The nonhistone, N-terminal tail of an essential, chimeric H2A variant regulates mitotic H3-S10 dephosphorylation

Xiaoyuan Song,¹ Josephine Bowen, Wei Miao,² Yifan Liu,³ and Martin A. Gorovsky⁴

Department of Biology, University of Rochester, Rochester, New York 14627, USA

H2A.Y is an essential, divergent *Tetrahymena thermophila* histone variant. It has a long nonhistone N terminus that contains leucine-rich repeats (LRR) and an LRR cap domain with similarity to Sds22p, a regulator of yeast protein phosphatase 1 (PP1) activity in the nucleus. In growing cells, H2A.Y is incorporated into micronuclei only during S phase, which occurs immediately after micronuclear mitosis. Depletion of H2A.Y causes prolonged retention of mitosis-associated histone H3-S10 phosphorylation and mitotic abnormalities that mimic S10E mutation. In cells where H2A.Y is depleted, an inducible chimeric gene, in which the H2A.Y N terminus is attached to H2A.X, is shown to regulate micronuclear H3-S10 phosphorylation. H2A.Y can also be specifically coimmunoprecipitated with a *Tetrahymena* PP1 ortholog (Ppo1p). Taken together, these results argue that the N terminus of H2A.Y functions to regulate H3-S10 dephosphorylation. This striking *in vivo* case of “cross-talk” between a H2A variant and a specific post-translational modification of another histone demonstrates a novel function for a histone variant.

[**Keywords:** dephosphorylation; H2A variant; H3; mitosis; S10]

Supplemental material is available for this article.

Received November 5, 2011; revised version accepted February 6, 2012.

Two of each of the core histones H2A, H2B, H3, and H4 wrap around ~147 base pair (bp) of DNA and form nucleosomes, the fundamental repeating units of eukaryotic chromatin. Chromatin is a dynamic entity that is involved in regulating different cellular processes, such as replication, transcription, and repair. This functional heterogeneity of chromatin at the nucleosome level is achieved mainly by four mechanisms that likely act in concert: ATP-dependent chromatin remodeling (Eberharter and Becker 2004; Eberharter et al. 2005; Varga-Weisz and Becker 2006; Flaus and Owen-Hughes 2011), covalent post-translational modifications of histones (Margueron et al. 2005; Bannister and Kouzarides 2011), the incorporation/exchange of histone variants into chromatin (Talbert and Henikoff 2010), and the binding of activators/repressors (Quina et al. 2006). Major, or canonical, replication-coupled, histone isotypes are synthesized and deposited onto chromatin in S phase, make up the bulk of the histones in the cell, and are usually encoded by

multiple genes. Minor, or variant, histones are less abundant, are often replication-independent, and are usually encoded by single-copy genes. All core histone isotypes contain a conserved, type-specific histone fold domain (HFD) involved in histone-histone interactions required to form the nucleosome core, as well as a type-specific N-terminal tail that extends from the nucleosome core and contains specific sites of post-translational modifications. Some minor variants, such as H3.3, H2A.X, H2A.Z, and CenH3, are found in most organisms. Others, mainly H2A variants, can be species-specific or even cell type-specific (Talbert and Henikoff 2010). Histone variants play key roles in a number of biological and developmental processes, such as chromosome segregation, transcriptional regulation, DNA repair (Talbert and Henikoff 2010), and the initiation and maintenance of pericentric heterochromatin, X-inactivation, and germ cell differentiation (Banaszynski et al. 2010).

Among the four core histones, H2As exhibit the largest, most heterogeneous family of known variants (Talbert and Henikoff 2010). They differ from major H2As and from each other mainly in their nonglobular C-terminal tails, most of which are short. However, the vertebrate-specific macroH2A variant (unrelated to macronuclear H2A in *Tetrahymena thermophila*) has been unique in containing a large, globular, nonhistone C-terminal (macro) domain about twice as long as the N-terminal histone

Present addresses: ¹School of Medicine, University of California at San Diego, La Jolla, CA 92093, USA; ²State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, the Chinese Academy of Science, Wuhan 430072, China; ³Pathology Department, University of Michigan Medical School, Ann Arbor, MI 48109, USA.

⁴Corresponding author.

E-mail goro@mail.rochester.edu.

Article is online at <http://www.genesdev.org/cgi/doi/10.1101/gad.182683.111>.

domain (Gamble and Kraus 2010; Talbert and Henikoff 2010). This macrodomain is the founding member of an evolutionarily ancient ADP-ribose-binding structural domain that occurs in a number of proteins, some of which can convert ADP-ribose-1''-monophosphate to ADP-ribose. MacroH2A is generally associated with transcriptionally repressed chromatin: It associates with the inactive X chromosome, is involved in X-chromosome inactivation (Costanzi and Pehrson 1998), and is enriched in the specialized domains of transcriptionally silent, senescence-associated heterochromatic foci (Zhang et al. 2005). Both its HFD and long nonhistone tail may have roles in transcriptional repression, consistent with the ability of macroH2A to bind histone deacetylases and inhibit histone acetylation and nucleosome remodeling, as well as PARP1 activity, *in vitro*. Genome-wide localization analyses also demonstrate a broad role for macroH2A on autosomes (Buschbeck et al. 2009), and recent studies suggest that macroH2A has a role in fine-tuning the expression of some key genes during development, serving as either a transcriptional repressor or activator, depending on context (Gamble and Kraus 2010; Gamble et al. 2010; Talbert and Henikoff 2010). In addition, macroH2A has been implicated in regulating cell proliferation (Sporn et al. 2009) and in tumor suppression, which is mediated through direct transcriptional regulation of CDK8 (Kapoor et al. 2010).

Few variants identified so far differ from major H2As in their N termini. An exception is the vertebrate-specific H2A.Bbd, which has a short, arginine-rich N terminus. It also lacks a typical H2A C-terminal tail and destabilizes nucleosomes (Talbert and Henikoff 2010). H2A.Bbd is depleted in the inactive X chromosome and is present in the advanced spermiogenic region of mammalian testis and in mature human sperm (Ishibashi et al. 2010). *In vitro* experiments suggest that this variant functions in transcriptional activation, possibly by destabilizing nucleosomes in an acetylation-independent fashion (Gonzalez-Romero et al. 2008).

We have been analyzing the histones of *T. thermophila*, exploiting the fact that, like most ciliates, *Tetrahymena* exhibits a remarkable nuclear dimorphism in which distinct functions of histones are found in different nuclei. Each *T. thermophila* cell contains a germline micronucleus (MIC) and a somatic macronucleus (MAC) that are different in both structure and function (Gorovsky 1973, 1980). During vegetative growth, the diploid MIC divides mitotically and is transcriptionally inert. The polyploid (45C) MAC divides amitotically without chromosome condensation or segregation of sister chromatids and is transcriptionally active during vegetative growth. Thus, the two nuclei in *T. thermophila* appear to differ in two of the essential functions of chromatin: the mechanism of chromosome transmission (mitotic vs. amitotic) and the level of expression of genetic information (Coyne et al. 1996). Despite these remarkable differences, the two nuclei have a common origin during conjugation, the sexual stage of the life cycle. When starved cells with different mating types are mixed, pairs form, and MICs undergo meiosis and a prezygotic mitosis to produce

pronuclei. Thus, genes in the MIC are transmitted to the next sexual generation in typical Mendelian fashion (Hamilton and Orias 2000). After pronuclear exchange and fertilization, two post-zygotic MIC divisions occur, followed by differentiation of a new MIC and a new MAC (referred to as a developing MAC, or anlage); the old MAC is destroyed and is not transmitted to the next sexual generation (for details, see Ray 1956; Sugai and Hiwatashi 1974; Martindale et al. 1982).

Multiple histone variants have been identified in *Tetrahymena*. We report here that, in addition to the conserved H2As (H2A1, H2A.X, and H2A.Z), *T. thermophila* contains H2A.Y, a novel H2A variant with a long (258-residue) nonhistone sequence at its N terminus. Thus, the chimeric nature of H2A.Y, with the HFD connected to a long, nonhistone domain, resembles macroH2A, although their nonhistone domains are unrelated and are located at opposite ends of the proteins.

The N-terminal domain of H2A.Y resembles Sds22p, a yeast regulatory subunit of protein phosphatase 1 (PP1). In yeast, Sds22p interacts with the sole PP1 catalytic subunit, Glc7p, localizing it to nuclei to stimulate dephosphorylation of its nuclear substrates, including phosphorylated H3 (Hsu et al. 2000), and is required for proper chromosome transmission during mitosis (Hisamoto et al. 1995; MacKelvie et al. 1995; Peggie et al. 2002). Depletion of Sds22p results in many large-budded cells containing two nuclei in one cell body (Hisamoto et al. 1995). Mutations in *SDS22* can suppress mutations in *IPL1*, the H3-S10 kinase, which, together with PP1, regulates the level of mitotic phosphorylation of histone H3 in yeast and *Caenorhabditis elegans* (Hsu et al. 2000; Peggie et al. 2002). In Chinese hamster cells, a H3 mutation (S10E) that mimics S10 phosphorylation results in abnormal chromosome segregation similar to that of Sds22p-depleted yeast cells (Ota et al. 2002).

In vegetative *Tetrahymena* cells, phosphorylated S10 (S10-P) of H3 is detected only during MIC divisions (meiosis and mitoses), but not in amitotic MACs at any stage of the life cycle (Wei et al. 1998). When *Tetrahymena* H3-S10 is mutated to S10A, S10 phosphorylation is eliminated and abnormal micronuclear mitosis occurs (Wei et al. 1999). Taken together, these studies argue that S10 phosphorylation has a conserved function in chromosome segregation and that Sds22p plays a role in the regulation of H3 dephosphorylation.

Here we show that H2A.Y is essential, is deposited in MICs at the end of mitosis, and is required for efficient micronuclear DNA replication and efficient histone H3-S10 dephosphorylation when MICs exit mitosis. We also demonstrate that the H2A.Y N-terminal tail is responsible for the effect of H2A.Y on H3 dephosphorylation.

Results

H2A.Y is a novel leucine-rich repeat (LRR) domain-containing H2A chimeric protein

A search of the *T. thermophila* macronuclear genome database (*Tetrahymena* Genome Database [TGD],

<http://www.ciliate.org>) revealed *HTAY* (221.m00055/TTHERM_01079200) in addition to three previously known H2A genes (*HTAX*, *HTA1*, and *HTAZ*). 5' RLM-RACE and 3' RACE identified two transcription start sites 73 bp or 66 bp upstream of the ATG and a 225-bp 3' untranslated region (UTR), which has a polyA tail like other *Tetrahymena* histone gene transcripts (Bannon et al. 1983; Liu and Gorovsky 1993). Comparing the cDNA (AY706656) and the genomic DNA (see TGD, <http://www.ciliate.org>) sequences revealed an identical 1167-bp ORF lacking introns and predicted a 43-kDa, 388-amino-acid peptide (H2A.Y). Alignment of the four *Tetrahymena* H2As showed that H2A.Y has a long, non-histone, N terminus (amino acids 1–258) twice as long as the C-terminal HFD (amino acids 259–388) (Fig. 1A), making H2A.Y different from other H2A variants. When compared with *Tetrahymena* major H2As H2A.X and H2A.1, the sequence of the H2A.Y HFD is even more divergent (48% identical) than that of H2A.Z (55% identical). Phylogenetic analysis indicates that the H2A.Y HFD is highly diverged from those of other organisms (Fig. 1B,C).

A sequence domain analysis (SMART, <http://smart.embl-heidelberg.de>) of the H2A.Y N terminus revealed the existence of LRR domains (amino acids 54–163) followed by a LRR cap domain (amino acids 139–157) (Fig. 1A), a motif frequently found C-terminal to LRRs (Kobe and Kajava 2001) in Sds22-like proteins. When the H2A.Y N terminus was used to query the PSIPRED Protein Structure Prediction Server (<http://bioinf.cs.ucl.ac.uk/psipred/>), hits were obtained to a number of LRR domain proteins, including Sds22p.

HTAY is constitutively expressed at low levels

Quantitative microarray analyses (Miao et al. 2009) of *HTAY* expression (*Tetrahymena* Functional Genomics Database [TetraFGD], http://tfgd.ihb.ac.cn/search/detail/gene/TTHERM_01079200) showed that *HTAY* is expressed at a much lower mRNA level than other *Tetrahymena* *HTA* genes (Fig. 2A). Expression of *HTAY* at 9 h of starvation is about two times the level during growth and peaks at 4–6 h of conjugation, when MICs are undergoing rapid meiosis and post-meiotic mitoses, at about five times the level of expression during growth (Fig. 2B). Using RACE, we identified full-length *HTAY* cDNAs from growing cells whose sequences coincided completely with the genomic sequence.

We created strains with the *HTAY* ORF at the *HTAY* locus fused to different tags (HA, Flag, and GFP at either the N terminus or C terminus), but no tagged protein was detectable by Western blotting (WB) of whole-cell extracts (WCE) or immunofluorescence (IF) (data not shown). A polyclonal antibody against a specific peptide in the H2A.Y N terminus recognized an ~43-kDa protein, the expected size for H2A.Y, when large numbers of isolated nuclei (including both MACs and MICs) from 2.5×10^6 cells (Gorovsky et al. 1975) were analyzed by WB (Supplemental Fig. S2). WB showed that H2A.Y was detected at similar levels in different physiological conditions (Fig. 2C). The

H2A.Y antibody failed to detect endogenous H2A.Y by IF analysis in all conditions tested using a number of different fixation methods. These results indicate that *HTAY* is expressed, albeit at low levels, under all physiological conditions analyzed and is likely to be functional.

HTAY is essential

We attempted to create *HTAY* knockout strains by transforming cells with $\Delta htay$ (Fig. 3A, panel a), in which the whole *HTAY* ORF was replaced with the *neo3* cassette (Shang et al. 2002), which confers resistance to paromomycin sulfate (pm). The number of *HTAY* genes in the polycopy MAC could be greatly reduced but not completely replaced in cells growing at the highest pm concentration (500 $\mu\text{g}/\text{mL}$ with 0.5 $\mu\text{g}/\text{mL}$ Cd^{2+}) at which they survive (Fig. 3B, lanes 2,3), indicating that *HTAY* is an essential gene. Incomplete somatic knockout cells growing under these conditions are referred to as $i\Delta htay$, or knockdowns of *HTAY*.

A standard approach to analyze the phenotypes of essential genes in *Tetrahymena* is to study progeny of conjugation between two germline knockout heterokaryon strains, which have wild-type MACs and homozygous knockout MICs, before they die. However we were unable to generate *HTAY* germline knockouts. As an alternative approach, we created a *HTAY* conditional knockout strain ($c\Delta htay$) lacking most, if not all, endogenous *HTAY* genes in the MAC (Fig. 3B, lanes 4,5) and containing an HA₂-tagged *HTAY* gene (*M-HA₂-Y*) (Fig. 3A, panel d) regulated by the Cd^{2+} -inducible *MTT1* promoter (Shang et al. 2002). As noted above, *HTAY* expression during log growth is very low (Fig. 2A), so very little gene product is sufficient for normal function. Because of trace heavy metals in the growth medium, the *MTT1* locus is expressed at very low levels even in the absence of exogenous Cd^{2+} (X Song and MA Gorovsky, unpubl.), and *Tetrahymena* cells accumulate and can retain Cd^{2+} after they are transferred to Cd^{2+} -free medium (T Clark, pers. comm.). Therefore, it was necessary to deplete heavy metals in the growth medium to turn off the *MTT1* promoter in order to determine the true $c\Delta htay$ phenotype. In Cd^{2+} -depleted medium, growth of $c\Delta htay$ cells was inhibited, and few cells survived four serial transfers in Cd^{2+} -depleted medium. Addition of Cd^{2+} after the third transfer rescued the few surviving cells (data not shown). Wild-type cells grew well under the same treatment (data not shown). Upon prolonged starvation, $c\Delta htay$ cells die more rapidly than wild-type cells in the nonpermissive condition (Supplemental Fig. S3A), while addition of Cd^{2+} to starved cells rescued them (Supplemental Fig. S3B). Starvation-induced death also was observed in knockdown $i\Delta htay$ cells (data not shown). These results argue that *HTAY* is an essential gene required for growth and cell viability during starvation. Starvation is a condition that is likely found frequently in *Tetrahymena*'s freshwater environment and is required for the sexual process of conjugation, which occurs often in wild populations (Doerder et al. 1995). These studies also demonstrate that the *M-HA₂-Y* gene is functional.

Figure 1. *T. thermophila* H2A.Y is a novel H2A. (A) Comparison of H2A.Y with the known *T. thermophila* H2As using Clustal X. *Tetrahymena* H2A.X (previously H2A.1, AAC37291), H2A.1 (previously H2A.2, AAC37292), and H2A.Z (hvl, CAA33554) sequences were obtained from GenBank. The red boxes show the five LRR domains, and the green box shows the LRR cap. (B) Alignment of the HFD and the typical N- and C-terminal regions of H2As from different species by Clustal X. Comparable extents (cores) of variants with unusually long tails are also shown. The accession numbers for the different H2As obtained from GenBank are TtH2A.Y (AAU87547; only the histone region, referred to as TtH2A.Ycore), TtH2A.X (previously H2A.1, AAC37291), TtH2A.1 (previously H2A.2, AAC37292), TtH2A.Z (hvl, CAA33554), plasmoh2A (AAA29612), ArabH2A (NP_200275), hH2A.1 (P02261), hH2A.2 (P28001), hH2A.X (P16104), hH2A.Z (P17317), hmH2A1 (AAC33434, human macroH2A.1; only the histone region, referred to as hmH2A1core), GiardiaH2A (AAF00590), and hH2A-Bbd (AAL01652). The domain structure is adapted by permission from Macmillan Publishers Ltd. from Malik and Henikoff (2003); © 2003. The HFD sequences between the vertical red arrows were used for the phylogenetic analysis in C. (C) Phylogenetic tree of the H2A HFDs generated by TreeView.

H2A.Y regulates H3-S10 dephosphorylation

Figure 2. *HTAY* is expressed at low levels in different physiological conditions. (A) Microarray data (TetraFGD, http://tfgd.ihb.ac.cn/search/detail/gene/TTHERM_01079200) showing gene expression patterns of all four *T. thermophila* H2A genes during the three physiological stages: vegetative growth ([L-1] low cell density [100,000 cells per milliliter]; [L-m] medium density [350,000 cells per milliliter]; [L-h] high cell density [1,000,000 cells per milliliter], starvation ([S-0] 0 h; [S-3] 3 h; [S-6] 6 h; [S-9] 9 h; [S-12] 12 h; [S-15] 15 h; [S-24] 24 h), and conjugation ([C-0] 0 h; [C-2] 2 h; [C-4] 4 h; [C-6] 6 h; [C-8] 8 h; [C-10] 10 h; [C-12] 12 h; [C-14] 14 h; [C-16] 16 h; [C-18] 18 h). Note the large difference between the level of expression of *HTAY* and the three other H2A genes. (B) Expanded scale view of microarray data showing gene expression patterns of the *T. thermophila* *HTAY* gene during the three physiological stages as described in A. (C) Nuclei isolated from wild-type CU428 (CU) or B2086 (B) cells in log phase (Log) after 24 h starvation (Sta) or from different time points during conjugation of CU428 × B2086 cells ($\sim 3.5 \times 10^6$ nuclei per lane) were subjected to 12% SDS-PAGE, blotted, and probed with an antibody against a specific peptide in the H2A.Y N terminus. See Supplemental Figure S1 for Ponceau S staining as a loading control.

H2A.Y is in nucleosomes and is complexed with H2B

Because of its unusual structure and the divergence of its HFD from other H2As, we sought to demonstrate that

H2A.Y has properties of a functional histone. Due to the low level of endogenous *H2A.Y* expression, we moderately overexpressed HA₂-tagged *H2A.Y* using the functional *M-HA₂-Y* gene, which can rescue the essential

Figure 3. *HTAY* knockout and rescue strains. (A) *HTAY* knockout and *M-HA₂-Y* rescue constructs were constructed as described in the Supplemental Material. During transformation, homologous recombination occurs in the *HTAY* 5'- and 3'-flanking regions to insert the *neo3* construct (panel a) into the endogenous *HTAY* gene locus (panel b). The *neo2-HA₂-HTAY* fragment (panel d) with a *MTT1* 5'- and 3'-flanking sequence was similarly inserted into the endogenous *MTT1* gene locus (panel c). The primers used in B are shown as black arrows. (B) Genomic PCR from wild-type cells, $\Delta htay$ (Δ) cells grown in medium with the highest pm concentration in which they can survive, or $c\Delta htay$ ($c\Delta$) cells grown in 2 mg/mL pm with 0.5 μ g/mL Cd^{2+} . Primers HTAY far 5', HTAY 999-T-A, and MTT 1848 were used to monitor the *HTAY* and/or *htay::neo3* genes in the cells. Lanes 2 and 3 and lanes 4 and 5 are two clones of $\Delta htay$ or $c\Delta htay$ strains, respectively. Shown are the EB-stained products on 1% agarose gel.

functions of *HTAY*. We created the *M-HA₂-Y* strain containing a wild-type *HTAY* at the endogenous locus and a *HA₂-tagged HTAY* gene at the Cd^{2+} -inducible *MTT1* locus. To determine whether H2A.Y is an H2A-type histone and is in nucleosomes, *M-HA₂-Y* cells were induced by Cd^{2+} for the shortest time that enabled detection of HA-tagged H2A.Y in biochemical fractions. H2A.Y was detected mainly in the chromatin-enriched fraction (P3) and was solubilized by micrococcal nuclease (MNase) digestion (Fig. 4A; Wysocka et al. 2001). The S4 fraction containing mainly di- and mononucleosomes (Fig. 4A) was immunoprecipitated with monoclonal anti-HA-agarose beads and analyzed by WB probed with anti-H2B antibody. H2B coimmunoprecipitated with anti-HA (Fig. 4B) in extracts of *M-HA₂-Y* cells, but not from wild-type cells, indicating that H2A.Y is in a complex with H2B. To show that the complex with H2B was not an artifact of overexpression and/or of the *HA₂* tag, we prepared nuclear extracts from large numbers of isolated nuclei by two different methods and used anti-H2A.Y antibody to immunoprecipitate endogenous H2A.Y (and its associated proteins). For one method (Fig. 4C, panels a–c), nuclei were sonicated and treated with Benzonase (a nuclease that digests both RNAs and DNAs; Sigma), while for the other (Fig. 4C, panels d–f), the nuclei were extracted with 0.4 M NaCl, and the salt extracts were treated with Benzonase and used for the immunoprecipitation. The immunoprecipitate was analyzed by WB. Although there is very little H2A.Y present to interact with the H2B, and the anti-H2A.Y is a relatively weak antibody, it is clear that H2B was coimmunoprecipitated by anti-H2A.Y antibody but not by preimmune serum (Fig. 4C).

MNase-digested nuclei from these *M-HA₂-Y* cells were also run on native PAGE, blotted, and probed with anti-HA. *HA₂-H2A.Y* comigrated with mononucleosomes, the

same as major H2A and H3 (data not shown). H2A.Y was also released from chromatin at salt concentrations similar to that of yeast H2A.Z (although lower than that for most of the major *Tetrahymena* H2A and H2B) (Supplemental Fig S4; Zhang et al. 2005). Taken together, the above evidence argues that H2A.Y functions as a histone.

H2A.Y deposition in MICs is replication-dependent

Using IF, *HA₂-tagged H2A.Y* was detected in MACs of *M-HA₂-Y* cells at all cell cycle stages in log cells as early as 50 min after induction by 0.25 μ g/mL Cd^{2+} (Fig. 5A), indicating that deposition into the MAC is replication-independent. A detailed analysis of H2A.Y and its essential function in transcriptionally active MACs will be presented elsewhere (X Song and MA Gorovsky, unpubl.).

HA₂-H2A.Y was deposited in MICs only in dividing or recently divided cells during vegetative growth (Figs. 5A [panel e], C [panel c]), when MICs are known to be in S phase, which follows immediately after mitosis due to the lack of a distinct G1 phase in *Tetrahymena* MICs (Wu et al. 1988). These results argue that deposition of *HA₂-H2A.Y* into MICs is cell cycle-dependent and is likely restricted to S phase. During conjugation, *HA₂-H2A.Y* also appears in meiotic and prezygotic and post-zygotic mitotic MICs (X Song and MA Gorovsky, unpubl.). The timing of appearance of H2A.Y in meiotic and mitotic MICs is similar to that of MIC-specific H3-S10 phosphorylation (Wei et al. 1998).

The S-phase-specific localization of H2A.Y in MICs suggested that H2A.Y deposition might require MIC DNA synthesis and/or that H2A.Y might function in MIC DNA replication. To test the first possibility, we starved *M-HA₂-Y* cells to stop growth and then refed them in the presence of 20 mM hydroxyurea (HU) for 10 h to completely block DNA synthesis. *HA₂-H2A.Y* synthesis was

Figure 4. H2A.Y exhibits the properties of a nucleosomal H2A histone. (A) Biochemical fractionation was performed from 3.5×10^7 log-phase wild-type or induced *M-HA₂-Y* (0.25 μ g/mL Cd²⁺ for 50 min) cells. P3 fractions were treated with 12 U/mL MNase for different time points as indicated. Different fractions and WCEs were separated on 12% SDS-PAGE, blotted, and probed with anti-HA monoclonal antibody. The 2-min S4 fraction (black box on the bottom right part of A) was determined to have mainly di- and mononucleosomes by analyzing the purified DNA on agarose gels (black box on the bottom left part of A) and was used for the co-IP shown in B. (B) S4 fractions containing mono- and dinucleosomes were mixed with monoclonal anti-HA-agarose beads and subjected to immunoprecipitation. Immunoprecipitation samples (4.8×10^6 cells) and the input (4.8×10^4 cells) were loaded on 12% SDS-PAGE, blotted, and probed with anti-HA monoclonal antibody, anti-H2B antibody, or control anti-polyglutamic acid (Poly-E) antibody (Shang et al. 2002). (C) *Tetrahymena* nuclei (6×10^7) (including MACs and MICs) were sonicated and treated with Benzonase, and the resultant lysate were used to immunoprecipitate endogenous H2A.Y with the anti-H2A.Y peptide antibody (Imm) or preimmune antiserum (Pre) as a control. Immunoprecipitation samples (1.5×10^7 cells) and the input (1.6×10^6 cells) were loaded on 15% SDS-PAGE, blotted, and probed with anti-H2A.Y (panel a) or anti-H2B (panels b,c) antibodies; panel c is a longer exposure of panel b. In another method, the nuclei (6×10^7) were extracted with 0.4 M NaCl, treated with Benzonase, and then used for immunoprecipitation. The immunoprecipitation samples (1.0×10^7 cells) and the input (1.2×10^6 cells) were analyzed by WB and probed with anti-H2A.Y (panel d) or anti-H2B (panels e,f) antibodies; panel f is a longer exposure of panel e.

then induced with 0.25 μ g/mL Cd²⁺ for 50 min. After HU treatment, HA₂-H2A.Y failed to accumulate in S-phase MICs (Fig. 5B, cf. panels a and b), but localized to MACs as in untreated cells (Fig. 5B). This result demonstrated that H2A.Y deposition in MICs (but not in MACs) requires DNA synthesis.

H2A.Y is required for proper MIC division and DNA replication

We analyzed MICs in slowly growing *cΔhtay* cells under nonpermissive growth conditions. Thirty-six percent of dividing cells had delayed or failed MIC mitosis (Fig. 5C, panels b,d), while mitotic defects were detected in only ~8% of dividing cells in wild-type cells or *cΔhtay* cells under permissive conditions (Fig. 5C, panels a,d). Since the MIC division cycle lacks a distinct G1 phase in *Tetrahymena* (Wu et al. 1988), in wild-type *Tetrahymena* cells when MAC division and cytokinesis start, MICs have already finished their mitotic division (Gavin 1965) and have entered S phase. They can be visualized as two DAPI-stained dots, one located near each end of the cell, prior to one MIC entering each daughter cell when cytokinesis is completed (Fig. 5C, panels a,c). In *cΔhtay* cells exhibiting division defects, MICs appeared to be blocked at different stages of mitosis or had failed to divide when the MACs started to divide, and sometimes both daughter MICs entered one daughter cell (Fig. 5C, panel b). Consistent with these apparent segregation defects, ~10% of *cΔhtay* cells grown in Cd²⁺-depleted medium lack MICs (data not shown). These observations indicate that *HTAY* plays a role in MIC division.

To investigate the relationship of MIC division defects in *cΔhtay* cells with DNA replication, log-phase wild-type or *cΔhtay* cells grown in Cd²⁺-depleted medium were incubated with 200 μ M BrdU for 1.5 h and then stained with α -BrdU monoclonal antibody. Under the nonpermissive condition, no BrdU incorporation was detected in the MICs of *cΔhtay* cells that exhibited division defects (data not shown). In >90% of the *cΔhtay* cells with more normal appearing, recently divided MICs, there was either no or less BrdU staining than in wild-type cells (Fig. 5D, cf. panels a–c and e–g), suggesting that DNA synthesis is slowed or blocked in these cells. Coupled with the observations that H2A.Y is deposited into MICs during S phase, these results argue that H2A.Y is a micronuclear replication-coupled histone whose deposition is required for efficient MIC DNA replication. This effect of H2A.Y depletion on DNA replication is specific to MICs, since BrdU incorporation into MACs was not affected (Fig. 5D, panels d,h).

H2A.Y is involved in regulation of histone H3-S10 dephosphorylation after mitosis

The defects in MIC mitotic division caused by H2A.Y depletion are similar to the phenotype of Sds22p depletion in yeast (Hisamoto et al. 1995) and to that of a histone H3 mutation (S10E) that mimics constitutive S10-P in mammalian cells (Ota et al. 2002). Since Sds22p

has been suggested to play a role in the regulation of H3-S10 dephosphorylation, and H2A.Y is deposited only in S-phase MICs of vegetative cells, around the time when *Tetrahymena* S10-P starts to disappear (Wei et al. 1998), we hypothesized that H2A.Y, like Sds22p, plays a role in regulation of H3-S10 dephosphorylation. To test this hypothesis, we compared anti-S10-P antibody staining of MICs in dividing wild-type and *cΔhtay* cells under nonpermissive conditions (Fig. 6A–C). *cΔhtay* and wild-type cells have similar maximal levels of H3-S10 phosphorylation at metaphase/early anaphase (Fig. 6, panels

c,d,n). In anaphase, the H3-S10 phosphorylation signal appears to decrease a little in wild-type cells; however, the difference is not significant compared with that in the *cΔhtay* cells (Fig. 6, panels e,f,o). Strikingly, the normal-appearing, recently divided (S-phase) MICs of *cΔhtay* cells (selected because they have normal shapes and appear to have normal DNA contents, allowing comparison with the euploid wild-type MICs) (see Supplemental Fig. S5 for division defective MICs) stained more strongly than the S-phase MICs of wild-type cells (Fig. 6, panels g–j,p). As noted previously, the MIC division cycle lacks a distinct G1 phase in *Tetrahymena*, and MICs enter S phase immediately after mitosis (Wu et al. 1988). Thus, while S10 phosphorylation is normally reduced after wild-type MICs complete mitosis, the *cΔhtay* cells under nonpermissive conditions appear to retain the higher mitotic levels of H3-S10-P, suggesting that there is less S10 phosphatase activity in *cΔhtay* cells than in wild-type cells when cells exit mitosis.

MICs in *cΔhtay* cells in G2 phase (Fig. 6, panels k,l,q) and early mitosis (Fig. 6, panels a,b,m), also have higher levels of S10 phosphorylation than wild-type cells at similar stages, probably due to retention of the S10 phosphorylation from the preceding mitosis. We conclude that *HTAY* has little effect on the maximal level of H3-S10-P reached in mitotic MICs, but plays a positive role in the normal dephosphorylation of S10 as MICs exit mitosis. In contrast, cells in which one of the two major *Tetrahymena*

Figure 5. H2A.Y deposition in MICs is replication-dependent and is required for normal MIC division. (A, panels a–e) Log-phase *M-HA₂-Y* cells were treated with 0.25 μ g/mL Cd²⁺ for 50 min. Cells were fixed and stained with anti-HA monoclonal antibody and DAPI. Shown are cells with MICs in G2 phase (panel a), prophase (panel b), metaphase/early anaphase (panel c), anaphase (panel d), and S phase (panel e). MICs are indicated with white arrowheads. (B, panel a) *M-HA₂-Y* cells starved for 25 h were refed with 1 $\times$ SPP containing 20 mM HU and grown for 10 h, then induced with 0.25 μ g/mL Cd²⁺ before being stained with anti-HA monoclonal antibody and DAPI. (Panel b) The same cell treatment with no HU added. MICs are indicated with white arrowheads. (C) Wild-type (panel a) or *cΔhtay* (panel b) cells grown in Cd²⁺-depleted medium for 15 h (to a density of 1.4×10^5 cells per milliliter) were fixed and stained with DAPI. Shown are representative dividing cells. (Panel c) Stages of *Tetrahymena* vegetative cell cycle (adapted from Wang and Allis 1992 by permission of Oxford University Press). Nuclei in S phase are shaded. (Panel d) The percentage of MIC division defects (delays or failures) in wild-type or *cΔhtay* cells with or without Cd²⁺. (D) Wild-type (panels a–d) or *cΔhtay* (panels e–h) cells were grown in Cd²⁺-depleted medium to a density of 2.2×10^5 cells per milliliter, then grown another 1.5 h after addition of BrdU to final 200 μ M. Cells were fixed and reacted with anti-BrdU monoclonal antibody. Shown are dividing cells when MICs are visualized as two DAPI-stained dots near the ends of the cell and should be in S phase (panels a–c,e–g), and nondividing cells when MACs are in S phase (panels d,h). MICs are indicated with white arrowheads. Images in A were obtained using a confocal microscope. Images in B–D were obtained using an Olympus BH-2 fluorescence microscope. Bars: A, 8 μ m, B–D, 10 μ m.

Figure 6. H2A.Y or its N terminus attached to H2A.X is required for efficient dephosphorylation of H3-S10 after mitosis. (A–C) Wild-type (A) or *cΔhtay* (B) cells were grown in Cd²⁺-depleted medium to log phase, fixed, and stained with anti-S10-P antibody, which was preincubated with (unphosphorylated) H3 peptide for 1 h at 37°C. Shown are cells with MICs in prophase (panels *a,b*), metaphase/early anaphase (panels *c,d*), anaphase (panels *e,f*), S phase (panels *g–j*), and G2 phase (panels *k,l*). (C) S10-P intensities (panels *m–p*) or percentages of S10-P-positive interphase cells (panel *q*; *n* = 100) of wild type and *cΔhtay*. Bar, 10 μ m. The ImageJ program was used to measure mean intensities (arbitrary units) from 10 different S10-P-stained MICs, which were averaged and plotted with Excel (error bars show the standard deviation, some of which are too small to see). See also Supplemental Figure S5. (D) The *YN-HTAX* construct with a HA-tagged *HTAY* N terminus connected to the *HTAX* gene with *MTT1*-flanking sequences was inserted into the *MTT1* locus (see the Supplemental Material for details). (E) After selection in 30 mg/mL pm with 0.5 μ g/mL Cd²⁺, *YN-HTAX* single-cell transformant lines were assayed for the ability of the chimeric *YN-HTAX* gene in the *MTT1* locus to rescue *HTAY* function. PCR of genomic DNA with primers in the flanking regions of the *HTAY* gene (*HTAY* far 5'f and *HTAY* 3'f R) (see Fig. 3A, panel b) revealed almost complete elimination of the *HTAY* gene and replacement with the *htay::neo3* knockout allele at the endogenous *HTAY* locus in the transformants (#1, #3, and #7). Wild-type (WT) cells had only the *HTAY* band, *cΔhtay* cells (*cΔ*) had only the knockout band, and the original host cells for the transformation, *iΔhtay* (*iΔ*), had both bands present. Shown are the EB-stained products on 1% agarose gel. (F) Using the same staining conditions as in B, *YN-HTAX* cells grown in Cd²⁺-depleted medium (thus reducing expression of the N terminus of *HTAY*) showed a larger number of anti-S10-P-stained MICs than when the cells were grown with Cd²⁺. (G) Separate *YN-HTAX* cell lines were transferred in medium with or without Cd²⁺ for 6 d, fixed, and stained with DAPI and anti-S10-P as described above. DAPI-stained MACs were counted to give the total cells present in a field, then the anti-S10-P-stained MICs were counted for the same field. Several field results were combined, with at least 650–900 cells total for each sample used to determine the percentage stained.

mena H2A genes (*HTA1*) (Liu and Gorovsky 1996) was knocked out failed to exhibit detectable differences in S10-P staining compared with wild-type cells (Supplemental Fig. S6), demonstrating the specificity of this phenotype for H2A.Y deficiency.

The N-terminal tail of H2A.Y regulates post-mitotic dephosphorylation of H3-S10

To determine whether it is the Sds22p-like N terminus of H2A.Y that regulates micronuclear H3-S10 dephosphorylation, we created a gene encoding a chimeric H2A protein consisting of the N-terminal amino acids 1–258 of H2A.Y connected to histone H2A.X (amino acids 1–138). Note that H2A.X is one of the two *Tetrahymena* major, replication-dependent, H2A variants found in both MACs and MICs. The DNA sequence coding for this chimeric protein was used to replace the *HTAY* coding region in *M-HA₂-Y*, creating *YN-HTAX*, which has two HA tags and *MTT1*-flanking sequences (Fig. 6D). This construct was then used for transformation of strain *iΔhtay*, which contains the incomplete knockdown of *HTAY*, thereby inserting the *YN-HTAX* chimeric gene into the Cd²⁺-inducible *MTT1* locus (Fig. 6D). If the YN-H2A.X protein (generated from the *MTT1* locus) can replace the essential function of wild-type H2A.Y, it should be possible for the transformants to grow in Cd²⁺-containing medium at higher pm concentrations than the host *iΔhtay* strain, since the remaining copies of the wild-type *HTAY* will no longer be essential, enabling the *neo3* knockout allele to replace the wild-type allele at the endogenous *HTAY* locus as a result of phenotypic assortment. The original *iΔhtay* cells were only able to grow in 0.5 μg/mL Cd²⁺ medium at 500 μg/mL pm. Strikingly, after serial passage at increasing concentrations of pm, the *YN-HTAX* transformants could be grown in medium with 0.5 μg/mL Cd²⁺ at 30 mg/mL pm. PCR analysis of DNA isolated from these transformants indicated that their wild-type *HTAY* had been almost completely replaced by the *neo3* knockout cassette (Fig. 6E). This result shows that the essential function of H2A.Y resides in the nonhistone N-terminal tail.

Since these *YN-HTAX* cells contain mainly the YN-H2A.X protein produced by the chimeric N terminus *HTAY-HTAX* gene that is regulated by the Cd²⁺-inducible *MTT1* promoter, we could compare micronuclear H3-S10-P of these cells grown in permissive medium (with Cd²⁺) with those grown in nonpermissive (Cd²⁺-depleted) medium where the YN-H2A.X protein is depleted, as had been done to study the effects of depletion of H2A.Y using the *cΔhtay* cells. Three separate *YN-HTAX* cell lines were serially transferred in medium with 0.5 μg/mL Cd²⁺ or in Cd²⁺-depleted medium for 6 d, and then staining of MICs with the anti-S10-P antibody was examined. As was seen for the *cΔhtay* cells, when cells were grown without Cd²⁺, there is a marked increase in the fraction of cells showing S10-P staining as compared with cells grown in Cd²⁺ or with wild-type cells (Fig. 6F, G). Some of the *YN-HTAX* cells grown without Cd²⁺ also exhibited abnormal micronuclear division patterns similar to those

seen when H2A.Y was deficient in the *cΔhtay* cells. Thus, the Sds22p-like H2A.Y N terminus, when attached to another H2A isoform, can replace the function of the full-length endogenous H2A.Y, and its absence results in retention of the H3-S10-P. The simplest interpretation of these results is that the H2A.Y N terminus is acting in MICs by facilitating the activity of a phosphatase that removes phosphate groups from H3-S10 at the end of mitosis.

H2A.Y interacts specifically with a Tetrahymena ortholog of yeast Glc7p

Sds22p plays a role in the regulation of H3 dephosphorylation by interacting with Glc7p, the only PP1 in yeast (Hsu et al. 2000). Thus, if H2A.Y functions by regulating PP1, it should be possible to demonstrate a physical interaction between these two proteins. A BLAST search of the *Tetrahymena* genome (TGD, <http://www.ciliate.org>) using yeast Glc7p as the query sequence identified a number of candidates with strong sequence homology. All corresponded to predicted *Tetrahymena* genes, except that two adjacent ORFs, identified as TTHERM_00865290 and TTHERM_00865300 in the TGD, were actually parts of a single gene whose sequences could be combined into a 2055-bp gene encoding a predicted ~36-kDa protein, with several regions showing complete identity to both yeast and worm Glc7-like protein phosphatases (Supplemental Fig. S7A). We named this gene *PPO1*. Phylogenetic analysis indicated that conceptual translation products of the two top BLAST hits, TTHERM_00238830 and *PPO1*, clustered with Glc7p and three *C. elegans* homologs (Supplemental Fig. S7B). A tagged version of the TTHERM_00238830 protein product localized only to the nucleolar regions of the MAC (data not shown) and was not studied further. An HA-tagged conceptual translation product of the *PPO1* gene (Ppo1p) did not specifically stain either the MAC or the MIC in vegetative cells, but the significant level of cytoplasmic staining made it impossible to rule out the possibility there were low levels of nuclear staining (data not shown).

Because of the close phylogenetic relationship between Ppo1p and Glc7 (Supplemental Fig. S7B), we sought to use coimmunoprecipitation (co-IP) to demonstrate a physical association between H2A.Y and Ppo1p. A peptide-specific polyclonal antibody against the C terminus of Ppo1p (Supplemental Fig. S8; Supplemental Material) was used to stain WBs of co-IP experiments from moderately induced *M-HA₂-Y* cells. Following the fractionation scheme used to obtain the results in Figure 4, we were unable to detect Ppo1p in the HA-IP-bound fraction. Since we kept induction of the tagged HA₂-H2A.Y to a minimum and its interaction with the protein phosphatase is probably transient, we repeated this experiment using formaldehyde fixation, which was shown previously in *Tetrahymena* to detect proteins that interact dynamically in MICs (Wang and Allis 1993). *M-HA₂-Y* cells were formaldehyde-fixed (Sutherland et al. 2008; Smart et al. 2009), sonicated to release chromatin-bound proteins, and combined with

anti-HA agarose for immunoprecipitation. Using this method, Ppo1p coimmunoprecipitated with HA-tagged H2A.Y during vegetative growth when we used anti-HA antibody in the immunoprecipitation procedure (Fig. 7A). In a reciprocal procedure, HA-tagged H2A.Y could also be coimmunoprecipitated with Ppo1p when we used anti-Ppo1p immune serum, while preimmune serum failed to precipitate HA₂-H2A.Y (Fig. 7B). These results argue that this *Tetrahymena* Glc7p ortholog likely interacts with HA₂-H2A.Y. Formaldehyde fixation co-IP experiments in vegetative *Tetrahymena* cells overexpressing HA-tagged *Ichthyophthirius multifiliis* asparaginyl endopeptidase (Asn-E) from the *MTT1* locus did not detect co-IP of Ppo1p (Fig. 7A), indicating a specific interaction between H2A.Y and Ppo1p. Interaction between H2A.Y and Ppo1 is also observed by co-IP in 9-h-conjugating cells (Supplemental Fig. S9), when MICs exit post-zygotic divisions (Ray 1956; Sugai and Hiwatashi 1974; Martindale et al. 1982).

Discussion

Several lines of evidence argue that *Tetrahymena* H2A.Y is a newly identified H2A variant. It contains a HFD that is phylogenetically related to those of other H2As with which it shares a large number of completely conserved residues (Fig. 1) and it complexes with H2B. Functional HA-tagged, moderately overexpressed H2A.Y localizes

specifically to nuclei, coimmunoprecipitates with H2B, and comigrates with solubilized mononucleosomes. It is released from chromatin at salt concentrations similar to that of yeast H2A.Z. H2A.Y differs from most other H2As by having a long, nonhistone sequence at its N terminus, which has five LRRs, a binding motif involved in protein-protein interactions (Kobe and Kajava 2001), and a LRR cap domain (Ceulemans et al. 1999). Thus, *Tetrahymena* H2A.Y joins macroH2A as the only chimeric H2As whose nonhistone parts contain a known structural motif (Karras et al. 2005).

Due to its low expression, we were unable to localize H2A.Y expressed from the *HTAY* locus using IF, either by protein tagging or with an H2A.Y antibody. However, microarray analyses and WB of heavily loaded nuclear proteins detected the endogenous RNA and protein (Fig. 2), and *HTAY* depletion is lethal, arguing that it is a functional gene.

We were able to localize moderately overexpressed, tagged H2A.Y and believe that this localization reflects the wild-type condition. First, all localizations were done at the earliest induction stage at which the overexpressed protein was first detectable by IF, minimizing the likelihood that incorrectly bound protein was being observed. Second, the overexpressed HA₂-*HTAY* gene is functional—it can rescue the essential functions of the *HTAY* gene, making cells dependent on its conditional expression for normal growth and survival during starvation. Third,

Figure 7. H2A.Y interacts with Ppo1p in vivo. (A) Cd²⁺-induced *M-HA₂-Y* and *Asn-E* cells (CU428 cells expressing HA-tagged *I. multifiliis* asparaginyl endopeptidase from the *MTT1* locus) were formaldehyde-fixed and coimmunoprecipitated with anti-HA agarose. (Left) Input and immunoprecipitation samples were run on 12% SDS-PAGE, blotted, and probed with rabbit anti-Ppo1p antibody. (Right) The blot was stripped and stained with rabbit anti-HA antibody. See Supplemental Figure S8 and the Supplemental Material. (B) Cd²⁺-induced *M-HA₂-Y* cells were formaldehyde-fixed and coimmunoprecipitated with 3, 5, or 7 μ L of anti-Ppo1p immune or preimmune antiserum. (Left) Input and immunoprecipitation samples were run on 12% SDS-PAGE, blotted, and probed with mouse anti-HA monoclonal antibody and donkey anti-mouse HRP secondary antibody. (Right) The blot was stripped and stained with rabbit anti-Ppo1p antibody with goat anti-rabbit HRP secondary antibody. See the Supplemental Material for details.

continuous overexpression of the HA₂-HTAY gene did not produce any detectable abnormal phenotypes (data not shown). Fourth, the properties of overexpressed HA₂-H2A.Y are those expected of histone H2A. Fifth, we performed similar localization studies using an overexpressed *HTAY-Flag* gene and observed the same localization as HA₂-H2A.Y (data not shown). Sixth, the MIC-specific localization of HA₂-H2A.Y during MIC S phase correlates with the MIC phenotypes observed when *HTAY* is depleted.

We noted a similarity between the long N terminus of H2A.Y and yeast Sds22p, which, in yeast, complexes with the PP1 catalytic subunit Glc7p and localizes it to nuclei (Hsu et al. 2000). Based on the timing of H2A.Y deposition in post-mitotic S-phase MICs when S10 is being dephosphorylated (Wei et al. 1998), and on the properties of Sds22p, we hypothesized that, like Sds22p, H2A.Y functions to regulate mitotic dephosphorylation of H3-S10 and that its N-terminal tail is responsible for this function. We obtained a number of experimental results that support this hypothesis. First, we showed that depletion of the *HTAY* gene resulted in reduced H3-S10 dephosphorylation in MICs exiting mitosis and in mitotic segregation defects that are consistent with the function of H3-S10-P determined by mutational analyses in *Tetrahymena* (Wei et al. 1999) and in hamsters (Ota et al. 2002). Second, we showed that when a gene encoding the N terminus of H2A.Y attached to H2A.X was used to transform a partially deleted *HTAY* strain, it enabled complete deletion of the essential *HTAY* gene. Third, we showed that when this chimeric *YN-HTAX* gene was placed under the regulation of the inducible *MTT1* gene (in cells lacking the endogenous *HTAY* gene), its reduced expression in the absence of Cd²⁺ greatly inhibited normal post-mitotic dephosphorylation of H3 at S10. Finally, using reciprocal co-IP, we demonstrated a physical interaction of H2A.Y with Ppo1p, a *Tetrahymena* ortholog of Glc7p. In MICs of growing cells, H2A.Y deposition requires MIC DNA replication. Given the absence of a distinct G1 phase in the *Tetrahymena* MIC division cycle, this replication-coupled deposition ensures that H2A.Y will be present in MICs as they exit mitosis, providing a novel, cell cycle-dependent mechanism for targeting a regulator of histone modification to MICs when it is needed to function in dephosphorylating H3-S10 after mitosis. Interestingly, deposition of H2A.Y in MACs is not dependent on DNA synthesis, indicating that H2A.Y can also function as a replication-independent variant.

It should be noted that there is an alternative hypothesis that the Sds22p-like tail of H2A.Y and its associated phosphatase could regulate the kinase activity by regulating phosphorylation of the kinase itself, as has been shown for kinetochore-associated aurora B activity in mammalian cells (Posch et al. 2010). In this scenario, depletion of H2A.Y would result in failure of the H3 kinase to be dephosphorylated at the end of mitosis, causing it to remain active. Our results cannot distinguish between these mechanisms (or other possible indirect mechanisms) and the more direct one we proposed above. We favor the direct mechanism because H2A.Y is

located in nucleosomes, in proximity to the H3 substrate, and because depletion of H2A.Y had no detectable effect on the timing or extent of MIC H3 phosphorylation during mitosis, only on its dephosphorylation.

We provided evidence that *Tetrahymena* H2A.Y contains two distinct domains: one that likely confers the properties of a typical histone, and another that likely functions to localize or activate an H3-S10-P phosphatase specifically in the MIC. Vertebrate histone macroH2A also contains two domains: a HFD and a macrodomain. The macrodomains of macroH2A isoforms likely play both ligand-independent and ligand-dependent roles in the cell (Gamble and Kraus 2010). The properties of H2A.Y and macroH2A suggest that the formation of H2A variants by fusion of a typical H2A and a functionally distinct nonhistone protein has occurred at least twice in evolution and could represent a general mechanism for either targeting a nonhistone function to chromatin or adding new functionality to histones. Another example of dual functionality in a single histone is CENP-A (CenH3), whose conserved C terminus has been shown to be necessary and sufficient for centromere and kinetochore protein recruitment and function (Guse et al. 2011) and whose CENP-A targeting domain is required for new CENP-A histone assembly (Black et al. 2004). This suggests that other chimeric histones might also represent evolutionary fusions between histone and nonhistone domains with highly specific functions that act, directly or indirectly, on other histones.

Histone “cross-talk” (Fischle et al. 2003) has generally referred to signaling mechanisms in which a particular post-translational modification on a histone acts either in *cis* or in *trans* to affect other histone modifications. The studies described here provide a novel example of cross-talk in vivo in which a specific core histone (H2A) variant plays a role in regulating the cell cycle-dependent phosphorylation level of another core histone (H3). Although vertebrate histone macroH2A can bind ADP-ribose and inhibit transcription initiation by affecting both histone acetylation and nucleosome remodeling in vitro (Gamble and Kraus 2010; Talbert and Henikoff 2010), *Tetrahymena* H2A.Y is the first chimeric histone variant for which a specific effect on a histone modification has been demonstrated in vivo.

Materials and methods

Strains, culture conditions, and viability assay

Wild-type strains CU428 and B2086 were provided by P.J. Bruns (Cornell University). The Asn-E strain, CU428 cells expressing HA-tagged *I. multifiliis* asparaginyl endopeptidase from the *MTT1* locus, was provided by Donna Cassidy-Hanley (Cornell University). Cells were grown in 1× SPP (1% proteose peptone) medium with shaking at 30°C (Gorovsky et al. 1975). Cells were counted using a cell counter (Coulter Electronics, Inc.). For starvation, growing cells were washed and resuspended in 10 mM Tris (pH 7.5) for 24 h at 30°C without shaking unless specified otherwise. Standard conjugation methods were used (Bruns and Brussard 1974). For H2A.Y depletion in strains *cΔhtay* and *YN-HTAX*, heavy metals were removed from SPP medium using

Chelex 100 resin (D Dave and J Gaertig, pers. comm.). For BrdU incorporation, 200 μ M BrdU (Sigma) was added to log-phase cells ($\sim 2 \times 10^5$ cells per milliliter) and incubated for an additional 1.5 h. To assay viability, 70 μ L of growing or starved cells was mixed with 30 μ L of trypan blue stock (0.4% in H₂O; Sigma), ≥ 300 cells were counted, and the fraction of intact, uniformly stained (dead) cells was determined.

Gene replacement

See the Supplemental Material for the details of methods used to create *HTAY* over-expression (*M-HA₂-Y*) cells, knockdown (*iΔhtay*) cells, conditional knockout cells (*cΔhtay*), and *YN-HTAX*-containing cells. It is possible to perform DNA-mediated biolistic transformation (Cassidy-Hanley et al. 1997) of either MACs (somatic) or MICs (germline) in *T. thermophila*. Almost all transformation in *Tetrahymena* occurs by homologous integration. Since MACs are polycopy and divide amitotically, when a selectable marker is transformed into a MAC chromosome, it will be assorted randomly during vegetative division (phenotypic assortment), and cells containing increased numbers of the transformed gene can be selected. For an essential gene, the wild-type copies in the MAC cannot be completely replaced by a nonfunctional allele, providing a test for essential genes.

Primer sequences

For primer sequences, see the Supplemental Material.

Biochemical fractionation, chromatin isolation, and co-IP

M-HA₂-Y cells grown to 1.3×10^5 cells per milliliter in $1 \times$ SPP medium were exposed to 0.25 μ g/mL Cd²⁺ for 50 min. Cells (3.5×10^7) were collected and washed with 10 mM Tris (pH 7.5). Biochemical fractionation and MNase (Worthington) treatment were performed as described (Wysocka et al. 2001) with modifications (details in the Supplemental Material). Aliquots of fractions S2, S3, and P3 (5×10^5 cells), and S4 and P4 (2.5×10^5 cells) from *M-HA₂-Y* cells were analyzed by WB with anti-HA monoclonal antibody. For the co-IP assays, the S4 fraction containing di- and mononucleosomes was mixed with 40 μ L of monoclonal anti-HA-agarose beads (Sigma) and nutated overnight at 4°C. Beads were washed and eluted, and concentrated immunoprecipitation samples (4.75×10^6 cells) and 1.6% of input (7.5×10^4 cells) were separated on 12% SDS-PAGE and subjected to WB with anti-H2B antibody (1:2,000; a gift from Dr. C.D. Allis) or anti-HA monoclonal antibody (1:5000; clone 16B12, Covance Research Products). Blotting and immunostaining procedures were as described (Gallagher et al. 2004). Wild-type cells were treated the same as *M-HA₂-Y* cells, except no Cd²⁺ was added.

For the co-IP of endogenous H2A.Y and associated proteins, lysates of nuclei from 3×10^7 cells were prepared by salt extraction or by sonication and used with protein A agarose for co-IP with 10 μ L of either anti-H2A.Y immune or preimmune serum. See the Supplemental Material for details. Fifteen percent SDS-PAGE and WB were as described above.

Formaldehyde fixation and co-IP of Ppo1p

Asn-E cells, expressing an HA-tagged asparaginyl endopeptidase protein as a negative control, were grown overnight with 1.0 μ g/mL Cd²⁺, and growing *M-HA₂-Y* cells were induced with 0.5 μ g/mL Cd²⁺ for 1 h. The cross-linking and co-IP procedures are modifications of a published method (Smart et al. 2009) and are detailed in the Supplemental Material. Since the amounts of HA-H2A.Y in the immunoprecipitation samples were much

greater than the Ppo1p, two concentrations of each immunoprecipitation sample were loaded on the same 12% acrylamide SDS-PAGE gel used for WB shown in Figure 7A. The blot was first stained with anti-Ppo1p antibody and then stripped and stained with rabbit anti-HA antibody. See the Supplemental Material for gel loading and WB conditions.

Immunocytochemistry

For immunocytochemistry, see the Supplemental Material.

Anti-H2A.Y and anti-Ppo1p antibodies

For the anti-H2A.Y and anti-Ppo1p antibodies used, see the Supplemental Material.

Acknowledgments

We thank Drashti Dave and Dr. Jacek Gaertig (University of Georgia) for the method for depleting growth medium of heavy metals, Dr. Theodore Clark (Cornell University) for information on retention of Cd²⁺ in *Tetrahymena* cells, Dr. C. David Allis (Rockefeller University) for providing the anti-H2A and anti-H2B antibodies, Dr. Andreas Mayer (Université de Lausanne, Switzerland) for providing anti-yeast Glc7p antibody for initial analysis of H2A.Y-PP1 interaction, Dr. Donna Cassidy-Hanley (Cornell University) for discussion and providing the Asn-E strain, Dr. Janna Bednenko (University of Rochester) for assistance with the chromatin immunoprecipitation protocol, Dr. Zhe Wang for providing RLM-RACE cDNA, Dr. Subhojit Sen (NIH) for helpful suggestions on native PAGE gel transfer and salt washes of nuclei, and Deana Benson (UCSD) for assistance with generating figures. This work was supported by NIH grants GM 021793 and GM 072752.

References

- Banaszynski LA, Allis CD, Lewis PW. 2010. Histone variants in metazoan development. *Dev Cell* **19**: 662–674.
- Bannister AJ, Kouzarides T. 2011. Regulation of chromatin by histone modifications. *Cell Res* **21**: 381–395.
- Bannon GA, Calzone FJ, Bowen JK, Allis CD, Gorovsky MA. 1983. Multiple, independently regulated, polyadenylated messages for histone H3 and H4 in *Tetrahymena*. *Nucleic Acids Res* **11**: 3903–3917.
- Black BE, Foltz DR, Chakravarthy S, Luger K, Woods VL Jr, Cleveland DW. 2004. Structural determinants for generating centromeric chromatin. *Nature* **430**: 578–582.
- Bruns PJ, Brussard TB. 1974. Pair formation in *Tetrahymena pyriformis*, an inducible developmental system. *J Exp Zool* **188**: 337–344.
- Buschbeck M, Uribealago I, Wibowo I, Rue P, Martin D, Gutierrez A, Morel L, Guigo R, Lopez-Schier H, Di Croce L. 2009. The histone variant macroH2A is an epigenetic regulator of key developmental genes. *Nat Struct Mol Biol* **16**: 1074–1079.
- Cassidy-Hanley D, Bowen J, Lee J, Cole ES, VerPlank LA, Gaertig J, Gorovsky MA, Bruns PJ. 1997. Germline and somatic transformation of mating *Tetrahymena thermophila* by particle bombardment. *Genetics* **146**: 135–147.
- Ceulemans H, De Maeyer M, Stalmans W, Bollen M. 1999. A capping domain for LRR protein interaction modules. *FEBS Lett* **456**: 349–351.
- Costanzi C, Pehrson JR. 1998. Histone macroH2A1 is concentrated in the inactive X chromosome of female mammals. *Nature* **393**: 599–601.

- Coyne RS, Chalker DL, Yao MC. 1996. Genome downsizing during ciliate development: Nuclear division of labor through chromosome restructuring. *Annu Rev Genet* **30**: 557–578.
- Doerder FP, Gates MA, Eberhardt FP, Arslanyolu M. 1995. High frequency of sex and equal frequencies of mating types in natural populations of the ciliate *Tetrahymena thermophila*. *Proc Natl Acad Sci* **92**: 8715–8718.
- Eberharther A, Becker PB. 2004. ATP-dependent nucleosome remodelling: Factors and functions. *J Cell Sci* **117**: 3707–3711.
- Eberharther A, Ferreira R, Becker P. 2005. Dynamic chromatin: Concerted nucleosome remodelling and acetylation. *Biol Chem* **386**: 745–751.
- Fischle W, Wang Y, Allis CD. 2003. Histone and chromatin cross-talk. *Curr Opin Cell Biol* **15**: 172–183.
- Flaus A, Owen-Hughes T. 2011. Mechanisms for ATP-dependent chromatin remodelling: The means to the end. *FEBS J* **278**: 3579–3595.
- Gallagher S, Winston SE, Fuller SA, Hurrell JG. 2004. Immunoblotting and immunodetection. *Curr Protoc Mol Biol* **83**: 10.8.1–10.8.24.
- Gamble MJ, Kraus WL. 2010. Multiple facets of the unique histone variant macroH2A: From genomics to cell biology. *Cell Cycle* **9**: 2568–2574.
- Gamble MJ, Frizzell KM, Yang C, Krishnakumar R, Kraus WL. 2010. The histone variant macroH2A1 marks repressed autosomal chromatin, but protects a subset of its target genes from silencing. *Genes Dev* **24**: 21–32.
- Gavin RH. 1965. The effects of heat and cold on cellular development in synchronized *Tetrahymena pyriformis* WH-6. *J Protozool* **12**: 307–318.
- Gonzalez-Romero R, Mendez J, Ausio J, Eirin-Lopez JM. 2008. Quickly evolving histones, nucleosome stability and chromatin folding: All about histone H2A. *Bbd. Gene* **413**: 1–7.
- Gorovsky MA. 1973. Macro- and micronuclei of *Tetrahymena pyriformis*: A model system for studying the structure and function of eukaryotic nuclei. *J Protozool* **20**: 19–25.
- Gorovsky MA. 1980. Genome organization and reorganization in *Tetrahymena*. *Annu Rev Genet* **14**: 203–239.
- Gorovsky MA, Yao M-C, Keevert JB, Pleger GL. 1975. Isolation of micro- and macronuclei of *Tetrahymena pyriformis*. *Methods Cell Biol* **IX**: 311–327.
- Guse A, Carroll CW, Moree B, Fuller CJ, Straight AF. 2011. In vitro centromere and kinetochore assembly on defined chromatin templates. *Nature* **477**: 354–358.
- Hamilton EP, Orias E. 2000. Genetically mapping new mutants and cloned genes. *Methods Cell Biol* **62**: 265–280.
- Hisamoto N, Frederick DL, Sugimoto K, Tatchell K, Matsumoto K. 1995. The EGP1 gene may be a positive regulator of protein phosphatase type 1 in the growth control of *Saccharomyces cerevisiae*. *Mol Cell Biol* **15**: 3767–3776.
- Hsu JY, Sun ZW, Li XM, Reuben M, Tatchell K, Bishop DK, Grushcow JM, Brame CJ, Caldwell JA, Hunt DF, et al. 2000. Mitotic phosphorylation of histone H3 is governed by Ip11/aurora kinase and Glc7/PP1 phosphatase in budding yeast and nematodes. *Cell* **102**: 279–291.
- Ishibashi T, Li A, Eirin-López JM, Zhao M, Missiaen K, Abbott DW, Meistrich M, Hendzel MJ, Ausió J. 2010. H2A.Bbd: An X-chromosome-encoded histone involved in mammalian spermiogenesis. *Nucleic Acids Res* **38**: 1780–1789.
- Kapoor A, Goldberg MS, Cumberland LK, Ratnakumar K, Segura MF, Emanuel PO, Menendez S, Vardabasso C, Leroy G, Vidal CI, et al. 2010. The histone variant macroH2A suppresses melanoma progression through regulation of CDK8. *Nature* **468**: 1105–1109.
- Karras GI, Kustatscher G, Buhecha HR, Allen MD, Pugieux C, Sait F, Bycroft M, Ladurner AG. 2005. The macro domain is an ADP-ribose binding module. *EMBO J* **24**: 1911–1920.
- Kobe B, Kajava AV. 2001. The leucine-rich repeat as a protein recognition motif. *Curr Opin Struct Biol* **11**: 725–732.
- Liu X, Gorovsky MA. 1993. Mapping the 5' and 3' ends of *Tetrahymena thermophila* mRNAs using RNA ligase mediated amplification of cDNA ends (RLM-RACE). *Nucleic Acids Res* **21**: 4954–4960.
- Liu X, Gorovsky MA. 1996. Cloning and characterization of the major histone H2A genes completes the cloning and sequencing of known histone genes of *Tetrahymena thermophila*. *Nucleic Acids Res* **24**: 3023–3030.
- MacKelvie SH, Andrews PD, Stark MJ. 1995. The *Saccharomyces cerevisiae* gene *SDS22* encodes a potential regulator of the mitotic function of yeast type 1 protein phosphatase. *Mol Cell Biol* **15**: 3777–3785.
- Malik HS, Henikoff S. 2003. Phylogenomics of the nucleosome. *Nat Struct Biol* **10**: 882–891.
- Margueron R, Trojer P, Reinberg D. 2005. The key to development: Interpreting the histone code? *Curr Opin Genet Dev* **15**: 163–176.
- Martindale DW, Allis CD, Bruns PJ. 1982. Conjugation in *Tetrahymena thermophila*. A temporal analysis of cytological stages. *Exp Cell Res* **140**: 227–236.
- Miao W, Xiong J, Bowen J, Wang W, Liu Y, Braguinets O, Grigull J, Pearlman RE, Orias E, Gorovsky MA. 2009. Microarray analyses of gene expression during the *Tetrahymena thermophila* life cycle. *PLoS ONE* **4**: e4429. doi: 10.1371/journal.pone.0004429.
- Ota T, Suto S, Katayama H, Han ZB, Suzuki F, Maeda M, Tanino M, Terada Y, Tatsuka M. 2002. Increased mitotic phosphorylation of histone H3 attributable to AIM-1/Aurora-B overexpression contributes to chromosome number instability. *Cancer Res* **62**: 5168–5177.
- Peggie MW, MacKelvie SH, Bloecher A, Knatko EV, Tatchell K, Stark MJ. 2002. Essential functions of Sds22p in chromosome stability and nuclear localization of PP1. *J Cell Sci* **115**: 195–206.
- Posch M, Khoudoli GA, Swift S, King EM, Deluca JG, Swedlow JR. 2010. Sds22 regulates aurora B activity and microtubule-kinetochore interactions at mitosis. *J Cell Biol* **191**: 61–74.
- Quina AS, Buschbeck M, Di Croce L. 2006. Chromatin structure and epigenetics. *Biochem Pharmacol* **72**: 1563–1569.
- Ray C. 1956. Meiosis and nuclear behavior in *Tetrahymena pyriformis*. *J Eukaryotic Microbiol* **3**: 88–96.
- Shang Y, Li B, Gorovsky MA. 2002. *Tetrahymena thermophila* contains a conventional γ -tubulin that is differentially required for the maintenance of different microtubule-organizing centers. *J Cell Biol* **158**: 1195–1206.
- Smart SK, Mackintosh SG, Edmondson RD, Taverna SD, Tackett AJ. 2009. Mapping the local protein interactome of the NuA3 histone acetyltransferase. *Protein Sci* **18**: 1987–1997.
- Sporn JC, Kustatscher G, Hothorn T, Collado M, Serrano M, Muley T, Schnabel P, Ladurner AG. 2009. Histone macroH2A isoforms predict the risk of lung cancer recurrence. *Oncogene* **28**: 3423–3428.
- Sugai T, Hiwatashi K. 1974. Cytologic and autoradiographic studies of the micronucleus at meiotic prophase in *Tetrahymena pyriformis*. *J Protozool* **21**: 542–548.
- Sutherland BW, Toews J, Kast J. 2008. Utility of formaldehyde cross-linking and mass spectrometry in the study of protein-protein interactions. *J Mass Spectrom* **43**: 699–715.
- Talbert PB, Henikoff S. 2010. Histone variants—ancient wrap artists of the epigenome. *Nat Rev Mol Cell Biol* **11**: 264–275.

- Varga-Weisz PD, Becker PB. 2006. Regulation of higher-order chromatin structures by nucleosome-remodelling factors. *Curr Opin Genet Dev* **16**: 151–156.
- Wang T, Allis CD. 1992. Replication-dependent and independent regulation of HMG expression during the cell cycle and conjugation in *Tetrahymena*. *Nucleic Acids Res* **20**: 6525–6533.
- Wang T, Allis CD. 1993. An abundant high-mobility-group-like protein is targeted to micronuclei in a cell cycle-dependent and developmentally regulated fashion in *Tetrahymena thermophila*. *Mol Cell Biol* **13**: 163–173.
- Wei Y, Mizzen CA, Cook RG, Gorovsky MA, Allis CD. 1998. Phosphorylation of histone H3 at serine 10 is correlated with chromosome condensation during mitosis and meiosis in *Tetrahymena*. *Proc Natl Acad Sci* **95**: 7480–7484.
- Wei Y, Yu L, Bowen J, Gorovsky MA, Allis CD. 1999. Phosphorylation of histone H3 is required for proper chromosome condensation and segregation. *Cell* **97**: 99–109.
- Wu M, Allis CD, Gorovsky MA. 1988. Cell-cycle regulation as a mechanism for targeting proteins to specific DNA sequences in *Tetrahymena thermophila*. *Proc Natl Acad Sci* **85**: 2205–2209.
- Wysocka J, Reilly PT, Herr W. 2001. Loss of HCF-1-chromatin association precedes temperature-induced growth arrest of tsBN67 cells. *Mol Cell Biol* **21**: 3820–3829.
- Zhang R, Poustovoitov MV, Ye X, Santos HA, Chen W, Daganzo SM, Erzberger JP, Serebriiskii IG, Canutescu AA, Dunbrack RL, et al. 2005. Formation of MacroH2A-containing senescence-associated heterochromatin foci and senescence driven by ASF1a and HIRA. *Dev Cell* **8**: 19–30.

The nonhistone, N-terminal tail of an essential, chimeric H2A variant regulates mitotic H3-S10 dephosphorylation

Xiaoyuan Song, Josephine Bowen, Wei Miao, et al.

Genes Dev. 2012, **26**:

Access the most recent version at doi:[10.1101/gad.182683.111](https://doi.org/10.1101/gad.182683.111)

Supplemental Material

<http://genesdev.cshlp.org/content/suppl/2012/03/16/26.6.615.DC1>

References

This article cites 57 articles, 14 of which can be accessed free at:
<http://genesdev.cshlp.org/content/26/6/615.full.html#ref-list-1>

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).