with the following modifications. Fifty milliliters of culture was grown to an optical density at 600 nm (OD600) of ~1.0. The cells were washed with 50 ml of sterile water once and with 25 ml of Tris-EDTA (TE) plus 0.1 M lithium acetate and incubated for 1 h on a roller drum at 32°C. Cells (0.1 ml) were incubated on a roller drum at 32°C for 30 min after adding 5 µl of 10 mg/ml carrier DNA (salmon sperm DNA) and 1.0 µg of DNA. Then, 0.7 ml of polyethylene glycol solution (40% polyethylene glycol 4000 in 0.1 M lithium acetate and TE) was added to the cells and incubated at 32°C for 1 h. The cells were heat shocked for 5 min at 42°C, resuspended in 0.2 ml of water, and plated on EMM plates supplemented with appropriate amino acids.

Generation of growth curve. Logarithmically growing asynchronous cultures of wild-type (LPY3279) and hst4Δ (LPY3278) mutant strains were used to inoculate YES medium at an OD600 of 0.05, the cells were collected every 2 hours, and the OD600 was measured. To generate the growth curve, OD600 and time were plotted on a graph.

Septation index. The cdc25-22 hst4Δ (ROP216) strains were generated by crossing hst4Δ (LPY3739) with temperature-sensitive mutant cdc25-22 (ROP204) strain. The cdc25-22 (ROP204) and cdc25-22 hst4Δ (ROP216) strains were grown at 25°C to log phase and synchronized in G2 by shifting the cells to 36°C for 4 h. Cells were then shifted back to 25°C, and mitotic progression was determined by 4’,6-diamidino-2-phenylindole (DAPI) and calcofluor staining and counting binucleated/septated cells (at least 300 cells for each point). To generate the septation index plot, the percentage of septate cells and time were plotted on a graph.

DAPI staining. DAPI staining was carried out as described previously (18) with modifications. S. pombe cells were prepared for DAPI staining by fixing 1 ml of a culture in 1 ml of 30% methanol/70% acetone for at least 20 min at 20°C. Cells were then rehydrated with 5-min washes in 75, 50, and 25% methanol in phosphate-buffered saline. Cells were then resuspended in 30 µl of phosphate-buffered saline. Two microliters of cells was mixed with 2 µl DAPI (Vector shield mounting medium with DAPI; Vector Lab, Inc.) on a slide immediately before viewing.

Chk1p mobility shift assays. Protein extraction from wild-type (ROP165) and Chkl-HA (ROP183) strains was performed by glass bead disruption in buffer containing 50 mM HEPES (pH 7.5), 250 mM NaCl, 80 mM β-glycerophosphate, 5 mM EDTA, 0.1% NP-40, 10% glycerol, and protease inhibitors. Thirty-five micrograms of total protein was analyzed for the Chk1 mobility shift by blotting in sodium dodecyl sulfate (SDS) sample buffer and was separated on an 8% SDS-acrylamide gel followed by immunoblotting with the antihemagglutinin (anti-HA) mouse monoclonal antibody 12CA5.

The phosphorylated and unphosphorylated HA-tagged Chkl (Chkl-HA) hands on the autoradiographs of the above Western blots were quantified using Image J software. The ratio of phosphorylated Chkl to unphosphorylated Chkl was determined for wild-type cells not treated with MMS was normalized to 1, and the rest of the ratios were calculated and plotted accordingly.

Treatment with HU, UV, gamma ray, and MMS. Sensitivities to DNA-damaging agents were assayed by using cells in log phase (A600 of 0.8 to 1.0). To assay viability in MMS (0.015%) and hydroxyurea (HU) (25 mM), cells were washed, rehydrated, and plated to determine the number of viable CFU as a function of time of exposure. In spot assays, 10-fold or fivefold serial dilutions from 1 OD600/ml for each culture were spotted onto a set of YES control plates or a set of plates containing YES plus either HU (10 mM), CPT (10 µM), or MMS (0.015%) using a pin replicator. UV sensitivity was measured by growing cells of each strain in YES medium to a concentration of 5 × 109 to 1 × 1010 cells/ml and then diluting and plating to determine the number of viable CFU for each indicated dose of UV radiation (see Fig. 1B) in joules/m2. UV irradiation was carried out using a Stratagene Stratalinker (UV Stratalinker 2400). Plates were incubated for 4 days in the dark at 32°C.

For gamma irradiation, cells were grown in YES medium to a concentration of 5 × 109 to 1 × 1010 cells/ml and aliquots containing 1,000 cells in a volume of 100 µl were made of each strain for each dose. Cells were plated in a rotating cylinder and exposed to a 137Cs source emitting 3.3 Gy/min for different lengths of time to expose them to different doses of gamma radiation. Viability was determined by plating different numbers of cells (200 and 800) and counting CFU as a function of irradiation.

Construction of epitope-tagged Hst4p. A strain expressing a carboxy-termi
dally tandem affinity purification (TAP)-tagged Hst4 (ROP238) was con- structed by a PCR-based gene targeting method as described previously (4). To construct this strain, a construct carrying a TAP tag and KanMX6 gene flanked by a 160-bp homology region to the 3′ end of the hst4Δ chromosomal locus, on either end, was constructed by PCR using pKG1810 (51) as the template. This PCR product was transformed into S. pombe strain ROP192. After transformation, cells were plated on a YES plate containing 200 µg/ml G418. The 3′ end of the TAP tag contained a KanMX6 gene, which conferred resistance to G418.

Histone preparations and Western blot analysis of bulk histones. Histones were prepared as described previously (15). Histone preparations (5 µg) from wild-type and hst4Δ and sir2Δ mutant strains were run on 15% SDS-polyacryl-
amide gels and electroblotted onto polyvinylidene difluoride membranes. Filters were blocked with 5% milk in Tris-buffered saline with 0.1% Tween 20 and incubated with rabbit antibodies against histone H3 acetylated at position K9 or K14, K56 or histone H4 acetylated at position K12 or K16 purchased from Upstate at 1:3,000 dilution in the same buffer. Rabbit immunoglobulin G was detected by using an ECL kit (Amersham) using horseradish peroxidase-conju-
gated anti-rabbit immunoglobulin G (anti-PAP; Sigma), and the ab1791 antibody from Abcam was used to detect histone H3.

Cell cycle analysis. The cdc25-22 mutant strain (ROP204) was crossed with a strain containing TAP-tagged Hst4p (ROP238) to generate a cdc25-22 mutant strain with TAP-tagged Hst4p (ROP266). The ROP266 strain was grown at 23°C to log phase and shifted to 36°C for 4 h to synchronize cells in the G2 phase of cell cycle. Cells were released from G2 by shifting the cells to 23°C, and cells were collected every 20 min for 260 min. Extracts were made and processed for immunoblotting using antibody against acetylated K56 on H3 (anti-AcK56 H3) (07-677; Upstate) and anti-TAP antibodies (anti-PAP; Sigma). Fluorescence-
TABLE 1. *Schizosaccharomyces pombe* strains used in this study

| Strain       | Genotype                                      | Source          |
|--------------|-----------------------------------------------|-----------------|
| LPY3279      | h⁵ ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18 | Lorraine Pillus |
| LPY3278      | h⁵ ade6-216 arg3-D4 his3-D1 leu1-32 ura4-D18 his4²::his3⁺ | Lorraine Pillus |
| HL6383       | h⁵ ade6-216 his3-D1 leu1-32 ura4-D18          | Henry Levin     |
| ROP238       | h⁵ ade6-216 arg3-D4 his3-D1 leu1-32 ura4-D18 his4-TAP-KANMX6 |               |
| ROP91        | h⁵ his3-D1 leu1-32 ura4-D18 his4-TAP-KANMX6   |                 |
| ROP266       | h⁵ leu1-32 ura4-D18 his3-D1 ade6-216 (or ade6-704) rad3³::ura4⁺ |               |
| ROP277       | h⁵ leu1-32 ura4-D18 his3-D1 ade6-216          |                 |
| ROP95        | h⁵ leu1-32 ade6-216 his3-D1 ura4-D18 his4-TAP-KANMX6 |               |
| ROP153       | h⁵ leu1-32 ade6-210 his3-D1 ura4-D18 his4-TAP-KANMX6 |               |
| FS236        | h⁵ ade6-216 his3-D1 leu1-32 ura4-D18 rad50Δ::KANMX6 | Nick Rhind    |
| ROP98        | h⁵ leu1-32 his3-D1 ura4-D18 his4-TAP-KANMX6   |                 |
| ROP174       | h⁵ rad1::ura4⁺ ura4-D18 leu1-32 ade6-216 (or ade6-704) his3-D1 arg3-D4 |               |
| ROP248       | h⁵ leu1-32 ura4-D18 (or ura4-D5::LEU2) ade6-216 his3-D1 bub1Δ::ura4⁺ | Nick Rhind    |
| TMN3309      | h³ ade6-216 ura4-D18 ade6-216 his3-D1 chk¹⁻::9myc-2HA-6His-ura4⁺ |               |
| Y21          | h⁵ ade6-M216 ura4-D18 leu1-32 rad3Δ::ura4⁺ | Shoji Yasuhara |
| FO726        | h⁵ maglΔ::ura4⁺ ura4-D18 leu1-32 his3-D1       | Magnus Bjoras  |
| FO260        | h⁵ rad2Δ::LEU2 ura4-D18 leu1-32 his3-D1        | Magnus Bjoras  |
| RHI103       | h⁵ apm2Δ::KANMX ura4-D18 leu1-32 his3-D1       | Magnus Bjoras  |
| FY4754       | his3Δ::ura4⁺ ade6-216 ade6-210 his3-D1 arg3-D4 adele-210 |              |
| ROP239       | his2Δ::ura4 his3Δ::his3⁺ his3Δ::his3 arg3⁺ ade6-210 his3-D1 arg3-D4 | Robin Allshire|
| ROP199       | h⁵ ura4-D18 rad17Δ::ura4⁺ leu1-32 ade6-706 (or ade6-210) his3-D1 arg3-D4 |               |
| ROP210       | h⁵ his4Δ::his3⁺ ura4-D18 rad17Δ::ura4⁺ leu1-32 ade6-210 his3-D1 arg3-D4 |               |
| ROP231       | h⁵ his4Δ::his3⁺ rad17Δ::ura4⁺ ura4-D18 leu1-32 ade6-210 his3-D1 |               |
| ROP183       | h¹⁵::his3⁺ ade6-216 arg3-D4 his3-D1 leu1-32 ura4-D18 his4-TAP-KANMX6 |               |
| ROP204       | h³ cdc25-22 ura4-D18 ade6-210 arg3-D4 his3-D1 leu1-32 |               |
| ROP216       | h³ his4Δ::his3⁺ cdc25-22 ura4-D18 ade6-210 arg3-D4 his3-D1 leu1-32 |               |
| ROP100       | h³ his4Δ::his3⁺ bub1Δ::ura4⁺ ade6-210 ura4-D18 his3-D1 arg3-D4 |               |
| ROP198       | h³ his4Δ::his3⁺ rad13Δ::ura4⁺ ura4-D18 leu1-32 ade6-M216 his3-D1 arg3-D4 |               |
| ROP256       | h³ his4Δ::his3⁺ maglΔ::ura4⁺ ura4-D18 leu1-32 ade6-M210 his3-D1 arg3-D4 |               |
| ROP258       | h³ his4Δ::his3⁺ apm2Δ::KANMX ura4-D18 leu1-32 his3-D1 arg3-D4 |               |
| ROP261       | h³ his4Δ::his3⁺ rad2Δ::LEU2 ura4-D18 leu1-32 his3-D1 arg3-D4 |               |
| ROP245       | h³ K56R ura1-32 ura4-D18 his3-D1 arg3-D4 adele-210 |               |
| ROP246       | h³ K56Q ura1-32 ura4-D18 his3-D1 arg3-D4 adele-210 |               |
| ROP247       | h³ K56R h³ his4Δ::his3⁺ h³ his4Δ::his3 arg3⁺ adele-210 his3-D1 arg3-D4 |               |
| ROP253       | h³ K56Q h³ his4Δ::his3⁺ h³ his4Δ::his3 arg3⁺ adele-210 his3-D1 arg3-D4 |               |
| ROP263       | h³ K56Q h³ his4Δ::his3⁺ adele-210 his3-D1 leu1-32 ura4-D18 rad50Δ::KANMX6 |               |
| ROP268       | h³ cdc25-22 ade6-216 arg3-D4 his3-D1 leu1-32 ura4-D18 his4-TAP-KANMX6 |               |
| ROP275       | h³ K56R h³ his4Δ::his3⁺ h³ his4Δ::his3 arg3⁺ adele-210 his3-D1 arg3-D4 |               |
| ROP278       | h³ K56Q h³ his4Δ::his3⁺ h³ his4Δ::his3 arg3⁺ adele-210 his3-D1 arg3-D4 |               |
| ROP128       | h⁵ leu1-32 his3-D1 ura4-D18 mad2Δ::ura4⁺ adele-210 |               |
| ROP130       | h⁵ leu1-32 his3-D1 ura4-D18 bub1Δ::ura4⁺ adele-210 |               |
| ROP133       | h⁵ leu1-32 his3-D1 ura4-D18 mad2Δ::ura4⁺ adele-210 |               |
| ROP145       | h¹⁵::his3⁺ mad2Δ::ura4⁺ ura4-D18 leu1-32 adele-210 (or adele-210) his3-D1 |               |
| ROP162       | h¹⁵::his3⁺ mad2Δ::ura4⁺ ura4-D18 leu1-32 adele-210 (or adele-210) his3-D1 |               |
| ROP144       | h¹⁵::his3⁺ mad2Δ::ura4⁺ ura4-D18 leu1-32 adele-210 (or adele-210) his3-D1 |               |
| ROP161       | h¹⁵::his3⁺ bub1Δ::ura4⁺ ura4-D18 leu1-32 adele-210 (or adele-210) his3-D1 |               |

activated cell sorting (FACS) analysis of each time point was carried out to monitor cell cycle progression.

FACS analysis. Cells were fixed in 70% ethanol, and samples were prepared using propidium iodide for DNA staining. DNA content was measured on a Becton Dickinson FACS Calibur with Cell Quest software. Flowjo software was used for data analysis.

Generation of plasmids expressing his4 and histone H4-H184V mutant. A plasmid (pR071) expressing his4 under its own promoter was generated as described previously (1). The phs4-H184V mutant was generated by overlapping PCR. A TAP tag was introduced at the N termini of these genes by PCR and cloning. The PCR product was then cloned into autonomously replicating plasmid pRS314. These plasmids expressed the his4⁺ gene, which could rescue the long cell phenotype associated with the his4A strain, but the his4-H184V mutant gene was also expressed and detected using anti-TAP antibodies, but it could not rescue the long cell phenotype associated with the his4A mutant.

Generation of histone mutants. Histone mutants were generated as described previously (37). To generate specific histone H3 mutations, a strain (FY4754; provided by Robin Allshire) with just the h3.2 gene deleted by insertion of ura4⁴⁻ that retained both the h3.1/h4.1 and h3.3/h4.3 gene pairs was used. This ura4⁴⁻ gene was then replaced with PCR-amplified DNA containing the histone H3 lysine 56 point mutation to arginine or glutamine by using homologous recombination. The strains with a point mutation were obtained by fluoroacetate acid selection. Once obtained, strains with mutant h3.2 were crossed with a strain (ROP259) in which both the h3.1/h4.1 and h3.3/h4.3 loci were replaced with his3⁴⁻ and arg3⁴⁻ markers and in which the wild-type histone h3.2/h4.2 locus was marked with the ura4⁺ gene. Recombinant progeny with mutant histone h3.2 as the sole source of histone H3 were obtained by selection on media containing fluoroacetate acid (to select against the wild-type h3.2, which is linked to ura4⁺) but lacking arginine and histidine and were verified by PCR analysis and sequencing.
RESULTS

Hst4 has a role in fission yeast DNA damage response. Hst4 is a Sir2p homolog in S. pombe, and its loss has previously been shown to result in a fragmented chromatin phenotype (18). To confirm whether hst4 deletion indeed resulted in fragmented DNA, we first analyzed the nuclear morphology of cells lacking Hst4 by DAPI staining. Our fluorescence microscopic analysis also clearly indicated that a significant percentage of the hst4/H9004 cells in culture had fragmented DNA (see Fig. S1 in the supplemental material). This result indicated that lack of Hst4 directly or indirectly induces DNA damage. Therefore, we hypothesized that Hst4 functions in the DNA damage response pathway and was required for maintenance of genomic integrity. To test our hypothesis, we then investigated whether lack of Hst4 makes cells more sensitive to DNA-damaging agents. The sensitivities of cells lacking Hst4 to different genotoxic agents (MMS, CPT, and hydroxyurea) were analyzed. These agents lead to DNA damage via distinct mechanisms. Serial dilutions of wild-type and mutant cells were spotted onto plates containing these drugs, and growth of the cells was analyzed. Compared to wild-type cells, hst4Δ cells were not significantly sensitive to HU. Interestingly, hst4Δ cells were sensitive to MMS and CPT (Fig. 1A) and were unable to grow on plates containing these drugs. The sensitivity of hst4Δ cells to UV light was also tested by determining their ability to survive following exposure to UV. Wild-type and hst4Δ mutant cells were first plated onto YES plates and then exposed to various doses of UV light, following which the percentage of survival was analyzed. The ability of hst4Δ cells to survive UV-induced damage was less than that of wild-type cells but higher than that of checkpoint mutant rad3Δ cells (Fig. 1B).

Exposure of cells to MMS and CPT results in single-strand breaks, which lead to double-strand breaks during the S phase. Gamma irradiation also causes double-strand breaks, but these are not restricted to the S phase. To determine whether hst4Δ mutants were able to efficiently recover from exposure to gamma irradiation, wild-type and hst4Δ mutant cells were irradiated with various doses of gamma rays and then plated onto YES plates. Formation of colonies was used as a measure of recovery from irradiation. Wild-type cells were able to efficiently recover from the irradiation, whereas the control rad3Δ

FIG. 1. Hst4p is required for DNA damage tolerance. (A) Fivefold (MMS and CPT treatment) or 10-fold (HU treatment) serial dilutions of the wild-type (LPY3279) and hst4Δ (LPY3278) strains were spotted onto rich media either lacking (control) or containing the indicated amount of each compound (shown below the panels) and incubated at 32°C. (B) Survival curves for the wild-type, hst4Δ, and rad3Δ strains after treatment with indicated doses of UV radiation. (C) Survival curves for the wild-type, hst4Δ, and rad3Δ strains after gamma irradiation (IR) at the indicated doses. (D) Survival curves for the wild-type, hst4Δ, and rad3Δ strains after exposure to MMS (0.015%) in rich media for the time indicated. wt, wild type.
cells were very sensitive to gamma irradiation. The Rad3 protein is required to generate the signal to delay the cell cycle when DNA gets damaged. If this signal is not generated, cells go through the cell cycle with damage and eventually die. Interestingly, hst4Δ cells behaved as wild-type cells did and were able to survive exposure to gamma irradiation (Fig. 1C). These results suggested that Hst4 is necessary for fission yeast cells to survive in the presence of specific types of double-strand break-generating DNA-damage agents (MMS and CPT). The ability of hst4Δ cells to survive damage induced by HU was also tested. The hst4Δ cells behaved as wild-type cells did and were able to survive exposure to HU (see Fig. S3 in the supplemental material). This specificity is interesting and intriguing, since it suggests that this histone deacetylase might be a crucial component of a specific S-phase DNA damage response machinery.

To determine whether hst4Δ cells were proficient in surviving DNA damage induced by MMS, we analyzed their ability to survive following exposure to MMS (Fig. 1D). Wild-type and hst4Δ and rad3Δ mutant cells were first treated with 0.015% MMS for various lengths of time and then plated onto YES plates to determine cell survival. Wild-type cells were able to survive drug-induced DNA damage efficiently once the drug was withdrawn. The absence of the checkpoint protein Rad5 resulted in a significant decrease in the viability of these cells following exposure to MMS. The ability of hst4Δ cells to survive MMS-induced damage was less than that of wild-type cells but higher than that of rad3Δ cells. Thus, the ability of hst4Δ mutant cells to survive MMS-induced damage was reduced compared to that of wild-type cells. This analysis revealed that Hst4 is required for cell survival following exposure to MMS.

Several components of DNA damage checkpoint machinery are essential for the survival of hst4Δ mutants. DNA damage leads to cell cycle arrest by DNA damage checkpoint activation, followed by repair of the damage (35, 47). To investigate whether the cause of the DNA damage sensitivity of the hst4Δ mutant is due to its interaction with the checkpoint pathway, resulting in defects in damage checkpoint activation, we generated and analyzed double mutants with the hst4Δ mutation and mutations in various checkpoint genes. We systematically crossed the hst4Δ strain with strains harboring deletions in the genes known to be involved in DNA damage, S phase, and spindle checkpoint control (Table 2). Quantitative analysis after tetrad dissection identified several genes involved in the DNA damage checkpoint pathway to be synthetically lethal with the hst4Δ mutant. Tetrad analysis demonstrating synthetic lethality of representatives of the DNA damage checkpoint mutant ctb2Δ and rad3Δ are shown in Fig. 2A (the genotypes of the double mutants were deduced from marker analysis). The bub1Δ tetrad analysis is an example of a nonlethal spindle checkpoint gene. The double mutant spores (spores with hst4Δ and one of the following mutations: ctb2Δ or rad3Δ) germinated but died after a few divisions as determined by microscopic analysis of the dissection plates (data not shown). We also identified synthetic lethality between the checkpoint mediator and effectors mre1Δ, cdc15Δ, and chk1Δ with hst4Δ (Table 2). These results demonstrate that several proteins involved in the DNA damage checkpoint pathway were essential for survival in the absence of Hst4p, suggesting this mutant experienced greater DNA damage and needed a functional checkpoint system for survival.

The DNA damage checkpoint is activated in hst4Δ mutants. Genetic analysis showed that some of the damage checkpoint genes were required for survival of mutants lacking Hst4, indicating the presence of an activated DNA damage checkpoint in these mutants. Therefore, we investigated whether the lack of Hst4 activated the DNA damage checkpoint by determining the phosphorylation status of the checkpoint kinase Chk1, as phosphorylation of the checkpoint kinase Chk1 is a specific marker for DNA damage checkpoint activation (8). We generated wild-type and hst4Δ strains containing HA-tagged Chk1 and analyzed phosphorylation of Chk1 by immunoblot analysis using antibodies against the HA epitope in the absence and presence of MMS (which is known to cause DNA damage and activate the checkpoint). In wild-type cells without damage, Chk1 was present as a single band of the expected size (Fig. 2B). In the presence of MMS, a slower-migrating band representing phosphorylated Chk1 was observed. In cells lacking Hst4, Chk1 was phosphorylated even in the absence of MMS exposure, and this phosphorylation did not significantly increase upon exposure to MMS (Fig. 2B). To determine the relative change in the amount of Chk1 shifted, we have quantified the signal ratio between phosphorylated and unphosphorylated bands. We have normalized the ratio of phosphorylated Chk1 to unphosphorylated Chk1 in the wild-type strain not treated with MMS by setting it at 1 and calculated and plotted the other ratios accordingly. These data suggest that cells lacking Hst4 have an activated checkpoint, presumably due to increased damage to their DNA (Fig. 2C). However, there is very little further damage-induced Chk1 shift in the hst4Δ mutant (the ratios are 5.92 without MMS treatment and 6.68 with MMS treatment).

| Pathway and deletion mutant | Function | Interaction |
|-----------------------------|----------|------------|
| DNA damage checkpoint       |          |            |
| Rad3                        | Checkpoint kinase | Synthetic lethal |
| Ctb2                        | Checkpoint component | Viable |
| Mre1                        | Checkpoint component | Viable |
| Cdc15                       | Checkpoint component | Viable |
| Cdh1                        | Checkpoint component | Viable |
| Chk1                        | Checkpoint kinase | Synthetic lethal |
| Spindle checkpoint          |          |            |
| Bub1                        | Checkpoint kinase | Viable |
| Bub3                        | Checkpoint component | Viable |
| Mad1                        | Checkpoint component | Viable |
| Mad2                        | Checkpoint component | Viable |
| Mad3                        | Checkpoint component | Viable |
| Mph1                        | Checkpoint component | Viable |
| DNA repair                  |          |            |
| Rad3                        | Nucleotide excision repair | Viable |
| Rad2                        | UV damage excision repair | Viable |
| Mag1                        | Base excision repair | Viable |
| Apn2                        | Base excision repair | Viable |
| Rad50                       | Homologous repair | Viable |

*Results of crossing the hst4 mutant with strains harboring deletions in the genes known to be involved in DNA damage, spindle checkpoint pathways, and various DNA repair pathways.
DNA damage checkpoint activation results in cell cycle arrest. To determine whether the cell cycle was arrested in the \textit{hst4} mutant, growth rate and cell cycle progression analyses were carried out. The growth of wild-type and \textit{hst4} mutant strains in YES medium was monitored at regular intervals over a 32-h period. At the end of 32 h, wild-type cells had stopped dividing and were in stationary phase, while the \textit{hst4} mutant strain failed to reach the wild-type level of cell density (Fig. 2D). The \textit{hst4} mutant had an initial lag and then entered logarithmic growth dividing at a rate significantly lower than that of wild-type cells. To further characterize the growth defect, we monitored the cell cycle progression of wild-type and mutant \textit{hst4} cells. To synchronize cells, we employed a temperature-sensitive allele of \textit{cdc25} (\textit{cdc25}-22) that causes cells to arrest in the G2 phase of the cell cycle when these cells are shifted to 36°C. Wild-type and mutant \textit{hst4} strains with the \textit{cdc25}-22 mutation were grown to log phase and shifted to 36°C. Following arrest and synchronization of the cells in G2, cells were released into YES medium and grown at 25°C. Aliquots of cells were collected every 20 min over a 200-min period, cells were stained with Calcofluor, and the number of cells with septa (indicative of cells that had completed mitosis) were analyzed to monitor mitotic progression. Wild-type cells showed a peak of septation approximately 80 min after release, whereas the \textit{hst4} cells showed a delayed peak of septation at 120 min (see Fig. S2 in the supplemental material). Furthermore, the number of cells undergoing septation was much lower in \textit{hst4} cells than in wild-type cells. These data provide further evidence for acti-
vation of the damage checkpoint resulting in cell cycle delay in cells lacking Hst4.

**Genetic interaction between hst4 and DNA repair genes.** The hst4Δ mutants contain damaged DNA in spite of having an activated DNA damage checkpoint. It is possible that cells lacking Hst4 cannot repair certain types of DNA damage, indicating that Hst4 may play a role in the repair of damaged DNA. To determine whether Hst4 functions in any of the known DNA repair pathways, we next monitored genetic interactions between this histone deacetylase and deletion mutants of repair genes that function in different repair pathways (Fig. 3), such as rad13, mag1, apn2, rad2, and rad50 (25, 46). This analysis demonstrated that mutations in the repair protein genes were not synthetically lethal with hst4Δ (Table 2). When strains were grown on rich medium (YES), we also did not observe very significant growth defects in any of the double mutants compared to the single mutant except the rad50Δ single mutant. However, every single repair mutant that we tested was synthetically sick in combination with hst4Δ when cells were grown on medium containing MMS (Fig. 3). These results suggest that the absence of Hst4 exacerbates defects in any of the double mutants compared to the single mutant except the rad50Δ single mutant. Hence, every single repair mutant that we tested was synthetically sick in combination with hst4Δ when cells were grown on medium containing MMS (Fig. 3). These results suggest that the absence of Hst4 exacerbates defects in any of the double mutants indicated that Hst4 is required for repair of DNA damage caused by MMS. Furthermore, it also suggested that Hst4 affects multiple DNA damage repair pathways simultaneously. However, Hst4 is not epistatic to any of the above repair genes tested here because it did not act through mag1 or apn1 in the base excision repair (BER) pathway, as hst4Δ mag1Δ and hst4Δ apn2Δ double mutants were more sensitive to MMS than the single mutants were. The same is true for nucleotide excision repair (NER) and recombinational repair pathways. Therefore, it is also possible that Hst4 is part of a different, nonoverlapping repair pathway that is crucially necessary for the repair of MMS-induced damage. Alternatively, it could affect all the repair pathways simultaneously through its functions in modulating chromatin structure.

**Hst4 regulates histone H3 lysine 56 acetylation during the cell cycle.** Although Hst4 is a member of the Sir2 family of histone deacetylases, its substrates are not known and deacetylase activity has not been demonstrated. If substrates of Hst4 are identified, they could be used to further understand its role in DNA damage response. We hypothesized that histones were among its substrates and that deletion of this histone deacetylase would result in an increase in acetylation of those acetylated histone residues which were its substrates. To determine the substrates of Hst4, we looked for histone residues whose acetylation increased in hst4Δ mutants. We initially compared the levels of acetylation of specific residues in wild-type and hst4Δ mutant strains using an array of the commercially available histone acetylation antibodies. To compare changes in histone acetylation, bulk histones were prepared from wild-type cells and hst4Δ and sir2Δ cells, and the proteins were subjected to immunoblot analysis using antibodies that recognized histones acetylated at specific residues. Unmodified histone H3 was used as a loading control. The hst4Δ mutant did not show significant changes in the acetylation levels of histone H3 K9 or K14 and histone H4 K16 compared to the wild-type control (Fig. 4A). There was a slight increase in the acetylation of histone H4 K12 in hst4Δ cells. In contrast, the hst4Δ mutant had strikingly elevated levels of acetylation of histone H3 K56, suggesting that Hst4 either directly or indirectly deacetylated the histone H3 K56 residue. The sir2Δ mutant acted as a positive control. In the absence of Sir2, we observed elevated levels of histone H3 K9 and histone H4 K16, which is consistent with previous reports (17, 48). The specificity of the anti-AcK56 antibody was checked by immunoblotting histones from a strain where histone H3 was mutated at K56, converting this residue to arginine. In this strain, K56 was not acetylated.
and the antibody failed to recognize the epitope (Fig. 4B). These results suggest that acetylated histone H3 K56 could be the substrate of Hst4.

Since hst4 mutants were sensitive to only those agents which are genotoxic during the S phase of the cell cycle, we were interested in finding whether there is a correlation between K56 acetylation/deacetylation and Hst4 expression during specific phases of the cell cycle in S. pombe. It has been demonstrated that histone H3 K56 is acetylated in a cell cycle-dependent manner in S. cerevisiae (34). We therefore investigated the kinetics of K56 acetylation and the levels of Hst4 during the cell cycle in S. pombe. We constructed a strain bearing a TAP-tagged Hst4 and cdc25-22. This strain behaved as the wild-type strain did and had no growth defects, suggesting that the attachment of the TAP tag to Hst4 did not affect the function of this enzyme. Cells were synchronized in the G2 phase of the cell cycle using the temperature-sensitive cdc25 allele. Following their arrest, cells were released from the arrest by lowering the temperature from 36°C to 23°C, and aliquots of cells were removed every 20 min. Cells were monitored for progression through the cell cycle using standard FACS protocols. Simultaneously, cell extracts were prepared and analyzed by immunoblotting using antibodies that recognized either the tagged Hst4 or histone H3 acetylated on K56. A FACS profile showing cell cycle progression of each time point is presented along with the immunoblot.
growing culture of cells to 36°C for 4 h and released from the arrest by shifting the cells to 23°C. Acetylation of histone H3 K56 was monitored as described above. The cell cycle regulation of histone H3 K56 acetylation was lost in the hst4/H9004 mutant, and H3 K56 remained acetylated throughout the cell cycle (Fig. 4D). Once again, we used FACS analysis as a means to monitor progression through the cell cycle. These results demonstrated that Hst4 regulated the acetylation of histone H3 K56 during the cell cycle.

Hst4 functions in DNA damage response by regulating acetylation of histone H3 K56. The chemistry of deacetylation of histones by Sir2 is well understood. A critical histidine residue is required for the NAD-dependent deacetylation activity of these enzymes (3, 9, 39), and this residue is conserved in Hst4. If Hst4 were functioning as a histone deacetylase, then it is likely that a mutation in this conserved histidine would lead to loss of enzymatic activity. To test whether the function of Hst4 in DNA damage response is dependent on its histone deacetylase, then it is likely that a mutation in this conserved histidine would lead to loss of enzymatic activity. To test whether the function of Hst4 in DNA damage response is dependent on its histone deacetylase, we cloned hst4 into a multicopy plasmid (pRO741) in hst4 mutant results in deacetylation of histone H3 K56. The hst4Δ mutants were transformed with autonomously replicating plasmid (phst4 and phst4H184Y). Extracts were prepared from the transformant and run on a 15% SDS-polyacrylamide gel, and immunoblots was performed with antibodies specific for histone H3 acetylated at K56 (anti-H3 K56ac) and histone H3.

DNA damage up regulates K56 acetylation and down regulates Hst4 expression. It has been proposed that histone H3 K56 acetylation creates a more open chromatin environment for DNA repair (34). We hypothesized that histone H3 K56 acetylation and the open chromatin environment are brought about through Hst4 in response to DNA damage. This hypothesis would mean that acetylation of histone H3 on K56 is up regulated upon DNA damage and that this effect is brought about by down regulation of Hst4. To test this experimentally, we monitored the acetylation of K56 in wild-type and hst4Δ mutant cells following exposure of the cells to MMS. Strains were exposed to MMS for 2 h following which the drug was

![Image](https://example.com/image.png)

**FIG. 5.** Hst4p activity regulates histone H3 K56 acetylation. (A) Fivefold serial dilutions of the strains indicated to the left of the figure were spotted onto selective media (EMM with no Ura [EMM −Ura]) either lacking (control) or containing the indicated amount of MMS (0.01%) and incubated at 32°C. (B) Expression of Hst4p from a multicopy plasmid (pRO741) in hst4Δ mutants results in deacetylation of histone H3 K56. The hst4Δ mutants were transformed with autonomously replicating plasmid (phst4 and phst4H184Y). Extracts were prepared from the transformant and run on a 15% SDS-polyacrylamide gel, and immunoblots was performed with antibodies specific for histone H3 acetylated at K56 (anti-H3 K56ac) and histone H3.
neutralized and the acetylation status of the histones was monitored. We compared histone H3 K56 acetylation levels of MMS-treated wild-type and hst4Δ cells by immunoblotting whole-cell lysates with anti-AcK56 H3 antibody. In wild-type cells, upon MMS treatment, the acetylation of K56 increased. The hst4Δ mutants had constitutively elevated levels of histone H3 K56 acetylation, which did not change significantly upon MMS treatment (Fig. 6A). To determine whether the elevated levels of histone H3 K56 acetylation on MMS treatment were due to altered Hst4 levels, we prepared total cell lysates from a strain expressing TAP-tagged Hst4 and monitored the level of Hst4 by immunoblot analysis using antibody specific to the protein A epitope tag (Fig. 6B). Upon MMS treatment, the levels of Hst4 decreased significantly, correlating with the increase in acetylation of H3 K56. These results collectively suggest that alterations in K56 acetylation are dependent upon the presence of Hst4 in the cell, and more importantly, they suggest that the levels of Hst4 are regulated in response to cell cycle progression and DNA damage.

The histone H3 lysine 56 mutant exhibits the phenotype of the hst4Δ mutant. Our results show that histone H3 acetylated on K56 was the major substrate of Hst4. These results also suggest that Hst4 functions in the DNA damage response pathway through the deacetylation of histone H3 K56. If this were true, the histone H3 K56 mutants should show phenotypes similar to those of the hst4Δ mutant.

S. pombe has three copies of the core histones H3 and H4. We deleted two copies of histone H3 and generated strains carrying a mutation in the third copy of histone H3 at residue K56 where this residue was converted either into an arginine (K56R) or a glutamine (K56Q). The K56R mutant is expected to mimic the constitutively deacetylated state, while the K56Q mutant is expected to mimic constitutively acetylated histone H3 lysine 56. Both mutant strains were viable and able to grow at 32°C. However, like the hst4Δ strain, the histone H3 K56R mutant strains had slight growth defects compared to the strain containing a single copy of the H3 histone (Fig. 7B, first panel). The K56Q strain grew almost as the wild-type strain did and showed negligible growth defect. We also analyzed the morphology of cells with a phase-contrast microscope. Like hst4Δ cells, both histone H3 mutant cells were very elongated, and a percentage of these cells exhibited abnormal DAPI staining (Fig. 7A). The K56Q strain had 5% elongated cells, while the K56R strain had 10%. These results suggest that histone H3 constitutively acetylated or deacetylated on residue 56 affects chromosome morphology.

We next examined the effects of these mutations on the ability of cells to grow in the presence of DNA-damaging drugs. Serial dilutions of the appropriate strains were spotted onto plates containing MMS or CPT, and growth was monitored. Interestingly, similar to the hst4Δ mutant, both K56R and K56Q mutants were sensitive to MMS and CPT. However, the histone H3 K56Q mutant, which mimics the constitutively acetylated state, was less sensitive to MMS and CPT than the K56R mutant, which mimics the constitutively deacetylated state. The hst4Δ strain in which histone H3 was constitutively acetylated on K56 was more sensitive to CPT than the K56Q mutant, which mimics the constitutively acetylated state (Fig. 7B). These results suggest that changes in the acetylation state of histone H3 K56 following DNA damage and during DNA damage response are important for cell survival. This is similar to the Nua4- and Sin3p-dependent acetylation and deacetylation of histone H4 at DNA lesions in S. cerevisiae (5).

The histone H3 K56R mutation is epistatic to hst4Δ. The histone H3 mutations act as mimics of the acetylated and deacetylated states, and both the hst4Δ mutant and the K56R mutants were sensitive to MMS and CPT. To test whether Hst4 was functioning solely via changes in modification of histone H3 K56, we constructed double mutants (histone H3 K56R and hst4Δ double mutants and H3 K56Q and hst4Δ double mutants). All these strains contained a single copy of the histone H3. We then checked the sensitivities of these double mutant strains to damage compared to the single mutant strains. As shown in Fig. 7C, a combination of H3 K56R and hst4Δ mutations did not result in increased MMS sensitivity, indicating that these two mutations act in the same genetic pathway. This provides further evidence supporting the fact that Hst4 functions in the DNA damage response pathway through deacetylation of histone H3 lysine 56. Interestingly, the H3 K56Q mutation, which mimics constitutively the acetylated state, combined with the hst4Δ mutation, resulted in increased sensitivity of the cells to both MMS and CPT. This could be because the hst4 deletion affects additional pathways contributing to damage sensitivity. Further experiments need to be done to study this.

DISCUSSION

Here we show that Hst4, a Sir2 family histone deacetylase (HDAC) in Schizosaccharomyces pombe, promotes cell survival in response to genotoxic agents that interfere with DNA replication by regulating histone H3 K56 acetylation. Hst4 is nec-
necessary for the deacetylation of histone H3 K56 in vivo. The absence of Hst4 or the presence of a catalytic domain point mutation, hst4-H184Y, leads to an increase in the cellular levels of acetylated-K56 histone H3 (Fig. 5). We further show that Hst4 levels and histone H3 K56 acetylation are cell cycle regulated in S. pombe. Histone H3 K56 acetylation occurs at a point in cell cycle during the S phase, which coincides with the down regulation of Hst4 expression (Fig. 4C). Upon DNA damage, Hst4 is repressed and H3 K56 acetylation is elevated. In S. pombe, Rtt109 acetylates histone H3 K56 (56), while we now show that Hst4 participates in DNA damage response by deacetylating this residue. Therefore, this appears to be a conserved mechanism, as acetylation and deacetylation of histone H3 lysine 56 have already been linked to the DNA damage response in S. cerevisiae (9, 31, 34).

The hst4Δ mutants are specifically sensitive to MMS and CPT but not sensitive to ionizing radiation (Fig. 1). This differential sensitivity indicates that it is critically necessary for the tolerance of S-phase-specific damage. Ionizing radiation generates DNA breaks outside of S phase (25, 46). MMS alkylates DNA and can cause a range of lesions from alkylated bases to abasic sites to single- or double-strand breaks (28). MMS-damaged DNA causes replication forks to stall that in turn can lead to double-strand breaks which are repaired during the S phase of the cell cycle (52). HU stalls replication forks by reducing nucleotide pools which also result in DNA breaks. While both HU and MMS cause stalling of the replication fork, there are mechanisms that distinguish between damage caused by HU and MMS (10). MMS-induced DNA damage is repaired during the S phase by enzymes of the NER, BER, or recombinational repair pathways (24). Our genetic analysis suggests that the rad13Δ hst4Δ double mutant with a NER
gene was more sensitive to MMS than either single mutant was (Fig. 3). The same was true for \( hst4^{\Delta} mag^{\Delta}, hst4^{\Delta} apn2^{\Delta}, hst4^{\Delta} rad2^{\Delta}, \) and \( hst4^{\Delta} rad50^{\Delta} \) double mutants of genes involved in BER and homologous recombination repair pathways (Fig. 3). Therefore, it is tempting to speculate that Hst4 deacetylase may function in multiple DNA damage repair pathways simultaneously. A recent study shows that the SIR16 deacetylase promotes resistance to DNA damage and suppresses genomic instability through the BER pathway (41).

Our data suggest that Hst4 plays a crucial role in the tolerance of MMS-induced damage by regulating histone H3 K56 acetylation (Fig. 5). Histone H3 is acetylated on K56 during replication-coupled deposition (34). One possibility is that the cycle of H3 K56 acetylation/deacetylation is important for the removal and redeposition of histone octamers during replication. A multiplicity suppressor screen for growth defects and MMS sensitivity of the \( hst4^{\Delta} \) mutant has led to the isolation of two clones encoding proteins involved in DNA replication (unpublished results), and further experiments should shed light on whether and how these proteins function in this pathway. It is possible that during the S phase and DNA damage response, the chromatin structure needs to be made more accessible, and suppression of Hst4 levels could be one mechanism by which the cell achieves this opening of chromatin. However, decondensed chromatin is deleterious for cells, and we propose that Hst4 functions to restore the native condensed chromatin following DNA damage response and after completion of the S phase.

Activation of the DNA damage checkpoint either by external DNA-damaging agents or during normal cellular metabolism arrests cell cycle progression to allow time for DNA repair. Cells lacking Hst4 require an activated checkpoint pathway for survival. Our tetrad analysis indicates that double mutants of \( hst4 \) and checkpoint mutants were able to divide a few times before dying, suggesting that accumulation of damage overwhelms the capacity of the cell to repair damage, leading to death. In \( S. cerevisiae \), it was shown that even subtle perturbations in DNA replication are lethal for cells with H3 K56 hyperacetylation, and DNA damage checkpoint was critical for viability of \( hst3^{\Delta} hst4^{\Delta} \) double mutants (9). Interestingly, deletion of \( rtt109 \), the histone acetyltransferase which acetylates histone H3 K56 also results in activation of damage checkpoint (13). In \( S. cerevisiae \), the checkpoint kinase Rad3p homolog Mec1p is involved in down regulating Hst3 and Hst4 upon DNA damage (31, 38). In \( S. pombe \), CPT-induced DNA damage results in a prolonged delay in H3 K56 deacetylation (34, 56), indicating that regulation of H3 K56 deacetylase by damage checkpoint is likely to be conserved between these two yeasts.

Damage tolerance by organisms has been closely linked to histone modifications (55). Phosphorylation of H2A by checkpoint kinases Tel1 (hATM) and rad3 (hATR) is the best studied key histone modification that occurs when cell encounter DNA damage (50). Several studies indicate that acetylation of histones is also critical for conferring resistance to DNA-damaging agents. Acetylation of N-terminal tails of H3 and H4 has been shown to be important for cell survival following DNA damage (5, 26, 27, 55). Recently, it was shown that acetylation of histone H3 core domain residue K56 plays an important role in the DNA damage response (9, 13, 20, 31, 54). In this report, we extend these studies by demonstrating that acetylation/deacetylation of this globular domain residue of histone H3 is also important for maintenance of genomic integrity in \( S. pombe \). Importantly, we show that this important function is mediated through the HDAC Hst4 which alone can carry out the functions carried out by both Hst3 and Hst4 in \( S. cerevisiae \).

The fission yeast \( S. pombe \) carries two homologs of HDAC sir2, \( hst2 \) and \( hst4 \). Hst4 belongs to a subfamily that is distinct from Sir2 and Hst2, and its closest functional homologs are \( S. cerevisiae \) Hst3 and Hst4. We demonstrate that H3 K56 acetylation increases in the presence of MMS-induced damage and that this effect is brought about by down regulation of Hst4 expression (Fig. 6). The H3 K56 residue is present in the globular domain of histone H3 near the entry and exit points of the DNA in the nucleosome (29, 34). Acetylation/deacetylation of this residue could be important for mobility of nucleosomes, either for the removal of nucleosomes or their reformation during chromatin assembly. Loss of the H3 K56 acetyltransferase Rtt109 results in increase in genomic instability and gross chromosomal rearrangements (13, 20, 34, 43). It is also critical in survival following replicative DNA damage. Recently, other globular domain residues of histone H3 have also been shown to be required for survival from DNA damage. These are H3 K115 and H3 K79 (21). Cell cycle-regulated methylation of H3 K79 has been shown to recruit checkpoint adaptor protein 53BP1 (16). Interestingly, this methylation is highest in mitosis and excluded from S phase when H3 K56 is acetylated. It will be interesting to study the interplay between these core domain modifications to determine whether their functions in damage response are overlapping or unique.

The DNA damage response is a well-choreographed event with a specific order of recruitment of proteins to the damaged site. The occlusion of the genome within chromatin fibers necessitates utilization of additional factors to facilitate access to damaged DNA and to restore chromatin structure after their action (2, 45). Chromatin structure changes are one of the first steps in the initiation of DNA repair pathways, and it is possible that the acetylation of histone H3 K56 by Rtt109 creates a favorable chromatin structure for damage repair machineries to access damaged DNA. In \( S. cerevisiae \), nucleosomes from H3 K56Q mutant cells were extensively digested by micrococcal nuclease, supporting this hypothesis (34). At the end of DNA repair, it is necessary to restore the original chromatin structure, which could be achieved by the deacetylation of H3 K56 following its deposition. This model is consistent with the fact that the nucleosomal DNA from wild-type and K56R mutant is less readily digested by micrococcal nuclease (34). Thus, regulating the levels of Hst4 allows the cell to combat DNA damage.

In summary, our data and those of other groups suggest that Hst4 is likely to function in cell cycle progression and DNA damage tolerance by directly deacetylating H3 K56 and thereby affecting chromatin structure. This has emerged as a novel pathway for the regulation of a histone modification by a specific class of sirtuins which is critical for normal cell cycle progression and DNA damage tolerance. Nevertheless, many questions remain to be answered. The presence of the acetylase Rtt109 and the deacetylase Hst4 and the roles played by the acetylation and deacetylation of histone H3 K56 in \( S. pombe \) suggest that this modification could be conserved during evolution. However, this modification has been reported to...
be absent in human cells, though H3 K56 is conserved (57). One possibility is that technical limitations may prevent the detection of this modification in mammalian cells. The new family of HAT Rtt109 from yeast does not also share obvious homology with any protein in higher eukaryotes. Since H3 K56 acetylation is present in Drosophila melanogaster (57), the H3 K56 HAT must have diverged in sequence. Discovery of this HAT may provide clues for the H3 K56 puzzle in mammalian cells. It also remains to be established which of the seven human sirtuins (SIRT1 to SIRT7) correspond to Hst4. It will be exciting to know whether a functional homolog of Hst4 in humans also exhibits similar functions in DNA damage response and if so, its contributions to the manifestation of diseases like cancer.

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