# Histone H2B Ubiquitylation Is Not Required for Histone H3 Methylation at Lysine 4 in *Tetrahymena*[\*][s]

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Ubiquitylation of histone H2B and/or a component of the system that ubiquitylates H2B is required for methylation of histone H3 at lysine 4 (H3K4) in yeasts and probably in humans. In this study, the single ubiquitylation site was mapped to conserved lysine 115 of the C-terminal region of histone H2B in the single-cell model organism *Tetrahymena thermophila*. In strains lacking H2B ubiquitylation, H3K4 methylation was not detectably affected. As in other organisms, the E2 ubiquitin-conjugating enzyme Ubc2 and the E3 ubiquitin ligase Bre1 were required for H2B ubiquitylation. However, neither enzyme was required for H3K4 methylation. These studies argue that, in *T. thermophila*, the histone ubiquitylation mechanism is not required for H3K4 methylation, demonstrating that different organisms can speak different languages in the "cross-talk" among post-translational modifications on different histones.

In the nuclei of eukaryotic cells, DNA is highly compacted into chromatin, a nucleoprotein complex, whose major protein components are the histones. The basic unit of chromatin is the nucleosome core in which 146 base pairs of double-stranded DNA are wrapped in ~1.75 left-handed superhelical turns around an octamer containing two molecules of each of the four conserved core histones H2A, H2B, H3, and H4 (1, 2). A fifth, less conserved linker histone H1, associates with a variable length of linker DNA between nucleosome cores. Site-specific histone post-translational modifications, such as acetylation, methylation, phosphorylation, and ubiquitylation, are correlated with diverse chromatin functions, including DNA replication, DNA repair, chromatin assembly, gene transcription, and other chromatin-based processes (3, 4). It has been suggested that at least some distinct histone modifications act sequentially or in combination to form what has been variously referred to as a histone, epigenetic, or nucleosome "code" that is read by other proteins or protein complexes to alter chromatin structure and regulate distinct downstream events (5, 6). Deciphering this code, which is likely to have complex redundant, combinatorial, and multivalent features, is essential for understanding the *in vivo* function of the diverse post-translational modifications on histones.

Ubiquitin is a 76-amino acid globular protein that is conserved in eukaryotes. Unlike proteins that are targeted by polyubiquitylation to the proteosome for degradation, histones are reversibly modified by covalent attachment of one ubiquitin or a short ubiquitin chain. The bulk of histone ubiquitylation occurs on chromatin by the addition of a single ubiquitin molecule via an isopeptide linkage to a specific lysine residue in the C-terminal tail of histones H2A and H2B (7, 8). To a lesser extent, histones H1 (9), H3, and H4 (10, 11) also can be ubiquitylated *in vivo*. Ubiquitylation on different histones has distinct functions (12).

A number of recent studies have focused on ubiquitylation of H2B. In the budding yeast *Saccharomyces cerevisiae*, H2B is monoubiquitylated at lysine 123 by an evolutionarily conserved E2 ubiquitin-conjugating enzyme, Rad6/Ubc2 (13). Initial reports indicated that Ubc2-mediated H2B monoubiquitylation is a prerequisite for methylation of histone H3 at lysine 4 and lysine 79 by the histone methyltransferases Set1 and Dot1, respectively (14–17), providing the first evidence of "cross-talk" between different post-translational modifications on different histones. Bre1 was identified as an E3 ubiquitin ligase that associates with Rad6/Ubc2 and directs it for H2B ubiquitylation (18, 19). In budding yeast, Ubc2-Bre1-mediated H2B ubiquitylation is highly dynamic, and the sequential ubiquitylation and deubiquitylation of H2B helps to establish the appropriate level of H3K4[3] methylation and regulates transcriptional initiation (20, 21). Ubc2-Bre1-mediated H2B ubiquitylation is dependent on the transcriptional elongation complex Paf1 and promotes efficient RNA polymerase II elongation (22–24). In human cells, H2B ubiquitylation is also involved in transcriptional activation and appears to affect H3K4 methylation, as shown by knockdown of human orthologs of yeast *BRE1* (25, 26). It has been shown that Rad6/Ubc2 is also required for H2B ubiquitylation and efficient H3K4 di- and trimethylation in human cells (27). In the fission yeast *Schizosaccharomyces pombe*, Ubc2-Bre1-mediated H2B monoubiquitylation is conserved and is required for H3K4 methylation (28, 29). The apparent conservation of the relationship between H2B ubiquitylation and H3K4 methylation in yeasts and humans has led to the general belief that this pattern of cross-talk is likely to be universal. However, a recent study (30) has called some of the initial results on budding yeast into question. It was shown that

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[3] The abbreviations used are: H3K4, histone H3 lysine 4; H3K4me3, trimethyl-histone H3 lysine 4; H3K4me2, dimethyl-histone H3 lysine 4; H3K4me1, monomethyl-histone H3 lysine 4.

**TABLE 1**
**Tetrahymena strains used in this study**

| Strain | Mic genotype[a] | Mac genotype[a] | Mac phenotype[b] |
|---|---|---|---|
| B2086 | WT[c] | WT | II |
| CU428 | *mpr1-1/mpr1-1* | WT | mp-s, VII |
| CU427 | *chx1-1/chx1-1* | WT | cy-s, VI |
| B*VI | Genetically unfunctional mic (star) | WT | VI |
| B*VII | Genetically unfunctional mic (star) | WT | VII |
| hDΔ*HTB*-86B4A6 | *htb1-1::neo2/htb1-1::neo2; htb2-1::neo2/htb2-1::neo2* | WT | pm-s |
| hDΔ*HTB*-86D2A2 | *htb1-1::neo2/htb1-1::neo2; htb2-1::neo2/htb2-1::neo2* | WT | pm-s |
| *HTB1* | *htb1-1::neo2/htb1-1::neo2; htb2-1::neo2/htb2-1::neo2* | *HTB1/htb1-1::neo2; htb2-1::neo2/htb2-1::neo2* | pm-r |
| *htb1-K115R* | *htb1-1::neo2/htb1-1::neo2; htb2-1::neo2/htb2-1::neo2* | *htb1-2K115R/htb1-1::neo2; htb2-1::neo2/htb2-1::neo2* | pm-r |
| *htb1-K111R* | *htb1-1::neo2/htb1-1::neo2; htb2-1::neo2/htb2-1::neo2* | *htb1-3K111R/htb1-1::neo2; htb2-1::neo2/htb2-1::neo2* | pm-r |
| *htb1-K(111, 115)R* | *htb1-1::neo2/htb1-1::neo2; htb2-1::neo2/htb2-1::neo2* | *htb1-4K(111, 115)R/htb1-1::neo2; htb2-1::neo2/htb2-1::neo2* | pm-r |
| Δ*HHT3*Δ*HHT4* | *hht3-1::neo2, hht4-1::bsr1/hht3-1::neo2, hht4-1::bsr1* | *hht3-1::neo2, hht4-1::bsr1/hht3-1::neo2, hht4-1::bsr1* | pm-r, bs-r |
| *HHT3* | *hht1-1::neo2/hht1-1::neo2; hht2-1::neo2, hhf2-1::neo2/hht2-1::neo2, hhf2-1::neo2; hhf1-1::neo2/hhf1-1::neo2* | *hht1-1::neo2/hht1-1::neo2; HHT3, HHF2/hht2-1::neo2, hhf2-1::neo2; hhf1-1::neo2/hhf1-1::neo2* | pm-r |
| *hht2-K4Q* | *hht1-1::neo2/hht1-1::neo2; hht2-1::neo2, hhf2-1::neo2/hht2-1::neo2, hhf2-1::neo2; hhf1-1::neo2/hhf1-1::neo2* | *hht1-1::neo2/hht1-1::neo2; hht2-2K4Q, HHF2/hht2-1::neo2, hhf2-1::neo2; hhf1-1::neo2/hhf1-1::neo2* | pm-r |
| Δ*UBC2* | *ubc2-1::neo2/ubc2-1::neo2* | *ubc2-1::neo2/ubc2-1::neo2* | pm-r |
| Δ*BRE1* | *bre1-1::neo2/bre1-1::neo2* | *bre1-1::neo2/bre1-1::neo2* | pm-r |

[a] Only showing disrupted or mutant alleles.
[b] Including drug phenotype and mating type.
[c] WT, wild type.

in *S. cerevisiae*, H3K4 methylation is not solely dependent on H2B ubiquitylation and that elimination of the H2B ubiquitylation site, combined with an additional mutation on H2B, which was not identified in previous studies, are required to eliminate H3K4 methylation. This study also confirmed that *BRE1* is required for H3K4 methylation in *S. cerevisiae*, suggesting that components of the H2B ubiquitylation mechanism but not ubiquitylation itself were essential for H3K4 methylation. In addition, in both budding and fission yeast, H2B ubiquitylation has functions in transcription that are independent of H3K4 methylation (28, 29). H2B ubiquitylation in fission yeast also functions in cell growth, nuclear structure, and heterochromatin derepression by unknown mechanisms that are independent of H3K4 methylation, and H2B ubiquitylation regulates the expression of more genes than does H3K4 methylation (28).

In this study, the single ubiquitylation site in *Tetrahymena thermophila* histone H2B was mapped to the conserved lysine 115 of its C-terminal region. In mutant strains lacking H2B ubiquitylation, H3K4 methylation is not detectably affected. *Tetrahymena* orthologs of Ubc2 and Bre1 were identified as the conserved E2 and E3 enzymes specific for H2B ubiquitylation at Lys[115]. Neither enzyme is required for H3K4 methylation. These studies strongly suggest that, unlike in yeasts and mammals, neither Ubc2-Bre1-mediated H2B ubiquitylation nor the major components of the H2B ubiquitylation mechanism are required for H3K4 methylation in *Tetrahymena*, demonstrating that different organisms speak different languages and use different components in the cross-talk between post-translational modifications on different histones.

**EXPERIMENTAL PROCEDURES**

*Tetrahymena Strains and Culture Conditions—T. thermophila* wild-type strains B2086, CU428, and CU427 were kindly provided by P. J. Bruns (Cornell University, Ithaca, NY). Strains used in this study are listed in Table 1. Cells were grown in super proteose peptone medium (1% proteose peptone, 0.1%

yeast extract, 0.2% glucose, 90 $\mu$M EDTA ferric sodium salt) at 30 °C (31). For conjugation, midlogarithmic phase growing cultures of different mating types were washed, starved (16–24 h at 30 °C), and mated in 10 mM Tris-HCl (pH 7.5) (32).

*Making HTB1 and HTB2 Knock-out Constructs—*The *HTB1* gene was cloned from *Tetrahymena* genomic DNA, and plasmid pHTB1 was constructed. It is a pBluescript KS(+) (Stratagene) derivative and contains 1.7 kb of *HTB1* 5′-flanking sequence, the *HTB1* coding region, and 2.2 kb of the *HTB1* 3′-flanking sequence. Plasmid p4T2-1 (33) is a pBluescript KS(+) derivative and contains the *neo2* cassette, which consists of 0.3 kb of 5′-flanking sequence of the *HHF1* gene (encoding histone H4); the *neo* gene coding region, which confers resistance to paromomycin when expressed in *Tetrahymena*; and 0.3 kb of the 3′-flanking sequence of the *BTU2* gene (encoding β-tubulin). To make the *HTB1* knock-out construct (supplemental Fig. S2A), a 1.7-kb EcoRI-BstXI restriction fragment of the *HTB1* 5′-flanking sequence from pHTB1 was inserted into the EcoRV site of plasmid p4T2-1. A 1.4-kb fragment of the *HTB1* 3′-flanking sequence (from the TGA stop codon to a SpeI site) was PCR-amplified from pHTB1 and inserted into the SpeI site of p4T2-1. The final construct, referred to as pHTB1-KO, was digested with KpnI and SacI, and a 4.5-kb insert containing *HTB1* 5′-flanking sequence, the *neo2* cassette, and *HTB1* 3′-flanking sequence was released and used for germ line transformation. All constructs were verified by sequencing.

The *HTB2* gene was cloned from *Tetrahymena* genomic DNA, and plasmid pHTB2 was constructed. It is a pBluescript KS(+) (Stratagene) derivative and contains 1.7 kb of *HTB2* 5′-flanking sequence, the *HTB2* coding region, and 2.4 kb of *HTB2* 3′-flanking sequence. To make the *HTB2* knock-out construct (supplemental Fig. S2B), a 1.7-kb fragment of the *HTB2* 5′-flanking sequence (from a HindIII site to 33 bp upstream of the ATG start codon) was PCR-amplified from pHTB2 and inserted into the EcoRV site of p4T2-1. A 2.4-kb fragment of the *HTB2* 3′-flanking sequence (from the TGA

stop codon to an EcoRI site) was PCR-amplified from pHTB2 and inserted into the SmaI site of p4T2-1. The final construct, referred to as pHTB2-KO, was digested with XhoI and SpeI, and a 5.5-kb insert containing *HTB2* 5′-flanking sequence, *neo2* cassette, and *HTB2* 3′-flanking sequence was released and used for germ line transformation. All constructs were verified by sequencing.

*Creation of HTB1 or HTB2 Single Knock-out Heterokaryons*—To obtain germ line transformants in which a single allele in the micronucleus had been knocked out, B2086 and CU428 cells were mated and transformed at 2.5–3.5 h postmixing with either the *HTB1* or *HTB2* knock-out construct using the PDS-1000/He biolistic particle delivery system (Bio-Rad) as described previously (34). Germ line knock-out homozygous heterokaryon strains, in which both copies of either the *HTB1* or *HTB2* gene are replaced by the *neo2* cassette in the germ line micronucleus but retain wild-type genes in the somatic macronucleus, were then created as described previously (35).

*Creation of HTB1 and HTB2 Double Knock-out Heterokaryons*—*HTB1* and *HTB2* double knock-out heterokaryon strains hΔΔHTB-86B4A6 and hΔΔHTB-86D2A2 (Table 1) were created by crossing the *HTB1* and *HTB2* single knock-out heterokaryon strains as described previously (35).

*Making HTB1 Somatic Rescue Constructs and Creation of HTB1 Somatic Rescue Strains*—PCR-directed mutagenesis was performed as described previously (36) on pHTB1-M, which is identical to pHTB1 except that it contains a slightly shorter 3′-flanking sequence of *HTB1*. At a specific residue, the codon for lysine was changed to arginine. The resulting plasmids or pHTB1-M were digested with PstI and XhoI, and the 4.1-kb inserts containing 1.7 kb of *HTB1* 5′-flanking sequence, the wild-type or mutated *HTB1* gene, and 2.1 kb of *HTB1* 3′-flanking sequence were released and used for somatic rescue transformation. All constructs were verified by sequencing.

As expected, the progeny of two *HTB* double knock-out heterokaryon strains were not viable because they lacked both genes for H2B, an essential, major component of the nucleosome core. Somatic rescue strains were created by transforming mating double knock-out heterokaryon strains hΔΔHTB-86B4A6 and -86D2A2 at late conjugation (20–24 h postmixing) by biolistic transformation with a wild-type or mutated copy of the *HTB1* gene and selecting paromomycin-resistant progeny (34).

*Cloning the Tetrahymena UBC2 Gene and Creation of UBC2 Knock-out Strains*—A gene (TTHERM_00550720) encoding a putative Ubc2 was identified in the *Tetrahymena* Genome Database (available on the World Wide Web) using *S. cerevisiae* Rad6/Ubc2 as the query and named *UBC2*. A 3.0-kb HindIII-HincII fragment containing 0.8 kb of 5′-flanking sequence, the 456 bp *UBC2* coding region, and 1.7 kb of 3′-flanking sequence was amplified by PCR from genomic DNA and cloned into the pBluescript KS(+) vector (Stratagene). The construct, named pUBC2, was verified by sequencing.

To make the *UBC2* knock-out construct pUBC2-KO, the 456-bp *UBC2* coding region in pUBC2 was replaced by the *neo2* cassette (33). pUBC2-KO was digested with HindIII and SpeI to release a 4.0-kb *ubc2-1*::*neo2* insert, which was used for germ line transformation. Germ line knock-out homozygous hetero-

karyon strains, in which both copies of the *UBC2* gene are replaced by the *neo2* cassette in the germ line micronucleus but retain wild-type genes in the somatic macronucleus, were then created as described previously (35). The *UBC2* knock-out homozygous homokaryon strains (ΔUBC2), in which *ubc2-1*::*neo2* had completely replaced all micronuclear and macronuclear *UBC2* copies, were created by mating *UBC2* germ line knock-out heterokaryon strains and selecting their paromomycin-resistant progeny. The ΔUBC2 strains were verified by PCR.

*Cloning the Tetrahymena BRE1 Gene and Creation of BRE1 Knock-out Strains*—A gene (TTHERM_00790530) encoding a putative Bre1 was identified in the *Tetrahymena* Genome Database and named *BRE1*. A 5.7-kb fragment containing 1.2 kb of 5′-flanking sequence, the 3.2-kb *BRE1* coding region, and 1.3 kb of 3′-flanking sequence was amplified by PCR from genomic DNA and cloned into the pBluescript KS(+) vector (Stratagene). The construct, named pBRE1, was verified by sequencing.

To make the *BRE1* knock-out construct, the *BRE1* coding region, except for 80 bp of 3′ sequence, in pBRE1 was replaced by the *neo2* cassette (33). The final construct, referred to as pBRE1-KO, was digested with KpnI and SacI, and a 4.0-kb *bre1-1*::*neo2* insert was released and used for germ line transformation. Germ line knock-out homozygous heterokaryon strains, in which both copies of the *BRE1* gene are replaced by the *neo2* cassette in the germ line micronucleus but retain wild-type genes in the somatic macronucleus, were then created as described previously (35). The *BRE1* knock-out homozygous homokaryon strains (ΔBRE1), in which *bre1-1*::*neo2* had completely replaced all micronuclear and macronuclear *BRE1* copies, were created by mating *BRE1* germ line knock-out heterokaryon strains and selecting their paromomycin-resistant progeny. The ΔBRE1 strains were verified by PCR.

*PCR Analysis*—Total genomic DNA was isolated from cells of wild-type, ΔUBC2, and ΔBRE1 strains, as described previously (37), and used for PCR. The PCR products were separated on agarose gels. The primers used for the *UBC2* locus were UBC2-04 (5′-GAATTAAACTTACTTATGTCTATC-3′), UBC2-seqC (5′-GAACGAGTGATTGAACTTG-3′), and neo-sense (5′-GAATGGGCTGACCGCTTCCTCGTGC-3′). The primers used for the *BRE1* locus were BRE1-18 (5′-TTTACAGATAGATTGATTGAGTG-3′), BRE1-19 (5′-TTCCATATCTATCTATCTATCTTG-3′), and neo-253 (5′-CAGCCAGTCCCTTCCCGCTTCAGTGAC-3′).

*Growth Analysis*—Cells from each strain were used to inoculate 50 ml of super proteose peptone medium at starting densities of $1 \times 10^4$ cells/ml. Cultures were grown at 30 °C with vigorous shaking (150 rpm). Cells were counted by a Coulter counter (model ZBI, Coulter Electronics, Inc.) at frequent intervals. Growth data were plotted using Cricket Graph III (Computer Associates). Doubling times were calculated using the linear portion of the logarithmic growth curve.

*Fluorescence Microscopy*—Cells from each strain were grown to $2–3 \times 10^5$ cells/ml and fixed in 1.8% formaldehyde at room temperature, followed by staining with 0.4 $\mu$g/ml of the DNA-specific dye 4′,6-diamidine-2-phenylindole dihydrochloride (Roche Applied Science). Macro- and micronuclei were visual-

ized using an Olympus BH-2 fluorescence microscope. Images were recorded using a CFW-1310 M Grayscale digital camera (Scion Corp.) and processed using Adobe Photoshop.

*Nuclear Isolation and Histone Extraction*—Macronuclei were isolated from growing cells as described previously (31) and extracted with 0.2 M $H_2SO_4$ for 12–18 h at 4 °C, and the acid-soluble material was precipitated with 20% trichloroacetic acid (38).

*CNBr Cleavage*—Acid-extracted histones of isolated macronuclei were incubated with 1 mg/ml CNBr in 70% formic acid for 24 h at room temperature in the dark (39). For control, the samples were incubated with 70% formic acid only.

*Protein Electrophoresis and Western Blotting*—~15–20 $\mu$g of acid-extracted macronuclear histones were separated by 15% SDS-PAGE (40) and transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore) using a Trans-Blot SD semidry electrophoretic transfer cell (Bio-Rad). Blots were blocked in 5% nonfat dry milk in TBST1 (100 mM Tris-HCl (pH 7.5), 0.9% NaCl, 0.1% Tween 20) for 1 h at room temperature and incubated overnight at 4 °C with primary rabbit anti-ubiquitin antiserum (Sigma) diluted 1:1,000 in blocking buffer. Membranes were washed in TBST1 and incubated with a 1:5,000 dilution of goat anti-rabbit immunoglobulin (IgG) horseradish peroxidase-conjugated secondary antibody (Sigma) in 1% bovine serum albumin/TBST1 for 1 h at room temperature. Blots were processed using the Western Lightning chemiluminescence reagent (PerkinElmer Life Sciences) and exposed to Blue Sensitive Autoradiographic Film (Marsh Bio Products, Inc.). Before reprobing with other antisera, blots were incubated for 30 min at 50 °C in stripping buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 100 mM $\beta$-mercaptoethanol). The stripped blots were blocked in blocking buffer and probed with rabbit antisera made against *Tetrahymena* histones diluted in 1% bovine serum albumin/TBST1. Blots were then processed as described above for the anti-ubiquitin antiserum, except that a 1:20,000 dilution of the secondary antibody was used. The following histone antisera were used: anti-H2B (1:15,000 dilution), anti-H2A (1:20,000 dilution), anti-H4 (1:4,000 dilution) antisera (provided by Dr. C. David Allis, Rockefeller University, New York, NY) and anti-H2A.Z antiserum (1:10,000 dilution) (41).

~10 $\mu$g of CNBr-cleaved macronuclear histones were separated by 15% SDS-PAGE and transferred as above, except that Immobilon-P$^{SQ}$ polyvinylidene difluoride membranes (Millipore) were used for blotting. For the rabbit anti-trimethyl-histone H3 Lys[4] (anti-H3K4me3) antiserum (Abcam), which was preabsorbed with 1 $\mu$g/ml dimethyl-histone H3 Lys[4] (H3K4me2) peptide (Abcam) for 1 h at 37 °C, blots were blocked in 5% bovine serum albumin in TBST2 (TBST1 with Tween 20 at 0.5%) for 1 h at room temperature and incubated overnight at 4 °C with the primary rabbit antiserum diluted 1:5,000 in the blocking buffer. Membranes were washed in TBST2 and incubated with 1:10,000 dilution of goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Sigma) in the blocking buffer for 1 h at room temperature. Blots were then processed as above. For the rabbit anti-monomethyl-histone H3 Lys[4] (anti-H3K4me1) and anti-H3K4me2 antisera (Upstate), blots were blocked in 5% nonfat dry milk in TBST1 and incubated with the primary rabbit antiserum (1:2,000 dilution for anti-H3K4me1 antiserum, 1:5,000 dilution for anti-H3K4me2 antiserum) in the blocking buffer. Membranes were then incubated with a 1:5,000 dilution of goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Sigma) in the blocking buffer. Blots were stripped and reprobed with the anti-H3 antiserum (1:6,000 dilution) as described above for the other histone antisera.

*Microarray Analysis*—In each experiment, total RNAs were extracted from wild-type CU428 and mutant strains at a density of $2 \times 10^5$ cells/ml and purified further with Qiagen RNeasy columns. Gene expression microarray analysis was performed as described (42).

## RESULTS

*The Two Tetrahymena H2B Genes Function Redundantly*—In *Tetrahymena*, histone H2B.1 and H2B.2 (encoded by *HTB1* and *HTB2*, respectively) are non-allelic variants of H2B that differ in three amino acids (26) (supplemental Fig. S1). *HTB* single knock-out homozygous homokaryon strains, Δ*HTB1* and Δ*HTB2*, in which either the *HTB1* or *HTB2* gene, respectively, was disrupted in both macronuclear and micronuclear genomes, were viable and grew normally (data not shown), demonstrating that neither of two *HTB* genes is essential. *HTB* double knock-out heterokaryon strains were created, in which both copies of *HTB1* and *HTB2* genes were replaced by the *neo2* cassette in the germ line micronucleus while wild-type genes were retained in the somatic macronucleus. When two *HTB* double knock-out heterokaryon strains with different mating types were mated, all of their exconjugant progeny failed to grow. The progeny of *HTB* double knock-out heterokaryons could be rescued by somatic transformation with the wild-type *HTB1* gene, demonstrating that the growth defect was due to the absence of any copies of the *HTB* genes in the newly developed macronuclei of these exconjugant progeny. Therefore, like the other non-allelic genes encoding variants of the major core histones H2A, H3, and H4 (43, 44), the two genes encoding H2B are functionally redundant in *T. thermophila*.

*Lysine 115 Is the Only Residue of H2B That Can Be Ubiquitylated in Tetrahymena*—There are only two lysine residues, Lys[111] and Lys[115], in the *Tetrahymena* H2B C-terminal region. Lys[115] corresponds to a conserved C-terminal lysine residue that is the only ubiquitylation site of H2B in mammals (45), *S. cerevisiae* (13), *S. pombe* (28, 29), and *Arabidopsis* (46). To identify the ubiquitylation site(s) in *Tetrahymena* H2B and eliminate the possibility that, in the absence of the preferred H2B ubiquitylation site, the other lysine residue could be ubiquitylated, single or double lysine to arginine (which cannot be ubiquitylated) mutations were introduced (Fig. 1*A*). The wild-type *HTB1* gene and three mutated copies of the *HTB1* gene, *htb1-K111R*, *htb1-K115R*, and *htb1-K(111,115)R*, were used to rescue the progeny of mating between two *HTB1* and *HTB2* double knock-out heterokaryons that would otherwise die due to the absence of a functional gene encoding H2B in their macronucleus. All three mutated *HTB1s* could rescue the progeny, suggesting that neither Lys[111] nor Lys[115] is essential.

Monoubiquitylated H2B can be easily distinguished from unmodified H2B by its slow migration on one-dimensional
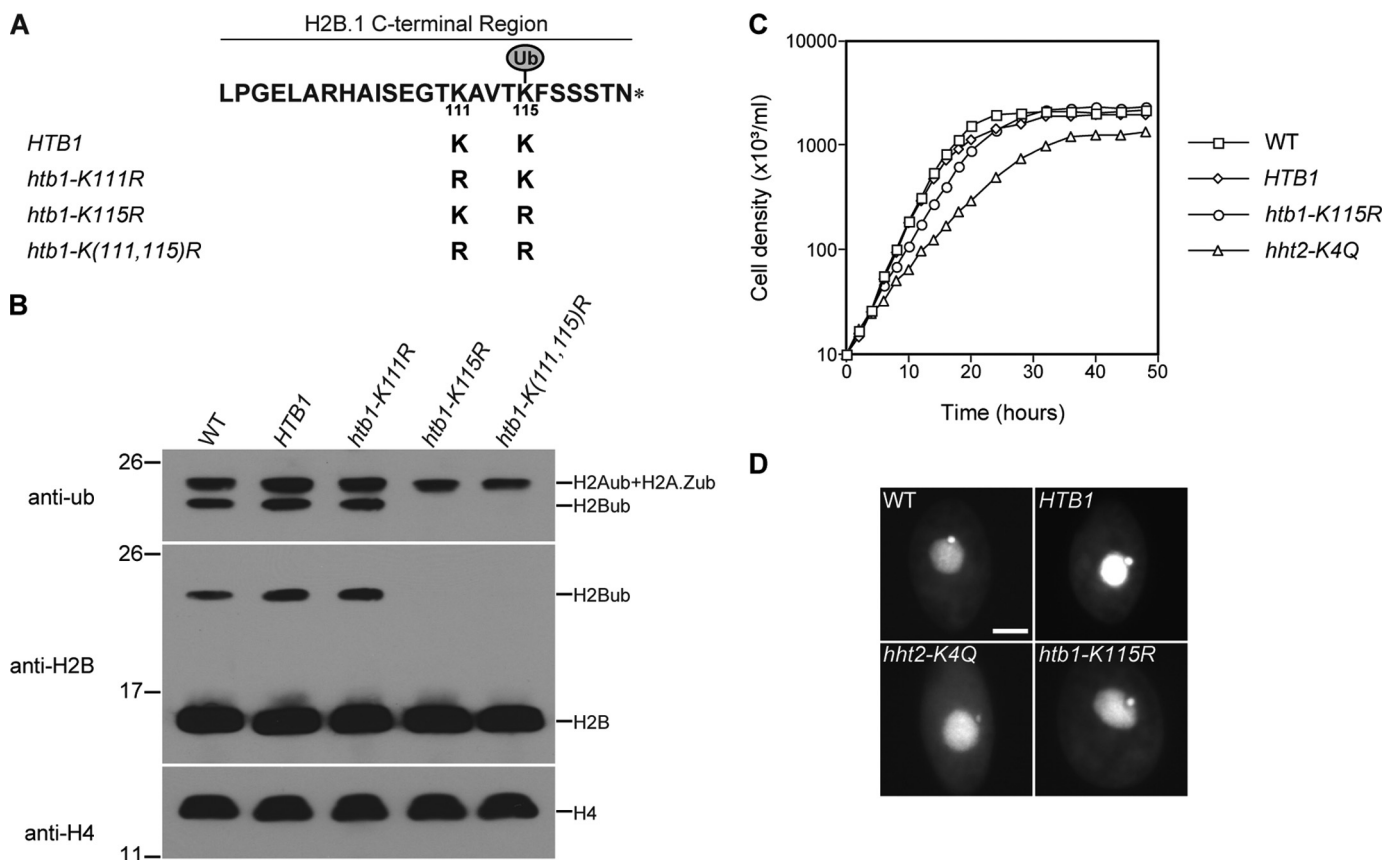
FIGURE 1. **Identification of Lys[115] as the only ubiquitylation site of histone H2B and demonstration that H2B ubiquitylation is not required for vegetative growth in Tetrahymena.** *A*, C-terminal sequences of proteins encoded by the wild-type and mutant *HTB1* genes used to rescue *HTB* knock-out strains. The two lysines in the C-terminal region of H2B.1 were changed, either separately or together, to arginines, which cannot be ubiquitylated. *B*, acid-extracted macronuclear histones of wild-type (*WT*) CU428, *HTB1*, and three *htb1* mutant strains were analyzed by Western blotting using anti-ubiquitin (*anti-ub*) and anti-H2B antisera. Anti-H4 antiserum was used to monitor histone loading. The slower migrating band stained with anti-H2B antiserum in the *middle panel* and the faster band stained with anti-ubiquitin antiserum in the *upper panel* (from which the unstained lower portion has been removed) co-migrate, enabling them to be identified as ubiquitylated H2B (*H2Bub*). Molecular mass markers (in kDa) are indicated on the *left*. *C*, growth curves of wild-type (*WT*) CU428, *HTB1*, *htb1-K115R*, and *hht2-K4Q* strains. The doubling times are as follows: wild type, 2.3 h; *HTB1*, 2.2 h; *htb1-K115R*, 2.9 h; *hht2-K4Q*, 4.3 h. *D*, small micronuclei were observed in *hht2-K4Q* but not in *htb1-K115R* cells. Wild-type CU428, *HTB1*, *htb1-K115R*, and *hht2-K4Q* cells were stained with 4',6-diamidine-2-phenylindole dihydrochloride. *Scale bar*, 10 $\mu$m.

SDS-PAGE gels (Fig. 1*B*). In *htb1-K115R* and *htb1-K(111,115)R*, but not *htb1-K111R* mutant strains, monoubiquitylated H2B was not detectable (Fig. 1*B*), demonstrating that Lys[115] is the only ubiquitylation site on histone H2B in *Tetrahymena*.

The *htb1-K115R* strain, lacking H2B ubiquitylation, grew a little more slowly than the wild-type strain (Fig. 1*C*) and had morphologically normal germ line micronuclei and somatic macronuclei when viewed in the light microscope (Fig. 1*D*). In contrast, *hht2-K4Q* cells (47), in which the only major H3 contains a K4Q mutation and thus cannot be methylated, showed severe defects in vegetative growth and micronuclear morphology, suggesting that H2B ubiquitylation may not be required for H3K4 methylation in *Tetrahymena*.

*Both Major and Minor H3s Are Methylated at Lys[4] in Tetrahymena*—*Tetrahymena* has four genes encoding non-allelic variants of histone H3; *HHT1* and *HHT2* encode the same major H3 variant, and *HHT3* and *HHT4* encode minor variants H3.3 and H3.4, respectively, which cannot be separated by one-dimensional SDS-PAGE (48). Growing cells of *Tetrahymena* contain the major H3 and the minor H3.3. *HHT4* is only expressed when *HHT3* is knocked out. Both major and minor

H3s are detected by antibodies specific for H3K4 methylation. Therefore, to determine whether H2B ubiquitylation is required for H3K4 methylation in *Tetrahymena*, both major and minor H3s need to be examined. The major H3 and the minor H3.3 have 2 and 3 methionine residues, respectively (Fig. 2*A*), only one of which is at the same position. CNBr cleaves proteins at methionine residues, so that, after CNBr cleavage, major and minor H3s generate different patterns of peptides, enabling the separation of the N-terminal fragments of major and minor H3 variants (39) and distinction of Lys[4] methylation of major and minor H3s. Macronuclear histones from wild-type, $\Delta HHT3\Delta HHT4$, *HHT2*, and *hht2-K4Q* cells were incubated with CNBr and separated on SDS-polyacrylamide gel, followed by Western blotting with anti-H3K4me3 (Fig. 2*B*), anti-H3K4me2 (Fig. 2*C*), or anti-H3K4me1 (Fig. 2*D*) antisera to check the trimethylation, dimethylation, or monomethylation of major and minor H3s at Lys[4], respectively ([supplemental Fig. S3](#)). Antisera specific for H3K4 methylation detected the 120-amino acid peptide (H3-(1–120)) of major H3 and the 76-amino acid peptide (H3.3-(1–76)) of minor H3.3, both of which contain lysine 4, indicating that both major and minor H3 variants can be methylated at Lys[4] in
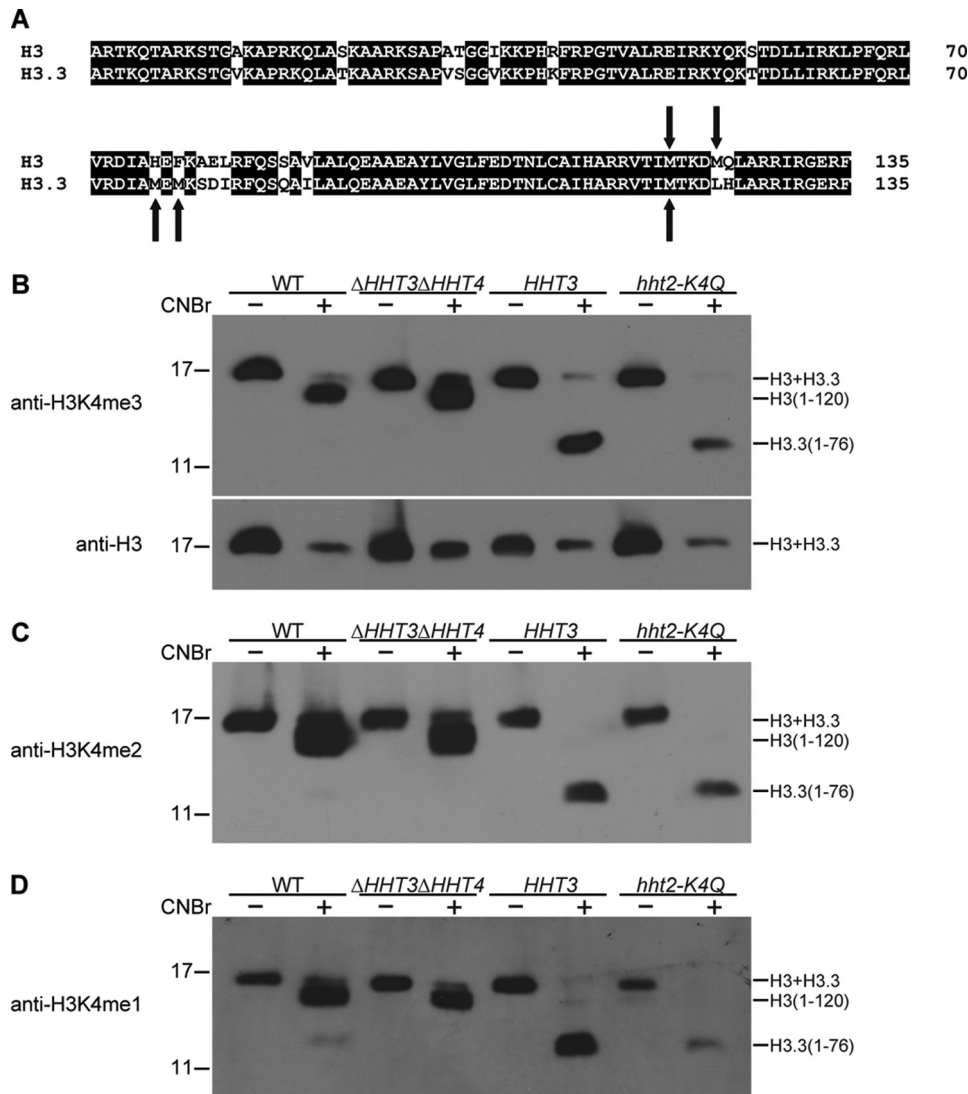
FIGURE 2. **Major and minor H3s can be distinguished by CNBr cleavage.** *A*, alignment of sequences of *Tetrahymena* major H3 and minor H3.3. The *arrows* indicate methionine residues that can be cleaved by CNBr. *B*, acid-extracted macronuclear histones from wild-type (*WT*) CU428 cells, from cells (Δ*HHT3*Δ*HHT4*) in which both minor H3s were knocked out and the only H3 in the cells is major H3, and cells (*HHT3*) containing only the minor H3.3 created by rescuing progeny of knock-out heterokaryons lacking all major H3s with the gene encoding H3.3, and *hht2-K4Q* cells in which the only major H3 contained a K4Q mutation. The histones were incubated without or with CNBr (1 mg/ml) for 24 h at room temperature and then were separated on a 15% SDS-polyacrylamide gel, followed by Western blotting with anti-H3K4me3 (preabsorbed with the H3K4me2 peptide to block nonspecific binding) and anti-H3 antisera. Bands corresponding to major H3, minor H3.3, major H3 peptide (H3-(1–120)), and minor H3.3 peptide (H3.3-(1–76)) are indicated on the *right*. Molecular mass markers (in kDa) are indicated on the *left*. *C* and *D*, the same analyses as in *B* were performed with anti-H3K4me2 or anti-H3K4me1 antisera, respectively.

results demonstrate that Lys[4] methylation of major and minor H3s can be distinguished by CNBr cleavage.

*Ubiquitylation of H2B Is Not Required for Methylation of H3 at Lysine 4*—To investigate whether H2B ubiquitylation at Lys[115] is required for H3K4 methylation of either H3 or H3.3, macronuclear histones of wild-type, *HTB1*, *htb1-K111R*, *htb1-K115R* and *htb1-K(111,115)R* strains were cleaved by CNBr and separated on SDS-polyacrylamide gel, followed by Western blotting with anti-H3K4me3, anti-H3K4me2, and anti-H3K4me1 antisera (Fig. 3, *A–C*). Lys[4] methylation of neither major H3 nor minor H3.3 (visualized by overexposing these Western blots; data not shown) was abolished in any of these strains. Therefore, mutating the ubiquitylation site on H2B did not detectably affect H3K4 methylation. These results indicate that H2B ubiquitylation at Lys[115] is not required for mono-, di-, or trimethylation of H3 at Lys[4] in *Tetrahymena*, suggesting that the crosstalk of H2B ubiquitylation and H3K4 methylation observed in yeast and mammals may not be conserved for either major or minor H3s of *Tetrahymena*.

*Ubc2 and Bre1 Are the Conserved E2 Ubiquitin Conjugation Enzyme and E3 Ubiquitin Ligase, Respectively, Specific for H2B Ubiquitylation in Tetrahymena*—In addition to preventing ubiquitylation of H2B, mutating Lys[115] to arginine might also disrupt some structural feature of *Tetrahymena* H2B that is required for the regulation of H3K4

*Tetrahymena*, although Lys[4] methylation of major H3 is much more abundant than Lys[4] methylation of minor H3.3 in wild-type cells. In Δ*HHT3*Δ*HHT4* cells, both genes encoding minor H3s were knocked out (48), and antisera only detected Lys[4] methylation of major H3. In *HHT3* cells, minor H3.3 is the only H3 variant (48), and antisera only detected Lys[4] methylation of minor H3.3. However, when minor H3.3 is the only H3 in the cells, its methylation is increased but does not appear to fully reach the wild-type level of Lys[4] methylation of total H3s. In *hht2-K4Q* mutant cells, major H3 cannot be methylated at Lys[4], and the level of Lys[4] methylation of minor H3.3 increases, as in *HHT3* cells. These

methylation. As an alternative approach, we sought to identify and mutate the enzymes responsible for H2B ubiquitylation. A putative *Tetrahymena* Ubc2 gene (*UBC2*) was identified by searching the *Tetrahymena* Genome Database using *S. cerevisiae* Rad6/Ubc2 as the query. Southern blotting indicated that *Tetrahymena UBC2* is a single copy gene (data not shown). Comparison of cDNA and genomic DNA sequences indicated that *UBC2* contains no introns and encodes a putative protein of 151 amino acids (data not shown). The sequence alignment of Ubc2 orthologs of *Tetrahymena*, yeasts, and other eukaryotes (supplemental Fig. S4) demonstrates that Ubc2 proteins are highly conserved among diverse organisms. Phyloge-
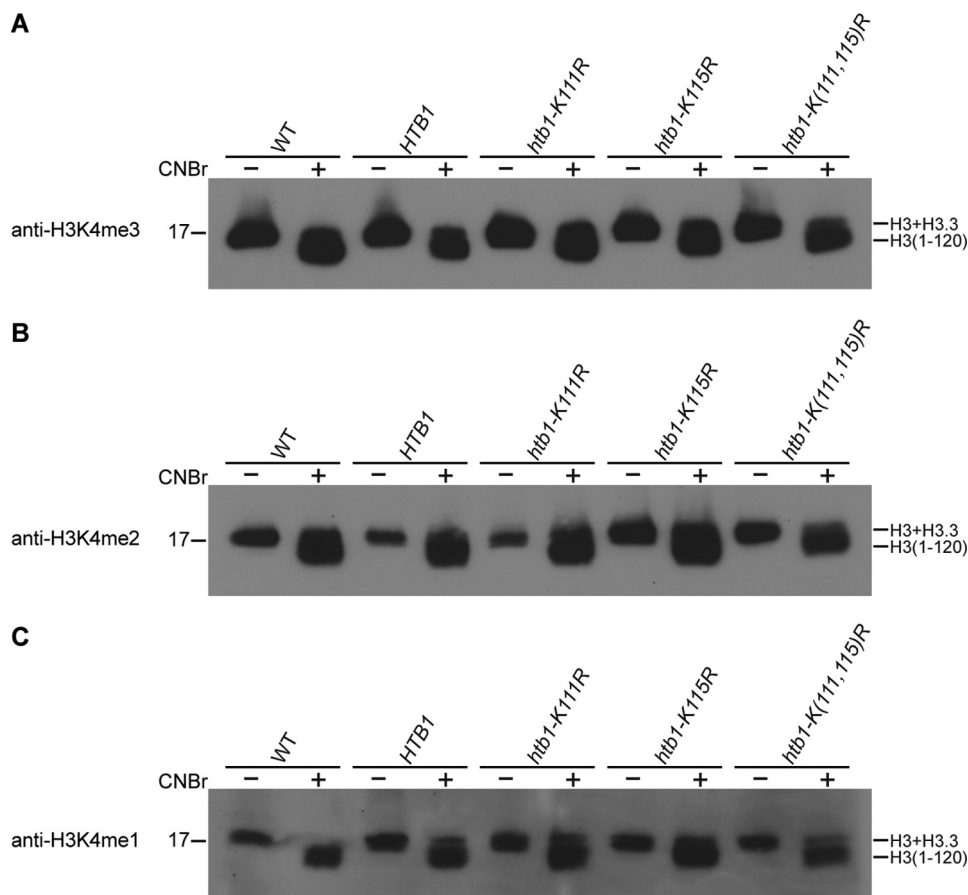
FIGURE 3. **H2B ubiquitylation is not required for H3K4 methylation in *Tetrahymena*.** Acid-extracted macronuclear histones from wild-type (*WT*) CU428, *HTB1*, *htb1-K111R*, *htb1-K115R*, and *htb1-K(111,115)R* strains were cleaved by CNBr and separated on 15% SDS-polyacrylamide gel, followed by Western blotting with anti-H3K4me3 (*A*), anti-H3K4me2 (*B*), or anti-H3K4me1 (*C*) antisera. Bands corresponding to major H3, minor H3.3, and major H3 peptide (H3-(1–120)) are indicated on the *right*. Bands corresponding to minor H3.3 peptide (H3.3-(1–76)) are not shown. Molecular mass markers (in kDa) are indicated on the *left*.

contain multiple predicted coiled-coil domains (18). Northern blotting failed to detect the mRNA of *BRE1*, but RT-PCR analysis showed that *BRE1* is expressed in growing, starved, and conjugating cells of *Tetrahymena* (data not shown). To study the *in vivo* function of *BRE1*, *BRE1* knock-out strains (Δ*BRE1*) were created, in which all micronuclear and macronuclear *BRE1* copies were replaced by the *neo2* cassette (supplemental Fig. S8*A*). Replacement in Δ*BRE1* strains was confirmed by PCR (supplemental Fig. S8, *B* and *C*).

To study their *in vivo* function, complete knock-out strains of *UBC2* and *BRE1* were created, and the ubiquitylation levels of macronuclear histones of Δ*UBC2* and Δ*BRE1* cells were analyzed (Fig. 4*A*). In both Δ*UBC2* and Δ*BRE1* cells, H2B ubiquitylation was abolished, whereas ubiquitylation of major or minor H2As was not detectably affected. These results demonstrate that, as in other organisms, *UBC2* and *BRE1* in *Tetrahymena* encode the conserved E2 ubiquitin-conjugating enzyme and E3 ubiquitin ligase, respectively, that are specifically responsible for H2B ubiquitylation *in vivo*. Δ*UBC2* and Δ*BRE1*

netic analysis (supplemental Fig. S5) indicates that *Tetrahymena* Ubc2 is most closely related to the putative Ubc2 ortholog of another ciliate, *Paramecium tetraurelia*. *UBC2* is expressed in growing, starved, and conjugating cells of *Tetrahymena*, especially strongly at 4–8 h postmixing in conjugation, after meiosis and during the earlier stages of nuclear differentiation (data not shown). To study the *in vivo* function of *UBC2*, *UBC2* knock-out strains (Δ*UBC2*) were created, in which all micronuclear and macronuclear *UBC2* copies were replaced by the *neo2* cassette (supplemental Fig. S6*A*). Replacement in Δ*UBC2* strains was confirmed by PCR (supplemental Fig. S6, *B* and *C*).

A putative *Tetrahymena* Bre1 gene (*BRE1*) was also identified by searching the *Tetrahymena* Genome Database using the RING finger domain sequence of *S. cerevisiae* Bre1 as the query. Southern blotting indicated that *Tetrahymena BRE1* is a single copy gene (data not shown). Comparison of cDNA and genomic DNA sequences showed that *BRE1* contains no introns and encodes a putative protein of 1075 amino acids (data not shown). Bre1 orthologs of *Tetrahymena*, yeasts, and other organisms (supplemental Fig. S7) contain a conserved C3HC4 RING finger domain in their C-terminal region, which is characteristic of a class of ubiquitin ligases (49). Other parts of Bre1s share low sequence homology (data not shown) and

cells grew much more slowly than the wild-type strain (Fig. 4*B*), suggesting that *UBC2* and *BRE1* are required for normal vegetative growth of *Tetrahymena*. Importantly, this slow growth phenotype was not observed when H2B ubiquitylation was abolished by mutating Lys[115], indicating that *UBC2* and *BRE1* in *Tetrahymena* may have functions in addition to H2B ubiquitylation.

To determine whether H2B ubiquitylation was required for H3K4 methylation in wild-type (as opposed to mutated) *Tetrahymena* and whether the enzymes responsible for H2B ubiquitylation might themselves regulate H3K4 methylation through another pathway, we examined H3K4 methylation in the Δ*UBC2* and Δ*BRE1* cells. Macronuclear histones of Δ*UBC2* and Δ*BRE1* cells were cleaved by CNBr and analyzed by Western blotting with antibodies specific for tri-, di-, or monomethylation of H3K4. Lys[4] methylation of major H3 (Fig. 4*C*) and minor H3.3 (data not shown) was not detectably affected in Δ*UBC2* or Δ*BRE1* cells. These results demonstrate that neither ubiquitylation of H2B nor *UBC2* or *BRE1* is required for H3K4 methylation in *Tetrahymena*.

*Microarray Analysis*—Microarray analyses were performed to compare the gene expression profiles of wild-type and mutant strains (Fig. 5 and supplemental Fig. S9). Although 92 genes showed similar changes in expression in *htb1-K115R*,
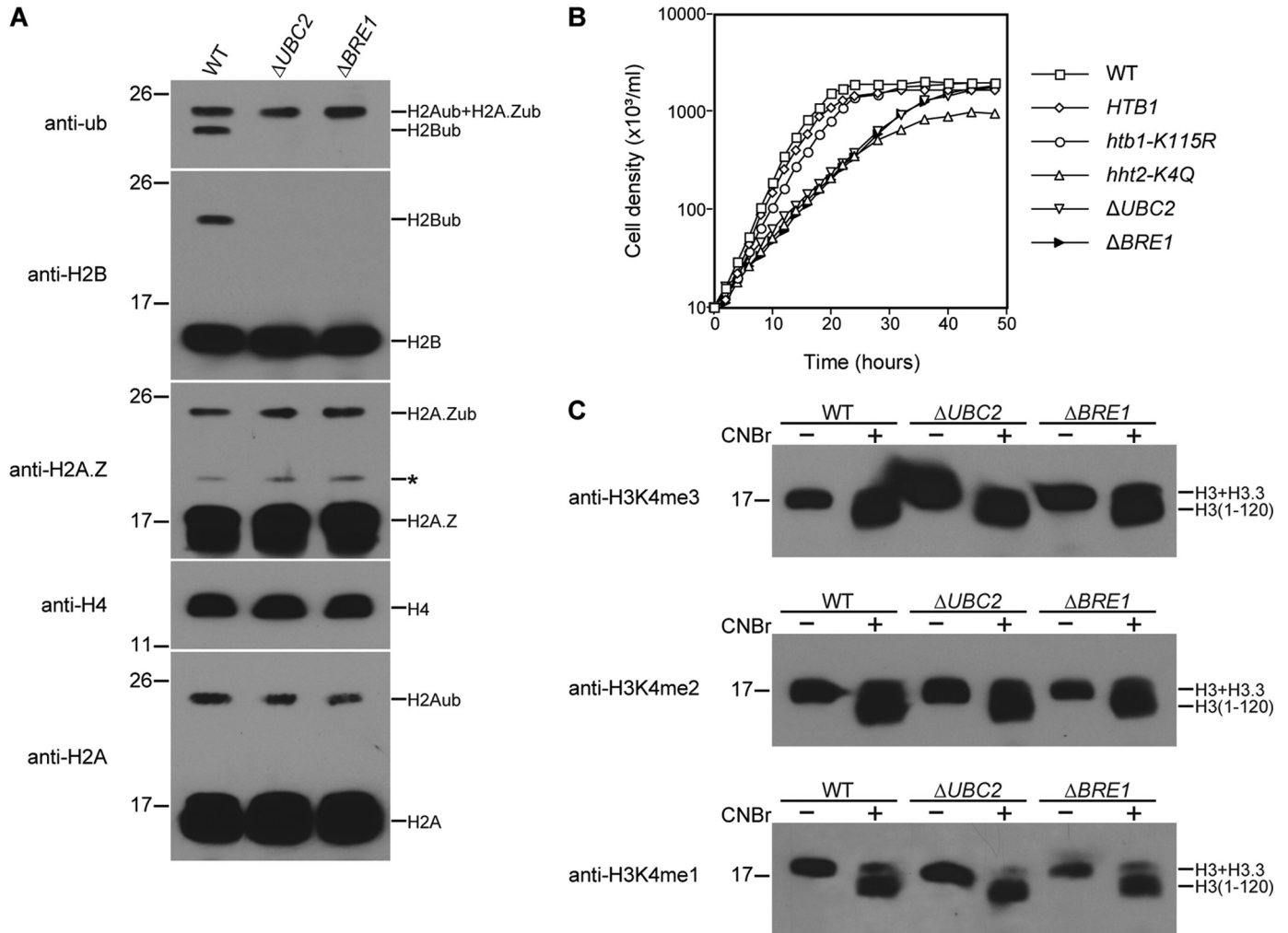
FIGURE 4. **UBC2 and *BRE1* are required for H2B ubiquitylation but not for H3K4 methylation in *Tetrahymena*.** *A*, acid-extracted macronuclear histones of wild-type (*WT*) CU428, *UBC2* knock-out (Δ*UBC2*), and *BRE1* knock-out (Δ*BRE1*) strains were analyzed by Western blotting using anti-ubiquitin (*anti-ub*), anti-H2B, anti-H2A.Z, anti-H4, and anti-H2A antisera. Anti-H4 antiserum was used to monitor histone loading. The *asterisk* denotes an unknown protein that cross-reacts with anti-H2A.Z antiserum. Molecular mass markers (in kDa) are indicated on the *left*. *B*, growth curves of wild-type CU428, *HTB1*, *htb1-K115*, *hht2-K4Q*, Δ*UBC2*, and Δ*BRE1* strains. The doubling times are as follows: wild type, 2.2 h; *HTB1*, 2.3 h; *htb1-K115R*, 2.6 h; *hht2-K4Q*, 4.1 h; Δ*UBC2*, 4.6 h; Δ*BRE*, 4.5 h. *C*, acid-extracted macronuclear histones of wild-type CU428, Δ*UBC2*, and Δ*BRE1* strains were cleaved by CNBr and separated on 15% SDS-polyacrylamide gel, followed by Western blotting with anti-H3K4me3, anti-H3K4me2, or anti-H3K4me1 antisera. Bands corresponding to major H3 and minor H3.3 or major H3 peptide (H3-(1–120)) are indicated on the *right*. Bands corresponding to minor H3.3 peptide (H3.3-(1–76)) are not shown. Molecular mass markers (in kDa) are indicated on the *left*.

Δ*BRE1*, and *hht2-K4Q* strains, unlike in yeast, these genes cannot be regulated by cross-talk between H2B ubiquitylation and H3K4 methylation. Only 71 genes were similarly affected in all three mutations (*htb1-K115R*, Δ*UBC2*, and Δ*BRE1*) that eliminated H2B ubiquitylation; many of these genes are likely to be regulated by H2B ubiquitylation. Consistent with our observations that mutation of the H3K4 site, knock-out of *UBC2*, or knock-out of *BRE1* had greater effects on growth than mutation of the H2B ubiquitylation site, elimination of H3K4 methylation and knock-out of either of the ubiquitylation enzymes affects a broader spectrum of genes than elimination of H2B ubiquitylation. Taken together, the large differences in growth and in the number of genes whose expression is altered in the respective mutants argue strongly that *UBC2* and *BRE1* are involved in pathways other than H2B ubiquitylation and that *UBC2* and *BRE1* mainly function independently of each other.

## DISCUSSION

In this study, we have shown that, in *Tetrahymena*, mutant strains lacking H2B ubiquitylation could be created by rescuing the progeny of *HTB* double knock-out heterokaryons with a mutated *HTB1* gene lacking the conserved ubiquitylation site, making *Tetrahymena* the only organism besides yeasts in which the *in vivo* function of H2B ubiquitylation has been studied directly by mutating the ubiquitylation site.

We have also shown that genes encoding *Tetrahymena* Ubc2 and Bre1 are the E2 ubiquitin-conjugating enzyme and E3 ubiquitin ligase, respectively, for H2B ubiquitylation, indicating that, as in yeasts and human cells, Ubc2-Bre1-mediated H2B ubiquitylation is conserved in *Tetrahymena*. It was suggested that, in human cells, H2B ubiquitylation requires UbcH6, which has E2 ubiquitin-conjugating enzyme activity *in vitro* (26). However, compared with the previously identified human HR6A and HR6B enzymes (50, 51), UbcH6 has less sequence homology
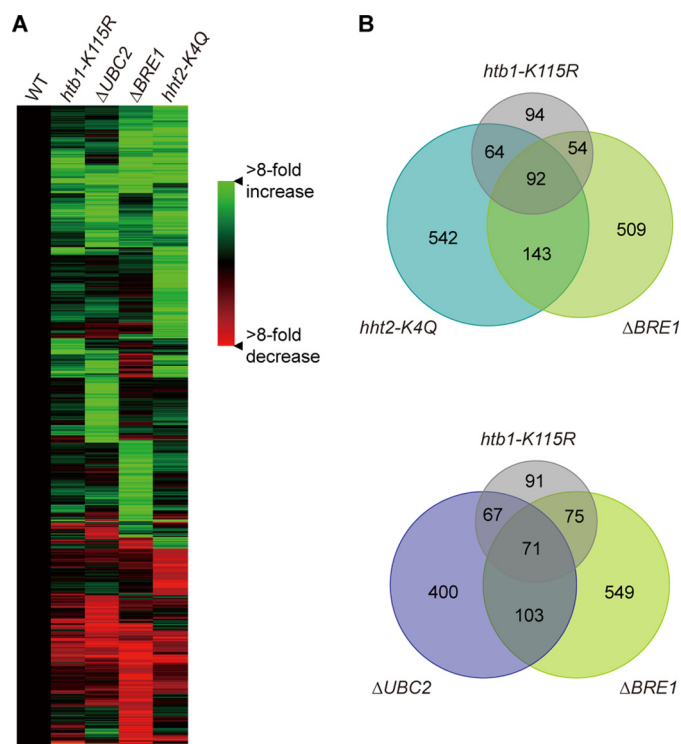
FIGURE 5. **Microarray analysis of gene expression profiles of wild-type (*WT*) CU428, *HTB1*, *htb1-K115*, Δ*UBC2*, Δ*BRE1*, and *hht2-K4Q* strains.** *A*, genes whose expression levels were altered by at least 4-fold in one or more mutant strains (supplemental Table S1) were clustered using a hierarchical clustering algorithm. All data sets are normalized to the values of a wild-type CU428 strain. *B*, Venn diagrams show overlap among *htb1-K115*, *hht2-K4Q*, Δ*UBC2*, and Δ*BRE1* data sets. The number of genes whose expression level had over 4-fold change is indicated.

to Ubc2s of *S. cerevisiae* and *S. pombe* (supplemental Figs. S4 and S5). Together with the evidence that Ubc2 is required for H2B ubiquitylation in *Tetrahymena* and yeasts, these results suggest that Ubc6H is not a true Ubc2 ortholog. A recent study (27) also showed that Rad6/Ubc2, not Ubc6H, is required for H2B ubiquitylation in human cells.

We have also shown that H3K4 mono-, di-, and trimethylation occur in *Tetrahymena*. However, although H2B ubiquitylation is required for H3K4 methylation in some strains of *S. cerevisiae* (15, 17), in *S. pombe* (28, 29), and probably in mammals (25, 26), none of the levels of H3K4 methylation were detectably affected in the *HTB* ubiquitylation mutant strain of *Tetrahymena*. Our study has shown that none of the levels of H3K4 methylation were detectably affected in *UBC2* or *BRE1* knock-out strains of *Tetrahymena*. These results strongly argue that, despite the conservation of the site of H2B monoubiquitylation and H3K4 mono-, di-, and trimethylation and the fact that the genes involved in the H2B ubiquitylation pathway are highly conserved, the cross-talk between Ubc2-Bre1-mediated H2B ubiquitylation and H3K4 methylation is not universally conserved among all organisms.

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