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Phosphorylation of the SQ H2A.X Motif Is Required for Proper Meiosis and Mitosis in *Tetrahymena thermophila*▽

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Phosphorylation of the C terminus SQ motif that defines H2A.X variants is required for efficient DNA double-strand break (DSB) repair in diverse organisms but has not been studied in ciliated protozoa. *Tetrahymena* H2A.X is one of two similarly expressed major H2As, thereby differing both from mammals, where H2A.X is a quantitatively minor component, and from *Saccharomyces cerevisiae* where it is the only type of major H2A. *Tetrahymena* H2A.X is phosphorylated in the SQ motif in both the mitotic micronucleus and the amitotic macronucleus in response to DSBs induced by chemical agents and in the micronucleus during prophase of meiosis, which occurs in the absence of a synaptonemal complex. H2A.X is phosphorylated when programmed DNA rearrangements occur in developing macronuclei, as for immunoglobulin gene rearrangements in mammals, but not during the DNA fragmentation that accompanies breakdown of the parental macronucleus during conjugation, correcting the previous interpretation that this process is apoptosis-like. Using strains containing a mutated (S134A) SQ motif, we demonstrate that phosphorylation of this motif is important for *Tetrahymena* cells to recover from exogenous DNA damage and is required for normal micronuclear meiosis and mitosis and, to a lesser extent, for normal amitotic macronuclear division; its absence, while not lethal, leads to the accumulation of DSBs in both micro- and macronuclei. These results demonstrate multiple roles of H2A.X phosphorylation in maintaining genomic integrity in different phases of the *Tetrahymena* life cycle.

Histone H2A.X is defined by the presence of a conserved SQ motif at the C terminus of a histone H2A, regardless of whether this H2A is a minor variant that is distinct and longer than the major H2A, as in mammals (49, 55), or whether it is the "major" H2A, as in *Saccharomyces cerevisiae* (16, 61), or is on another conserved variant (H2A.Z), as in *Drosophila* (83). The SQ motif is invariant and, in all cases but one (a predicted H2A.X in *Gallus*, accession no. XP_416906, derived from an annotated genomic sequence NW_060235), it localizes 3 to 4 residues from the C terminus, followed by a penultimate acidic residue (E/D) and then a terminal hydrophobic residue (Y/F/I/L) (61). Defined in this way, the H2A.X motif is found in most, if not all, eukaryotes from primitive ones, like *Giardia* (89), to higher organisms, like humans (49), although the histone it is on may not always have been named H2A.X.

DNA double strand breaks (DSBs), whether induced by external sources (ionizing radiation or drugs), by endogenous damage (free-radicals or replication fork collapse), or by developmentally programmed events [V(D)J joining, mating type switching, meiotic recombination, or apoptosis], invariably cause the serine in the SQ motif to become phosphorylated within minutes to produce an isoform commonly referred to as γ-H2A.X (28, 29, 44). The enzymes that phosphorylate the SQ motif are phosphatidylinositol 3-kinase-like kinase family members, Mec1 and Tel1 in *S. cerevisiae* (23, 74) and ATM, ATR, and DNA-dependent protein kinase (DNA-PK) in higher eukaryotes (9, 58, 77, 84).

Formation of γ-H2A.X is an evolutionarily conserved response to DSBs, as indicated by the fact that an anti-γ-H2A.X antibody raised against a synthetic phosphorylated peptide containing the mammalian γ-H2A.X sequence can recognize DSB-induced γ-H2A.X from diverse species (67). H2A.X phosphorylation in response to DSBs extends for megabases in sequences flanking the DSB sites in mammalian cells (67) and for 50 to 100 kb surrounding a single induced DSB in budding yeast (81). Thus, H2A.X phosphorylation is a highly sensitive DNA damage sensor and is required for efficient DSB repair (12, 23). In addition, γ-H2A.X is required to maintain genome stability (12) and has a role in condensing and inactivating sex chromosomes in male meiosis in mice (27).

Many proteins interact, either directly or indirectly, with γ-H2A.X and appear at the break sites or in broader areas surrounding DNA breaks after the appearance of γ-H2A.X (reviewed in references 28 and 29). These proteins include histone modifiers like the histone acetyltransferase NuA4 (22), the histone deacetylase Sin3 (34), chromatin remodelers like Ino80 (22, 53, 82) and SwrC (22), the Tip60 complex which has both histone acetyltransferase and ATP-dependent chromatin remodeling activities (43), DNA repair complexes (Mre11/Rad50/Nbs1 in mammals, and Mre11/Rad50/Xrs2 in budding yeast) (12, 36, 58), checkpoint proteins 53BP1 (85) and Crb2

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(56), and cohesin (81). Despite initial speculation that it recruits repair proteins to DNA breaks, γ-H2A.X appears to function in DSB repair by concentrating or retaining the modifying, remodeling, and repair proteins (11, 53), which may be recruited to the damage sites by redundant or alternative mechanisms (53). Removing γ-H2A.X after DNA repair also is required for cells to recover from the DNA damage checkpoint and resume their normal functions (35). By recruitment or retention of the SwrC complex (22) and Tip60 (43), γ-H2A.X may mediate its own removal from the altered chromatin structure produced by these recruited remodeling or modifying complexes. The proteasome also localizes at DNA damage sites and is required for proper DNA-damage responses (41), providing another possible mechanism for turning off the pathway initiated by γ-H2A.X. Recently, protein phosphatase 2A was shown to dephosphorylate γ-H2A.X in mammalian cells (17), and phosphatase Pph3 in *S. cerevisiae* was identified in a phosphatase complex responsible for γ-H2A.X dephosphorylation in yeast (35).

Repairing DSBs is crucial to maintain genome integrity in eukaryotes, as a failure to do so will result in acentric chromosome fragments that will be lost during mitosis. DSBs are mainly repaired either by homologous recombination (HR), in which two broken DNA ends join together based on homologous DNA pairing and strand exchange (79), or by nonhomologous end-joining (NHEJ), in which the broken DNA ends are joined together without using long homologous regions (for a review, see reference 70). Absence of γ-H2A.X or loss of H2A.X results in inefficient NHEJ (23) and HR (12). Defects in NHEJ or HR activities result in sensitivity to genotoxic agents, mitotic and meiotic chromosome aberrations, and destabilization of the genome (39, 64, 73, 80).

We have been studying the function of histones and their modifications in the ciliated protozoan, *Tetrahymena thermophila*. As in most ciliates, cells in this organism contain two highly dimorphic nuclei: a germ line micronucleus (MIC) and a somatic macronucleus (MAC). The diploid MIC contains five pairs of chromosomes, divides mitotically, and is transcriptionally inactive during vegetative growth. In contrast, the MAC is transcriptionally active, contains ~225 acentric chromosomes (13, 19, 24), each in ~45 copies, which are derived by fragmentation, telomere addition, and endoreplication from the MIC chromosomes during the sexual process of conjugation. MACs divide by amitosis, a process in which chromosomes assort randomly without condensing or attaching to a mitotic spindle. Because amitosis routinely assorts previously fragmented chromosomes without deleterious consequences, it is not clear whether MACs require mechanisms to efficiently repair DSBs.

The currently held models for meiotic recombination assume that a DSB is an essential recombinogenic substrate in DNA (42, 57, 76), and γ-H2A.X is associated with meiotic DSBs (47) which precede synapsis in mouse germ cell development and which are thought to initiate meiotic recombination, as in yeast (65, 95). During *Tetrahymena* conjugation, micronuclei undergo meiosis, adopting a highly elongate crescent shape (60). During crescent formation the round MIC elongates gradually to the crescent form, which, when maximally extended, can be up to twice as long as the cell, and then shortens and condenses at metaphase I. Because it precedes the meiotic divisions, the crescent stage is thought to be analogous to most of the prophase of meiosis I. However, while crescents in *Tetrahymena* exhibit some features of meiotic prophase found in other organisms, such as bouquet-like clustering of both telomeres (45) and centromeres (19), synaptonemal complexes (SCs) have not been detected (45, 88), making it difficult to correlate the different stages of micronuclear meiotic prophase to the key events in meiosis such as homologous chromosome pairing and recombination. Thus, different mechanisms could be used in *Tetrahymena*. Also, during conjugation, the parental MAC is destroyed by a process that has been suggested to be related to apoptosis in higher eukaryotes (20, 26), and phosphorylation of H2A.X accompanies formation of the DSBs associated with DNA fragmentation during apoptosis in mammals (46, 54, 68).

Based on the above considerations, we sought to investigate the role of H2A.X phosphorylation in amitosis, in the unusual meiosis, in chromosome rearrangement, and in MAC degeneration in *Tetrahymena.* We also sought to utilize the timing of H2A.X phosphorylation during meiosis to help determine when meiotic recombination occurred. In *T. thermophila* there are four H2As, and only one of the two major H2As has the SQ motif (44; X. Song and M. A. Gorovsky, unpublished data). Using a mammalian γ-H2A.X-specific monoclonal antibody (MAb) that recognizes phosphorylated *T. thermophila* H2A.X (formerly H2A.1), we determined that serine 134 in the SQ motif of *T. thermophila* H2A.X is phosphorylated in both MACs and MICs in response to DSBs induced by chemical agents. We show that *Tetrahymena* γ-H2A.X appears in meiotic MICs at early stage II (when the MICs just start to elongate) (18, 45, 78), indicating that DNA DSBs occur before the MICs acquire Rad51 (45, 72) and during MAC development when chromosome rearrangements occur. Surprisingly, H2A.X is not phosphorylated when parental MACs are being degraded. We also provide evidence that the *HTAX S134A* mutation abolishes SQ motif phosphorylation and causes accumulation of DNA DSBs in both meiotic and mitotic MICs and in amitotic MACs. This mutation makes cells sensitive to chemical agents causing DNA DSBs, causes mitotic delays and DNA loss, and produces meiotic defects, including chromosome loss at metaphase I and lagging chromosomes in anaphase I and II, leading to premature cessation of conjugation. These studies argue that H2A.X SQ motif phosphorylation functions in DSB repair in mitosis, meiosis, and amitosis but not during programmed nuclear death in *Tetrahymena*.

MATERIALS AND METHODS

Strains, culture, and conjugation. Table 1 lists the *T. thermophila* strains used in this study. Strains CU428, CU427, and B2086 were provided by P. J. Bruns (Cornell University). Major histone H2A (H2A.X and H2A.1) germ line double knockout heterokaryon strains G4A1F14A and G4B1G6A and all mutant strains were generated as previously described (62). For studies of vegetative growth, *Tetrahymena* cells were grown in super proteose peptone (SPP) medium (31) containing 1% proteose peptone (1× SPP). For conjugation, two strains of different mating types were washed, starved (15 to 24 h, without shaking at 30°C), and mated in 10 mM Tris-HCl (pH 7.5) as previously described (3). Major H2A genes (*HTAX* and *HTA1*) germ line double knockout heterokaryons and site-directed mutagenesis were generated and performed as described (62).

Transformation and gene replacement. Constructs containing the wild-type (WT) or mutated *HTAX* gene were digested with XhoI and BamH and transformed into 24-h conjugating *HTA* double knockout heterokaryons (for germ line rescue) or 15- to 17-h conjugating CU428 and B2086 cells (for somatic

TABLE 1. Strains used in this study

Strain	Genotype (micronuclei)	Phenotype (macronuclei)
CU428	*CHX1/CHX1 mpr1-1/mpr1-1 HTAX/HTAX HTA1/HTA1*	WT; Cy^s Mp^s Pm^s; VII
CU427	*chx1-1/chx1-1 MPR1/MPR1 HTAX/HTAX HTA1/HTA1*	WT; Cy^s Mp^s Pm^s; VI
B2086	*CHX1/CHX1 MPR1/MPR1 HTAX/HTAX HTA1/HTA1*	WT; Cy^s Mp^s Pm^s; II
HTAX S1P+5R	*Δhtax/Δhtax Δhta1/Δhta1*[a] *CHX1/CHX1 mpr1?/mpr1?*[b]	H2A.X PRRRRR, ΔH2A.1; Pm^r Cy^s Mp?[c,d]
HTAX S1P+5R+(AAAAS)$_C$	*Δhtax/Δhtax Δhta1/Δhta1*[a] *CHX1/CHX1 mpr1?/mpr1?*	H2A.X PRRRRR+$(AAAAS)_C$, ΔH2A.1; Pm^r Cy^s Mp?[d]
HTAX S134A rescued	*Δhtax/Δhtax Δhta1/Δhta1*[a] *CHX1/CHX1 mpr1?/mpr1?*	H2A.X S134A, ΔH2A.1; Pm^r Cy^s Mp?[d]
Rejuvenated *HTAX S134A* rescued	*chx1-1/chx1-1 MPR1/MPR1 HTAX/HTAX HTA1/HTA1*	H2A.X S134A, ΔH2A.1; Pm^r Cy^s Mp?[d]
HTAX rescued	*Δhtax/Δhtax Δhta1/Δhta1*[a] *CHX1/CHX1 mpr1?/mpr1?*	H2A.X, ΔH2A.1; Pm^r Cy^s Mp?[d]
Somatic *HTAX S134A*	*CHX1/CHX1 mpr1-1/mpr1-1 HTAX/HTAX HTA1/HTA1* or *CHX1/CHX1 MPR1/MPR1 HTAX/HTAX HTA1/HTA1*	H2A.X S134A, H2A.1; Pm^r Cy^s Mp^s; VII or II
Somatic *HTAX*	*CHX1/CHX1 mpr1-1/mpr1-1 HTAX/HTAX HTA1/HTA1* or *CHX1/CHX1 MPR1/MPR1 HTAX/HTAX HTA1/HTA1*	H2A.X, H2A.1; Pm^r Cy^s Mp^s; VII or II
HTAX-neo3 somatic replacing *S134A* in *S134A* rescued	*Δhtax/Δhtax Δhta1/Δhta1*[a] *CHX1/CHX1 mpr1?/mpr1?*	H2A.X, ΔH2A.1; Pm^r Cy^s Mp?[d]

[a] The full genetic nomenclature for *Δhtax/Δhtax Δhta1/Δhta1* is htax-1::*neo2*/*htax-1*::*neo2 hta1-1*::*neo2*/*hta1-1*::*neo2* (2); the abbreviation has been used to conserve space.
[b] Question mark indicates undetermined genotype.
[c] Mp?, 6-methyl-purine sensitivity not determined.
[d] Mating type not determined.

transformation) as previously described (10). Germ line rescued progeny or somatic transformants were initially selected with paramomycin sulfate (Sigma) at 60 μg/ml and serially transferred every 2 to 3 days to fresh medium with increasing concentrations of paromomycin. The genotypes of all transformed cells were confirmed by sequencing the PCR products using *HTAX*-specific primers from genomic DNA of the transformants.

To replace the mutated *HTAX S134A* gene in the MACs of the S134A rescued strain, the *HTAX* gene with a selectable marker inserted in the 5′ flanking region was somatically transformed into the paromomycin-sensitive (Pm^s) S134A rescued cells.

Rejuvenation of the double H2A knockout MICS in the S134A rescued strain with a WT MIC through round I genomic exclusion. Round I genomic exclusion (1, 8, 21) is a special type of abortive mating between WT and star (*) strains which have defective, hypodiploid MICs. Star strains can form pairs with WT but are not able to produce pronuclei during nuclear exchange and fertilization stages of conjugation (18, 50, 78). As a result, both partners of the pairs have only haploid MICs received from the WT cell, which are then endoreplicated to form homozygous diploid MICs in both cells. After this step, conjugation is aborted, and the pairs separate as two round I exconjugants, with each cell retaining its original MAC but obtaining a new homozygous MIC, whose genotype depends on which meiotic product was provided from the normal parent. Cells with defective (star) MICs that have received a WT MIC produced by this process are often referred to as rejuvenated because they obtain a new MIC that should be competent for conjugation. Note also that, because these cells do not form a new MAC, they retain their original mating type and, unlike cells that complete normal conjugation, which are immature for ~65 fissions (69), can mate immediately.

S134A or WT rescued cells were mated with CU427 cells, and single pairs were picked into drops of 1× SPP medium at 5 h postmixing. Individual, separated round I exconjugants were picked from each drop into fresh drops of 1× SPP medium at about 11 h postmixing. After cells grew up in the drops, they were transferred to 1× SPP medium in 96-well plates and tested for sensitivity at 120 μg/ml paromomycin. Pm^r round I exconjugants from the S134A rescued cells were starved and mated with CU428. The progeny were then tested for cycloheximide resistance (Cy^r) as well as paromomycin sensitivity. The cells whose progeny are Cy^r Pm^s are the rejuvenated cells, which have the WT *HTAX* and *HTA1* genes (instead of the disrupted versions of those genes) in their MICs but retain the *H2AX* S134A mutation or *H2AX* WT copy in their MACs.

Short-circuit genomic exclusion. Short-circuit genomic exclusion (7) occurs in a small fraction of cells during the same type of matings described above for round I genomic exclusion when, instead of simply aborting conjugation, a small percentage of cells form a new MAC. The genotype of this new MIC (and the phenotype of the cell) is determined by the genetic makeup of the cell with the functional MIC.

Indirect immunofluorescence microscopy. γ-H2A.X was immunostained with anti-phospho H2A.X MAb (Upstate), a monoclonal antibody raised against a phosphorylated peptide corresponding to residues 134 to 142 (KATQA[pS]QE Y) of human H2A.X. Growing or mating cells were fixed as previously described (87) with some modifications. Briefly, 5 μl of partial Schaudin's fixative (two parts saturated $HgCl_2$ to one part 100% ethanol) was added directly to 1.5 ml of cells (2×10^5 cells/ml of growing cells or the same cell density of mating cells in 10 mM Tris, pH 7.5), hand mixed, and incubated for 5 min at room temperature (RT). Cells were gently pelleted (130 × *g* for 30 sec), resuspended in 3 ml of RT methanol, repelleted, and resuspended in 1 ml of RT methanol. A total of 30 to 50 μl of cells was spread onto a coverslip and air dried for 30 min. Cells were stained with anti-γH2A.X (1:100) followed by incubation with AlexaFluor 568 goat anti-mouse immunoglobulin G (IgG; 1:500) (Invitrogen). Nuclei were stained with the DNA-specific dye 4′,6′-diamidino-2-phenylindole (DAPI; Roche) at 10 ng/ml for 10 min. Images were obtained with an Olympus BH-2 fluorescence microscope equipped with filters specific for AlexaFluor and DAPI using a 100× or 40× lens and the Scion VisiCapture and Scion TWAIN 1394 camera import programs (Scion Corporation). Adobe Photoshop software was used for coloring images.

Nucleus isolation, histone extraction, and phosphatase treatment. Log-phase (2×10^5 cells/ml) or 3.5-h conjugating cells were used to isolate MACs and/or MICs as described previously (31). Histones were extracted from MACs with 0.4 N H_2SO_4 (4) and precipitated with 20% trichloroacetic acid. Aliquots of 25 μg of histones were treated with λ protein phosphatase (New England Biolabs, Inc.) at 10 U/μl for 5 h at 30°C and precipitated with 20% trichloroacetic acid.

AU-PAGE. Acid-urea polyacrylamide gel electrophoresis (AU-PAGE) was performed as described previously (5, 62).

Immunoblotting. Histones from mutated and WT *HTAX* rescued strains, with or without pretreatment with λ protein phosphatase, were separated on long AU-PAGE. Macronuclear and micronuclear extracts from WT cells from log phase and 3.5-h conjugation were separated on 12% sodium dodecyl sulfate-PAGE. Separated histones or nuclear extracts were transferred to Immobilon-P membranes. After being blocked in 5% nonfat milk, the blot was incubated with anti-H2A (1:5,000) or anti-γ-H2AX (1:1,000, Upstate) overnight at 4°C. A 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) or goat anti-mouse IgG-IgA-IgM (Zymed Labs Inc.) was used as secondary antibody. Blots were developed using an ECL Western blotting detection kit (Perkin-Elmer) according to the manufacturer's instructions.

DAPI stain for photomicroscopy. One microliter of 0.1 mg/ml DAPI was added to 1 ml of log-phase cells fixed with 50 μl of formaldehyde (37% solution; J. T. Baker). Cells were stained for 5 min. Images were obtained using an Olympus BH-2 fluorescence microscope with the Scion VisiCapture and Scion TWAIN 1394 camera import programs (Scion Corporation).

Comet assay. A neutral comet assay was performed as previously described (75, 92) with minor modifications. Briefly, 40 μl of 0.3% low-melting-point agarose (Sigma) in phosphate-buffered saline was coated onto the frosted area (18 by 18 mm) of a double-frosted slide (Fisher) and air dried for several days before being used. Five microliters of 2×10^5 to 5×10^5 cells/ml was mixed with 35 μl of 1% low-melting-point agarose in phosphate-buffered saline and spread onto the precoated area, solidified by being immediately put onto a metal tray on ice for 3 min, to form microgels. From that point, all steps were performed in dimmed light or in the dark to reduce DNA damage, and slides were set horizontally during lysis and the following treatments. Microgels were lysed with freshly made, cold (at 4°C for 0.5 h) lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10) with 1% Triton for 2 h at 4°C. They were then treated with 10

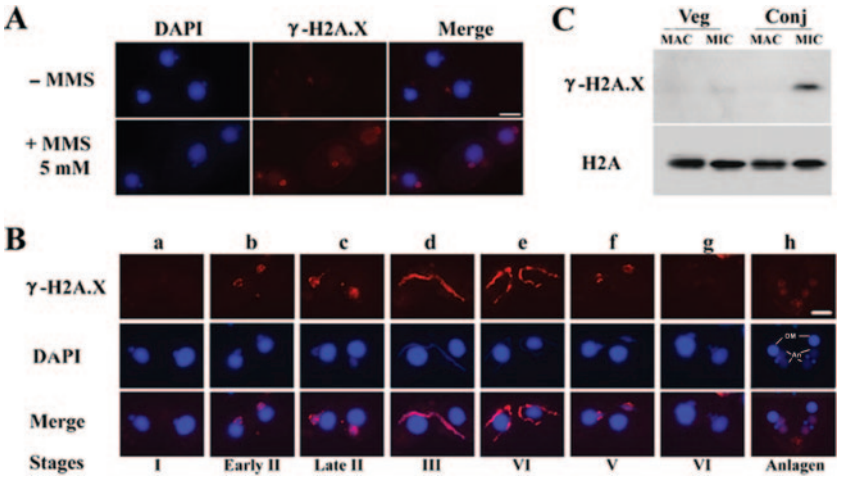

FIG. 1. Human anti-γ-H2A.X antibody detects chemical induced and meiotic DSBs in *T. thermophila*. (A) IF analysis of WT *Tetrahymena* cells with a specific MAb raised against the phosphorylated C-terminal region of human H2A.X (γ-H2A.X) shows that MMS treatment induces macro- and micronuclear staining. Scale bar, 10 μm. (B) IF analysis of different stages during conjugation in *Tetrahymena*. DAPI stain shows the nuclei, and the anti-γ-H2A.X MAb staining indicates that DSBs, indicative of meiotic recombination, appear as the MICs begin to elongate in early stage II (b) and disappear at stage VI (diakinasis/metaphase, g). γ-H2A.X also appears in the newly developing MACs (anlagen) undergoing DNA fragmentation and rearrangement (An, h;), but not in the parental MAC (OM, h) undergoing programmed nuclear death. Scale bar, 10 μm. (C) Western blot of a sodium dodecyl sulfate-PAGE gel of macro- and micronuclear extracts, probed with anti-γ-H2A.X or anti-H2A antibodies. Only MICs from early conjugating cells contain a significant amount of γ-H2A.X.

μg/ml RNase A in lysis buffer without Triton for 2 h at 37°C, followed by 2 h at 37°C in 1 mg/ml proteinase K in lysis buffer without Triton, and equilibrated in 1 liter of freshly made electrophoresis buffer (300 mM sodium acetate, 100 mM Tris, pH 9.0) in an electrophoresis apparatus for 20 min at RT (slides were put side by side tightly and at one end of the apparatus). Electrophoresis was carried out in the same buffer at 12 V (0.6 V/cm) and 100 mA at RT for 1 h. After electrophoresis, microgels were neutralized with 0.4 M Tris, pH 7.4 (drop-wise added on top of the microgels and drained; the procedure was repeated three times), dehydrated with 100% ethanol, and air dried. DNA in microgels was stained with DAPI (20 ng/ml) in 1× Tris-acetate-EDTA buffer for 5 min, followed by destaining for 5 min in 1× Tris-acetate-EDTA buffer. Images were obtained with an Olympus BH-2 microscope equipped for fluorescence, with the Scion VisiCapture and Scion TWAIN 1394 camera import programs (Scion Corp.). The Image J program was used to measure the tail lengths and total DNA content.

RESULTS

H2A.X in *T. thermophila* is phosphorylated in response to induced DSBs. To determine whether phosphorylation of H2A.X in *Tetrahymena* occurs in response to induced DSB formation, we carried out immunofluorescence (IF) analyses using a specific MAb to human γ-H2A.X that reacts across species to examine *Tetrahymena* cells treated with methyl methanesulfonate (MMS), an alkylating agent that causes base alkylations and DNA lesions that are converted to DSBs similar to those produced by ionizing radiation (40, 71, 90, 91). No signal was detected in nuclei of untreated cells (Fig. 1A, −MMS), demonstrating that this antibody does not recognize an epitope in untreated vegetative cells. In contrast, MICs and, to a lesser extent, MACs were stained with the anti-γ-H2A.X MAb after MMS treatment (Fig. 1A, +MMS), indicating that γ-H2A.X is produced in nuclei from vegetative cells when DSBs are induced by exogenous DNA-damaging agents. The difference in γ-H2A.X staining intensity in MICs and MACs from MMS-treated cells could be due to the differential activities of the phosphorylation machinery in the different nuclei or it could reflect the previously observed higher levels of H2A.X (formerly H2A.1) as a fraction of total H2A in MICs than in MACs (5).

H2A.X in *T. thermophila* is phosphorylated during meiosis. As noted above, *Tetrahymena* meiosis is unusual in lacking synaptonemal complexes. In addition, previously observed histone steady-state levels in *Tetrahymena* show enrichment of histone H2A.X in the MICs for reasons that were unclear (5). We reasoned that these differences in relative levels of H2A forms between nuclei could suggest distinct functional roles for different *Tetrahymena* H2As. In particular, higher micronuclear levels of H2A.X might correlate with the specialized function of MICs during conjugation, i.e., its potential to undergo homologous recombination during meiosis. These considerations led us to examine the role of H2A.X phosphorylation during meiosis in *Tetrahymena*.

Based on morphological changes, *Tetrahymena* meiotic prophase has been divided into six stages, with stages II to V being the crescent stages (18, 45, 78). Soon after conjugation starts, the MIC moves away from the macronuclear pocket where it resides during interphase and begins to elongate into a teardrop shape (early stage II), then a spindle-shape (late stage II) (45), and then a head-neck-trunk crescent (stage III), followed by bidirectional elongation to become fully elongated in stage IV, where the five bivalent chromosomes are in parallel arrangement, with telomeres at one end of the crescent (45) and the centromeres at the other end (19). MICs stain with the anti-γ-H2A.X MAb beginning at early stage II, when they have just started to elongate (Fig. 1B, frame b). The signal continues through stage V (Fig. 1B, frames b to f) and disappears or is only barely visible at stage VI (diakinesis/metaphase I) (Fig. 1B, frame g). Immunoblotting analysis of extracts from purified MACs and MICs isolated from either vegetative cells or early conjugation also confirmed the IF analyses showing

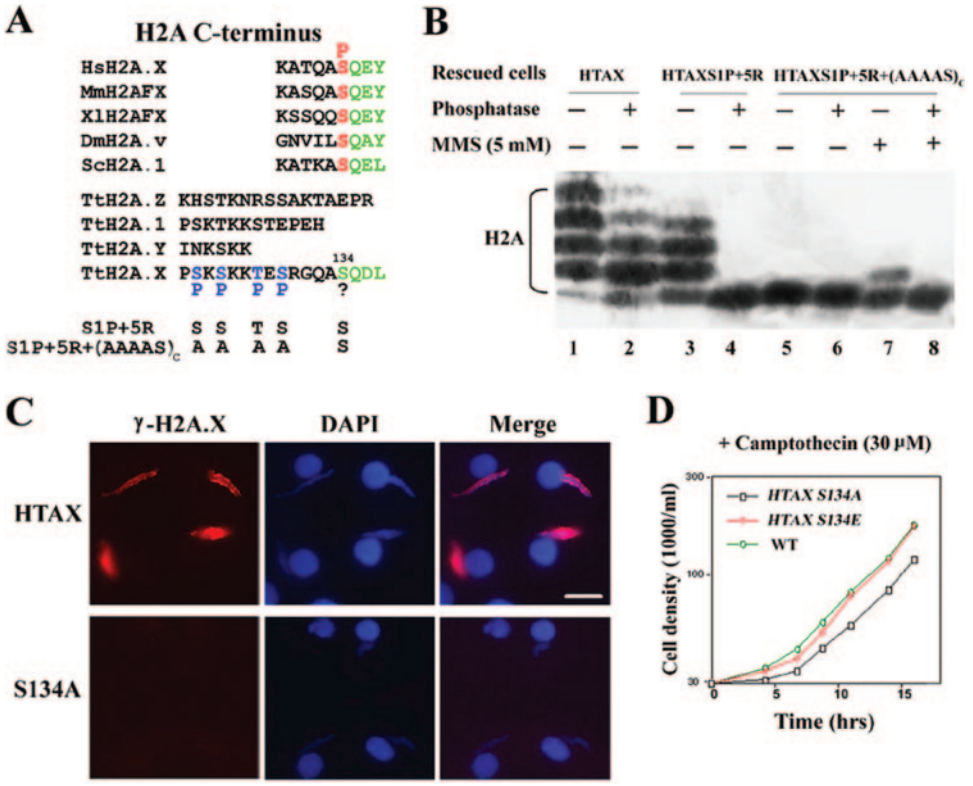

FIG. 2. H2A.X S134 residue in the SQ motif is responsible for the γ-H2A.X signal induced by DSBs. (A) Alignment of the C-terminal tails of H2A.Xs in different organisms and the four H2As in *T. thermophila* using Clustal X. The sequences were obtained from GenBank. The accession numbers are as follows: HsH2A.X, NP_002096; MmH2AFX, NP_034566; XlH2AFX, Q6GM86; DmH2A.v, NP_524519; ScH2A.1, CAA24611; TtH2A.Z (hv1), CAA33554; TtH2A.1 (previously H2A.2), AAC37292; TtH2A.Y, AAU87547; TtH2A.X (previously H2A.1), AAC37291. The serine in the SQ motif that is known to be phosphorylated upon γ irradiation is labeled in red. The conserved SQ motif is labeled in green. TtH2A.X is the only H2A in *T. thermophila* that has an SQ motif, and it has four serine/threonine residues upstream of the SQ motif (labeled in blue) which are the sites for normal phosphorylation of the protein in vegetative growth (see panel B). (B) Western blot of an AU-PAGE gel separating nuclear histones from WT or mutated *HTAX* rescues of HTA double knockout heterokaryons, stained by anti-H2A polyclonal antibody. Lanes 1 and 2 show that WT H2A.X is phosphorylated but that it is impossible to determine the phosphorylation status in detail due to the presence of acetylation on the protein. Lanes 3 and 4 show that there are three phosphatase-sensitive isoforms of the H2A.X in the N-terminal mutation strain (HTAX S1P+5R) that abolished the acetylation sites. Lanes 5 and 6 show that changing the four serine/threonine residues upstream of the SQ motif to alanines eliminates the three phosphatase-sensitive isoforms. Lanes 7 and 8 show that an additional phosphatase-sensitive isoform is detected upon MMS treatment in the mutant *Tetrahymena* strain [HTAX S1P+5R+$(AAAAS)_C$] lacking all other known acetylation and phosphorylation sites. (C) IF analysis of the HTAX or S134A rescued cells during conjugation (4 h). γ-H2A.X staining is not detectable in S134A rescued cells. Scale bar, 10 μm. (D) Growth curve of WT, S134A, and S134E rescued cells in 1× SPP medium with 30 μM camptothecin.

that only MICs from early conjugation stages corresponding to meiotic prophase I contain substantial amount of γ-H2A.X (Fig. 1C).

γ-H2A.X is detectable in developing MACs undergoing DNA rearrangement but not in MACs undergoing programmed nuclear death. H2A.X phosphorylation accompanies V(D)J rejoining (15), and, in mouse cells, the formation of DNA ladders that accompanies apoptosis requires H2A.X phosphorylation (46). During conjugation in *Tetrahymena*, DNA breakage is known to occur at two stages: when chromosome fragmentation and DNA elimination are occurring in developing MACs (94) and when parental MACs undergo programmed nuclear death, which has been viewed as an apoptotic-like process during which DNA is degraded to produce oligonucleosome-sized DNA ladders (20). No staining of γ-H2A.X in the parental MAC was detected at any stage of conjugation, including late stages when the parental MAC was undergoing programmed nuclear death (Fig. 1B, frame h, OM). In contrast, γ-H2A.X staining was easily detectable in late-stage developing MACs when DNA rearrangement events were occurring (Fig. 1B, frame h, An).

H2A.X phosphorylation occurs on S134. To determine if phosphorylation at serine 134 in the SQ motif in *Tetrahymena* H2A.X (Fig. 2A) is associated with anti-γ-H2A.X staining, we performed site-directed mutagenesis together with gene replacement. When a WT *HTAX* gene was used to rescue *HTAX* and *HTA1* (formerly *HTA2*) germ line double knockout heterokaryons (32, 62), the histones isolated from viable progeny (*HTAX* rescued cells) exhibited phosphatase-resistant H2A isoforms when analyzed on acid-urea acrylamide gels (Fig. 2B, lanes 1 and 2), due to charge-altering acetylations in the N-terminal tail (62). When histones isolated from vegetatively growing cells that were rescued with a gene encoding H2A.X that was mutated to eliminate all acetylation sites were analyzed, the viable progeny (HTAX S1P+5R rescued) exhibited three phosphatase-sensitive isoforms in addition to unmodified

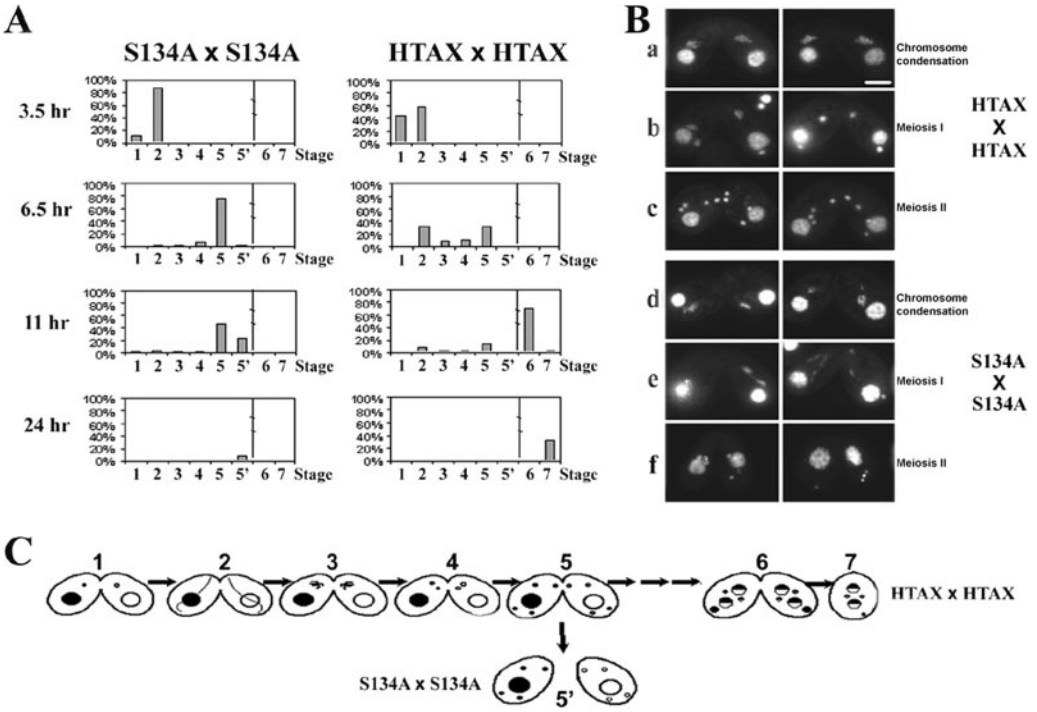

FIG. 3. Absence of S134 phosphorylation leads to meiosis defects and premature termination of conjugation. (A) Conjugation profiles of matings between two S134A rescued strains or two HTAX rescued strains. Different stages during conjugation (indicated in panel C) were scored in samples removed at different times. The vertical line between stage 5′ (see panel C) and 6 indicates that there are several noninformative stages in normal conjugation that were not precisely staged in this study but were counted in the total number of cells ($n \geq 346$) analyzed. (B) DAPI staining of the nuclei from the HTAX or S134A rescued strains at different stages of conjugation times as indicated. Scale bar, 10 μm. (C) Different stages of conjugation scored in panel A. S134A rescued matings prematurely terminated mating after meiosis II (stage 5), which was not seen in HTAX rescued matings and was denoted as stage 5′. There are several stages, indicated by several horizontal arrows, between stage 5 and 6 that were not plotted. Black and white nuclei indicate two mating types of the same genetic background cells.

H2A.X (Fig. 2B, lanes 3 and 4). Upon mutating four S/T residues (S122, S124, T127, and S129) in the H2A.X C terminus (Fig. 2A) to alanines [HTAX S1P+5R+(AAAAS)c rescued] (63), the three phosphorylated isoforms disappeared (Fig. 2B, lanes 5 and 6). These cells produce viable progeny and grow normally. These results indicate that three of the four mutated sites (S122, S124, T127, and S129) in H2A.X are phosphorylated in untreated vegetatively growing cells, yet they are dispensable (63).

HTAX S1P+5R+(AAAAS)c rescued cells, lacking any acetylation sites and in which the only remaining phosphorylatable serine is S134, were then treated with MMS to induce DSBs. A single, phosphatase-sensitive isoform (Fig. 2B, lanes 7 and 8) (63) was detected. These observations suggest that S134 in the SQ motif is responsible for this phosphorylation event in response to DSBs. To test this, we made an HTAX S134A rescued strain, in which an *HTAX* gene bearing a mutation of S to A at residue 134 was used to rescue major *HTA* double knockout heterokaryons (32, 62). The viable progeny will be referred to as S134A rescued cells; they have a MIC with both the *HTAX* and *HTA1* genes knocked out and a MAC containing the double knockout genes plus a mutated *HTAX S134A* gene. To examine whether the S134A mutation abolished DSB-induced phosphorylation, S134A rescued cells of different mating types were mated, fixed, and stained with the anti-γ-H2A.X MAb. No γ-H2A.X signal was detected in crescent MICs of the S134A rescued cells (Fig. 2C). Control HTAX rescued cells, in which a WT *HTAX* gene was used to rescue major *HTA* double knockout heterokaryons, stained like WT cells (Fig. 2C). Thus, the S134A mutation in H2A.X can specifically abolish γ-H2A.X staining in meiotic prophase crescent MICs, confirming that S134 in the H2A.X SQ motif is the site for phosphorylation in response to meiotic DSBs. This S134A mutation also was more sensitive to the DSB-producing chemicals camptothecin (Fig. 2D) (63) and MMS (data not shown) than WT cells or than an S134E mutant which is otherwise isogenic with S134A mutant cells, arguing that phosphorylation on S134 is important for the signaling and/or repair of DNA damage in *Tetrahymena*.

Absence of S134 phosphorylation leads to meiotic defects. To investigate the function(s) of SQ phosphorylation in meiosis in *Tetrahymena*, we examined whether the absence of S134 phosphorylation during homologous recombination in prophase of meiosis I leads to any conjugation defects. We monitored the conjugation process between two different mating types of HTAX or S134A rescued strains and scored the percentages of different stages at various time points after cells were mixed. Aliquots of the mating cells also were fixed and stained with the DNA-specific dye DAPI to examine nuclear morphology. HTAX rescued cells could go through conjugation to pair separation and formed 34% exconjugants after 24 h postmixing (Fig. 3A and C, stage 7). This result showed

TABLE 2. *HTAX-neo3* somatic replacing *HTAX S134A* mutant could not rescue its phenotype during conjugation[a]

Mating	Percentage of exconjugants by conjugation type					Pm^r/Cy^r (%)
	6-h pairing	10-h pairing	10-h anlagen	10-h exconjugants	29.5-h exconjugants	
CU427 × CU428	81.5	70.0	100.0	15.4	68.4	0
CU427 × S134A	52.0	23.0	3.0	0	6.7	16
CU427 × rejuvenated S134A (50-3)	23.2	18.2	9.5	0	5.9	16
CU427 × rejuvenated S134A (62-5)	48.6	10.6	5.9	0	2.5	10.4

[a] To determine whether the S134A mutation only causes the meiosis defect, the mutant *HTAX S134A* gene was replaced with the WT *HTAX-neo3* gene in MACs of the S134A rescued cells (Fig. 5A). These cells now contain WT *HTAX* genes in MACs but retain MICs that could have kept any DSBs accumulated earlier. Two such strains were made (50-3 and 62-5). The conjugation results showed that the strains that replaced the S134A mutation in MACs of S134A rescued cells did not reduce the meiotic defects, as indicated by the low percentage of exconjugants and low Pm^r/Cy^r ratio. Experiments were repeated twice with similar results.

that, although they do so less efficiently than WT cells, the H2A double knockout heterokaryon cells are able to complete conjugation with a transformed WT *HTAX* gene only in the MACs. Matings between S134A rescued cells appeared cytologically normal during prophase of meiosis I (data not shown), but at metaphase I, there is a decrease in micronuclear DAPI staining compared to HTAX rescued mating cells, and DAPI-stained fragments were seen near the condensed chromosomes (Fig. 3B, compare frames d and a), suggesting that there was (partial or whole) chromosome loss in S134A rescued cells. During anaphase I, lagging chromosomes were often observed in S134A mating cells (Fig. 3B, frames e). Although they could still enter meiosis II, the four meiotic MICs of S134A rescued cells were much smaller than those of HTAX rescued cells (Fig. 3B, compare frames f and c), and some of the meiotic MICs were missing. Conjugation of the S134A rescued cells was then aborted, and the two partners separated as single cells with four or fewer defective meiotic MICs (Fig. 3A and C, stage 5′). Occasionally, pairs separated after meiosis I, leaving single cells with MICs undergoing meiosis II (data not shown).

When S134A rescued cells were mated with WT CU427 cells, the WT cells could not fully rescue the *HTAX S134A* mutation. Since the S134A rescued strain has a homozygous double *HTA* knockout (both major H2A genes replaced by *neo2* cassettes) in its MIC (62) and since the CU427 MIC is homozygous for the *chx1-1* gene, which confers dominant Cy^r when these two cells mate, true progeny that have completed conjugation should be both Cy^r (since they received one of the *chx1-1* genes from CU427) and Pm^r (since they also obtained the *htax::neo2* and *hta1::neo2* genes, which confer Pm^r, from the S134A rescued parent). Note that progeny cells also can be obtained at low frequency from a process known as short-circuit genomic exclusion (7) which occurs when cells containing a functional MIC are mated to cells with defective MICs (see Materials and Methods for details). In the studies performed here, these cells will be Cy^r. Only a few exconjugants were observed after 30 h postmixing, and the Pm^r/Cy^r ratio was far below 100% (Table 2). Most of the pairs contained four MICs, indicating that they had stopped conjugation after meiosis was completed. In the following experiments, a low percentage of exconjugants and a low Pm^r/Cy^r ratio when cells were mated to CU427 cells were used as indicators to identify the S134A mutant phenotype in conjugation.

Because MICs develop into new MACs and MICs during conjugation and, in the rescued H2A double knockout strains, the MICs contain only knockout copies of the major H2A genes, there was a concern whether the conjugation defects observed for S134A rescued cells were caused by the absence of major H2A genes in the conjugating knockout heterokaryon cells before the mutated *HTAX S134A* gene was transformed into the cells. To clarify this issue, a WT MIC was reintroduced into the S134A rescued strain to create a "rejuvenated" strain. This is possible in *Tetrahymena* through round I genomic exclusion (1, 8, 21), a special type of abortive mating between WT and star (*) strains which have defective, hypodiploid MICs (see Materials and Methods for details). Since the S134A rescued cells stopped mating at meiosis II and did not produce pronuclei, they behaved like star cells, suggesting that they had defective MICs. S134A rescued cells were mated with WT CU427 cells, and two mating types of rejuvenated S134A rescued cells were obtained through round I genomic exclusion (Fig. 4A). These rejuvenated S134A rescued cells have WT *HTAX* and *HTA1* genes (instead of the double knockouts) in their MICs but retain the mutated *HTAX S134A* genes in their MACs. Rejuvenated S134A rescued cells of different mating types were then mated with each other or mated with CU427, and the conjugation process was monitored. Matings with rejuvenated S134A rescued cells showed the same conjugation phenotype as the original S134A rescued cells (data not shown), indicating that the phenotypes observed are due to the expression of the *HTAX S134A* gene in the parental MACs, not to defects in the MICs or the newly formed zygotic MAC.

As an alternative approach to analyze the phenotype produced by *HTAX S134A* in an otherwise normal background, the macronuclear *HTAX* genes were somatically replaced with *HTAX S134A-neo2* or *HTAX-neo2* (the selectable marker *neo2* cassette was inserted in *HTAX* 3′ flanking region) genes in WT cells of different mating types (Fig. 4B). We found that these somatic *HTAX S134A* mutants had the same conjugation phenotype as the S134A rescued cells (data not shown). These experiments demonstrate that expression of the H2A.X S134A mutation in MACs of conjugating cells produces meiotic defects and premature termination of conjugation.

Absence of S134 phosphorylation causes mitotic defects. To determine if the S134A mutant phenotype described above is the result of events that occur during meiosis or is the result of accumulated defects in the MICs during vegetative growth in

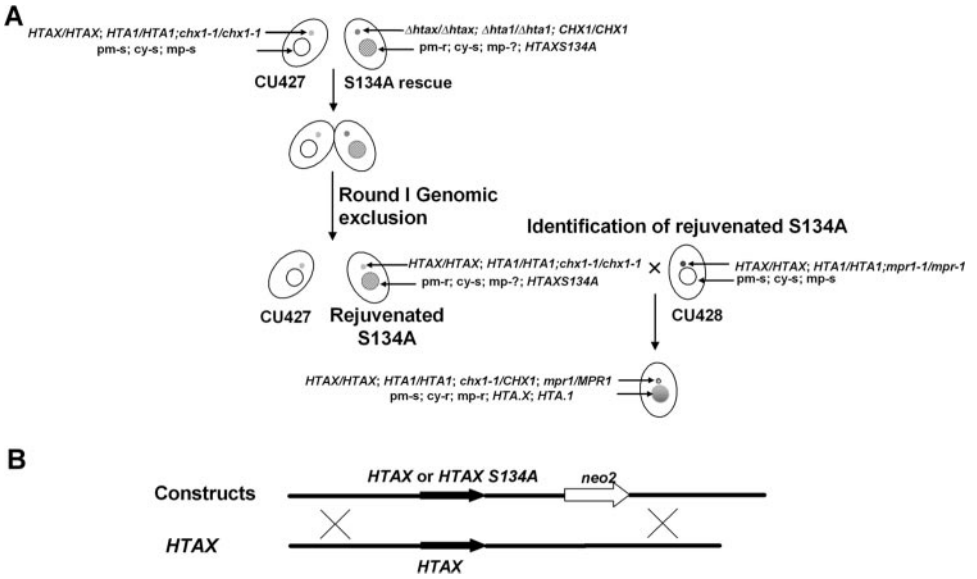

FIG. 4. Strategy to determine if *HTAX S134A* mutation phenotype is truly meiotic. (A) Genetic manipulations used to replace the *HTAX* and *HTA1* double knockout MIC in the S134A rescued strain with a WT MIC to generate the rejuvenated S134A strain. This was used to determine if the phenotype of the *HTAX S134A* mutation was truly meiotic (see text for description). (B) Diagram of somatic replacement of the *HTAX* gene in MACs of WT cells of different mating types with the *HTAX-neo2* or *HTAX S134A-neo2*.

the absence of phosphorylatable H2A.X, the mutated *HTAX S134A* genes in S134A rescued MACs were replaced with WT *HTAX* genes (Fig. 5A). If the S134A mutation causes only meiosis-specific defects in the MIC of a conjugating cell and if these cells now go through conjugation with a WT MAC, the conjugation phenotype should be rescued. On the other hand, if the S134A mutation also causes irreversible damage to mitotic MICs during vegetative growth, this damage should accumulate before the cells are somatically rescued by replacing the *HTAX S134A* gene with a WT *HTAX* gene. Therefore, although the reintroduced *HTAX* completely replaced the *HTAX S134A* genes (data not shown), these cells would retain damaged MICs that might not be able to complete conjugation. Table 2 shows that the S134A mutation phenotype in conjugation (determined as a low percentage of exconjugants and low Pm^r/Cy^r ratio when cells were mated with CU427 cells) is still present in S134A rescued cells in which the mutated genes in MACs had been replaced with WT *HTAX* genes, arguing that, in addition to affecting meiosis, the *HTAX S134A* mutation causes micronuclear defects during vegetative

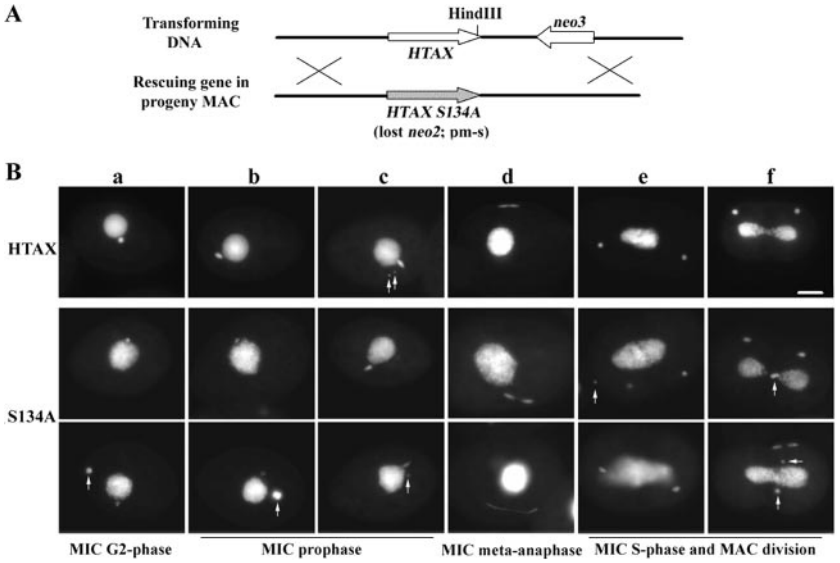

FIG. 5. Eliminating H2A.X SQ motif phosphorylation causes micronuclear DNA loss and abnormal mitosis in *T. thermophila*. (A) Strategy for replacing the mutant *HTAX S134A* gene with WT *HTAX-neo3* gene in MACs of S134A rescued cells. (B) Vegetatively growing HTAX rescued cells (top row) and S134A rescued cells (lower rows) were fixed and stained with DAPI. Shown are MICs and MACs at different stages of the vegetative cell cycle as indicated. Arrows indicate CEBs (see text for description). Scale bar, 10 μm.

growth. Therefore, H2A.X phosphorylation on the SQ motif is likely required for both normal meiosis and mitosis.

Because cells must be grown vegetatively before they can be conjugated, we cannot rule out the possibility that the meiotic defects we observed in both the rejuvenated S134A cells or somatic S134A cells described above were due to the accumulation of mitotic defects during the period of vegetative growth before the cells were conjugated. However, the behavior of other mitotic defect mutants or knockouts suggests that the mitotic defects of S134A cannot account for all of the meiotic defects. (i) *HTAY* conditional knockout cells in nonpermissive conditions have mitotic defects (X. Song and M. A. Gorovsky, submitted for publication) but can mate with WT cells and finish conjugation, producing 50% exconjugants (Song and Gorovsky, unpublished observations), while in the mating between S134A and WT CU427, only a low percentage (<7%) of exconjugants were produced and most of the paired cells stopped mating after meiosis II. (ii) *DCL1* knockout cells, which have severe defects in mitotic chromosome segregation, could finish mating at a low percentage (52), while in matings between two different mating types of S134A rescued cells stopped mating after meiosis II. For these reasons, we believe that, besides the mitotic defects, S134A has meiotic defects.

To confirm that the *HTAX S134A* mutation causes micronuclear damage during mitosis in vegetative growth, we examined micronuclear morphology by DAPI staining of log-phase S134A or HTAX rescued cells. MICs in more than 70% of the S134A rescued cells (Fig. 5B, lower rows) were smaller, more irregular, and less strongly stained than the bright and distinct MICs of HTAX rescued cells in both mitotically dividing and nondividing stages (Fig. 5B, top row), suggesting there was DNA loss during vegetative growth in S134A rescued cells. In HTAX rescued cells, as in WT cells (30), when macronuclear division and cytokinesis start, the MICs have already finished their mitotic division and are visualized as two round dots near the ends of the cells, each of which will enter one of the daughter cells (Fig. 5B, top row, frames e and f). In mitotic S134A rescued cells, lagging chromosomes (Fig. 5B, lower rows) were often observed along with delayed mitotic divisions in which MICs were still in different stages of mitosis when the MACs and cells were dividing (Fig. 5B, lower rows, frames e and f).

MACs in S134A cells also showed defects. There are more cells with chromatin exclusion bodies (CEBs) in the S134A rescued strain (Fig. 5B, lower rows, arrows in frames a, b, c, e and f) than in HTAX rescued cells. DNA loss by elimination of CEBs occurs normally in *Tetrahymena* and is thought to be a mechanism to maintain the level of macronuclear ploidy (6). Thus, H2A.X phosphorylation is required for normal micronuclear mitosis and probably also for normal macronuclear division.

***The HTAX S134A* mutation in the MAC causes DNA damage accumulation in both MACs and MICs.** Next, we studied whether the DSB repair machinery is affected in S134A rescued cells. It has been reported that foci of Rad51, a recombinase required for HR in both meiosis and mitosis (72), are present in vegetative as well as conjugating cell MACs (45). IF analyses showed that the Rad51 signal in MACs in vegetatively growing S134A or HTAX rescued cells was similar as was the appearance of Rad51 in meiotic MICs (data not shown). Thus, in *Tetrahymena*, Rad51 accumulation is not dependent on H2A.X phosphorylation. We then tested whether the S134A mutation causes less efficient repair and leads to accumulation of DNA damage. Since γ-H2AX is one of the earliest cellular responses to DNA DSBs, we first checked γ-H2AX expression in S134A rescued cells. To overcome the fact that, in S134A rescued cells, the mutated H2A.X S134A cannot be phosphorylated on S134 and thus cannot be stained by the anti-γ-H2AX MAb, we utilized the well-established phenomenon of conjugation-mediated transfer of protein and/or mRNA between two mating cells in *Tetrahymena* (51). A WT cell was mated with an S134A rescued cell whose nuclei, lacking WT H2A.X, cannot be stained with the anti-γ-H2A.X MAb. When the mutant cell receives WT H2A.X (or *HTAX* mRNA) from the WT cell by conjugation-mediated transfer (Fig. 6A), both cells in the pair should be stained with the anti-γ-H2AX if H2A.X is phosphorylated on SQ. The staining observed in the mutant cell then becomes an assay for the presence of DSBs in that cell.

IF staining by anti-γ-H2A.X of matings between WT and S134A or HTAX rescued cells are shown in Fig. 6B. In matings between WT and HTAX rescued cells, γ-H2A.X staining is observed initially in early stage II meiotic prophase cells (Fig. 6B, row c), as in WT mating cells (Fig. 1B). However, in matings between WT and S134A rescued cells, γ-H2A.X signal is detectable in the S134A cell in stage I, even before the MICs elongate (Fig. 6B, row b), indicating that DNA DSBs had accumulated in MICs before conjugation. In the S134A rescued cell, the MAC also shows a strong signal (Fig. 6B, row b) that is not observed in the WT partner. These differences between the cells containing WT H2A.X and the S134A mutant H2A.X distinguish the WT from S134A rescued cells in a pair. When MICs elongate, the γ-H2A.X staining appears in MICs of both WT and S134A rescued cells, and the macronuclear staining in S134A cells persists (Fig. 6B, rows d, f, and h). γ-H2A.X staining in parental MACs of conjugating WT cells is never observed. From the DAPI staining, as observed in vegetative cells, the MICs, and probably also the MACs, of S134A rescued cells contain less DNA than those in WT cells. As seen in matings of two S134A rescued cells (Fig. 3B), meiotic defects in matings between WT and S134A cells were also observed. γ-H2A.X signal was observed in the hypodiploid MICs as well as lagging chromosomes in metaphase I (Fig. 6C, rows a and b), anaphase I (Fig. 6C, row c), and anaphase II (Fig. 6C, rows d and e). These defects were observed only in the S134A cell in the pair but not in the WT partner, indicating that they reflected intrinsic defects in the S134A MICs. These abnormalities were not observed in matings between WT and HTAX rescued cells (data not shown). These results argue that DSBs exist in meiotic MICs before and after the period of HR as well as in MACs of the S134A cells. Although the major function of γ-H2AX appears to be associated with DSBs, it has also been suggested to have other functions (reviewed in reference 59). Note, however, that one commonly cited case where H2A.X phosphorylation appears to function independently of DSBs, its association with the inactive X chromosome, has recently been shown to occur during late replication, where it could also be associated with transient, replication-associated DSBs (14). To confirm that S134A cells accumulated DSBs, we used single-cell, neutral gel electrophoresis

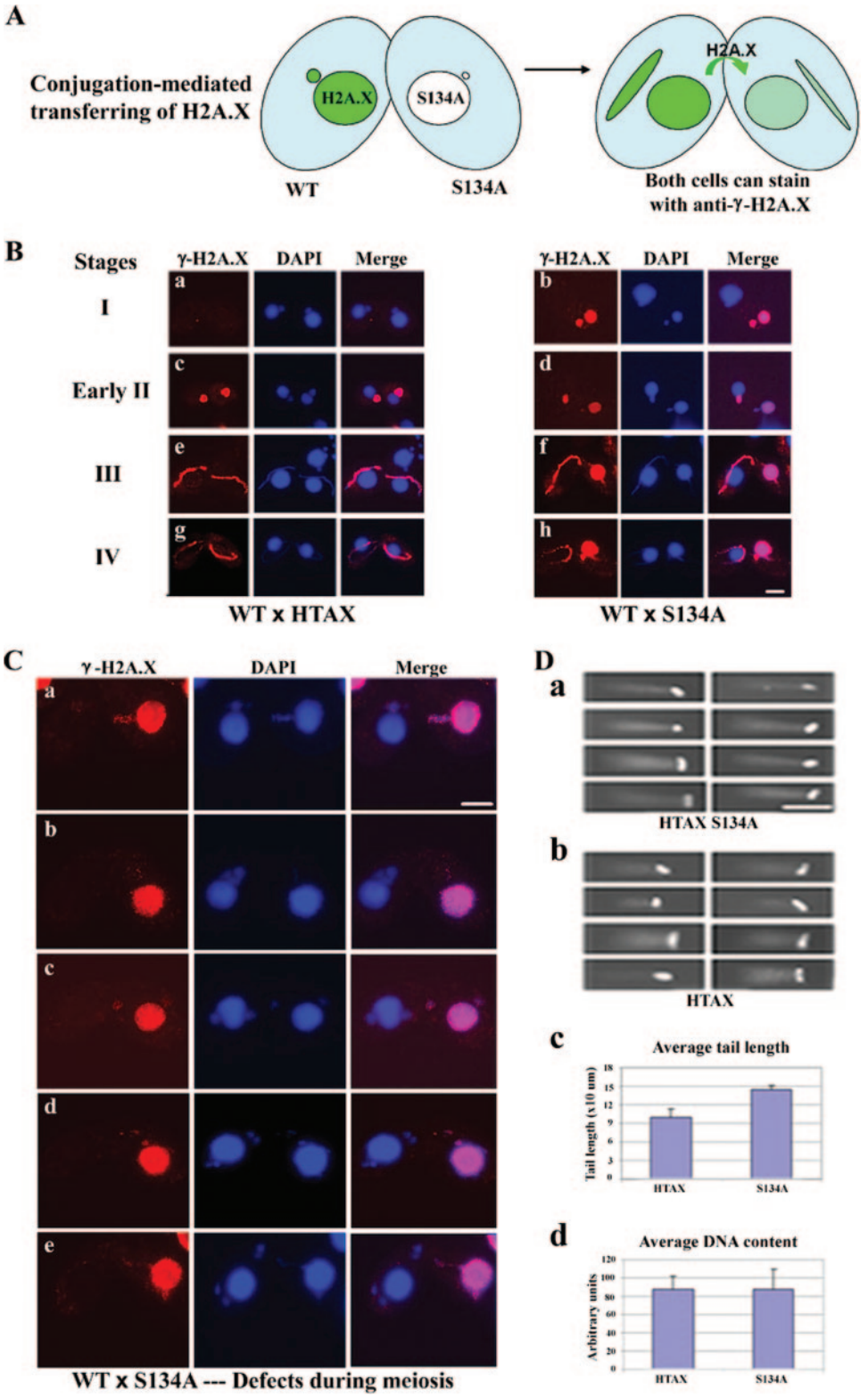

FIG. 6. *HTAX S134A* mutation in MACs causes DNA damage accumulation in both MICs and MACs. (A) Diagram of conjugation-mediated transfer of protein (or mRNA) between the two partners of a pair. (B) IF analysis of conjugation between WT and HTAX rescued cells (rows a, c, e, and g) or WT and S134A rescued cells (rows b, d, f, and h), stained with anti-γ-H2A.X and DAPI. WT cell is on the left in each pair of matings between WT and S134A rescued cells. WT cells and HTAX rescued cells are indistinguishable. (C) IF analysis of conjugation between WT and S134A rescued cells, stained with anti-γ-H2A.X and DAPI, showing the meiotic defects of S134A MICs (see text for details). WT cell is on the left in each pair. (D) Neutral comet assay showing the DAPI-stained nuclei (a and b) after neutral single-cell gel electrophoresis. Graphs show quantification of the average tail lengths (c) and total DNA contents (including tail and chromosomal DNA; d) from about 50 nuclei of S134A or HTAX rescued cells using the Image J program. Scale bars, 10 μm (B and C) and 100 μm (D).

(comet assay) (75). As shown in Fig. 6D, S134A rescued cells exhibited longer comet tails (average tail length, 145 μm; $n = 51$; standard deviation [SD], 6.7) than HTAX rescued cells (average tail length, 99 μm; $n = 50$; SD, 13.6) (Fig. 6D, a to c), while they have similar total (mostly macronuclear) DNA contents (Fig. 6D, d), indicating that they contain more DSBs in their macronuclear DNA.

DISCUSSION

We show in this study that one of the major histone H2As in *Tetrahymena* is a typical H2A.X. It can be phosphorylated at the serine 134 residue in its SQ motif in response to DSBs induced by chemical agents and during meiosis. Using a MAb specific to a phosphopeptide (KATQA[pS]QEY) corresponding to residues 134 to 142 of human H2A.X, together with a mutant strain (S134A) that abolished the phosphorylation site, we demonstrate that the SQ motif phosphorylation is important for cells to recover from exogenous DNA damage and to repair breaks associated with normal micronuclear meiosis and mitosis and macronuclear amitosis. The inability to phosphorylate this site leads to meiotic, mitotic, and amitotic defects and accumulation of DSBs in both MICs and MACs of *Tetrahymena* cells.

Although the H2A.X S134A mutation causes visible defects in mitosis of MICs, and appears to affect amitosis of MACs, the mutation is not lethal for vegetative growth. Most previously described mutations that affect *Tetrahymena* MICs are not lethal (52, 86), which is likely due to the transcriptional inertness of the MIC in vegetative growth. Mutations that abolish the SQ phosphorylation site or knock out H2AX in other organisms also are not lethal (12, 23), suggesting that either there are alternative, less efficient, pathways to repair DSBs in the absence of γ-H2A.X or that cells can tolerate DSBs. While our studies do not allow us to determine whether *Tetrahymena* has such alternative pathways, it is clear that this organism can tolerate unrepaired DSBs.

Since the S134A rescued strain grew but had severe meiosis defects and stopped conjugation prematurely, it appears that, in *Tetrahymena*, either the role of γ-H2A.X is more important in repair of meiotic DSBs than in repair of breaks created during mitosis or that meiosis is more sensitive to the existence of DSBs. During vegetative growth, the MIC is not transcribed, and the damage accumulated in vegetative MICs can cause mitotic defects but will not have a major phenotypic effect until the next round of conjugation. Since *Tetrahymena* cells can replicate indefinitely in the absence of functional MICs, it may have been evolutionarily advantageous to eliminate mitotic DNA damage checkpoints. In MACs, which are transcriptionally active and control the phenotype of vegetative cells, there are ~45 copies of each chromosome, so breaks in some genes could be compensated by other copies that are still intact, enabling cells to survive with unrepaired DSBs.

Given that the MIC is the germ line nucleus and will give rise to the new MIC and MAC in the process of conjugation, it seems reasonable that *Tetrahymena* cells would have meiotic checkpoints that protect the long-term survival of the cells by stopping the process of conjugation to prevent production of progeny with aneuploid macronuclei. However, although H2A.X phosphorylation at the SQ motif is essential for proper meiosis and leads to premature termination of conjugation after meiosis II, matings between the S134A rescued cells showed no evidence of a block in prophase I even though they show chromosome loss at metaphase I and chromosome segregation defects in anaphase I. These results suggest that the recombination (or pachytene) checkpoint observed in many organisms, which monitors DSBs in meiotic recombination and delays cell cycle progression in prophase I until all the DSBs have been repaired (33, 66), is either weak or nonexistent in the absence of γ-H2A.X in *Tetrahymena* cells. It is possible that an adaptation process, which renders cells capable of overcoming the checkpoint-dependent block and permits meiotic progression with unrepaired DSBs, could be operating in the S134A rescued cells. Such adaptations have been observed in the budding yeast recombination checkpoint (33, 48, 93). However, this seems unlikely since there is no detectable delay in the progress of S134A cells through meiosis until after meiosis II (Fig. 3). The arrest observed in later stages of conjugation, after meiosis II and before the third prezygotic mitosis in matings between S134A rescued cells, could be explained by the activity of a mitotic DNA damage checkpoint activated by the unrepaired DSBs that persist past meiosis II in S134A rescued cells. If this is the case, there is no specific meiotic checkpoint in *Tetrahymena* in the absence of γ-H2A.X. However, if such a mitotic checkpoint exists in *Tetrahymena*, it must be subject to adaptation in vegetative S134A cells, which continue to grow, but not in conjugating cells.

Meiotic recombination events in mouse and yeast are well studied, and γ-H2A.X appearance in these organisms precedes and is spatially distinct from synapsis (47, 65, 95). SCs have not been identified in *Tetrahymena*. Our results show that *Tetrahymena* γ-H2A.X follows a similar time line, even though it lacks SCs; i.e., γ-H2A.X appears in early stage II cells, before the reported appearance of Rad51 in MICs in late stage II and before the close pairing of homologous chromosomes in stage IV (45), and disappears when the micronuclear chromosomes become condensed in metaphase I. These studies provide the first evidence for the timing of the appearance of meiotic DSBs in *Tetrahymena*, demonstrating that they occur in the very early crescent stage of *Tetrahymena* conjugation at the beginning of prophase of meiosis I and that they likely persist until the end of the crescent stage, when meiotic crossing over is probably completed. Finally, our results shed new light on the nature of the programmed degradation of the parental MAC during conjugation. Previous studies showed that this process is accompanied by the production of oligonucleosome-sized DNA fragments (20) and the appearance of caspase 1-, 8-, and 9-like (25, 37) and endonuclease G-like activities (38), suggesting that it occurred by a mechanism resembling apoptosis in higher organisms. However, apoptosis in higher organisms is also accompanied by phosphorylation of H2A.X (46, 54, 68), and we were unable to demonstrate any γ-H2A.X staining in degenerating MACs. We also failed to detect any open reading frames with significant homology to caspases 1, 8, and 9 or to endonuclease G in a BLASTP search of the TIGR gene predictions based on the *Tetrahymena* macronuclear genome sequence (http://seq.ciliate.org/cgi-bin/BLAST-tgd.pl), making it highly unlikely that an apoptotic-like mechanism is involved.

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