which embryonic development is not affected (*19*). In contrast, DAF and MCP are heavily expressed in human placentas (*16*), and there is no direct human counterpart to Crry. Thus, human DAF or MCP should play a similar role as mouse Crry during early embryonic development by controlling effector components of natural immunity, in the form of complement regulation, to protect fetomaternal tissues from tissue inflammation and destruction.

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9. The murine *Crry* gene was isolated from a sv129 cell genomic library by the use of Crry cDNA as a probe (*5*). A 6.7-kb genomic Eco RI DNA fragment containing exons 5 and 6 of the *Crry* gene was subcloned into pBluescript KS (Stratagene). The coding sequence was interrupted by replacing a portion of exon 5 with the 1.6-kb neomycin resistance cassette, pGKneobpA [H. Molina *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 3357 (1996)]. In this targeting vector, the pGKneobpA is flanked by a 1.7-kb genomic *Crry* fragment on the 5′ side and a 5-kb genomic *Crry* fragment on the 3′ side. Transfection of ES cells and blastocyst injection were done as described [B. Hogan, F. Costantini, E. Lacey, *Manipulating the Mouse Embryo: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1986), pp. 152–203].
10. DNA from individual ES cell clones or from tails of 3-week-old mice was isolated [P. W. Laird *et al.*, *Nucleic Acids Res.* **19**, 4293 (1991)]. Southern blotting was standard [J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), pp. 9.31–9.57]. Filters were hybridized overnight with a 0.5-kb probe derived from the *Crry* intronic area 5′ of the targeting construct sequence.
11. Single cell suspensions were prepared from primary fibroblasts derived from 13.5-dpc embryos. Crry staining was done with rabbit antibody to mouse Crry (1 μg) [B. Li *et al.*, *J. Immunol.* **151**, 4295 (1993)]. Binding was detected by the addition of fluorescein isothiocyanate–conjugated goat anti-rabbit antibody (Southern Biotechnology Associates, Birmingham, AL). Flow cytometry was done with a FACScan (Becton-Dickinson).
12. Deciduae were removed and frozen quickly in OCT compound (Miles, Elkhart, IN), and 10-μm-thick sections were cut. Endogenous peroxidase was quenched with 0.2% H_2O_2 in methanol. Sections were first incubated with rabbit antibody to mouse Crry followed by goat anti-rabbit immunoglobulin G (IgG) conjugated to horseradish peroxidase (HRP) (Southern Biotechnology) or with goat anti-mouse C3 (Cappel, Aurora, OH) followed by rabbit anti-goat IgG conjugated to HRP (Southern Biotechnology). Bound HRP was detected with diaminobenzidine. Sections were counterstained with 1% methyl green and covered with Crystal/Mount (Biomeda, Foster City, CA).
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15. Genotype analysis of 41 littermates from $C3^{-/-}$ $Crry^{+/-}$ matings revealed that 13 (32%) were $Crry^{+/+}$, 17 (41%) were $Crry^{+/-}$, and 11 (27%) were $Crry^{-/-}$.
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20. We thank H. Colten for $C3^{-/-}$ mice, P. Morgan for antibody to mouse DAF, and W. Yokoyama for critical comments. All experimental procedures and care of the animals were carried out in compliance with guidelines established by NIH and approved by the Division of Comparative Medicine at Washington University School of Medicine. Supported by grants from the National Institute of Allergy and Infectious Diseases (grants RO1 AI40576-01 and RO1 AI44912-01) to H.M. and by the Veteran's Administration Merit Award to H.M.

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Rad6-Dependent Ubiquitination of Histone H2B in Yeast

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Although ubiquitinated histones are present in substantial levels in vertebrate cells, the roles they play in specific biological processes and the cellular factors that regulate this modification are not well characterized. Ubiquitinated H2B (uH2B) has been identified in the yeast *Saccharomyces cerevisiae*, and mutation of the conserved ubiquitination site is shown to confer defects in mitotic cell growth and meiosis. uH2B was not detected in *rad6* mutants, which are defective for the ubiquitin-conjugating enzyme Ubc2, thus identifying Rad6 as the major cellular activity that ubiquitinates H2B in yeast.

Modulation of chromatin structure by the posttranslational modification of histones has emerged as an important mechanism for regulating chromosome function in eukaryotes. Although acetylation of the histone NH_2-termini has been shown to be intimately connected to transcriptional regulation, the biological roles of other histone modifications remain obscure (*1*). A noteworthy modification is the conjugation of ubiquitin to the COOH-termini of the core histones H2A, H2B, and H3 (*2*). Ubiquitinated forms of these histones are stable in vivo, and their incorporation into nucleosomes has been proposed to alter chromatin structure locally (*2, 3*). Although the precise cellular roles of histone ubiquitination are unclear, this modification has been correlated with increased transcriptional activity, replication, and meiosis in higher eukaryotes (*3, 4*).

Ubiquitin is transferred to target proteins in a reaction catalyzed by members of a large group of ubiquitin-conjugating enzymes (Ubc's), which donate ubiquitin to the ε-amino group of specific lysine residues, often in a substrate-specific manner (*5*). Two evolutionarily conserved Ubc's, Rad6/Ubc2 and Cdc34/Ubc3, are able to ubiquitinate histones in vitro without the mediation of an E3 ubiquitin ligase (*6, 7*). However, neither protein has been demonstrated to ubiquitinate histones in vivo, and the Ubc that targets histones in cells remains to be identified. Here, we present evidence that histone H2B is ubiquitinated in yeast. We also show that attachment of ubiquitin to this core histone depends primarily on the activity of Rad6/Ubc2 and is required for both optimal mitotic cell growth and meiosis.

It has been reported that *Saccharomyces cerevisiae* contains little, if any, uH2A or uH2B (*8*). We reinvestigated this issue using combined genetic and immunological approaches. Lysine-to-arginine (K → R) substitutions were introduced at the conserved ubiquitination sites of both H2A and H2B (*9*). A single K → R substitution at Lys^{123} in H2B (*htb1-K123R*) was combined with four K → R substitutions at Lys^{119}, Lys^{120}, Lys^{123}, and Lys^{126} of H2A [*hta1-K119R, K120R, K123R, K126R* (hereafter *hta1-4K/R*)] to eliminate the possibility that, in the absence of the preferred H2A ubiquitination site (Lys^{119}), adjacent lysine residues (Lys^{120}, Lys^{123}, or Lys^{126}) could serve as acceptors for ubiquitin conjugation. Strains that contained the fully mutant forms of H2A plus H2B (*hta1-4K/R* + *htb1-K123R*) were viable (*9, 10*) but showed pronounced mitotic and meiotic defects. The mitotic phenotype was characterized by a small colony size on

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plates (*11*) and an ~30% increase in doubling time in liquid medium (Table 1). A slight increase (~10%) in the number of large budded cells additionally suggested a delay in the S or G_2/M phases of the cell cycle (*11*). The meiotic defect resulted in a failure of homozygous mutant diploids to form spores (Table 1). Mutant diploids had a single mass of DNA, indicating that neither meiotic division occurred and that the meiotic program was blocked at an early stage (Fig. 1). These results indicate that the lysine residues corresponding to the evolutionarily conserved ubiquitination sites in H2A and/or H2B are required for specific cellular processes in yeast.

To identify which K → R substitutions conferred these phenotypes, we examined the H2A and H2B mutants individually. The H2B mutant on its own showed the mitotic and meiotic defects of the quintuple mutant, whereas the quadruple H2A mutant was phenotypically wild type (Fig. 1 and Table 1)

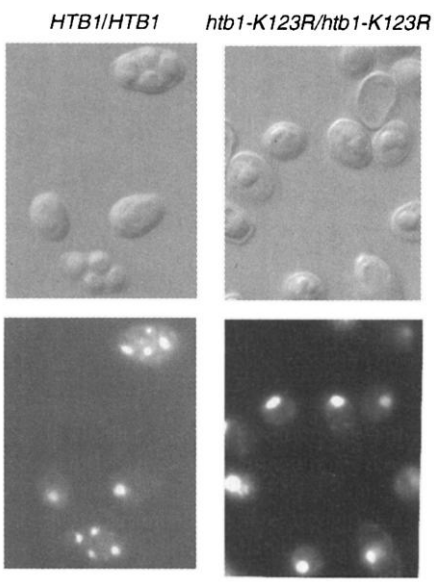

Fig. 1. Meiotic phenotype of H2B ubiquitination site mutant. Homozygous diploid strains YKR55 (*hta1-htb1Δhta2-htb2Δ* + *HTA1-HTB1*) and YKR57 (*hta1-htb1Δhta2-htb2Δ* + *HTA1-htb1-K123R*) were grown to stationary phase in YPD medium and induced to sporulate by transfer to sporulation medium (SPM) (1% potassium acetate). At 48 hours after transfer to SPM, aliquots of each culture were fixed with 3.7% formaldehyde for 1 hour, washed with distilled water, and resuspended in 70% ethanol for 30 min. After washing with distilled water, cells were resuspended in 1 μg/ml of the fluorescent dye 4′,6-diamidino-2-phenylindole for 30 min, then washed and resuspended in distilled water. Cells were spotted onto poly-lysine-coated glass slides and photographed with a Zeiss Axiophot fluorescence microscope. The top panels were imaged with Nomarski optics, and the bottom panels were imaged with DNA fluorescence. The wild-type diploid sporulated with ~50% efficiency, and the mutant diploid sporulated with 0% efficiency.

(*11*). Together, the data strongly support a role for the conserved ubiquitination site of H2B, but not that of H2A, in optimal cell growth and meiosis in yeast.

To determine if ubiquitin is conjugated to H2B in vivo, we developed a sensitive immunoassay to detect modified forms of this histone. We employed a yeast strain that contained Flag-H2B as the only source of this core histone (*12*). Flag-H2B can be quantitatively immunoprecipitated from yeast cell extracts (*11, 12*), allowing detection of even low levels of modified H2B. To further sensitize detection of ubiquitin-H2B conjugates, we next introduced a plasmid encoding a galactose-inducible, hemagglutinin (HA)-tagged ubiquitin gene (HA-Ub) into the Flag-H2B strain (*13*). When this strain was grown in galactose, high levels of HA-Ub were induced. Growth in dextrose prevented expression of tagged ubiquitin and served as a control.

Using a method that preserved only covalent interactions during cell lysis (*14*), we immunoprecipitated Flag-H2B from cells in which HA-Ub was either present (galactose)

Table 1. Phenotypes of H2A and H2B ubiquitination site mutants. Growth and sporulation were monitored in *hta1-htb1Δhta2-htb2Δ* homozygous diploids that carried the indicated *HTA1* or *HTB1* alleles on a plasmid. Growth was followed by measuring the optical density at 600 nm of cultures grown at 30°C. Sporulation was induced by transfer of stationary-phase YPD cells to SPM (1% potassium acetate). Percent sporulation was determined by counting the number of asci present among 200 to 500 cells using light microscopy. UV sensitivity was monitored by spotting 10-fold serial dilutions of cultures growing exponentially in YPD medium onto YPD plates, followed by irradiation at 50 J/m² and incubation at 30°C for 2 days in the dark.

Allele	Growth* (min)	Sporulation† (%)	UV‡	uH2B§
HTA1-HTB1	150	45	No	+
HTA1-htb1-K123R	195	0	No	–
hta1-4K/R-HTB1	150	40	No	ND
hta1-4K/R-htb1-K123R	195	0	No	ND

*Doubling time in synthetic medium minus uracil. †Number of asci present after 48 hours in SPM. ‡Sensitivity to 50 J/m² of UV light. §Presence (+) or absence (–) of uH2B; ND, not determined.

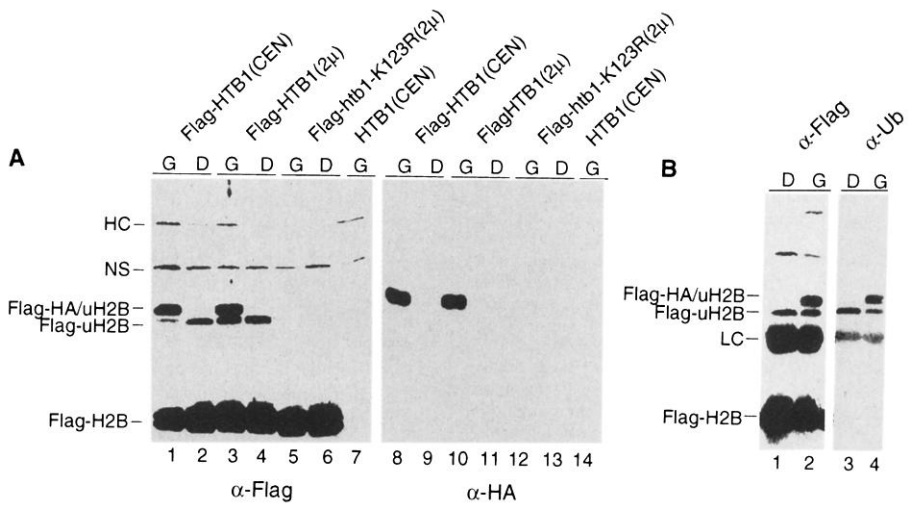

Fig. 2. Detection of uH2B in mitotically dividing yeast cells. (**A**) Haploid *htb1-1 htb2-1* strains contained a *CEN-URA3* (YKR30) or 2μm-*URA3* (YKR28) *Flag-HTB1* plasmid, a 2μm-*HIS3 Flag-htb1-K123R* plasmid (YKR42), or a *CEN-HIS3* plasmid with an untagged *HTB1* gene (YKR33). A 2μm-*TRP1* plasmid that contained a *GAL1*-regulated *HA-UBI4* gene (pKR41) was also present in each strain. Expression of HA-Ub was induced or repressed by growth in the presence of 2% galactose (G) or 2% dextrose (D), respectively. Lysates were prepared from equivalent numbers of cells and incubated with α-Flag monoclonal antibody coupled to Sepharose beads (*14*). Immunoprecipitates were collected, eluted with Flag peptide, and 20-μl (α-Flag) or 10-μl (α-HA) aliquots were subjected to 15% SDS-PAGE. After transfer to filters, the blots were probed with α-Flag (lanes 1 through 7) or α-HA (lanes 8 through 14) monoclonal antibodies. Flag-H2B migrates as a doublet, and preliminary data indicate that the top band of the doublet represents a phosphorylated species of H2B. HC, immunoglobulin heavy chain; NS, nonspecific band. (**B**) Haploid *htb1-1 htb2-1* strain YKR28 containing a *Flag-HTB1* gene on a 2μm-*URA3* plasmid and *GAL1*-regulated HA-Ub was grown in the presence of galactose (G) or dextrose (D). After the preparation of lysates and immunoprecipitation with α-Flag monoclonal antibody, the immunoprecipitates were eluted by boiling, and after SDS-PAGE, the blots were probed with α-Flag monoclonal antibody (lanes 1 and 2) or α-ubiquitin polyclonal antibody (lanes 3 and 4). LC, immunoglobulin light chain.

or absent (dextrose). After SDS–polyacrylamide gel electrophoresis (SDS-PAGE), we analyzed the immunoprecipitates by Western blot analysis with α-Flag or α-HA antibodies (Fig. 2A). Three bands were specifically detected by the α-Flag antibody when HA-Ub was expressed (Fig. 2A, lanes 1 and 3). The most abundant and rapidly migrating band represented unmodified Flag-H2B. The two less abundant and more slowly migrating bands represented ubiquitinated conjugates of Flag-H2B. The upper band was identified as HA-Ub conjugates of Flag-H2B (Flag-HA/uH2B) because it was also detected by the α-HA antibody (Fig. 2A, lanes 8 and 10) and was absent when HA-Ub was not expressed (Fig. 2A, lanes 2 and 4 and 9 and 11). The lower band represents endogenous ubiquitin conjugates of Flag-H2B (Flag-uH2B). This band was detected only by the α-Flag antibody, and its presence was independent of HA-Ub expression (Fig. 2A, lanes 1 through 4). These designations were confirmed by probing Western blots of α-Flag immunoprecipitates with polyclonal antibodies against native ubiquitin (Fig. 2B): Each of the two putative ubiquitinated Flag-H2B species, but not Flag-H2B itself, selectively reacted with this antibody.

To determine if ubiquitin was attached to H2B at Lys^{123}, we introduced the K123R mutation into a *Flag-HTB1* gene (*12*) and immunoprecipitated Flag-H2B (K123R) in the presence or absence of HA-Ub. Although unmodified Flag-H2B (K123R) was present in these immunoprecipitates, no ubiquitin conjugates could be detected (Fig. 2A, lanes 5 and 6 and 12 and13). Together, the results support the conclusion that ubiquitin is attached to yeast H2B at the same lysine residue to which ubiquitin is conjugated in vertebrate H2B (*2*).

Although proteins can be either mono- or polyubiquitinated (*5*), the mobility of the two ubiquitinated H2B species is consistent with monoubiquitination; moreover, a longer exposure of the immunoblots failed to reveal species that migrated more slowly than either Flag-uH2B or Flag-HA/uH2B (*11*). However, it is also possible that polyubiquitinated forms of H2B are unstable or present at levels too low to be detected by the immunoassay. Titration experiments suggest that the levels of monoubiquitinated H2B might represent as much as 10% of the total cellular H2B (*15*). This is significantly higher than the levels in fly or vertebrate cells, where uH2B accounts for 1 to 2% of the total cellular H2B (*16*).

Rad6/Ubc2 shows marked specificity for histones H2A and H2B in vitro, catalyzing predominantly monoubiqitinated forms of these histones (*7*). Because the H2B ubiquitination site mutant shares the sporulation defect of *rad6* mutants (*17*), it seemed likely that Rad6 might be the cellular Ubc that ubiquitinates H2B. To test this, we transferred the following three *rad6* mutations into the strain that was tagged with double epitopes (*10*): *rad6Δ*, which lacks Rad6 protein; *rad6-C88A*, which is defective in ubiquitin-conjugating activity because the site of ubiquitin linkage has been destroyed (C88A, $Cys^{88} \rightarrow Ala^{88}$); and *rad6-149*, which is catalytically active but unable to ubiquitinate histones efficiently in vitro because of the absence of acidic COOH-terminal residues (*18, 19*). Only unmodified Flag-H2B was present in these strains, despite the use of HA-Ub to sensitize the detection of uH2B (Fig. 3A). Together with the analysis of the H2B ubiquitin site mutant, the results support the conclusions that Rad6 ubiquitinates yeast histone H2B on Lys^{123} in mitotically growing cells and that, in its absence, no other Ubc's are able to substitute efficiently. This identifies H2B as a physiologically relevant substrate of Rad6/Ubc2 in yeast.

We next asked if Rad6 is also required to ubiquitinate H2B during meiosis. The presence of uH2B in meiotic cells was monitored at various times after homozygous Flag-H2B diploids had been induced to sporulate (Fig. 3B). In a *RAD6* diploid, Flag-uH2B was present throughout sporulation and decreased in amount only late in the meiotic program (Fig. 3B, lanes 2 through 6), when mature spores appeared (*11*). In a *rad6Δ* diploid, however, Flag-uH2B could not be detected in either premeiotic vegetative cells or in sporulating cultures (Fig. 3B, lanes 7 through 12), and spores were not formed (*11*). A meiosis-defective *rad6-149* diploid also contained no Flag-uH2B when induced to sporulate (*11*). Thus, the Rad6 dependence of budding yeast meiosis might result in part from the ubiquitination of H2B. Rad6 homologs are also required for meiosis in fission yeast and vertebrates (*4*), but it is not known whether uH2B is present in meiotic cells from these organisms.

Although these results indicate that H2B is a major target of Rad6 in both mitotic and meiotic cells, Rad6 must have additional targets as well. For example, the failure to ubiquitinate H2B (and H2A) cannot account for the ultraviolet (UV) sensitivity of a *rad6Δ* mutant because the quintuple H2A plus H2B ubiquitin site mutant is not UV sensitive (Table 1).

In higher eukaryotes, where ubiquitinated histones were first discovered, uH2A is the most abundant species (*3, 16*). This is apparently reversed in yeast, where uH2B is abundant and uH2A is either absent or present at levels that are too low to be detected by the Flag/HA–based detection system (*8, 11*). Thus, in yeast, uH2B might assume some of the roles played by uH2A in higher eukaryotic cells. Covalent attachment of ubiquitin to the H2B COOH-terminus could modulate the interaction of the histone tail with linker DNA, adjacent nucleosomes, or nonhistone

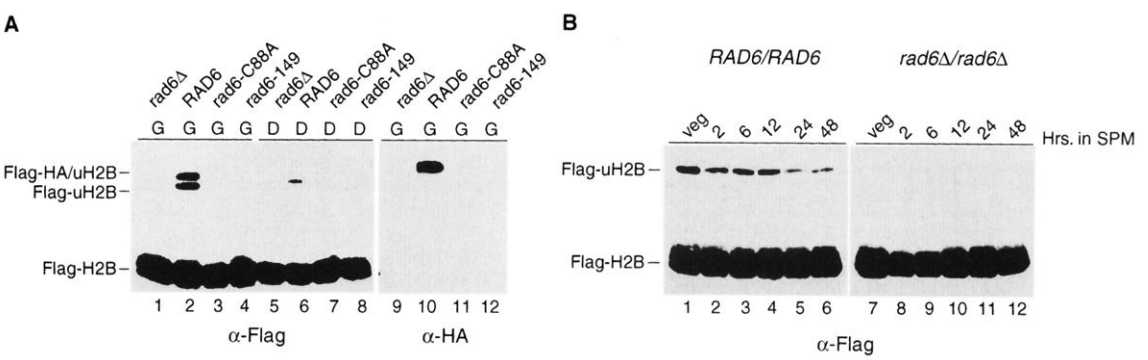

Fig. 3. Rad6/Ubc2 is required for ubiquitination of H2B in mitotically dividing cells and during meiosis. (**A**) *CEN-URA3* plasmids carrying *RAD6* (pKR57), *rad6-C88A* (pKR58), or *rad6-149* (pKR97) alleles were introduced into strain YKR36, a *rad6Δhtb1-1 htb2-1* haploid strain that contained both *Flag-HTB1* on a *CEN-TRP1* plasmid and *GAL1*-regulated HA-Ub on a 2μm-*HIS3* plasmid. After growth in the presence of galactose (G) or dextrose (D), lysates were prepared from equivalent cell numbers and incubated with α-Flag monoclonal antibody. The immunoprecipitates were eluted with Flag peptide, and after SDS-PAGE, the blots were probed with α-Flag (lanes 1 through 8) or α-HA (lanes 9 through 12) monoclonal antibodies. (**B**) *RAD6* (YKR49) or *rad6Δ* (YKR64) *htb1-1 htb2-1* homozygous diploids that contained *Flag-HTB1* on a 2μm-*URA3* or 2μm-*LEU2* plasmid were grown vegetatively in YPD medium (veg), allowed to reach stationary phase in the same medium, and then were induced to sporulate by transfer into SPM (1% potassium acetate). During vegetative growth and at the indicated times after sporulation was induced, lysates were prepared from equivalent numbers of cells and incubated with α-Flag monoclonal antibody. The immunoprecipitates were eluted with Flag peptide, and after SDS-PAGE, the Western blots were probed with α-Flag monoclonal antibody. At 48 hours after transfer into SPM, 52% of the *RAD6/RAD6* cells and 0% of the *rad6Δ/rad6Δ* cells had formed spores.

regulatory proteins (*20*). This could lead to a more open chromatin structure or mark chromatin for recognition by regulatory proteins and, in turn, permit the transcription of key genes during mitosis and meiosis.

The pleiotropic roles of yeast Rad6 in the DNA-mediated events of repair, mutagenesis, meiosis, retrotransposition, and gene silencing has long prompted the view that these events might result from Rad6-dependent histone ubiquitination (*7, 19, 21, 22*). Ubiquitin conjugation is required for all Rad6-dependent functions (*23*), and ubiquitination of H2B by Rad6 might indeed constitute an essential meiotic function because *rad6* mutants (*17, 24*) and the H2B K123R mutant fail to sporulate. The slow growth phenotype of the H2B K123R mutant also suggests that Rad6-dependent H2B ubiquitination helps to promote optimal mitotic cell growth. Although the H2B ubiquitination-defective *rad6-149* mutant has no growth defect (*24*), residual activity of rad6-149 protein might ubiquitinate H2B at low levels that are sufficient for cell growth (*11, 19*). At least two other Rad6-dependent processes, however, are unlikely to result from H2B ubiquitination: DNA repair, which is unaffected by the H2B K123R mutation, and telomeric silencing, which is maintained in tailless *rad6* mutants (*22*). Thus, the multifunctional Rad6 protein probably ubiquitinates other chromosomal proteins besides histones.

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9. Lysine-to-arginine substitution mutations were introduced at conserved ubiquitination sites in the *HTA1* and/or *HTB1* genes by polymerase chain reaction (PCR)–based mutagenesis combined with homologous recombination in vivo. The following four oligonucleotides were used as primers to construct the *HTA1* and *HTB1* alleles, with pTRT1 serving as the template: *hta1-4K/R* (*hta1-K119R, K120R, K123R, K126R*), 5′-TTATAATTCTTGAGAAGCCCTGGTAGCCCTGGCAGACCTCCTTGGCAACAAGTTTTGATG-3′ (primer A1-9036); *htb1-K123R*, 5′-TTATGCTTGAGTAGAGGAAGAGTACCTGGTAACAGCTCTAGTACC-3′ (primer B1-3); *HTA1*, 5′-TTATAATTCTTGAGAAGCCTTGGTAGAC-3′ (primer A1-WT); and *HTB1*, 5′-TTATGCTTGAGTAGAGGAAGAGTACTT-3′ (primer B1-WT). In all cases, the PCR primers also included 40 nucleotides at their 5′ ends that were homologous to polylinker sequences in plasmids pRS416 (*CEN-URA3*) or pRS426 (2μm-*URA3*). The PCR products were cotransformed with Bam HI–linearized plasmids pRS416 or pRS426 into strain YKR25 (*hta1-htb1Δhta2-htb2Δ*), which carried a *GAL1*-regulated *HTA2-HTB2* locus on a *CEN-HIS3* plasmid (pKR69). After selection for Ura$^+$ transformants on glucose, strains that had lost the resident *GAL1*-regulated *HTA2-HTB2* plasmid were identified; these strains were viable because they contained recombinant plasmids that carried mutant *HTA1* and/or *HTB1* alleles. The plasmids were recovered from yeast and subjected to DNA sequence analysis to confirm that the predicted substitution mutations were present.
10. The *S. cerevisiae* strains used in this study were derived from two genetic backgrounds: FY406 (*MATa hta1-htb1Δ::LEU2, hta2-htb2Δ::TRP1, leu2Δ1, ura3-52, trp1Δ63, his3Δ200* + pSAB6 [*CEN, URA3, HTA1-HTB1*], obtained from F. Winston, and JR5-2A (*MATa htb1-1 htb2-1 ura3-1 leu2-3,-112, his3, trp1, ade2-1, ssd1, can1-100* + [Ycp50-HTB1]). The *RAD6* gene was disrupted in the latter strain with a *rad6Δ::hisGURA3hisG* allele obtained from R. Kulka, and various *RAD6* alleles were introduced into this strain on centromeric plasmids. The *RAD6* and *rad6-C88A* alleles were obtained from M. Bryk and were present on plasmid YCp50 (*CEN-URA3*). The *rad6-149* allele was obtained from R. Kulka and was present on plasmid pRS416 (*CEN-URA3*).
11. K. Robzyk, unpublished data.
12. J. Recht and M. A. Osley, *EMBO J.* **18**, 229 (1999). *Flag-HTB1* was present on plasmids pKR75 (*CEN-TRP1*), pKR76 (2μm-*URA3*), or pKR100 (2μm-*LEU2*). We introduced the *htb1-K123R* substitution mutation into plasmid pRS423 (2μm-*HIS3*) by PCR-based mutagenesis with the primers B1-3 (*9*) (*htb1-K123R*) and TRT1-1780 (5′-GATCCACTGGCTGGCTTCGTGAACG-3′), using plasmid pKR76 (Ycp50-*Flag-HTB1*) as the template. The PCR product was cotransformed with Bam HI–linearized plasmid pRS323 into strain YKR28 (*htb1-1 htb2-1* + YCp50-*HTB1*), and strains that contained the recombined plasmid (pKR99) were selected after growth on 5-fluoroorotic acid plates.
13. The *GAL1-HA-UBI4* gene was present on plasmid pKR41 (2μm-*TRP1*) or pKR81 (2μm-*HIS3*). The 9–amino acid HA epitope was fused to the NH_2-terminus of the second open reading frame (ORF) of the *UBI4* gene by inserting the *UBI4* ORF into plasmid pJG4-6, a *GAL1*-HA fusion vector generously provided by R. Brent.
14. Cell lysate preparation was modified from a protocol described by Foiani *et al.* (*25*). Fifty-milliliter cultures were grown in supplemented minimal medium or in yeast extract, peptone, and dextrose (YPD) medium to a density of 2×10^7 cells/ml. Cells were collected and washed immediately with 20% trichloroacetic acid (TCA), and the pellet was frozen under liquid nitrogen and stored at −80°C. After thawing, the cell pellet was resuspended in 0.5 ml of 20% TCA and broken by vortexing for 2 min with glass beads. The cell lysate was combined with two 0.5-ml 5% TCA washes of the beads, and the TCA pellet was collected by centrifugation at 3000 rpm for 10 min in a microfuge. The pellet was resuspended in 0.2 ml of 1× Laemmli sample buffer containing fresh β-mercaptoethanol, and 50 μl of unbuffered 2 M tris were added to neutralize the pH. The suspension was boiled for 3 min, and after centrifugation at 3000 rpm for 10 min, the supernatant was removed for immunoprecipitation (IP). Two hundred microliters of supernatant were added to 800 μl of IP buffer [50 mM tris (pH 7.4), 150 mM NaCl, 0.5% NP-40, and bovine serum albumin (BSA) (0.5 mg/ml)] containing 30 μl of anti-Flag M2 affinity resin (Sigma). After mixing for 1 hour at 4°C, the resin was washed once with IP buffer containing BSA and three times with IP buffer without BSA. Proteins were either eluted directly into SDS sample buffer by boiling for 2 min or by incubation with 2 μl of a 4 mg/ml solution of Flag peptide (Kodak) in 40 μl of IP buffer. Twenty microliters of the Flag eluate were mixed with 20 μl of 2× SDS sample buffer and boiled for 2 min before electrophoresis through 15% SDS-PAGE gels. Typically, 20 and 10 μl of eluate were loaded for α-Flag and α-HA Western blot analysis, respectively. Proteins were transferred to polyvinylidene difluoride membranes (Immobilon, Fisher Scientific) for Western blot analysis, except when α-ubiquitin antibodies were used for detection. In this case, proteins were transferred to a reinforced 0.2-μm nitrocellulose membrane (Schleicher and Schuell, Keene, NH), and the membrane was boiled for 20 min in distilled water before incubation with antibody. Western blot analysis was performed with monoclonal antibody against the HA (1:1000 dilution) or Flag (1:300 dilution) epitope. Polyclonal rabbit antibody against ubiquitin was a generous gift of J. Davie and was used at a dilution of 1:500. Detection was performed by enhanced chemiluminescence, following manufacturer's directions (NEN Life Science Products, Boston, MA).
15. An estimate of the fraction of uH2B in mitotically dividing cells was obtained by performing Western blot analysis on a series of dilutions of a Flag-H2B immunoprecipitate (undiluted, 1:5, 1:10, 1:20, 1:50, and 1:100). The intensity of the band representing monoubiquitinated H2B in the undiluted and 1:5 dilution samples was visually compared to the band corresponding to unmodified H2B in the dilution series. This comparison indicated that uH2B was present at ~10% of the level of unmodified H2B.
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