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Histone H4 Lysine 20 of *Saccharomyces cerevisiae* Is Monomethylated and Functions in Subtelomeric Silencing

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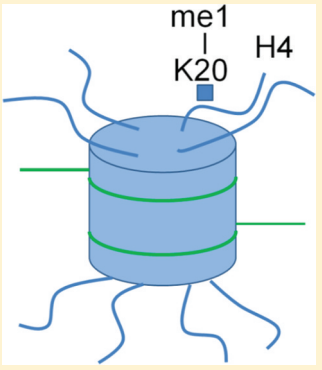
Supporting Information

ABSTRACT: Histones undergo post-translational modifications that are linked to important biological processes. Previous studies have indicated that lysine methylation correlating with closed or repressive chromatin is absent in the budding yeast *Saccharomyces cerevisiae*, including at H4 lysine 20 (K20). Here we provide functional evidence for H4 K20 monomethylation (K20me1) in budding yeast. H4 K20me1 is detectable on endogenous H4 by western analysis using methyl-specific antibodies, and the signal is abrogated by H4 K20 substitutions and by competition with H4 K20me1 peptides. Using chromatin immunoprecipitation, we show that H4 K20me1 levels are highest at heterochromatic locations, including subtelomeres, the silent mating type locus, and rDNA repeats, and lowest at centromeres within euchromatin. Further, an H4 K20A substitution strongly reduced heterochromatic reporter silencing at telomeres and the silent mating type locus and led to an increase in subtelomeric endogenous gene expression. The correlation between the location of H4 K20me1 and the effect of the H4 K20A substitution suggests that this modification plays a repressive function. Our findings reveal the first negative regulatory histone methylation in budding yeast and indicate that H4 K20me1 is evolutionarily conserved from simple to complex eukaryotes.



DNA exists within the cell wrapped around protein octamers composed of two copies each of histones H2A, H2B, H3, and H4. These histones participate in most DNA-related events such as transcription, replication, DNA repair, and chromatin compaction, and undergo numerous post-translational modifications (PTMs) that influence these processes. Lysine methylation is one such modification and occurs on six lysines (H3 lysines 4, 9, 27, 36, and 79 and H4 lysine 20) from the fission yeast *Schizosaccharomyces pombe* to humans, the only exception being H3 K27, which is not known to be methylated in fission yeast. Lysine can be reversibly mono-, di-, or trimethylated, and this modification is associated with different biological phenomena depending on the site and degree of methylation.[1]

H4 K20 is a particularly interesting residue since its methylation is linked to many physiological processes. The K20 methylated form recruits the methyllysine-binding protein L3MBTL1 to promote chromatin compaction[2−4] and also recruits the respective human and fission yeast DNA repair proteins 53BP1 and Crb2 to sites of DNA damage.[5,6] The methyltransferase Set8 (PrSet-7) localizes to replication forks in human cells to monomethylate H4 K20, and disrupting Set8 function results in replication defects.[7−9] H4 K20me1 is enriched at genes and linked to transcription, which may be associated with transcriptional attenuation.[10,11] In addition, in mammals, mono- and trimethylated H4 K20 localize respectively to the transcriptionally silent X chromosome Barr body and pericentromeric heterochromatin.[12]

In addition to the role of H4 K20 methylation, the residue itself may be linked to heterochromatin function as part of a patch of basic amino acids ($K_{16}RHRK_{20}$). In budding yeast, this patch, in particular the RHR motif, recruits or regulates several chromatin proteins, including Isw2 ATP nucleosome remodeling complex, Sir2/3/4 deacetylase complex, and Dot1 methylase.[13−15] Lysine 20, however, has been less well studied in budding yeast, and its role and modifications have not been elucidated.

While lysine methylation associated with active transcription (H3 K4, K36, K79) is conserved from budding yeast to humans, lysine methylation associated with gene repression (H3 K9 and K27, and H4 K20) is generally thought to be

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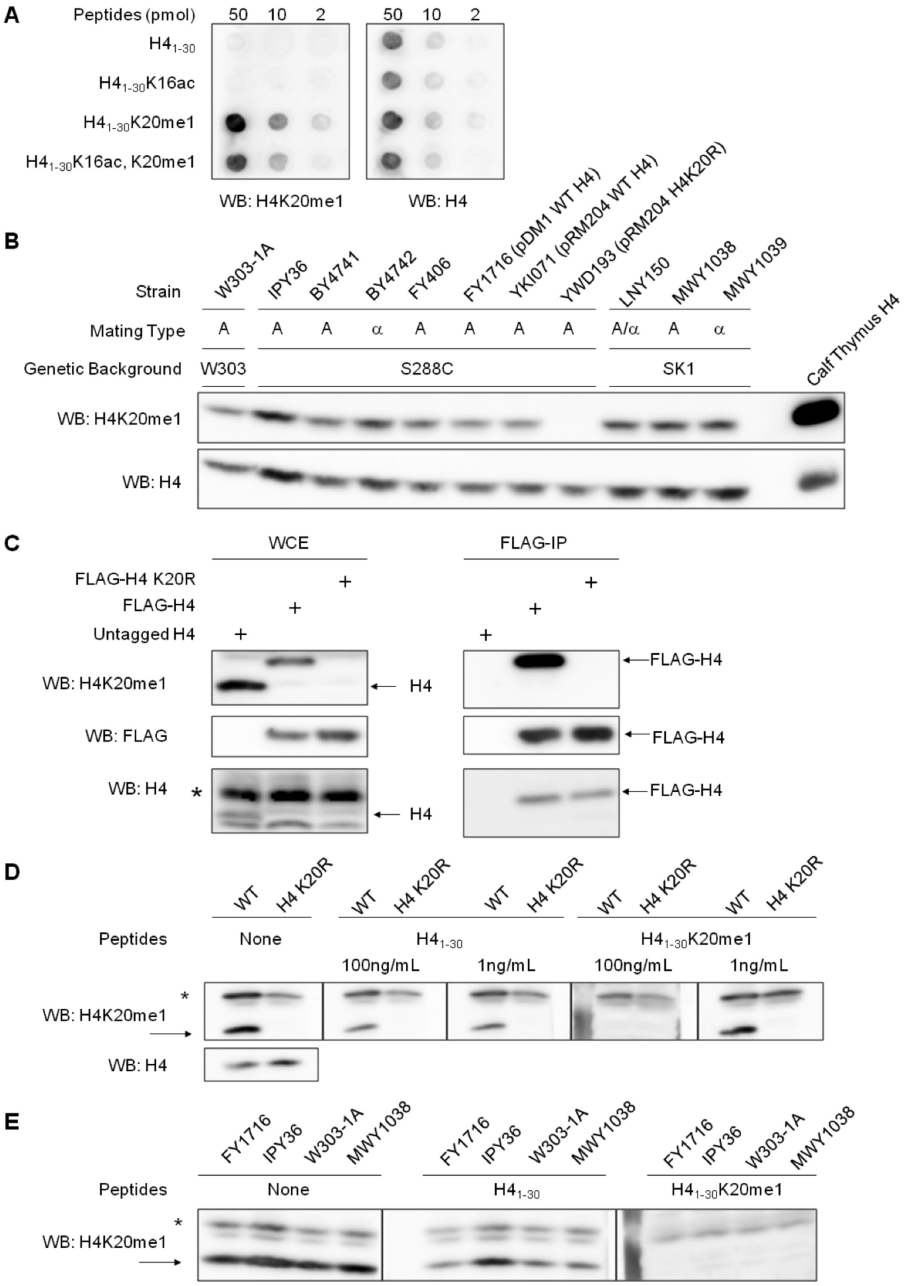

Figure 1. H4 K20me1 detectable in budding yeast. (A) Peptides matching the first 30 amino acids of H4 with or without acetylated lysine 16 or monomethylated lysine 20 were spotted onto PVDF and probed with antibodies. Peptides, peptide amounts, and antibodies are respectively indicated to the left, top, and bottom of the blots. (B) H4 K20me1 is detectable in whole-cell extracts (WCEs) from strains of different genetic backgrounds, mating types, and ploidies, is detectable whether H4 genes are present in the genome or on a plasmid, and is abrogated by an H4 K20R substitution. WCEs were analyzed on polyacrylamide gels, transferred to PVDF, and probed with antibodies. H4 levels are a loading control. Calf thymus H4 is a positive antibody control. Antibodies are indicated to the left of each blot. Strains, genetic backgrounds, and mating types are indicated above each lane. Strains FY1716, YKI071, and YWD193 have H3/H4 genes present only on a plasmid. (C) To confirm that the H4 K20me1 signal is H4 and not a similarly migrating protein, WCEs with untagged H4, FLAG-H4, or FLAG-H4 K20R were subjected to FLAG-affinity purification. WCEs and elutions were analyzed by western analyses. Antibodies are indicated to the left of each blot. The FLAG tag and K20R substitution are indicated above each lane. An asterisk indicates a nonspecific band that obscures FLAG-H4 in WCEs. Arrows indicate untagged H4 in WCEs and FLAG-H4 in eluates. (D, E) H4 K20me1 western signals preferentially competed by monomethylated rather than unmethylated H4 peptides. WCEs were analyzed on polyacrylamide gels, transferred to PVDF, and probed with anti-H4 K20me1 antibodies (1 mg/mL) that were preincubated with no peptides or H4 peptides (100 or 1 ng/mL in (D), 100 ng/mL in (E)) that were or were not monomethylated at lysine 20. H4 levels are a loading control. Antibodies are indicated to the left of each blot. Strains, peptides, and peptide concentrations are indicated above each blot. Arrow indicates H4 K20me1. Asterisk indicates a nonspecific band.

absent in *S. cerevisiae*.[12,16,17] However, intriguingly, mass spectrometry suggested that H4 K20me1 exists in budding yeast in low abundance.[18] Because of the important role of H4 K20 in histone−protein interactions, and the conservation of its methylation throughout higher organisms, we sought to confirm the presence of H4 K20 methylation in *S. cerevisiae* and to investigate possible functional roles for the modification and the K20 residue itself.

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MATERIALS AND METHODS

Plasmids. *SET4* was amplified by the Expand High Fidelity PCR System (Roche), cloned into pBM272 (GAL promoter, CEN, ARS, *URA3*), and sequenced. Amino acid substitutions were engineered into the H3/H4 plasmid pRM204 (*HHT2*, *HHF2*, CEN, ARS, *TRP1*) using the QuikChange site-directed mutagenesis kit (Agilent) and confirmed by sequencing. pBY011 (GAL promoter, CEN, ARS, *URA3*) overexpression plasmids were acquired from the Yeast FLEXGene Collection.[19]

Yeast Strains. Supporting Information Table S1 lists strains used in this study. Gene deletions and GAL promoter insertions were performed as described previously.[20] Plasmid transformations were performed using standard lithium acetate methods. Deletions, insertions, transformations, plasmid shufflings, and histone FLAG tags were confirmed by PCR, sequencing, and FLAG westerns as necessary.

Strains with ORFs deleted or overexpressed were created as follows. Heterozygous Diploid Deletion Collection[21] clones were sporulated to produce haploid methyltransferase deletion clones (YCE 002-016), after which mating tests and PCR confirmed ploidy and deletions, respectively. To acquire additional methyltransferase deletion strains (YCE EH A2-C4), genes were deleted in the H3/H4 shuffle strain JPY12, after which JPY12 and the deletion strains were transformed with FLAG-H4 plasmids (pRM204). Strains were then grown on synthetic complete (SC) media lacking tryptophan to select for pRM204 and dilute out the original JPY12 histone plasmid. *RKM2* and demethylase deletion strains were created by standard gene knockout methods. Overexpression strains were created by transforming pBM272 or FLEXGene Collection plasmids (pBY011) containing galactose-inducible genes into yeast or by integrating galactose-inducible promoters into the genome.

Strains with subtelomeric *URA3* and *ADE2* reporters plus wild-type or mutant histones were created as follows. pRM204 with wild-type or mutant H3/H4 genes was transformed into UCC1369 to create YCE UA1 to UA9, after which strains were grown on SC media lacking tryptophan to select for pRM204 and dilute out the original histone plasmid. Plasmids were similarly transformed into UCC7262 to make YCE UC1 to UC9 and UCC7266 to make YCE UD1 to UD9. UCC1369, UCC7262, and UCC7266 are reported elsewhere.[22]

All other mutant histone strains were created as follows. pRM204 (or derivatives of this) containing wild-type, FLAG-tagged, or mutant H3/H4 genes were transformed into the H3/H4 shuffling strain FY1716, after which SC media containing 5-FOA was used to select against the original FY1716 histone plasmid. Strains with either WT or K20 substitutions of H4 integrated into the genome were made as previously described.[23]

Whole-Cell Extract (WCE) Preparation and FLAG-Affinity Purification. Yeast were grown in YPD (or YP + galactose for overexpressions) to mid-log phase, resuspended in TENG-300 buffer (50 mM Tris-Cl pH 7.5, 300 mM NaCl, 0.5% NP-40, 1 mM EDTA, 10% glycerol, 0.5 mM PMSF, protease inhibitors), beat with silica beads, and sonicated, after which lysates were cleared by centrifugation at 14K rpm. Bradford assays determined protein concentrations. Anti-FLAG-agarose beads (Sigma) were incubated with WCEs overnight and then washed with TENG-300. FLAG peptides (Sigma) then competed off FLAG-tagged proteins.

Western Analyses. Samples were run on polyacrylamide gels, transferred to PVDF, and probed with antibodies followed by incubation with chemiluminescence reagent. Signals were visualized with a Fujifilm LAS-3000 Image Reader. Supporting Information Table S2 lists antibodies used in this study. Roche supplied calf thymus histone H4.

Dot Blots and Peptide Competitions. Peptides matching the first 30 amino acids of budding yeast histone H4 plus a C-terminal cysteine were synthesized with no modifications, acetylated K16, monomethylated K20, or both modifications (Baylor College of Medicine Protein Chemistry Core Laboratory). Peptides matching the higher eukaryote histone H4 lysine 20 epitope without modifications or with mono-, di-, or trimethylated lysine 20 were purchased from ABCam (ab2622, ab14964, ab17043, ab17567, ab21044). Ab21044 spans residues 16−25; all others span residues 17−24. For dot blots, known amounts of peptides were spotted onto PVDF and probed with antibodies. For peptide competitions, western analyses were performed as usual, except that antibodies were first incubated with peptides at room temperature for 1 h.

Phenotype Assays and Determination of Reporter Expression. Yeast from YPD cultures were washed and resuspended in water, serially diluted, and spotted onto media that were then incubated at 30 °C (37 °C for heat sensitivity). Yeast with a *URA3* reporter were spotted onto SC media, SC media lacking uracil, or SC media with 5-FOA followed by incubation at 30 °C. Yeast with an *ADE2* reporter were grown on YPD plates at 30 °C and then left at 4 °C for several days to allow pigment accumulation. Images were recorded with a scanner.

Replicative Lifespan Assay. Replicative lifespan assays and data analysis were performed as previously described.[23]

Chromatin Immunoprecipitation Assay and Determination of RNA Levels. For ChIP, yeasts were cross-linked with 1% formaldehyde at room temperature for 10 min followed by chromatin immunoprecipitation as described previously.[24] To extract RNA, yeasts were lysed via bead-beating, after which RNA was purified from extracts using the Qiagen RNeasy kit and converted to cDNA using random hexamers (IDT) and the Applied Biosystems Taqman reverse transcriptase kit. cDNAs and ChIP DNA were analyzed using the Applied Biosystems 7900HT Fast Real-Time PCR System.

RESULTS

Lysine 20 of Histone H4 Is Monomethylated in Budding Yeast. To determine whether lysine 20 of histone H4 is monomethylated in *S. cerevisiae*, we acquired a commercially available anti-H4 K20me1 polyclonal antibody (ABcam ab9051) and tested it in dot blots using $H4_{1-30}$ peptides that were unmodified, monomethylated at K20 (H4 K20me1), acetylated at K16 (H4 K16ac), or had both modifications. The antibodies preferentially recognized monomethylated versus unmethylated peptides and showed no affinity for H4 K16ac (Figure 1A). The antibody also comparably detected H4 K20me1 in the presence or absence of H4 K16ac.

We then determined whether H4 K20me1 is detectable by western analysis of whole-cell extracts (WCEs) from a variety of strains. An epitope was detected migrating similarly to calf thymus H4 in all strains tested regardless of genetic background (S288C, SK1, and W303), mating type, ploidy, or whether the H4 gene was present in the genome or on a plasmid (Figure 1B). Importantly, substitution of H4 K20 with the chemically

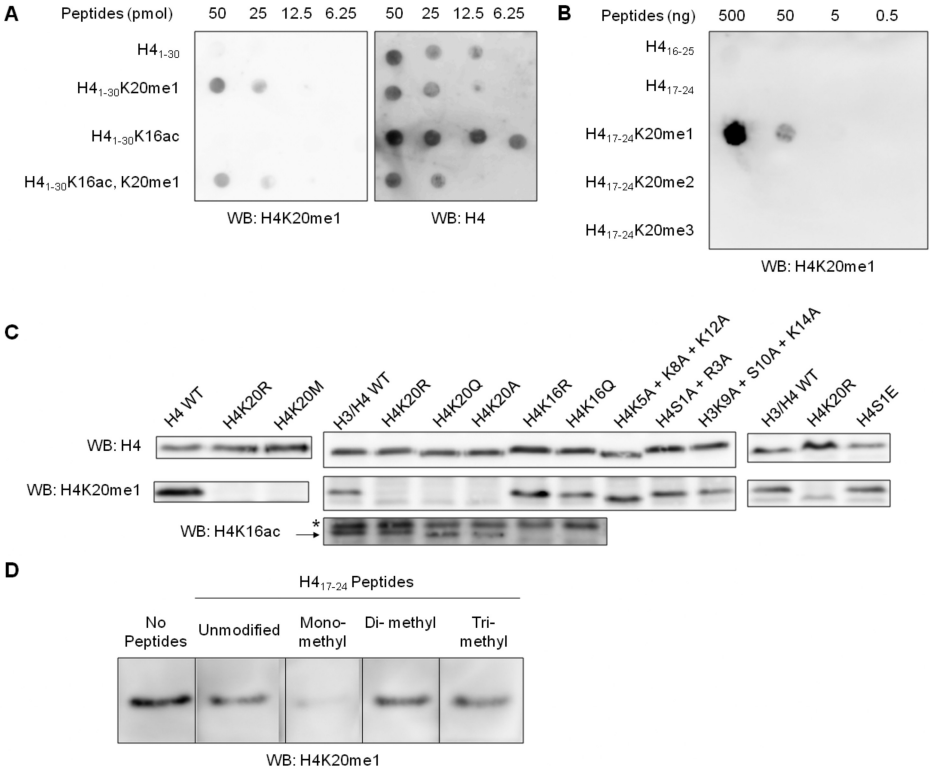

Figure 2. H4 K20me1 detectable with Millipore antibody 04-735. (A, B) Peptide dot blots as in Figure 1A. (C) Substitutions of modifiable histone residues do not affect H4 K20me1 levels and H4 K20 substitutions do not affect H4 K16ac levels. WCEs from yeast with wild-type or mutant histones were analyzed by western analyses. Antibodies and histone mutations are respectively indicated to the left of and above each blot. Asterisk indicates a nonspecific band present on blot probed for H4 K16ac. (D) Western blots with peptide competitions as in Figure 1D,E.

similar arginine (to mimic unmodified lysine) abrogated the signal. We also probed WCEs with anti-H4 K20me2, 3 antibodies but failed to detect these modifications (Figure S2).

To confirm that the western signal was from H4 rather than a similarly migrating protein, we performed westerns with WCEs from strains in which all copies of H4 were either FLAG-tagged or untagged (Figure 1C). WCEs containing untagged H4 had an H4 K20me1 western signal comigrating with calf thymus H4, whereas WCEs with FLAG-H4 had the H4 K20me1 signal migrating slower than calf thymus H4 (Figure 1C, left). A FLAG-H4 K20R substitution abrogated this signal confirming that the slower migrating species was FLAG-H4. These WCEs were also subjected to FLAG-affinity purification after which elutions were analyzed by western analysis. The H4 K20me1 epitope was purified from WCEs with FLAG-H4 but not from WCEs with untagged H4 or FLAG-H4 K20R (Figure 1C, right). These results show that the epitope is present on H4, and there is no cross-reaction with any other H3 methylation.

To further confirm the presence of the K20me1 modification, WCEs from wild-type or H4 K20R yeast were probed with anti-H4 K20me1 antibodies that had been preincubated without peptides (control) or with different concentrations of H4 peptides having or lacking K20me1. Control competition produced a western band with the wild-type but not mutant WCEs, and this epitope was competed in a dose-dependent manner by monomethylated but not unmodified peptides (Figure 1D). This preferential competition was seen with WCEs from yeast of three different genetic backgrounds (Figure 1E).

As further evidence for the existence of H4 K20me1, a second commercially available antibody (Millipore 04-735, formerly Upstate 05-735) was tested. This antibody showed specificity in dot blots for monomethylated rather than unmethylated, dimethylated, or trimethylated H4 peptides, and detection remained constant for the double modified K16ac and K20me1 peptide (Figure 2A,B). The antibody also detected budding yeast H4 in westerns of WCEs, comigrating with calf thymus H4, and yeast H4 detection was abrogated by alanine, arginine, glutamine, or methionine K20 substitutions but not by an H4 K16R substitution (Figure 2C). Further, this western signal was preferentially competed by peptides with monomethylated but not unmethylated, dimethylated, or trimethylated lysine 20 (Figure 2D). We conclude, using a wide variety of approaches and multiple antibodies, that H4 K20me1 is present in the budding yeast *S. cerevisiae*.

Approaches To Identify Enzymes That Modify H4 K20 in Budding Yeast. To identify histone methyltransferases (HMTs) and demethylases (HDMs) for this modification, a list of candidates was produced. Since most known histone lysine methyltransferases have a SET domain, we selected the 12 *S. cerevisiae* SET domain-containing proteins (*SET1* to *SET6*, *RKM1* to *RKM4*, *CTM1*, and *EFM1*). We also selected the non-SET domain-containing HMT *DOT1* and three putative arginine methyltransferases (*HMT1*, *RMT2*, and *HSL7*). We selected the five *S. cerevisiae* Jumonji-C (JmjC) domain-containing proteins (*JHD1*, *JHD2*, *RPH1*, *GIS1*, and *ECM5*), since this domain often has histone demethylase activity, and the proposed HDM *ELP3*.[25,26] These genes were individually

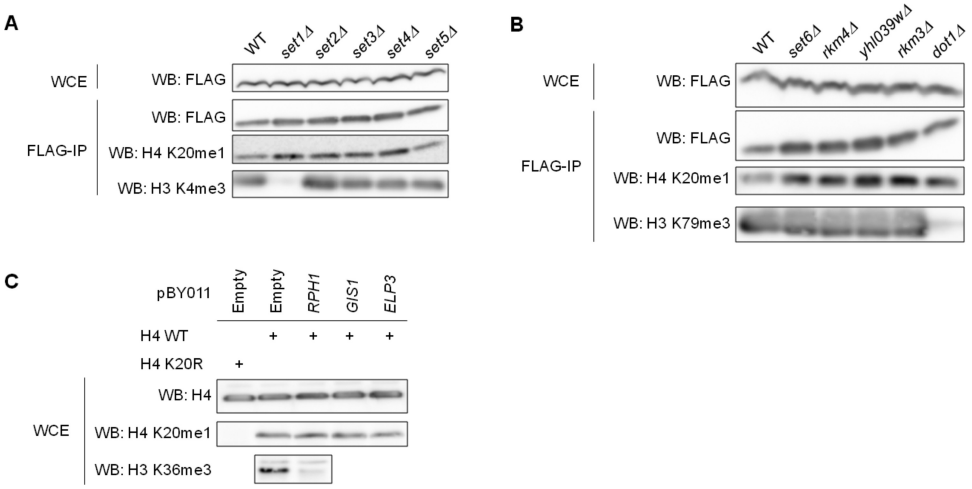

Figure 3. Deleting and overexpressing candidate genes does not change H4 K20me1 levels. (A, B) FLAG-H4 was purified by FLAG-affinity purification from strains with candidate HMTs deleted. WCEs and FLAG elutions were analyzed by western analysis. Antibodies and strains are respectively indicated to the left of and above each blot. FLAG levels are a loading control. (C) WCEs from strains with candidate HMTs overexpressed from a plasmid were analyzed by western analysis. Antibodies and strains are respectively indicated to the left of and above each blot. H4 levels are a loading control. An H4 K20R strain serves as a negative antibody control.

deleted or overexpressed, and H4 K20me1 levels were checked by western analysis.

H4 K20me1 levels in deletion strains were checked by western analyses of FLAG-purified histones from strains with FLAG-tagged H4 and WCEs from all other strains (Figure 3A,B and Figure S3). As positive controls, deleting *SET1* (Figure 3A), *SET2* (Figure S3A), or *DOT1* (Figure 3B and Figure S3A,B) decreased H3 K4me3, H3 K36me3, or H3 K79me3 levels, respectively. However, none of the deletion strains had significantly altered H4 K20me1 levels. Individual deletions of 14 additional ORFs whose protein products share homology with Dot1 (Table S3) also failed to abrogate the H4 K20me1 western signal.

Since enzyme redundancy might prevent H4 K20me1 levels from changing with individual or even double deletions, we overexpressed candidates individually and checked WCEs by western analysis (Figure 3C and Figure S4). As positive controls, overexpressing *RPH1* (Figure 3C and Figure S4B,C) and *JHD2* (Figure S4B) decreased H3 K36me3 and H3 K4me3 levels, respectively. However, H4 K20me1 levels did not change upon induction of any of the candidates. In summary, deletion or overexpression of known or potential HMTs or HDMs did not alter H4 K20me1 levels in yeast.

Examination of Crosstalk between H4 K20me1 and Other Modifications. To test whether other histone modifications affect H4 K20me1, WCEs from strains with substitutions of known modified histone H3 and H4 residues were probed with anti-H4 K20me1 antibodies. H4 K20 substitutions eliminated the H4 K20me1 signal, whereas all other substitutions (e.g., the triple substitution H4 K5R/K8R/K12R—all acetylated residues) had no effect (Figure 2C). Substitutions of H3 K4, H3 K36, and H3 K79 were not tested since deleting or overexpressing *SET1*, *SET2*, or *DOT1* did not affect H4 K20me1 levels (Figure 3 and Figures S3 and S4).

We then tested whether, conversely, H4 K20me1 altered H4 K16ac levels. H4 K16R and H4 K16Q mutations eliminated the K16ac signal, whereas H4 K20 substitutions showed no effect on K16ac (Figure 2C). H4 K16ac levels were also checked at various locations in the genome by ChIP and were not affected by H4 K20R substitution (data not shown). We conclude that H4 K20me1 is not altered by other abundant modifications (most of which correlate with transcription), nor does H4 K20 substitution affect H4 K16ac.

H4 K20me1 Abundance at Genomic Heterochromatin and Euchromatin. To determine the distribution of the methylation at key locations in the genome, we performed ChIP against H4 K20me1 and total H4 followed by qPCR of various locations. We checked heterochromatic locations and non-heterochromatic locations including upstream of and throughout the body of several genes as well as centromeres which, unlike in higher eukaryotes, are not known to contain heterochromatin.[27,28] We found that the input-normalized ChIP signal for H4 K20me1 was higher than IgG-mock ChIPs for all locations tested, but not in the H4 K20R substitution control ChIP (Figure 4B). We found a preferential distribution of H4 K20me1 levels within heterochromatic regions, including subtelomeres, rDNA, and the silent mating type loci *HML* (Figure 4A–C). The lowest level of H4 K20me1 was found at centromeres (Figure 4A–C). In general, genes showed an intermediate level between heterochromatin (highest levels) and centromeres (lowest levels) (Figure 4A–C). In addition, transcribed regions/open reading frames (ORFs) of genes were higher in K20me1 compared to the upstream promoter regions and at 5′ and 3′ ends of genes (Figure 4A,C,D).

As a further control for these results, we examined ChIP signals for H3 K4me3 normalized to total histone H4. As expected, and in contrast to the distribution of H4 K20me1, H3 K4me3 levels were lower at heterochromatic locations and higher at some euchromatic locations, particularly several centromeres and the 5′ region of an ORF (Figure 4C, compare upper panel with H3 K4me3 to lower panel with H4 K20me1).

Phenotypic Assays of H4 K20 Substitution Mutants in Heterochromatic Gene Silencing, Stress Pathways, and during Replicative Aging. As discussed above, H4 K20 is part of a patch of basic residues (K_{16}RHRK$_{20}$) that is linked to heterochromatin. Since we detected preferential localization of H4 K20me1 at heterochromatin, and since K20me1 in higher eukaryotes is linked to heterochromatin, chromatin compaction, and gene silencing, we investigated whether budding yeast H4 K20 and its methylation are linked to heterochromatic

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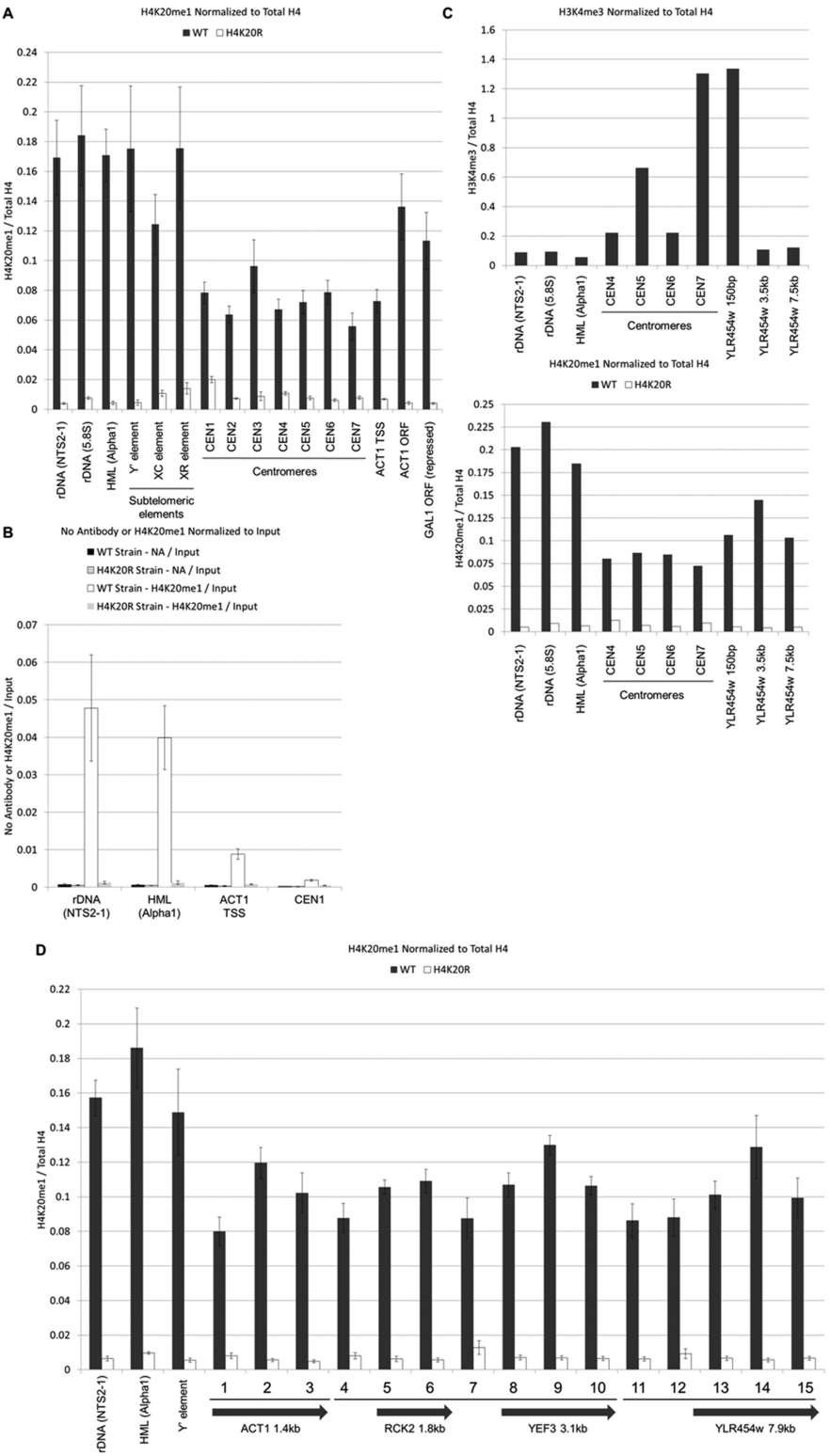

Figure 4. ChIP-qPCR analysis of H4 K20me1. Sonicated chromatin from yeast with WT or K20R histone H4 were immunoprecipitated by antibodies against H4 K20me1 or total H4 and copurified DNA was analyzed by qPCR. Bars indicate H4 K20me1 levels normalized to total H4 levels (A) or no antibody or H4 K20me1 levels normalized to input levels (B). Genomic locations are indicated below each pair of bars. (C) Comparison of H3 K4me3 versus H4 K20me1 levels relative to H4 levels at several loci. (D) H4 K20me1 levels relative to H4 levels across several regions: *ACT1* ORF, *RCK2-YEF3* region and *YLR454w* region. Horizontal bars and arrows underneath graph represent genome and genes, respectively. Position of bar graph bars above genes indicates locations of checked regions. A, B, and D represent means and standard error of the mean (SEM) of three experiments.

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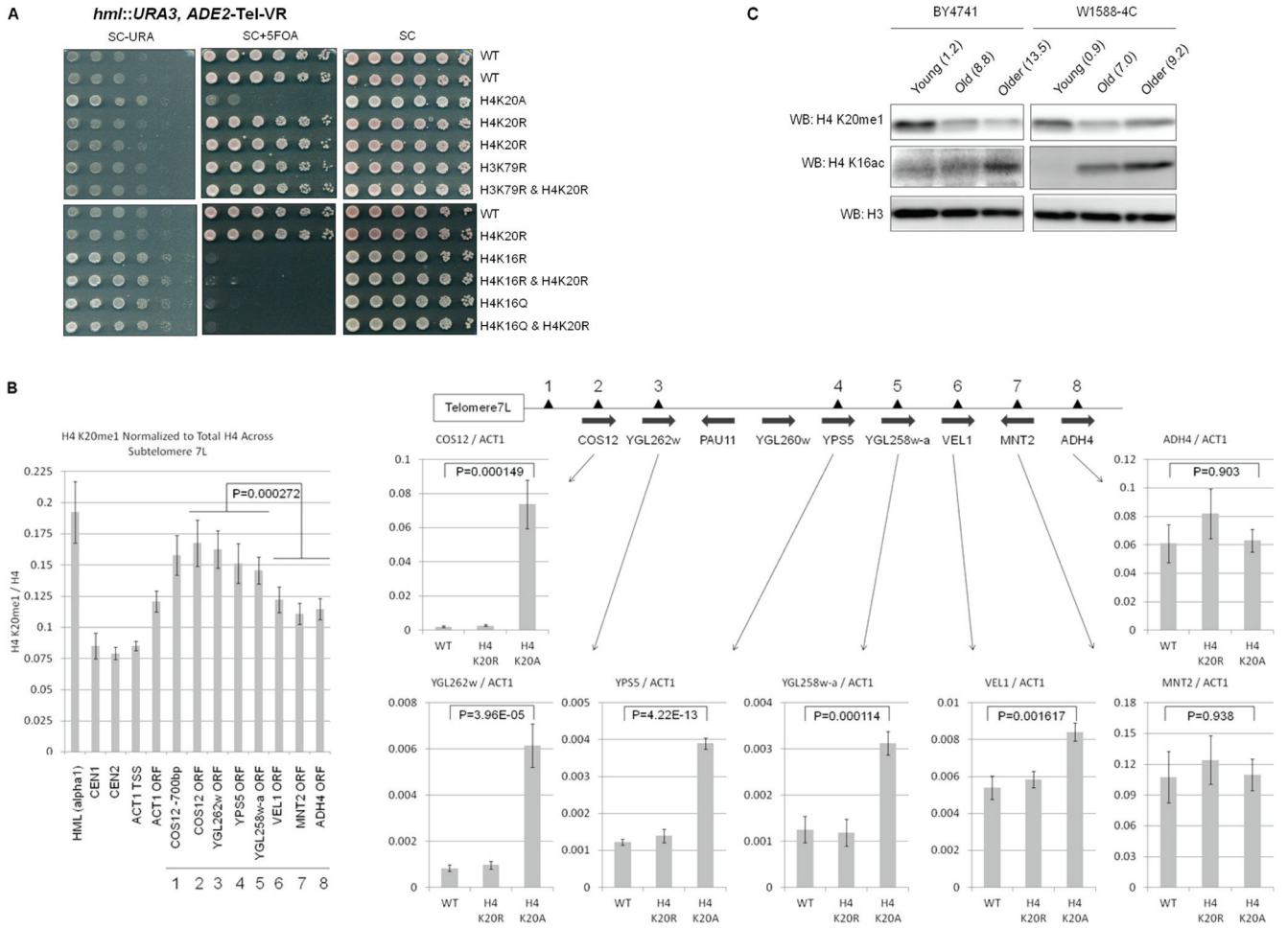

Figure 5. Phenotype analysis of H4 K20me1. (A) Dilutions of *hml::URA3 ADE2*-TEL-VR yeast with wild-type or mutant histones were grown on synthetic complete (SC), SC-URA, or SC+5FOA media. Media and strains are indicated above and to the right of each plate, respectively. Increased growth on SC-URA media and decreased growth on SC+5FOA media compared to SC media indicate increased *URA3* expression. White and red yeast coloration respectively indicates *ADE2* expression and silencing. (B) H4 K20me1 levels at and expression of genes proximal and distal to telomere 7L were determined by ChIP of H4 K20me1 and total H4 and by qPCR of harvested RNAs. (left) ChIP was as in Figure 4. H4 K20me1 levels per total H4 levels are shown with means and standard errors of the mean. ChIP locations residing along chromosome 7L are indicated in the map by black triangles while above numbers indicate which locations correspond to which bars in the graph. Locations 2−5 were compared to locations 6−8 using a Wilcoxon rank sum test and generated the indicated P-value. (right) RNA was extracted from yeast with WT, K20R, or K20A histone H4. mRNA levels of genes proximal and distal to telomere 7L were determined by qPCR and normalized to *ACT1* mRNA. Means and standard error of the means are shown. WT and H4 K20A samples were compared using a *t* test and generated the indicated *P*-values. (C) H4 K20me1 levels are lower in replicatively older than younger yeast. WCEs from replicatively young or old yeast were analyzed by western analyses. Antibodies are indicated to the left of each blot. Samples and strains are indicated above each lane. Average numbers of bud scars (cell divisions) for each yeast sample are indicated in parentheses. H3 levels are a loading control.

silencing in yeast. We used classic heterochromatic silencing reporters at telomeres and silent mating type loci and also assayed subtelomeric endogenous gene expression.

Expression of a *URA3* reporter integrated at heterochromatic regions subtelomere VIIL (Figure S5A), *HMR* (Figure S5B), or *HML* (Figure 5A) in yeast that have either wild-type or mutant histones was determined by comparing growth on nonselective SC plates to selective SC-URA plates (increased expression causes increased growth) or SC+5FOA plates (increased expression causes decreased growth). As positive controls, H4 K16R, H4 K16Q, and H3 K79R mutants caused silencing defects of subtelomeric *URA3*, and H4 K16R and H4 K16Q mutants caused silencing defects of *URA3* at *HMR* and *HML* loci (Figure 5A and Figure S5). We found that H4 K20A showed dramatic silencing defects at subtelomere VIIL, *HMR*, and *HML*, whereas H4 K20R did not change reporter expression at these tested locations. Strains with an *ADE2* reporter at subtelomere VR were also analyzed by observing yeast coloration (red and white respectively indicate silencing and expression). While H4 K16R, H4 K16Q, and H4 K20A mutations caused *ADE2* silencing defects, an H4 K20R substitution did not (Figure 5A and Figure S5). Importantly, H4 K20A substitution did not globally increase H4 K16ac levels in the cell (Figure 2C), indicating that the reduction of silencing was not due to an indirect effect on acetylation.

To determine if similar derepression occurs at endogenous genes, we checked expression levels of genes proximal and distal to the telomere of chromosome VIIL. The most telomere-proximal gene is *COS12*, and its location is similar to that of the subtelomeric *URA3* reporter used above[29] (Figure S5). While wild-type and H4 K20R strains had little or no detectable *COS12* expression, H4 K20A strains showed strong

derepression of this gene (Figure 5B, right panels). Interestingly, while these effects were seen at the telomere-proximal *COS12*, the distal *MNT2* and *ADH4* showed no such derepression with any H4 K20 substitutions, whereas numerous intermediate genes showed derepression with the H4 K20A substitution that became less intense as the distance from the telomere increased. Similar results were seen whether mRNA levels were normalized to *ACT1* mRNA (Figure 5B, right panels) or 18S rRNA (data not shown). Genes *PAU11* and *YGL260w* were not included due to qPCR difficulties resulting from their homologies to other genes in the genome.

To determine whether these K20A-mediated silencing defects correlate with levels of H4 K20me1, we performed ChIP for this mark across the chromosome VIIL subtelomeric region. Interestingly, the telomere-proximal locations contained higher H4 K20me1 relative to the telomere-distal locations, and the level graded at the genes in-between (Figure 5B, left panel). We noted that the four most telomere-proximal genes we tested (*COS12*, *YGL262w*, *YPS5*, and *YGL258w-a*) had H4 K20A-mediated derepression that was greater than 2-fold, whereas the three most telomere-distal genes we tested (*VEL1*, *MNT2*, and *ADH4*) had H4 K20A-mediated derepression that was less than 2-fold or nonexistent. While the H4 K20me1 levels decreased as distance from the telomere increased, we noted that H4 K20me1 levels among the telomere-distal group of genes were significantly lower than among the telomere-proximal group of genes (Figure 5B, left panel). The observations that H4 K20A, but not K20R, reduced heterochromatic silencing and increased subtelomeric transcription are discussed in detail below.

We assayed H4 K20 substitution mutants under a wide variety of growth conditions to test many different gene signaling pathways. Wild-type, H4 K20A, and H4 K20R yeast grew similarly on rich and synthetic media plates at 30 °C (Figure S6), and wild-type, H4 K20R, and H4 K20M yeast showed similar growth kinetics in liquid YPD media at 30 °C (data not shown). H4 K20A and H4 K20R mutants displayed no obvious growth advantages or disadvantages compared to wild-type yeast under a variety of stress conditions (Figure S6 and summarized in Table 1). Thus, any effects of H4 K20A do not appear to be the result of general defects in chromatin structure, stress response, or cell viability.

Acetylation of H4 K16, a residue proximal to H4 K20, increases at heterochromatin during replicative aging in budding yeast.[23] Since we observed heterochromatic localization of H4 K20me1, we investigated whether H4 K20me1 levels change during replicative aging. In two yeast strains, older cells had higher H4 K16ac levels by western analysis of histones, as expected.[23] Interestingly, H4 K20me1 levels were dramatically reduced during aging compared to young cells, opposite to K16ac (Figure 5C).

DISCUSSION

While previous analyses indicated that H4 K20 is not methylated in *S. cerevisiae*,[12,16,17] mass spectrometry data suggested that H4 K20me1 exists in low abundance.[18] Using multiple methods, we demonstrate that *S. cerevisiae* H4 K20 is, indeed, monomethylated. Anti-H4 K20me1 antibodies detected an H4 epitope in western blots of WCEs from various strains and purified H4. These western signals from endogenous histones were abrogated by a K20R substitution and were preferentially competed by H4 K20me1 peptides (Figures 1 and 2). In contrast, we detected neither dimethylated (consistent with previous reports[16,17]) nor trimethylated H4 K20 in budding yeast (Figure S2). Since antibodies are central to modification studies, we note that there was significant lot-to-lot variation in the efficacy of H4 K20me1 antibodies, typical of many modification antibodies.[30]

Table 1. Stress Conditions Tested[a]

conditions tested with H4 K20R	conditions tested with H4 K20A
YPD or SC at 37 °C (heat sensitivity)	YPD or SC at 37 °C (heat sensitivity)
YP + galactose (galactose utilization)	YP + galactose (galactose utilization)
YP + acetate (acetate utilization)	YP + acetate (acetate utilization)
YP + ethanol and glycerol (ethanol and glycerol utilization)	YP + ethanol and glycerol (ethanol and glycerol utilization)
YP + raffinose (raffinose utilization)	YP + sucrose (sucrose utilization)
YPD + 100−125 mM hydroxyurea (DNA replication stress)	YPD + 100−125 mM hydroxyurea (DNA replication stress)
YPD + 10−15 μg/mL camptothecin (topoisomerase inhibition stress)	YPD + 10−15 μg/mL camptothecin (topoisomerase inhibition stress)
YPD + 1 M NaCl (osmolarity stress)	YPD + 1 M NaCl (osmolarity stress)
SC + UV light (DNA damage)	SC + UV light (DNA damage)
YPD + 0.01−0.05% MMS (DNA damage)	
YPD + 0.02% H_2O_2 (oxidative stress)	
SC lacking inositol (inositol utilization)	
YP + low glucose (respiration)	

[a]Stress conditions used in phenotype assays. Growth of wild-type and H4 K20 substitution yeast were compared between stress and nonstress conditions.

Our evidence supporting the existence of budding yeast H4 K20me1 is not entirely surprising. First, studies reporting its absence according to western analysis actually used antibodies raised against H4 K20me2 peptides and not K20me1.[16,17] Second, although budding yeast lack orthologues to higher eukaryote H4 K20 methylase enzymes, chromatin modifying enzymes sometimes lack expected protein sequence homologies or domains. For example, most HMTs have a SET domain and yet the H3 K79 HMT Dot1 has a different catalytic domain.[31] Third, although the amino acid sequence surrounding budding yeast H4 K20 is not totally conserved in higher eukaryotes, it is fully conserved in fission yeast (Figure S1), which has K20 methylation.[5]

Functions of H4 K20 Monomethylation. H4 K20me1 is reported in higher eukaryotes to be associated with transcriptional silencing, chromatin compaction, and heterochromatin. We therefore hypothesized that this modification would preferentially associate with heterochromatic regions of the budding yeast genome. Levels of H4 K20me1 were significantly higher at heterochromatic locations and lower at euchromatic locations, including centromeres which, unlike in higher eukaryotes, are not heterochromatic (Figure 4A,C lower panel).[27,28] We also found H4 K20me1 to be higher in the middle of genes relative to upstream regions and 5′ or 3′ ends (Figure 4A,D), consistent with findings from mammalian studies showing enrichment of H4 K20me1 inside of genes.[10,11] H4 K20me1 may have a compaction function within genes, similar to H3 K36me3, which localizes to the 3′ half of genes and recruits HDACs to promote chromatin compaction.[32]

The previous relationship of H4 K20 methylation to heterochromatin, DNA replication, DNA repair, and chromatin compaction in higher eukaryotes, prompted testing of these pathways in budding yeast. It is intriguing that H4 K20A substitution produced dramatic silencing defects at subtelo-

meres (Figure S5A), including the most telomere 7L-proximal gene *COS12* and multiple adjacent subtelomeric genes (Figure 5B, right panels), and at the silent mating type loci *HMR* and *HML* (Figure 5A, S5B). Interestingly, H4 K20me1 levels showed a striking correlation with K20A-mediated silencing defects along regions proximal and distal to telomere VIIL (Figure 5B, compare left and right panels). The silencing defects are not an indirect effect of increasing acetylation at H4 K16 because global H4 K16ac levels were comparable in strains bearing wild-type H4 relative to K20A (Figure 2C) and were not due to general disruption of chromatin structure since the H4 K20A mutant did not show increased transcription at all genes examined (Figure 5B, right panels). Previous studies of the $K_{16}RHRK_{20}$ patch *in vitro* and *in vivo* have not identified a role for K20 in recruitment or regulation of heterochromatic or other complexes, such as Sir2/3/4, Isw2, or Dot1.[13−15] It will be interesting in future studies to determine the role of K20me1 in promoting repression at subtelomeres and at the silent mating type loci.

In contrast to K20A, the H4 K20R substitution did not show silencing defects at subtelomeres and silent mating type loci, nor did K20R exhibit phenotypes associated with DNA replication inhibitors, DNA damaging agents, or other stress conditions (Figure 5A, Figures S5 and S6, and Table 1). Nonetheless, we favor the view that there is a function for H4 K20me1, based on the strong correlation between the location of the modification at heterochromatic regions, and the highly specific defect of H4 K20A in telomere-proximal regulation. One possible explanation for the absence of an effect of K20R in silencing, or other processes, is that effector proteins may interact with this basic patch through binding to both the residues and K20me1, such that interactions are compromised by loss of either, but only sufficiently to produce a detectable phenotype with loss of the residues. In this view, K20A may alter the structure sufficiently to disrupt binding, but loss of methylation alone via K20R, would not disrupt binding.

An alternative possibility is that the methylation is redundant with other pathways. Such a scenario is not uncommon in budding yeast chromatin. Indeed, it is remarkable that certain well-known modifications, such as H2B K123 ubiquitylation, which is present on more than half the histone H2B proteins in the cell, has only a modest phenotype when abrogated by a K123R substitution. Yet, when K123R is combined with deletion of *GCN5*, the major H3 acetyltransferase (whose deletion also results in only minor reduction of transcription), there is a strong synthetic effect, causing very low transcription.[33]

Since acetylation of the adjacent H4 K16 participates in replicative aging,[23] we examined whether H4 K20me1 is also linked to aging. Interestingly, H4 K20me1 levels were reduced in older compared to younger yeast (Figure 5C). This lowered detection is not due to H4 K16ac obscuring the K20me1 signal because substituting K16 to arginine or glutamine does not globally alter K20me1 levels (Figure 2C), nor does K16ac prevent K20me1 recognition in peptide dot blots (Figures 1A and 2A). This result is consistent with association of H4 K20me1 and H4 K16ac with opposing chromatin states, i.e., the former with condensed and silent chromatin, whereas the latter with open and active chromatin. It is possible that the decrease in K20me1 in old cells might affect protein recruitment or chromatin structure. We note that we have not observed a change in lifespan in an H4 K20R substitution (data not shown); thus, a role of H4 K20me1 during aging is unclear. However, we do not believe that such a function would occur by altering H4 K16ac levels, since levels of this adjacent modification are not altered by H4 K20R substitutions (Figure 2C).

Unexpected Histone Modifications and Unidentified Enzymes in Budding Yeast. Budding yeast histones have additional lysines methylations, some of which are conserved in higher eukaryotes. Recently, H2B K37 methylation was identified at low levels in *S. cerevisiae*, and this residue and methylation are both conserved in complex eukaryotes; substitution mutations here (K37R and K37A) have no detectable phenotype, however.[34] Similarly, H3 K42 methylation was recently identified in budding yeast and may be linked to transcription; lysine to arginine substitution mutation here however also has no detectable phenotype.[35] These findings, along with our data on H4 K20 and its methylation, indicate that histone lysine methylation, including low abundance marks, may be more common than previously appreciated and may have pathway-specific functions that are difficult to reveal.

Furthermore, similar to H4 K20me1, individual deletions of *DOT1* and all known SET domain-containing genes do not abrogate H2B K37 or H3 K42 methylation,[34,35] and combinatorial deletions of three known arginine methyltransferases do not abrogate H3 R2 methylation.[36] While enzyme redundancies may be the reason in some cases, unidentified methyltransferases may instead be responsible for these previously unknown budding yeast modifications. Genome-wide deletion and overexpression screens and biochemical methods may help to discover the responsible enzymes.

While H4 K20 and H2B K37 are conserved and methylated from *S. cerevisiae* to higher eukaryotes, H3 K9 and H3 K27 are also conserved and methylated in higher organisms but reportedly unmethylated in *S. cerevisiae*. Our western analyses have not revealed H3 K9me in budding yeast (data not shown); however, our search for this mark has not been extensive. We note that recent mass spectrometry data detected low levels of this modification,[18] and the *S. cerevisiae* HDM Rph1 targets H3 K9me *in vitro*.[37] Whether any other "absent" marks are actually conserved in this model organism remains to be determined.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Tables listing yeast strains and antibodies used, a table listing additional deleted candidate methyltransferases, and additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

PTM, post-translational modification; HMT, histone methyltransferase; HDM, histone demethylase; WCE, whole-cell extract; ChIP, chromatin immunoprecipitation.

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